THE REGULATORY ROLE OF ACSDKP AND ANGIOTENSIN 1-CONVERTING ENZYME (ACE) INHIBITORS ON HAEMATOPOIETIC STEM AND PROGENITOR CELL PROLIFERATION

John Eugenes Chisi

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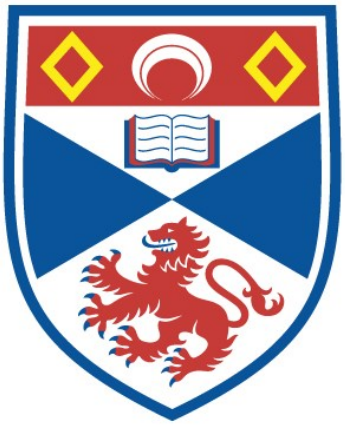
at the
University of St Andrews



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The regulatory role of AcSDKP and Angiotensin 1-converting enzyme (ACE) inhibitors on haematopoietic stem and progenitor cell proliferation.

Thesis submitted for the degree of Doctor of Philosophy to the University of St. Andrews

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Declaration

I, John Eugenes Musukuma Peter Chisi, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed: John Eugenes Chisi Date:

I was admitted to the faculty of Science of the University of St. Andrews under Ordinance General No. 12, and as a candidate for the degree of Ph.D. in September 1996.

Signed: John Eugenes Chisi Date:

Certificate:

I hereby certify that the candidate has fulfilled the conditions of the resolution and regulations appropriate to the degree of Ph.D.

Signature of Supervisor Date:

Dr. Andrew C. Riches

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THIS WORK IS DEDICATED TO MY WIFE, KATHRIN BROETZ, MY DAUGHTER TOWELA, MY SON TOBIAS, MY MOTHER, ESTER SOTWANE AND MY FATHER, PETER CHISI.

Abbreviations

2 Gy	2 Gy –γ-irradiation.
2 GyC	2 Gy-g-irradiation plus captopril
2 GyL	2 Gy-g-irradiation plus lisinopril
2 GyS	2 Gy-g-irradiation plus saline
5 FU	5 fluorouracil
5 FUC	5 fluorouracil plus captopril
5 FUL	5 fluorouracil plus lisinopril
5 FUS	5 fluorouracil plus saline
AC	Cytosine arabinoside plus captopril
ACE	Angiotensin 1-converting enzyme
ACEI	Angiotensin 1-converting enzyme inhibitor
AcSDKP	Acetyl-Ser-Asp-Lys-Pro
AcSDψKP	Acetyl-Ser-Asp ψ Lys-Pro , ψ = aminomethylene bond
AIDS	Acquired Immunity Deficiency Syndrome
AL	Cytosine arabinoside plus lisinopril
AML	Acute myeloid leukaemia
Ara-C	Cytosine arabinoside
AS	Cytosine arabinoside plus saline
AZT	3'-azido-3-deoxythymidine
Bl-CFC	Blast colony forming cell
bFGF	basic fibroblast growth factor
BFU-E	Burst forming unit-erythrocyte
CAFC	Cobble stone area forming cell
cAMP	cyclic adenosine monophosphate
Cap (C)	Captopril
CCE	Counterflow centrifugal elutriation
CD	Cluster of designation
CDK	Cyclin dependent kinase
CDP	Cytosine diphosphate

CFU-A	Colony forming unit- type A
CFU-C	Colony forming unit-culture (granulocyte colony)
CFU-E	Colony forming unit-erythroid
CFU-G	Colony forming unit-granulocyte
CFU-GEMM	Colony forming unit-granulocyte-erythrocyte -macrophage-megakaryocte
CFU-GMM	Colony forming unit-granulocyte-mast cells–macrophage/megakaryocyte
CFU-M(k)	Colony forming unit-megakaryocyte
CFU-S	Colony forming unit- spleen
CML	Chronic myeloid leukaemia
CMP	Cytosine monophosphate
Con-A	Concovalin-A
COX	Cyclooxygenase
CSF	Colony stimulating factor
C-src	Rous sarcoma virus protooncogene
CTP	Cytosine triphosphate
CTX	Cyclophosphamide
cyclic GMP	cyclic guanosine monophosphate
D FCS PS/G	Dulbeccos medium with foetal calf serum, penicillin, streptomycin and glutamine
D HS PS/G	Dulbeccos medium with horse serum, penicilline, streptomycine and glutamine
DAG	Diacylglycerol
DNA	Deoxyribose nucleic acid
DRTF1/E2F	A heterodimeric transcription factor DP/E2F
dUMP	d-uracil monophosphate
E2F	Adenovirus E2 gene product
Eo-CFC	Eosinophil colony forming cell

Epo	Erythropoietin
F HS PS/G	Fishers medium with horse serum, penicillin, streptomycin and glutamine.
F HS PS/G Hydro	Fishers medium with horse serum, penicillin, streptomycin, glutamine and hydrocortisone.
FACS	Fluorescence-activates cell sorting.
FAPGG	N-[3-(2-furyl)acryloyl-L-phenylalanylglycylglcine
FDC	Factor dependent cell
FITC	Fluorescein isothiocynate
FLS	Forward light scatter
GAGs	Glucosaminoglycans
G-CSF	Granulocyte- colony stimulating factor
GM-CFC (CFU-GM)	Granulocyte macrophage colony forming cells
GM-CSF	Granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
HiNF-D	Histone nuclear factor-D
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigent
Ho	Hoechst 33342
HPP-CFC	High proliferative potential colony forming cells
IFN	Interferon
IgG	Immunoglobulin-G
IL	Interleukin
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
IP_3	inositol triphosphate
JAK	Janus kinase
KDa	Kilodalton
L929	L929 fibroblast cell line conditioned medium
LAI	Leukaemia associated inhibitor
Lis	Lisinopril

LPP-CFC	Low proliferative potential colony forming cells
LPS	Lipopolysaccharide
LTBMC	Long-term bone marrow culture
LTCIC	Long-term culture initiating cells
MAPK	Mitogen activated protein kinase
M-CSF (CSF-1)	Macrophage colony stimulating factor
MEK	Mitogen activated protein kinase kinase
MEKK	Mitogen activated protein kinase kinase kinase
MHC	Major histocompatibility complex
MIP-1	Macrophage Inflammatory protein
MMP	Matrix Metalloproteinase
mRNA	Messenger ribose nucleic acid
NBME-IV	Normal bone marrow membrane extract-IV.
NC	Normal bone marrow plus captopril
NF	Nuclear factor
NFc	Normal femur cellularity
NGM	Normal granulocyte macrophage numbers.
NHPP	Normal high proliferative colony forming cell numbers
N-plasma	Normal plasma
NS	Normal bone marrow plus saline
N-spleen	Normal spleen
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
pEEDCK	p Glu-Glu-Asp-Cys-Lys
PG-E_2, F_1	Prostaglandin-E_2, F_1
P-gp	P-glycoprotein
PHA	Phytohaematoglutinin
PI4,5P_2	Inositol 4,5 diphosphate
PKC	Protein kinase C
PLC	Phospholipase C

PLS	Perpendicular light scatter
pRb	Retinoblastoma protein
PWM	Pokeweed mitogen
r h(u)	Recombinant human
r m(u)	Recombinant murine
RAA	Receptor associated antigen
Res. AcSDKP	AcSDψKP
Rho	Rhodamine 123
RTK	Receptor tyrosine kinase
S	Saline
SA2 (JMB1)	SA2 myeloid leukaemia cell line
SCF (SF)	Stem cell factor
SCID	Severre combined immunodefficency
SLE	Systemic lupus erythromytosis
STAT	Signal transducers and activators of transcription
TGF	Transforming growth factor
Thy-1Lin Sca	Thy-1 lineage stem cell antigen
TNF	Tumour necrosis factor
VP	Verapamil
WEHI-3B	WEHI-3B myelomonocytic leukaemia cell line conditioned medium
WGA	Wheat germ agglutinin

Abstract

Negative regulatory factors inhibit the proliferation of haematopoietic stem cells thus protecting them from differentiation pressures. One of the negative regulators of stem cell proliferation is the tetrapeptide Acetyl-Seryl-Aspartyl-Lysyl-Proline (AcSDKP). This peptide is endogenously produced *in vivo* and long term bone marrow cultures and is degraded by angiotensin 1-converting enzyme (ACE) both *in vivo* and *in vitro*. The aim of these investigations was to study the role of ACE on haematopoietic stem and progenitor cell proliferation. Since the N-domain ACE active has been implicated in AcSDKP degradation, an analysis of two ACE inhibitors (captopril and lisinopril) shown to have differential effects on the N-domain ACE active site was conducted.

Both captopril and lisinopril equally reduced ACE activity in plasma *in vitro*. However, captopril had a lesser effect on reducing serum ACE activity *in vitro* than lisinopril. Captopril and AcSDKP together reduced the proportion of GM-CFC in S-phase after 7 hours of *in vitro* incubation. In addition, ACE resistant AcSDKP analogue (AcSDψKP) when incubated with bone marrow cells in the absence of captopril also reduced the proportion of GM-CFC in S-phase. This finding suggest that the effect of captopril and AcSDKP on GM-CFC proliferation was due to AcSDKP alone.

Haematopoietic stem cells were induced into cell cycle by *in vivo* administration of either 2 Gy-γ–irradiation or the two cytotoxic drugs, cytosine arabinoside (Ara-C) (100 mg/kg i.p.) or 5 flourouracil (5 FU) (150 mg/kg i.v). Bone marrow cells were sampled and incubated *in vitro* for up to 24 hours. Captopril together with AcSDKP reduced the proportion of high proliferative colony forming cells-1 (HPP-CFC-1) in S-phase following 2 Gy-γ-irradiation. Lisinopril together with

AcSDKP had no such effect. In addition, captopril alone *in vitro* reduced the proportion of HPP-CFC-1 in S-phase induced into cell cycle by cytotoxic drugs. Lisinopril had no such effect. Incubation alone reduced the proportion of HPP-CFC-1 in S-phase in cytotoxic drug treated bone marrow cells. When cultures, which were incubated with captopril, were assayed for AcSDKP levels, captopril induced an increase in AcSDKP levels in both control normal bone marrow cells and cells derived from Ara-C treated mice. However, it did not affect AcSDKP levels in cultures derived from 5 FU and 2 Gy treated mice. AcSDKP together with captopril were shown to inhibit S-phase cell entry of HPP-CFC-1 when they were incubated with bone marrow cells derived from mice treated with either 2 Gy-γ-irradiation or cytotoxic drug insults. Interestingly, captopril was unable to reduce the proportion of SA2 leukaemic cells in S-phase

Captopril on its own at therapeutic doses reduced the proportion of HPP-CFC-1 in S-phase *in vivo* regardless of the insult used to induce HPP-CFC-1 into cell cycle. Lisinopril slightly reduced the proportion of HPP-CFC-1 in S-phase following Ara-C treatment only. Captopril induced an *in vivo* increase in AcSDKP levels in all the models tested. Captopril also reduced the proportion of HPP-CFC-1 and GM-CFC in S-phase following fractionated doses of Ara-C. Captopril's inhibitory effect on GM-CFC proliferation following fractionated dose of Ara-C was diminished after 7 days while it was sustained with HPP-CFC-1.

Long-term bone marrow cultures revealed that captopril and AcSDψKP had the same effect on cellularities of both layers and on the proliferation of HPP-CFC and GM-CFC in both layers. From the present investigations, it can be concluded that captopril is a potent inhibitor of HPP-CFC-1 proliferation. This effect may in part be mediated by AcSDKP mechanism.

CHAPTER 1

1.0 HAEMATOPOIESIS

1.0.1 *General haematopoiesis*

Blood cells are constantly produced and destroyed during an animal's life span. Despite a wide variation in the type of mature cells that make up the haematopoietic system, all haematopoietic cells originate from pluripotent stem cells. Stem cells have been defined as cells capable of self-renewal without diminishing in function and numbers and they furnish daughters to provide new functional cells of the haematopoietic tissue (Schofield, 1983). Others have suggested that a fixed number of stem cells is laid down during embryogenesis to supply the body's needs throughout its life time and these cells are randomly recruited into proliferation, differentiation, and development as required (Ogawa, 1993; Dexter, 1987). Haematopoietic stem cells originate in the foetus from the intra-embryonic mesodermal area close to the aorta, gonads, mesonephros and also from the yolk sac (Medvinsky et al., 1993). They then migrate to the foetal liver and finally settle in the bone marrow (Moore & Metcalf, 1970). It is possible to isolate cells with stem cell potential in all these organs. In humans, cord blood is also a source of cells with stem cell potential (Caux et al., 1989). Stem cells give rise to progenitor cells. Progenitor cells are lineage restricted and they form colonies when appropriate growth factors are added to *in vitro* cultures (Metcalf et al., 1993; Moser, 1991) and are usually committed to one or more lineages. The types of colonies formed by progenitor cells in agar cultures define them. In contrast to stem cells, a large proportion of progenitor cells is in S-phase of the cell cycle at any one time (Millard & Okell, 1975).

Progenitor cells divide to give rise to mature cells that carry out the function of the haematopoietic system. This can be achieved over a very short period of time according to the physiological need (Moser, 1991). Therefore under normal circumstances the number of functional haematopoietic cells remains constant.

This thesis will first introduce a general review of different stem cell assays and stem cell proliferation regulation mechanisms. Then factors involved in haematopoietic stem cell proliferation will be analysed with particular emphasis on negative regulators of stem cell proliferation. The major aim of this project is to define ways of preserving stem cell integrity during tumour therapy. The emphasis is on stem cell proliferation inhibitors.

1.0.2 *An overview of haematopoietic stem cell assays*

Several *in vivo* and *in vitro* assays have been devised for assaying cells with stem cell characteristics in both mice and man. The *in vivo* assays include transplantation of mouse bone marrow cells into lethally irradiated mice or human haematopoietic cells into immunocompromised (SCID) mice and preimmune foetal sheep (Bock, 1997). The *in vitro* stem cell assays include direct clonogenic assays and secondary clonogenic assays. Direct clonogenic assays are the most direct quantitative means of measuring haematopoietic stem cells by colony formation *in vitro*. On the other hand the secondary clonogenic assays are qualitative assays. These secondary clonogenic assays assay for cells that do not themselves form colonies in semisolid media but can produce clonogenic progeny if cultured for a certain period of time. In general test cells are cultured for several days/weeks and an analysis of the number of clonogenic cells is performed at set times during the culture period.

1.0.3 *Colony Forming Unit-Spleen (CFU-S) stem cell assay*

A subset of cells from normal bone marrow with the ability to protect lethally irradiated mice from haematotoxicity in the short term by forming colonies in the spleens after 10 days post transplantation was observed in 1961 by Till & McCulloch. They observed that the number of colonies in the spleens of rescued mice was directly proportional to the number of normal bone marrow cells transplanted. Moreover each colony originated from a single cell. The colony contained undifferentiated cells at day 10. Erythroblasts were observed in the centre of the colony and myeloid cells were observed at the peripheral of the colony. However at day 11-post transplantation, the process of differentiation was more marked with erythropoiesis, granulopoiesis and up to 5 megakaryocytes observed in the colony. Therefore cells responsible for the formation of colonies were deemed to have stem cell properties. Since a lot of bone marrow cells were needed to produce a few colonies, spleen colony forming cells responsible for individual colonies were called a unit. This procedure has become the *in vivo* haematopoietic stem cell assay called the "Colony Forming Unit-Spleen" (CFU-S) assay.

The potential of transplanted normal bone marrow cells to form spleen colonies can be assayed at different times from day 7 onwards. Cells obtained from spleen colonies that develop early are incapable of forming spleen colonies when reinjected in lethally irradiated mice and thus contain no CFU-S (Siminovitch et al., 1963). Moreover early CFU-S (Day 7) colonies are known to disappear from the spleen within 72 hours and they are mainly composed of erythroid cells (Magli et al., 1982). On the other hand spleen colonies sampled after 12 days contain cells capable of forming multilineage colonies (Ploemacher & Brons, 1988a). Therefore it can be

argued that early (CFU-S day 7) and the late (CFU-S day 14) spleen colonies originate from different colony forming cell populations.

The individual colony-forming cells, have a heterogeneous population of cells with regards to proliferation and differentiation potential (Lewis et al., 1968; Fowler et al., 1967; Mulder et al., 1987; Magli et al., 1982; Becker et al., 1963). Cells from an individual spleen colony can differentiate along the erythrocytic, granulocytic/monocytic and megakaryocytic series (Lewis et al., 1968). Day 7 and day 14 colony forming cells have different sensitivities to S-phase specific drugs as shown by the increased sensitivity of day 7 colony forming cells to 5 fluorouracil (5 FU) compared to day 14 colony forming cells (Hodgson & Bradley, 1979). In addition, day 7 colony forming cells have a short life span and they produce mainly cells of the erythroid series (Magli et al., 1982). Day 7 and day 12 colony forming cells express different cell surface antigens (Moore et al., 1980; Caux et al., 1989; Ploemacher & Brons, 1988a). Late CFU-S show heterogeneous Rho staining while early CFU-S are selectively labelled with Rho (Mulder et al., 1987). In addition day 7 colony forming cells have DNA that stains more strongly with Hoechst-33342 than day 14 CFU-S (Wolf et al., 1993). Therefore it can be concluded that cells responsible for CFU-S formation are heterogenous with varying stem cell potential. It seems that cells with a greater stem cell potential form colonies after a long time *in vivo* while cells with less stem cell potential form spleen colonies after a short time following transplantation. However in order to rescue mice from the cytotoxic effects on the stem cell population following irradiation for example, colony forming cells with short and long stem cell potential are needed to be transplanted together (Jones et al., 1990).

1.0.4 *An overview of in vitro stem cell assays*

There are four main *in vitro* stem cell assays. Two of these assays are direct stem cell clonogenic assays and they depend on the ability of different combinations of growth factors either in conditioned media or in a pure form to induce colony formation in agar cultures from haematopoietic bone marrow cells. These are the blast colony forming cell (Bl-CFC) assay and the high proliferative potential colony forming cell (HPP-CFC) assay. The secondary clonogenic assays assay cells with stem cell potential by their ability to give rise to clonogenic progenitor cells that can be assayed. These assays include the long-term bone marrow culture (LTBMC) and the long-term culture-initiating cells (LTCIC). These assays consist of two major components. These components are the haematopoietic cells and the stromal cells of the haematopoietic microenvironment. The other secondary clonogenic assay comprises a group of assays called the delta (Δ)-assays. In delta assays, bone marrow cells are sorted such that they are depleted of committed progenitors and enriched for primitive haematopoietic cells and are cultured in a seven-day suspension culture in the presence of a combination of various cytokines (Bock, 1997; Gordon, 1993; Bodine et al., 1991; Verfaillie & Miller, 1995). The effects of cytokines on these cells are measured in clonogenic assays for committed progenitor cells. There are several variants of delta assays utilising the property of early haematopoietic cells to adhere to stroma or plastic (Gordon, 1993).

1.0.5 *Blast-colony forming cell stem cell assay*

In mice, Bl-CFC assay is generated in the presence of medium conditioned by pokeweed mitogen-stimulated mouse spleen cells with or without erythropoietin (Suda et al., 1983; Nakahata & Ogawa, 1982, Humphries et al., 1981). Media

conditioned by leukocytes in the presence of phytohemagglutinin and erythropoietin on days 4 or 5 promotes the same cell population from human bone marrow cells (Frauser & Messner, 1978). Using mouse cells the Bl-CFC assay generates mainly macroscopic erythroid colonies on day 9. The multipotential characteristics of the Bl-CFC assay are characterised by individual colonies that contain granulocytes, macrophages, megakaryocytes and erythroid cells. These colonies are called CFU-GEMM colonies (CFU- Mixed).

A variation of Bl-CFC developed by Gordon et al., (1985) is assayed by adding bone marrow cells to preformed stromal layers. Stem cells bind to stromal cells within 2 hours of coincubation of bone cells to the preformed stroma. Bound stem cells produce colonies of undifferentiated blast cells. These blast colony-forming cells can self-renew as well as give rise to multipotential and lineage-committed colony-forming progenitor cells (Gordon et al., 1987).

Bl-CFC while originating from a single cell also contains a heterogeneous population of cells at different days of incubation period. Each Bl-CFC contains cells of different lineages (Suda et al., 1983). Most of Bl-CFC colonies show terminal differentiation at day 10 of culture towards the erythroid series. However if colonies are cultured over a long period of time, three types of colonies are observed on day 16. These three types of colonies are: the mast cell colonies, granulocyte-macrophage/megakaryocyte (GMM), and cells with greater self renewal potential, termed stem cells (Nakahata & Ogawa, 1982). Some macroscopic erythroid blasts contain cells capable of generating CFU-S and express multiple haematopoietic differentiation (Johnson, 1980). Humphries et al., (1979), assayed 25 macroscopic blasts and found that only 11 produced spleen colonies. There is also a heterogenous distribution of cells capable of forming CFU-S among Bl-CFC with some producing

up to four times the average number of CFU-S (Humpries et al., 1981). The number of daughter stem cells detectable in individual primary Bl-CFC clones varies markedly from one clone to the other (Humpries et al., 1981). These observations show that the cells responsible for Bl-CFC colony formation are as heterogeneous as the ones responsible for CFU-S colony formation. They also show that both these assays are multipotential.

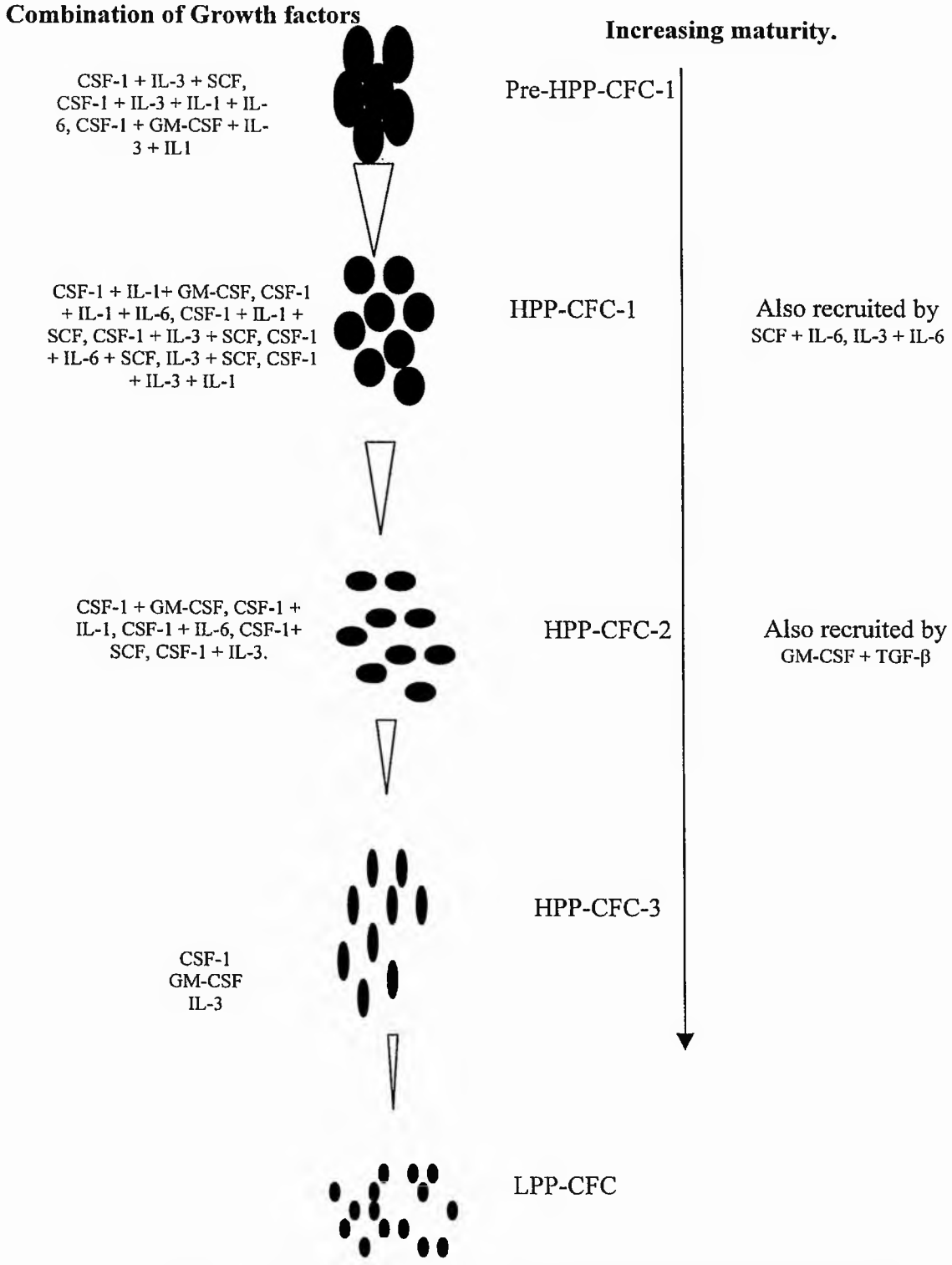
1.0.6 *High proliferative potential-colony forming cell stem cell assay*

As reviewed by Bertoncello (1992), HPP-CFCs require multiple haematopoietic growth factors acting in synergy in order to divide in agar cultures and form colonies greater than 0.5 mm in diameter containing at least 50,000 cells. Cells which form high proliferative potential colonies *in vitro* are relatively resistant to the cytotoxic effects of 5 FU *in vivo*. Different populations of cells with high proliferation potential can be recruited with different combinations of growth factors. One such cell population is called the colony forming unit type A (CFU-A). In this assay conditioned media from L929 fibroblasts cell line and AF1-19T a rat fibroblast cell line transformed with the malignant hystiocytosis sarcorma virus, are combined and colonies of diameter > 2 mm are scored at day 11 (Pragnell et al., 1988). McNiece et al, 1986, 1987, 1988a, 1988b, 1989, 1991; Falk et al., 1988, have identified a number of HPP-CFC from human and mouse bone marrow cells by culturing cells with different combinations of growth factors.

The heterogenity of HPP-CFC is recognised by the different composition of cells in colonies recruited by different combinations of growth factors. For example HPP-CFC surviving 5 FU treatment can only be detected in clonal agar cultures in the

presence of interleukin 1α (IL-1α) plus interleukin 3 (IL-3) plus macrophage colony stimulating factor (M-CSF, CSF-1) (Bartelmez et al., 1989). While HPP-CFC that require less than 3 growth factors are ablated by 5 FU, they re-emerge sequentially in the bone marrow during haematopoietic regeneration following treatment. McNiece et al., (1988b) demonstrated that granulocyte macrophage-colony-stimulating factor (GM-CSF) alone or in combination with M-CSF stimulated HPP-CFC colonies that were depleted *in vivo* but regenerated more rapidly than the ones stimulated by M-CSF alone. This was observed in cultures of bone marrow cells from mice treated with 5 FU 2, 4,and 8 days before sampling. If human stem cell factor (hSCF) is combined with GM-CSF, granulocyte-colony-stimulating factor (G-CSF), IL-3, or erythropoietin (hEpo) an increase in colony number and size is observed. When stained, cells have the morphology similar to the one recruited by single growth factors without hSCF (McNiece et al., 1991). Therefore hSCF responsive HPP-CFC are a heterogenous population of cells. Macrophage progenitor cells which respond to M-CSF alone form colonies with a diameter of <0.5 mm after 14 day of culture. These colonies have been called the low proliferative potential colony forming cells (LPP-CFC) (Bradley & Hodgson, 1979). From these observations Kriegler et al., 1994, observed a hierarchy of HPP-CFC according to stem cell potential and progenitor cell maturity by using regenerating bone marrow after 5 FU and cell sorting using Rho. (Fig.1.1 shows the proposed hierarchy and heterogeneous nature of the HPP-CFC compartment.)

Fig.1.1, HPP-CFC Relationships. Adapted from Kriegler et al., 1994



Note: A combination of more than 3 growth factors recruit pre-HPP-CFC, and more mature HPP-CFC require less growth factors to be recruited.

1.0.7 *Secondary clonogenic (qualitative) stem cell assays*

Bone marrow light density cells isolated from mice treated with 5 FU *in vivo* and cultured in the presence of SF+IL-3 have no clonogenic potential in soft agar assays but have myeloid-lymphoid potential as long-term culture-initiating cells (Bertolini et al., 1997). In LTBMCs, haematopoietic cells are placed into flasks and the stromal progenitor cells adhere to culture flasks and form a confluent layer consisting of fibroblasts, fat cells, macrophages and some endothelial cells (blanket cells) (Gordon, 1993; Gartner & Kaplan, 1980; Dexter et al., 1976, Hasthorpe et al., 1992). This enables a continuous production of haematopoietic cells. Some haematopoietic cells adhere to the stroma while others are released in the culture medium. Therefore progenitor cells developed from these cultures can be assayed in the adherent as well as the non-adherent layers. In human bone marrow cells, LTCICs assay haematopoietic cells with the ability to seed a preformed stroma that is normally irradiated. Bone marrow cells are put on an irradiated stroma and they produce precursor cells that are assayed from 5 week of culture (Sutherland et al., 1990). For a continual survival of cultures, they need to be fed at weekly intervals by fresh media replacement. Ploemacher et al. (1989) devised an *in vitro* limiting-dilution assay of long-term repopulating haematopoietic stem cells in mice by overlying bone marrow cells on irradiated stromal layers. Haematopoietic stem cells give rise to time-dependent clonal amplification under the stromal layers.

Non adherent cells from LTBMC which support mononuclear differentiation lose the ability to form CFU-S faster than cells from cultures which support granulocyte differentiation (Dexter & Lajtha, 1974). Once long-term bone marrow cultures have been established, proliferation of cells capable of forming CFU-S,

production of megakaryocyte precursor cells (CFU-M), and granulocyte precursor cells (CFU-G) and extensive granulopoiesis can be maintained for many months. However only immature erythroid progenitors (early BFU-E) are maintained in long term culture (Eliason et al., 1979; Dexter et al., 1976). In the presence of anaemic mouse serum or normal mouse serum plus erythropoietin (Epo), the most primitive erythroid progenitors (BFU-E) mature into colony-forming units-erythroid (CFU-E) with suppression of granulopoiesis (Dexter et al., 1981). CFU-E can also be produced in the presence of Epo incubated on a mechanical shaker (Eliason et al., 1979). Therefore, LTBMCs are capable of producing a heterogeneous population of haematopoietic progenitor cells. Additional evidence of mutipotentiality and heterogeneity of LTBMC is shown by the fact that CFU-C, BFU-E and CFU-G /E are consistently detectable in the adherent layer for at least 8 weeks. 70%-75% of BFU-E and CFU-G / E are located in the adherent layer at week 2-3 and more than 90% by week 7-8 (Coulombel et al., 1983).

From the above observations, all stem cell assays used to study haematopoiesis contain cells at different stages of maturation with regard to stem cell potential. Specific haematopoietic cell progeny can be derived from these stem cell assays. All *in vitro* assays can yield CFU-S if retransplanted into lethally irradiated mice (Nakahata & Ogawa, 1982; Dexter, 1979; Eckmann et al., 1988). In general these assays favour myeloid differentiation with limited or no lymphoid differentiation. However, it is possible to recruit cells of the lymphoid series from bone marrow cells following cell sorting for cells with stem cell potential (Sprangrude et al., 1988; Bertolini et al., 1997). The lymphoid cells (T lymphocytes) can be recruited from human Bl-CFC by addition of T-cell growth-promoting medium (Messner et al., 1981). Removal of corticosteroids and increasing incubation

temperature from 33°C to 37°C after establishment of LTBMC results in the development of B-lymphocytes (Denis & Witte, 1986; Dorskind, 1986).

1.0.8 *Stem cell self renewal*

The most important characteristic of stem cells is that they should be able to self renew. All available stem cell assays, whether qualitative or quantitative, show this to be the case (Williams et al., 1977; Schofield et al., 1980; McNiece et al., 1987; Mauch et al., 1980). Self-renewal is influenced by the primitiveness of cells and the amount of cell divisions they have gone through (Rosendaal et al., 1979). As shown in the long-term bone marrow cultures, CFU-S within the adherent stromal cell layers have a significantly greater self renewal capacity than CFU-S from the non adherent layer (Mauch et al., 1980). The self renewal capacity of the adherent layer CFU-S decreases as the total number of cells capable of forming CFU-S per flask increases (Mauch et al., 1980).

Murine Bl-CFC have been shown to self renew and it has been argued that the determination of self-renewal is intrinsic to stem cells themselves with growth factors playing a permissive rather than directive role (Humphries et al., 1981). The observation that CFU-GEMM colonies can be derived from CFU-GEMM suggests that early stem cells have a self-renewal capacity (Nakahata & Ogawa, 1982).

Stem cells have a finite probability of differentiating into committed lineage restricted progenitor cells. If stem cells are induced into cell division, they are likely to get committed to specific lineages and differentiate. For example stem cell self-renewal is decreased after recovery from the alkylating agents. Indeed there is a permanent defect in self-renewal capacity of the bone marrow stem cells after busulfan (Hellman et al., 1978). Stem cells are also at risk of being depleted with

prolonged chemotherapy (Blackett & Adams, 1972). A permanent loss in stem cell self renewal capacity has also been observed following weekly irradiation of mice for a total dose of 3,400 to 4,000 cGy (Mauch et al., 1988).

1.1 IDENTIFICATION AND CHARACTERIZATION OF HAEMATOPOIETIC STEM CELLS

It is difficult to isolate cells with all the characteristics of stem cells in normal bone marrow. However there are combinations of physical and immunological methods that are used to isolate a population of haematopoietic cells that have many if not all characteristics of stem cells. For example, the pretreatment of bone marrow cells with 5 FU spares cells with stem cell characteristics and diminishes progenitor cells with less stem cell potential (Hodgson & Bradley, 1979; Brandt et al., 1988; Bertolini et al., 1997; Harrison & Lerner, 1991).

The use of dyes like rhodamine 123 (Rho) and Hoechst 33342 that are selectively taken up by a subset of haematopoietic cells has also been crucial for isolating cells with stem cell characteristics. Rho is a supravital dye that is a substrate for transport mediated by the P-glycoprotein (P-gp), an efflux pump responsible for multidrug resistance in tumour cells. The highest P-gp levels are found in haematopoietic cells that show stem cell characteristics (Chaudhary & Roninson, 1991). For most stem cell assays in general, rhodamine dull cells show characteristics which places them closer to stem cells than rhodamine bright cells (Ploemacher & Brons, 1988b; Srour et al., 1993). Moreover rhodamine dull cells can be divided further into subsets using calcium channel blocker, verapamil. Most dull cells become positive for Rho when incubated in the presence of verapamil while a small proportion of cells remains negative. The Rho^{-}/VP^{-} have along term repopulating

ability while the Rho^{-}/VP^{+} have a short term repopulating ability (Zijlmans et al., 1995) when transplanted into lethally irradiated mice. Hoechst 33342 is a DNA binding dye. Cells with low levels of binding with Hoechst 33342 have properties which places them closer to cells with stem cell potential (Wolf et al., 1993).

In addition to chemical methods, physical methods are also available. These are counterflow centrifugal elutriation (CCE), light scatter and free flow electrophoresis methods. In the CFU-S and HPP-CFC assays, cells elutriated at lower flow rates resemble lymphocytes in appearance and have long-term marrow repopulating ability requiring more growth factors to clone than cells elutriated at high flow rates (Jones et al., 1990; Yoder et al., 1991). Light scattering properties of haematopoitic cells differ. Using light intensity measured in a forward (FLS) and perpendicular direction to the incident light (PLS), cell size and shape can be determined. During maturation, different spleen colony forming cells show an increase in PLS intensity (van den Engh & Visser, 1979). Free-flow electrophoresis sorts cells on the basis of electric charge. Cells are first treated with neuraminidase to remove sialic acid and are then labelled with a lectin called wheat germ agglutinin (WGA) (Visser & van Bekkum, 1990). This allows free-flow electrophoresis to be employed in isolating cells with stem cell potential.

The last commonly used method to identify and separate cells with stem cell potential from other cells is to utilise the ability of cells to lose and acquire specific cell surface antigens as haematopoietic cells progress in the hierarchy from stem cells to mature end cells. These antigens may be part of cytoadhesion molecules. The cytoadhesion molecules may represent integrins, selectins or immunoglobulins (Simmons et al., 1997). These cytoadhesion molecules allow stem cells and stromal cells of the microenvironment to interact. As cells progress from the stem cell

compartment to mature end cells, they progressively lose some of these antigens as they prepare to be released into the circulation.

In human progenitor cells, the CD34 (cluster of designation 34) antigen is crucial to the identity of progenitor cells with stem cell potential (Andrews et al., 1990). I antigens (Ia) in mice and DR antigens in humans, also known as class II major histocompatibility complex antigens (MHC-II), are absent on CD34 positive progenitor cells but get acquired as cells mature (Moore et al., 1980). The loss of CD34 expression precedes the expression of MHC-II (Caux et al., 1989). A subclass of human $CD34^{+}CD38^{-}$ progenitor cells has most characteristics of stem cells and is commonly assayed in human haematopoietic stem cell assays (Terstappen et al., 1991). Indeed haematopoietic cells expressing $CD34^{+}HLA^{-}DR^{+}CD38^{-}$ antigens can differentiate into haematopoietic precursors. Interestingly, haematopoietic cells expressing $CD34^{+}HLA^{-}DR^{-}CD38^{-}$ antigens can also give rise to stromal cells capable of supporting the differentiation of precursor cells (Huang & Terstappen, 1992) in addition to producing haematopoietic precursor cells.

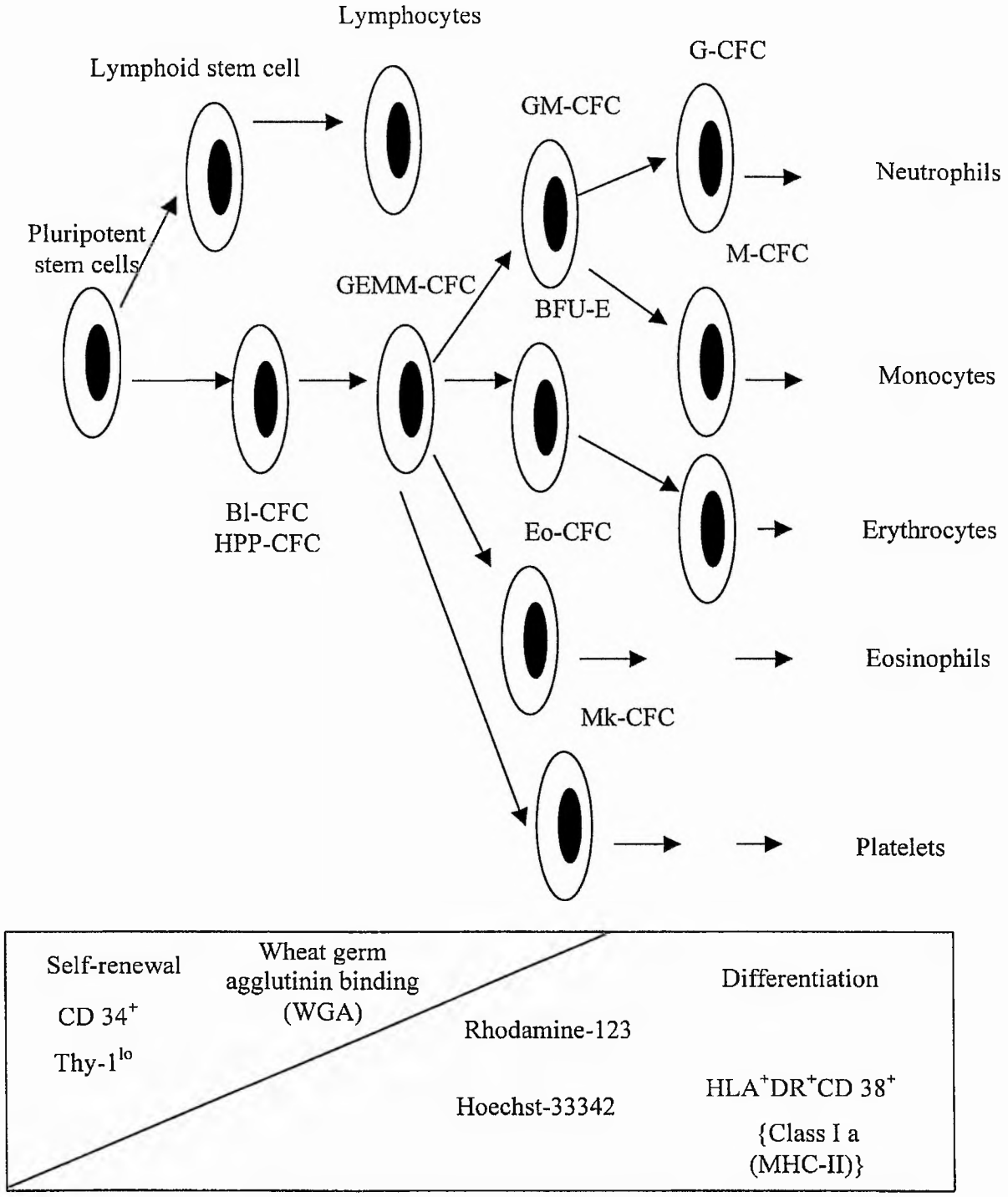
In mice HPP-CFC which express Qa-m7 (Ia) antigenic determinant have marked stem cell potential (Bertoncello et al., 1986). Moreover, the absence of the maturation antigens, B220 and 7/4 on cells with stem cell characteristics makes it possible to isolate a purified population of HPP-CFC (Bertoncello et al., 1989). The other antigen commonly used in isolating cells with stem cell potential is the Thy-1 agent. Thy-1 antigen was produced and identified from pre-T hybridomas (Aihara et al., 1986). While Thy-1 antigen is mainly used to isolate murine bone marrow cells with stem cell properties, it can sometimes be used in combination with the CD34 antigen (Andrews et al., 1990) to isolate human bone marrow cells with stem cell properties. The Thy-1 antigen is present on murine bone marrow cells which form

HPP-CFC colonies and those that have long term marrow repopulating ability (Boswell et al., 1984; Sprangrude et al., 1988).

Using a combination of the above methods, a large number of almost pure CFU-S day 12 (which have a strong affinity to bind to WGA) can be separated from lipopolysaccharide-(LPS)-stimulated mouse spleen using centrifugal elutriation, fluorescence-activated cell sorting (FACS) (light scatter) and WGA-fluorescein isothiocyanate (FITC) (Ploemacher & Brons, 1988d). When interleukin-3 (IL-3) receptor-associated antigen (IL-3 RAA) and WGA are combined, some day 12 CFU-S are IL-3RAA$^-$/WGA$^+$. This day 12 CFU-S cell population was also found to have long-term marrow repopulating ability (Ogata, 1992). Using a combination of CCE, WGA, FITC, FLS and PLS, Ploemacher & Brons (1988c; 1989) identified a cell population that was able to generate day-12 CFU-S and also had radioprotective ability (30-day survival) with the ability to repopulate the bone marrow or spleen over a 13-day period. They termed this cell a pre-CFU-S.

A sub-population of very primitive haematopoietic cells with long-term haematopoietic repopulating ability, HPP-CFC formation and the ability to completely repopulate the myeloid and the lymphoid B and T cell compartment after transplanting into lethally irradiated mice has also been isolated. This was achieved by using a combination of fluorescence-activated cell sorter (FACS), Rho and Hoechst 33342. Cells with low binding of both dyes are enriched with cells showing stem cell characteristics (Wolf et al., 1993). In addition haematopoietic cells with competitive long term marrow repopulating ability have also been purified from 5 FU treated bone marrow using Thy-1 and H-2K antigen expression and flow cytometry with sorting based on light scatter (Szilvassy et al., 1989). The combination of Thy-1 antigen and absence of lineage expression on haematopoietic cells have also enabled the isolation

Fig. 1.2. Relationship of different haematopoietic cell population as determined by cell sorting.



of cells with stem cell properties. Cells with Thy-1^{lo}Lineage^{-}Stem cell antigen-1^{+}(Thy-1^{lo}Lin^{-}Sca-1^{+}) antigenic expression have stem cell properties Indeed these cells are virtually a pure population of primitive myeloerythroid stem cells (Sprangrude et al., 1988). This cell population gives a rapid and sustained haematopoietic recovery in lethally irradiated mice after transplantation (Uchida et al., 1994). (Fig.1.2 summarises different relationships of haematopoietic cells as identified by cell sorting).

1.2 STEM CELL KINETICS

1.2.1 *Cell cycle regulation*

DNA content and the expression of Ki-67 antigen can be used to determine the phases of cell cycle. Ki-67 antigen is not expressed in quiescent cells, but is quickly up regulated as cells are induced into cycle (Jordan et al., 1996). As summarised by veer Reddy, (1994), there are four distinct phases of cell cycle called the $G_{0/1}$, S, G_2 and M phases. Cycle-dependent protein kinases (CDK) are crucial regulators of the timing and coordination of eukaryotic cell-cycle events. Two of these CDK1 (CDC2) and CDK2 are 34-KDa proteins containing a conserved kinase catalytic core. They are inactive as monomer and their activation requires binding to cyclins as well as phosphorylation by a CDK-activating kinase on threonine-161 or 160 respectively. There are five types of cyclins called A,B,C,D,E. Cyclin-A is first detected near G_1/S transition while cyclin-B is first synthesised during S-phase. Cyclins C, D1, and E are synthesised during G_1 phase. Cyclin E peaks at the G_1/S transition. Cyclin-D types are expressed when cells are activated by growth factors and their levels rise earlier than type E-Cyclins. CDK1 when complexed with B-type

cyclins is the major protein kinase acting at the S/G_2 and G_2/M transitions. CDK2 when complexed with cyclin-A act at the beginning of S-phase (Li et al., 1993). The rest of the cyclins regulate various CDKs and control various G_1, G_1/S, and G_2/M transition points.

Transition from G_0 to G_1 and S-phase is dependent on growth factors and the synthesis of enzymes of DNA replication and other signal molecules during G_1. In the absence of Ca^{2+}, cells at G_1/S boundary enter quiescence and there is a direct correlation between intracellular calmodulin levels and the ability of cells to replicate DNA (veer Reddy, 1994). Indeed it has been demonstrated that growth factor induced proliferation involves a 68 KDa calmodulin binding protein (veer Reddy et al., 1992). Crucial to G_1/S transition is the retinoblastoma gene protein (pRb). The product of the *Rb* gene is p105 with related proteins identified by their interaction with adenovirus E1A oncoproteins, p107 and pRb2/p130 (Baldi et al., 1995; Claudio et al., 1996). These gene products act at the restriction point (R) in late G_1 phase.

DNA replication-dependent histone H4 genes are regulated by a mechanism involving interaction of histone nuclear factor-D (HiNF-D) homeodomain protein CDP/cut with Rb-related proteins (van Wijnen et al., 1996). As reviewed by Weinberg, (1995), pRb is normally underphosphorylated but gets hyperphosphorylated at the R point enabling S-phase transition from G_1 phase of proliferating cells. pRb remains hyperphosphorylated through out the rest of the cell cycle. The underphosphorylated pRb binds the DRTF1/E2F transcription factor (Lam & La Thangue, 1994). In addition the hypophospharylated pp60 fraction of the pRB has been shown to translocate to the nucleolus (Rogalsky et al., 1993).

As reviewed by Lam & La Thangue, (1994), DRTF1/E2F is a group of DNA-binding heterodimers in which an E2F family interacts with a DP family

protein. E2F (Adenovirus E2 gene) is a family of transcription factors which comprises of five distinctly related factors all targeted to variants of the consensus nucleotide sequence TTTCGCGC. This sequence is present in genes whose products are involved in cell growth notably c-myc, B-myb, cdc2, dihydrofolate reductase, thymidine kinase and E2F-1 gene itself. E2F-1, E2F-2, and E2F-3 are under the direct control of pRb while E2F-4 and E2F-5 are under the control of p107 and p130. Cyclin D with CDK4/CDK6 and cyclin E with CDK2 kinases phosphorylate the Rb. This releases the E2F factors that initiate crucial steps towards S-phase progression. At S/G_2 transition, DP-1/E2F-1 binds to cyclin A-kinase thus stopping DNA replication (Krek et al., 1994, Lam & La Thangue, 1994). Cyclin dependent kinase inhibitors can affect the phosphorylation of the pRb. Since $p27^{kip}$ interacts with CDK2, CDK4 and CDK6 while p15 competes with Cyclin D for binding to CDK4/CDK6, high levels of these inhibitors will prevent pRb phosphorylation (Weinberg, 1995). Moreover, $p27^{kip}$ has been shown to be an essential component of the pathway that connects mitogenic signals to the cell cycle at the restriction point (Coats et al., 1996).

p53 has been shown to have an important role as a feedback control that keeps cells from entering S-phase when they contain damaged DNA by activating the expression of p21 which blocks the activities of both CDK2 and CDK4/CDK6 (Weinberg, 1995). Bcl-2 prevents aptoptosis without any effect on cell proliferation (Otani et al., 1993). Therefore the complexity of events that occur at G_1/S transitions ensures that only cells with intact DNA are able to replicate.

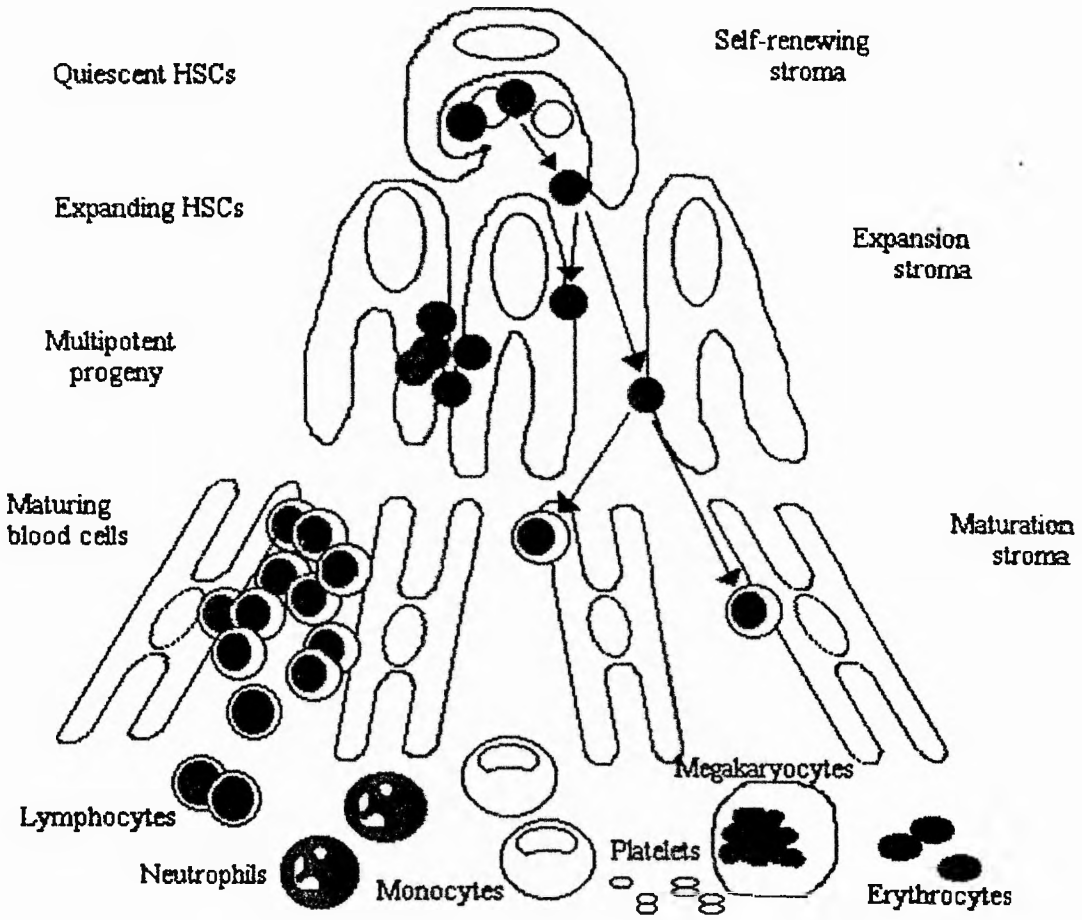
1.2.2 *Haematopoietic stem cell proliferation characteristics*

Using thymidine as an S-phase specific cytotoxic agent, it was found that the proportion of CFU-S in S-phase of cell cycle in normal murine bone marrow cells is less than 10% (Blackett et al.1974; Lahiri & van Putten, 1972). Using cytosine arabinoside (1-β-D-arabinofuranosylcytosine) (Ara-C) another S-phase specific agent, it was shown that the same proportion of both haematopoietic progenitor and stem cells are in S-phase (Millard & Okell, 1975). Ara-C enters cells through a nucleoside transporter by a process of facilitative diffusion (Cole & Gibson, 1997; Crisp et al., 1996). It is then phosphorylated by deoxycytidine kinase to Ara-CMP. Ara-CMP is further phosphorylated by deoxytidine monophosphate kinase to Ara-CDP. Finally, Ara-CDP is phosphorylated to the active cytotoxic metabolite Ara-CTP by nucleoside diphosphate kinase (Cole & Gibson, 1997; Crisp et al., 1996). Unlike cytosine, Ara-CTP incorporation into DNA hinders chain elongation and induces strand breakage resulting in cell death. *In vivo* Ara-CTP is converted into an inactive metabolite, uracil arabinoside (Ara-U) which is excreted, by cytosine deamase (Cole & Gibson, 1997).

It has however been shown recently that (30 ± 7)% of Rho/Ho dull primitive haematopoietic cells are actively cycling using continuous administration of bromodeoxyuridine over one week. The proportion of these cells in S-phase increased to (60 ± 14)% and (89 ± 3)% at 4 and 12 weeks respectively (Bradford et al., 1997). In general quiescent stem cells are much more refractory to recruitment into cell cycle and they require a combination of growth factors *in vitro* and at least 72 hours to be recruited (Jordan et al., 1996). 97% of Thy-1.1^{low} Lin^{-}Sca-1^{+} which exhibit a low level of staining with Rho are in G_0/G_1 and 70% Rho bright cells of the same cell

population are proliferating (Fleming et al., 1993). Interestingly, small erythroid colonies present in the nonadherent layer and granulocyte / macrophage colonies in

Fig 1.3 Progression of cells from the stem cell compartment to the mature cell compartment (adapted from Uchida et al 1993)



both adherent and nonadherent layers are actively dividing while big colonies of the same cell population are mainly quiescent in the adherent layer but are recruited into cycle following media change (Cashman et al., 1985). Uchida et al.(1997) showed that human bone marrow $CD34^{+}Thy\text{-}1^{+}Lin^{-}$ from individuals treated with cyclophosphamide and GM-CSF or G-CSF alone are proliferating, while the peripheral blood $CD34^{+}$ $Thy\text{-}1^{+}Lin^{-}$ from the same individuals were in G_0/G_1 phase.

About 30% of CFU-S are in S-phase 10 minutes after 150 rads irradiation in mice (Lahiri & van Putten., 1972). (Fig.1.3 gives a general view of the progression of cells from the stem cell compartment to the mature cell compartment.)

The G_0 phase of the cell cycle is crucial to the integrity of stem cells to maintain their stem cell potential. For example, prolongation of cells in G_1 phase of the cell cycle induces cellular differentiation (Carroll et al., 1995). There is evidence that suppression of apoptosis allows differentiation and development of multipotent haematopoietic cells in the absence of added growth factors (Fairbain et al., 1993). Therefore if stem cells are recruited from G_0 to G_1, they may differentiate if they are not allowed to self-renew and to return to G_0.

1.3 BONE MARROW MICROENVIRONMENT

1.3.1 *The role of the stromal cells*

In vitro, the microenvironment is characterised by the adherent stromal layer formed by LTBMCs. This layer consists of 'endothelial like cells', fat cells, macrophages and fibroblasts (Dexter et al., 1976). The microenvironment is transplantable as shown by the fact that people who receive a bone marrow transplant develop stromal cells which are derived from the donor in LTBMC (Keating et al., 1982). Stromal cells are strongly positive for alkaline phosphatases and smooth muscle actin (Liesveld et al., 1989). Haematopoietic cells and stromal cells following *in vitro* cultures may originate from the same cell population (Huang & Terstappen, 1992). One of the functions of the stromal cells is to support stem cell renewal (Zipori, 1989; Zuckerman et al., 1986).

1.3.2 *Stromal cells and extra cellular matrix*

Stromal cells are associated with the extra cellular matrix. Extra cellular matrix consists of ground substances in the form of glycoproteins or proteoglycans (glycosaminoglycans). These molecules may be utilised by haematopoietic cells in their maturation. For example, haemonectin a 60 KDa glycoprotein in bone marrow is a lineage and organ-specific attachment molecule for cells of the granulocytic lineage (Campbell et al., 1987). Moreover binding of CFU-S to marrow stromal cells is mediated by recognition of ground substances with galactosyl or mannosyl moieties (Aizawa & Tavassoli, 1987). The attachment of erythroid cells to fibronectin, a glycoprotein, increases with their degree of differentiation (Coulombel, et al., 1988). A 115-kD molecule on the erythroid cells is involved in the cellular interaction with fibronectin. As cells mature they lose this protein and the net result is cell detachment from stroma (Patel et al., 1986). There are other ground substances found in the extracellular matrix. For example, chondroitin 6- sulfate, a proteoglycan, is considered as one of the extra cellular matrix component which is involved in the construction of the haematopoietic microenvironment (Oguri et al., 1987, Okayama et al., 1988).

Ground substances may also be useful in the presentation of growth factors to haematopoietic cells. Heparan sulphate glycosaminoglycans have been shown to bind and protect basic Fibroblast Growth Factor (bFGF) from degradation by plasmin (Saksela et al., 1988). GM-CSF can be eluted from cultured stromal layers showing that this colony stimulating factor binds to stromal cells (Gordon et al., 1987a).

1.4 GROWTH FACTORS.

1.4.1 *Role of Growth Factors in haematopoiesis*

Most growth factors (cytokines) are single subunit glycoproteins containing disulfide bonds with a molecular weight of 14,000-28,000 daltons. However some of them are heterodimers with alpha and beta chains. M-CSF is unique in that it is a dimer of identical subunits (Nicola, 1987, Chiarugi et al., 1993). Individual growth factors display multiple activities (pleiotypic), they can interact with different cell types eliciting different functions (pleiotropy). They also have overlapping activities (redundancy)(Chiarugi et al., 1993; Metcalf, 1993). Growth factors exert their mitogenic effect by interacting with specific receptors on responsive cells. As reviewed by Chiarugi et al., 1993, there are five classes of haematopoietic and immune receptors characterised on the basis of molecular and type of signalling. Class 1-superpolymeric receptors activating both tyrosine kinases and phosphatases. Class 2-receptorial tyrosine kinases that dimerize and phosphorylate several substrates. Class 3-heterodimeric cytokine receptors consisting of alpha and beta chains that lack intrinsic tyrosine kinase activity but associates with src-like tyrosine kinases. Class 4-seven membrane-spanning domain receptors, which increase intracellular Ca^{2+}. Class 5-an IL-1 receptor induces formation of diacylglycerol.

In the haematopoietic system, growth factors can be categorised into 3 groups. There are late, intermediate, and early acting growth factors. This categorisation is based on the activity of growth factors on haematopoietic cells as they progress from the stem cell compartment to the mature precursor cell compartment (Ogawa, 1993). Growth factors in each category activate phosphotidylinostinol 3-kinase in haematopoietic cells (Gold et al., 1994). With SCF,

an early acting growth factor, having the greatest potency followed by IL-3, an intermediate acting growth factor. IL-5, a late acting growth factor having the least potency (Gold et al., 1994, Ogawa, 1993). In addition, haematopoietic stem cells contain high levels of receptors for early acting growth factors c-kit and IL-6R, IL-1R, G-CSF receptor with some receptors for intermediate acting growth factors like IL-3 receptors (Orlic et al., 1995, McKinstry et al., 1997). Pluripotent haematopoietic stem cells also contain high levels of the transcription factor GATA-2, p45, NF-E2 and c-myb. They do not express c-fms and do not have receptors for G-CSF and IL-5 or IL-7, all late acting growth factors (Orlic et al., 1995, Ogawa, 1993).

Synergistic effects of early factors are to shorten the G_0 phase of primitive haematopoietic cells. There is also similarity in function of some of the early acting growth factors as shown by biochemical mechanisms of their signal transduction (de Jong et al., 1997). As reviewed by Ogawa, (1993), intermediate acting growth factors influence multipotent progenitor cells after they exit from G_0. Cells lose responsiveness to some of as they terminally differentiate. Because these factors are intermediate, they can interact with both late and early growth factors to influence cell maturity and recruitment into cell cycle. Late growth factors allow lineage expression of progenitor cells (Ogawa, 1993).

1.4.2 *Growth Factors and haematopoietic microenvironment*

The type of growth factor produced by stromal cells is influenced by the microenvironment formed. For example stromal cells cultured and maintained for myelopoiesis produce growth factors responsible for myelopoiesis while the ones maintained for lymphopoiesis do not produce these growth factors. However if there is an alteration of conditions such that myelopoiesis cultures are switched to

lymphopoiesis, they lose their ability to produce growth factors responsible for myelopoiesis. The same is true for lymphopoiesis cultures switched to myelopoiesis (Johnson & Dorshkind, 1986). Gutierrez-Ramos et al.(1992), showed high mRNA levels of IL-1 to IL-7 with no IL-5 mRNA in three bone marrow and foetal liver stromal clones. A stromal cell line called, MC3T3-G2/PA6 (PA6) expressed SCF, M-CSF and high levels of Sca-1 in culture after 45 weeks (Satoh et al., 1997).

There may be a feedback loop in the production of growth factors by stromal cells. Indeed, exogenous as well endogenous IL-1 (β) induces human bone marrow stromal cells to produce IL-1β, IL-6, GM-CSF, G-CSF and M-CSF in LTBMC (Fibbe et al., 1988; Eaves et al., 1991). Moreover, there is also evidence that mononuclear phagocytes recruit stromal cells to produce multilineage growth factors *in vitro* (Broudy et al., 1986). Exposure of stromal cells to irradiation increases their production of GM-CSF (Alberico et al., 1986). Transforming Growth Factor-β (TFG-β) mRNA is constantly found at all times in LTBMC and is not influenced by media manipulation. However, addition of antibody against TGF-β to LTBMCs induces quiescent cells into the cell cycle (Eaves et al., 1991).

1.5 REGULATION OF STEM CELL PROLIFERATION

1.5.1 *Stimulators of stem cell proliferation*

Stimulatory factors of stem cell proliferation induce stem cells into cell cycle (Lord et al., 1977). Bone marrow cell suspensions taken from mice following cytotoxic insults are enriched with stimulatory factors for haematopoietic stem cell proliferation (Ali et al., 1989; Frindel et al., 1976; Lord et al., 1977; Lord, 1986; Wright & Lord, 1978). These factors are produced when mice are irradiated or given

cytotoxic drugs (Ali et al., 1989; Frindel et al., 1976). Cells that survive the insult and are not proliferating are responsible for the production of these factors. The concentration of stimulatory factors is seen to increase very rapidly after an insult to the haematopoietic system (Ali et al., 1989). These factors are not species specific (Cork et al., 1986) and they are produced by monocyte / macrophage lineage cells expressing the I a antigen (Wright et al., 1982; Ali et al., 1989). The nature of the feed back mechanism for the production of stimulatory factors is controlled both locally and by humoral factors (Ali et al., 1989; Frindel et al., 1976). Moreover, these factors can be separated following Amicon Diaflo membrane ultrafiltration in a molecular weight fraction of 30-50 KDa (Lord et al., 1977). The rate of stimulator production depends on the dose of the insult. The greater the insult the longer it takes for stimulators to be produced. For examples, 0.5Gy irradiation allows the production of stimulators within 30 minutes while it takes 6 hours for stimulatory factors to be produced following higher doses (Ali et al., 1989). Following hydroxyurea treatment to mice, there is no observable effect on haematopoietic stem cell cycling within 2 hours. Stem cells are recruited into cell cycle only after 4 hours (Hodgson & Blackett, 1977). Some tumour cells also produce stimulatory factors (Frindel et al., 1978). Stimulatory factors are produced very rapidly and the production of these factors is switched off as soon as they exert their influence on stem cell kinetics. It is suggested that an unknown mechanism triggers stimulator producing cells to secrete stimulators which are transported to responder cells which then initiate DNA synthesis (Frindel et al., 1979).

It is possible that cytokines may be the mediators of stimulatory effect on stem cell proliferation. This is confirmed by the fact that GM-CSF or G-CSF has been used as a stimulator to rescue murine haematopoietic system after cyclophosphamide

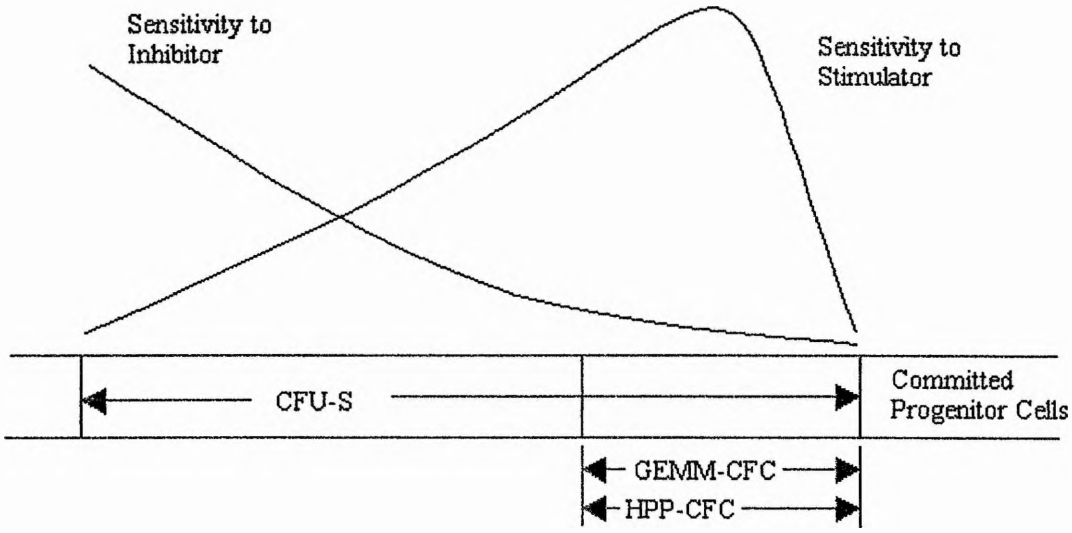
(Hornung & Longo, 1992). However the use of growth factors as exogenous haematopoietic stem cell stimulatory factors may result in some loss of stem cell ability to repopulate the bone marrow. This is because such treatments recruit more than necessary stem cells into a proliferative state hence induce them to differentiate. The net result is a permanent loss of some stem cells leading to bone marrow aplasia (Moser & Paukovits, 1991; Moore, 1992 Review paper).

Stem cells show different responses to the effect of stimulatory factors. Day 7 colony forming cells are more easily influenced by stimulatory factors than day 12 colony forming cells (Wright et al., 1985). In addition, cells that form CFU-S isolated from the axial parts of the femurs are less sensitive to stimulatory factors compared to cells that form CFU-S isolated from the marginal parts of the femurs (Lord, 1986). It has been observed before that mature cells with some stem cell characteristics have a higher probability of being influenced by stimulatory factors than primitive stem cells (Tejero et al., 1984).

1.5.2 *Inhibitors (Negative factors) of stem cell proliferation*

Negative regulatory factors lack cell specificity within the haematopoietic system, acting at different levels of differentiation and on several lineages. The mechanism involved in the control of murine and human stem cell proliferation is thought to be identical and inhibitors of stem proliferation are also not species specific (Wright et al., 1980a). In contrast to stimulators, inhibitors of stem cell proliferation have a great influence over the older CFU-S that form colonies on day 12 (Wright et al., 1985).

Fig.1.4 The influence of stimulators and inhibitors on haematopoietic cells. (Adapted from Tejero et al., 1984)



If inhibitors of stem cell proliferation can be maintained at a high enough concentration while phase specific cytotoxic therapy is given in the treatment of neoplastic diseases, haematopoietic stem cell proliferation can be suppressed. This can protect stem cells from the cytotoxic effects of these drugs (Tubiana et al., 1993 Review paper, Guigon & Bonnet, 1995 Review paper). Therefore patients might perhaps be spared from bone marrow failure which follows both radiotherapy and chemotherapy (Mauch & Hellman, 1989; Morley et al., 1975). (Fig.1.4 summarises the feed back loop imposed by stimulators and inhibitors of stem cell proliferation.)

1.6 SOME IMPORTANT INHIBITORS OF STEM CELL PROLIFERATION.

Inhibitors of stem cell proliferation include transforming growth factor-β (TGF-β), leukaemia associated inhibitor (LAI), haemoregulatory peptide 5b (pEEDCK), inhibin, normal bone marrow extract-IV (NBME-IV) which has been identified as macrophage inflammatory protein 1α (MIP 1α), tumour necrosis factor-α (TNF-α), and AcSDKP. These factors have been isolated separately from platelets, placenta, kidney, myeloid leukaemia cells, granulocytes, sertoli cells, adult and foetal bone marrow cells, and conditioned medium of normal blood and bone marrow cells (Axelrad, 1990 Review paper).

Glutathione inhibits GM-CFC with no effects *in vitro* on CFU-E. Oxidised glutathione has a stimulatory effect on colony growth of granulocyte (Fetsch & Maurer, 1990). Both prostaglandins and lactoferrin have an inhibitory effect on the proliferation of GM-CFC *in vitro*, prostaglandins being more inhibitory on macrophages while neutrophils are inhibited more by lactoferrin (Pelus et al., 1979). Interferons selectively inhibit CFU-E proliferation in mice and BFU-E proliferation in humans. This inhibitory effect is dose dependent but independent of erythropoietin (Ortega et al., 1979). Human gamma interferon (r-γ -IFN) suppresses colony formation by GM-CFC, BFU-E and multipotent cells (GEMM-CFC) in long-term bone marrow cultures with toxic effects on the stromal cells (Coutinho et al., 1986). Interferon-γ also selectively inhibits very primitive $CD34^{+}CD38^{-}$ but not $CD34^{+}CD38^{+}$ human haematopoietic progenitor cells (Snoek et al., 1994).

1.6.1 *Haemoregulatory pentapeptide pGlu-Glu-Asp-Cys-Lys (HP5b, or pEEDCK)*

This pentapeptide was isolated and purified from extracts of granulocytes (Paukovits et al., 1982). *In vitro,* pEEDCK inhibits CFU-C at a concentration of 10^{-7} M while *in vivo* it produces a non-significant reduction of CFU-S numbers per femur. The inhibitory effect of pEEDCK on CFU-S numbers is reversible (Laerum & Paukovits, 1984). Pre-CFU-S are protected by pEECDK from depletion following cytotoxic drug treatment (Paukovits et al., 1993). Purified pEEDCK from skin extracts inhibits proliferation and enhances terminal differentiation in cultured mouse epidermal cells (Reichelt et al., 1987; Elgjo et al., 1986). Surprisingly, the oxidised pEEDCK forms a dimer that has a stimulatory effect on colony formation (Laerum et al., 1988).

A single injection of 30μg/kg pEEDCK monomer given to mice 2 hours before a second Ara-C injection delays the onset of neutropenia by 2 to 3 days. Post chemotherapy infusion of the peptide in the form of a dimer at 1.4 μg/kg produces a four fold increase in progenitor cells 2 days post therapy. Animals given the two forms of the peptide together are protected from leukocyte depression by Ara-C (Paukovits et al., 1991). The dimer's stimulatory function is thought to be mediated through its effect on influencing stromal cells to produce colony stimulating activity (King et al., 1992, Veiby et al., 1992). A stable pentapeptide made by substituting the –SH with an isosteric methylene group has an inhibitory effect on cycling GM-CFC without any effect on colony numbers. It directly inhibits $Lin^{-}Sca1^{+}$ HPP-CFC stimulated by SCF+IL-3+IL-1 but not those stimulated with M-CSF+IL-3+IL-1 (Veiby et al., 1996). The pentapeptide

synergises with TNF-α and /or IFN-γ in its inhibitory effect at low concentrations (Lu et al., 1989).

pEEDCK protects mice from the cytotoxic effects of cytosine arabinoside (Ara-C) without changing Ara-C's cytotoxic effects on transformed leukaemic cell line (Paukovits et al., 1990). Surprisingly, the myeloid cell line (HL-60) which was directly inhibited by pEEDCK was killed by Ara-C in the presence of the pentapeptide (Paukovits et al., 1990). The fact that the pentapeptide has a direct inhibitory action on HL-60 leukaemic cells might result in residual disease after chemotherapy if pEEDCK has to be used as an adjuvant to chemotherapy.

1.6.2 *Transforming growth factor-β*

TGF-β inhibits Thy-1 expression on bone marrow cells induced by IL-3 (Keller et al., 1990). In addition TGF-β inhibits all subpopulations of HPP-CFC, multipotent and mixed colony cells with no effect on single granulocyte or macrophage colonies (Keller et al., 1990). TGF-β has a strong inhibitory effect on the proliferation of $CD34^{++}CD38^{-}$ and CFC generation of $CD34^{++}CD38^{-}$ progenitor cells with little effect on the proliferation of $CD34^{+}CD38^{+}$ cells (van Ranst et al., 1996). Moreover, TGF-β inhibits normal bone marrow myeloid precursor cells induced to proliferate by IL-6, IL-1 or lipopolysaccharide, but has little effect on the inhibition of precursor cells stimulated by IL-3, M-CSF or GM-CSF (Lotem & Sachs., 1990). TGF-β added at the time of media change, in long-term bone marrow cultures, inhibits primitive haematopoietic progenitor cells from entering S-phase (Cashman et al., 1990a). This inhibitory effect may involve apoptosis of haematopoietic progenitor cells (Hong et al., 1997). TGF-β has a

similar inhibitory effect on the proliferation of very primitive normal and chronic myeloid leukaemia progenitor cells (Cashman et al., 1990b). While this leukaemia inhibitory effect may be advantageous, these leukaemia cells may be protected from cytotoxic agents during therapy resulting in the recurrence of the disease.

1.6.3 *Tumour necrosis factor α*

TNFα pretreatment results in enhanced regeneration of circulating neutrophils in animals given cell cycle specific chemotherapeutic agents (Slordal et al., 1990). The effect of TNFα on cultures of L-cell line is cell cytostasis in the G_2 phase of cell cycle (Darzynkiewicz et al., 1984). In human long-term bone marrow cultures, TNFα strongly potentiates IL-3 induced short term proliferation of human $CD34^+$ haematopoietic progenitor cells which ceases after 10-12 days (Caux et al., 1991). After 10-12 days, TNFα inhibits granulocytes by arresting cells in G_0/G_1 phase (Caux et al., 1991) and stimulates monocyte proliferation. TNFα reversibly blocks differentiation of monocytes at the level of $CD13^-$ and $CD15^-$ (Caux et al., 1991). It has a rapid and transient inhibition on the non-adherent layer cell proliferation with a late effect on the inhibition of the adherent layer cells (Khoury et al., 1992). TNFα also induces production of IL-6 and the disappearance of adipocytes (Khoury et al., 1992) in the adherent layers of LTBMCs. This cytokine inhibits the proliferation of the CFU-S population and induces significant plasma CSF activity and confers radioprotection of CFU-S when administered to mice before irradiation (Warren et al., 1990). TNFα like TGF-β inhibits the proliferation of $CD34^{++}CD38^-$ and CFC generation of $CD38^{++}CD38^-$ progenitor cells with little effect on the proliferation of

$CD34^+CD38^+$ cells (van Ranst et al., 1996). However TNF inhibition of cell cycle may also involve programmed cell death of haematopoietic progenitor cells (Hong et al., 1997).

1.6.4 *Macrophage inflammatory protein 1α*

Macrophage inflammatory protein belongs to a group of cytokines called chemokines (Broxmeyer et al., 1993). As the name suggests macrophage inflammatory proteins are produced by macrophages and they are heparin binding proteins which cause localised inflammatory reactions after injection into foot pads of C3H/HeJ mice (Wolpe et al., 1988). Using the CFU-A assay for primitive stem cell proliferation, the stem cell inhibitor isolated by Amicon Diaflo membrane ultrafiltration in the molecular weight fraction 50-100KDa from bone marrow extracts was identified as MIP 1α (Graham et al., 1990). MIP 1α prevents recruitment of quiescent stem cells into cell cycle when added to regenerating bone marrow cells (Dunlop et al., 1992; Lord et al., 1992) and protects multipotent haematopoietic cells from the cytotoxic effects of hydroxyurea *in vivo* (Lord et al., 1992). MIP 1α has a weak inhibitory action on the proliferation of $CD34^{++}CD38^-$ cells and enhances primary proliferation of $CD34^+CD38^+$cells and generation of secondary CFC (van Ranst et al., 1996). MIP 1α suppresses the proliferation of cells that are responsible for day 12 CFU-S in a dose dependent manner with little effect on cells responsible for day 8 CFU-S (Dunlop et al., 1992).

MIP 1α, IL-3 and diffusible marrow-derived stromal factors maintain human haematopoietic stem cells for at least eight weeks *in vitro* by an unknown

mechanism (Verfaillie et al., 1994). In addition to inhibiting haematopoietic cell proliferation, MIP 1α enhances the stimulatory activity of GM-CSF or M-CSF on the mature bone marrow CFU-GM (Broxmeyer et al., 1990). The activity of this chemokine on haematopoietic cell proliferation can be antagonised by other members of the chemokine family. For example, MIP 1β abrogates the capacity of MIP 1α to suppress myeloid progenitor cell growth (Broxmeyer et al., 1991). Moreover, a large quantity of MIP 1α monomer is needed to exert inhibitory influence because MIP 1α aggregates to form inactive dimers that have no inhibitory effect on haematopoietic cell proliferation (Mantel et al., 1993). Interestingly, TGF-β inhibits MIP 1α gene expression in the bone marrow derived macrophages (Maltman et al., 1993).

The problem with most of the above inhibitors is that some of them have stimulatory effects on cell proliferation, others are not specific in their action while others are toxic. There is a growing interest in a tetrapeptide, Acetyl-Ser-Asp-Lys-Pro (AcSDKP), which has most of the desired properties as a selective inhibitor of normal cell proliferation without affecting tumour cell proliferation. The rest of the review will concentrate on the physiological properties of AcSDKP.

1.7 THE TETRAPEPTIDE Acetyl-Ser-Asp-Lys-Pro (AcSDKP)

1.7.1 *Sources of AcSDKP*

The first identification of a peptide with low molecular weight, which could inhibit murine haematopoietic stem cell recruitment into cell cycle after irradiation was in the extracts of foetal calf bone marrow (Frindel & Guigon,

1977). Furthermore foetal calf liver extracts were also identified as a source of a non species specific low molecular weight substance which prevents CFU-S entering S-phase after cytosine arabinoside (Ara-C) treatment (Guigon et al., 1984). Using the foetal calf bone marrow the structure of the inhibitory peptide was identified as a low molecular weight (Mw 487) tetrapeptide Acetyl-Ser-Asp-Lys-Pro (AcSDKP) (Lenfant et al., 1989b). The same molecular structure showing the same inhibitory phenomenon was also identified in human placental extracts (Lopez et al., 1991). It has been shown subsequently that AcSDKP is synthesised in mice under steady state condition and is secreted by long-term bone marrow cultures (Wdzieczak-Bakala et al., 1990).

Thymosin β4 has the SDKP sequence of amino acids at the beginning of its N-terminal sequence (Lenfant et al., 1989b; Voelter et al., 1995). Thymosin β4 is a ubiquitous polypeptide that is active in the regulation and differentiation of thymus dependent lymphocytes. High concentrations of thymosin β4 are found in peritoneal macrophages in mice (Hannappel et al., 1982). Interestingly AcSDKP is produced by macrophages in long-term bone marrow cultures (Li et al., 1997), showing a common source of these two peptides. Indeed studies have shown that AcSDKP can be generated from thymosin β4 both *in vivo* and *in vitro* (Grillon et al., 1990). AcSDKP like substance is present in all mouse tissues with a high concentration in the spleen with a poor correlation to thymosin β4 distribution (Pradelles et al., 1991). Therefore, further studies are needed to confirm that AcSDKP is cleaved from thymosin β4 under normal steady state *in vivo* in both mice and man.

While a possibility exists that thymosin β4 may be the precursor molecule for AcSDKP production *in vivo*, thymosin β4 may not be the only source of

AcSDKP as SDKP peptide sequence is also found in TNFα and rat liver phenylalanine hydroxylase (Lenfant et al., 1989b). Thymosin β4 inhibits actin assembly which disrupts cell division. However, the antiproliferative activity of AcSDKP in not mediated by a thymosin β4 like effect on actin assembly (Cheviron et al., 1996). In addition to this, thymosin β4 is a human haematopoietic stem cell ($CD34^+$) proliferation inhibitor in its own right (Bonnet et al., 1996). Studies have been carried out to compare the mode of action of AcSDKP and TNF α on haematopoietic stem cell proliferation (Bonnet et al., 1995). These two molecules also do not display the same mode of action.

1.7.2 *Mechanism of AcSDKP action*

Mice treated with Ara-C have reduced levels of AcSDKP. As a result the pluripotent haematopoietic stem cells (CFU-S) enter into cell cycle. However after some time a feed back mechanism causes an increase in AcSDKP levels and stem cells are switched to a quiescent state (Frindel et al., 1992b). Using HPP-CFC and CFU-S cell populations, it has been shown that AcSDKP does not have a direct inhibitory effect on cell proliferation after a short *in vitro* incubation. AcSDKP however, blocks the action of stimulators of stem cell proliferation that are found in regenerating haematopoietic tissues (Robinson et al., 1992; Monpezat & Frindel, 1989). AcSDKP shows a direct and reversible inhibitory effect on the growth of human $CD34^+$ subpopulations in response to growth factor stimulation (Bonnet et al., 1993). In the studies of Bonnet et al., (1993) AcSDKP was shown to reduce the numbers of both $CD34^+HLA\text{-}DR^{high}$ and $HLA\text{-}DR^{low}$ populations.

AcSDKP also demonstrated a greater inhibitory effect on the most primitive cells contained in the $CD34^{++}HLA\text{-}DR^{low}$ subpopulation.

While AcSDKP does not affect cycling murine haematopoietic progenitor cells after a short incubation period in mice (Robinson et al., 1992; Monpezat & Frindel, 1989), the peptide has an inhibitory effect on human progenitor cells (Guigon et al., 1990). This inhibition shows a bell shape response curve with no observable inhibitory effect at higher or lower AcSDKP concentration. Recent observations suggest that cycling murine progenitor cells may also be inhibited by the peptide (Jackson et al., 1996) after a prolonged incubation period. In addition to haematopoietic stem cells, AcSDKP inhibits the proliferation of lymphocytes isolated from both mammals and birds (Volkov et al., 1996a).

AcSDKP is active at the G_0 or early G_1 phases as shown by the response of CFU-S to Ara-C and AcSDKP (Monpezat & Frindel, 1989). AcSDKP inhibits the proliferation of hepatocytes after partial hepatectomy *in vivo* (Lombard et al., 1990) but has no such effect on the proliferation of the same cells *in vitro* (Lauret et al., 1989). Lymphocytes have been ruled out as intermediary cells in AcSDKP inhibitory pathway (Monpezat & Frindel, 1989). However, AcSDKP inhibits the cytotoxic effects of lymphocytes if added to mixed lymphocyte cultures from different donors (Lavignac et al., 1992). In mice, AcSDKP decreases the mixed lymphocyte reaction induced by H-2 incompatible allogeneic spleen cells (Frindel et al., 1992). The tetrapeptide has no inhibitory effect on the cell lines, 3T3, FDC-P2 and K562 *in vitro* (Lauret et al., 1989). However when continuous cell lines are partially synchronised into cell cycle by low serum concentration or serum deprivation, AcSDKP blocks or retards their entry into S-phase from early G_1 (Volkov et al., 1996).

AcSDKP does not affect the cycling of the HL-60 cell line and fresh leukaemic cells from patients with either chronic myeloid leukaemia (CML) or acute myeloid leukaemia (AML) (Bonnet et al., 1992b). This effect is independent of the dose of AcSDKP or the use of growth factors such as IL-3 or GM-CSF. No inhibitory effect of AcSDKP on neoplastic cells has been observed so far at concentrations which inhibit normal cell proliferation (Bonnet et al., 1992b, Cashman et al., 1994; Resnitzky et al., 1995; Defard et al., 1997). However very high doses of AcSDKP (10^{-5}M) may inhibit the proliferation of leukaemic cells *in vitro* (Defard et al., 1997). Therefore AcSDKP can achieve suppression of normal cells without affecting tumour cell proliferation, thereby potentially aiding intensification of cytotoxic therapy.

1.7.3 *Effect of AcSDKP on long-term bone marrow cultures*

Addition of AcSDKP at weekly intervals to long term bone marrow cultures has no effect on the proliferation of progenitor cells. However daily addition of the peptide to LTBMCs induces a reversible inhibition of the proliferation of non-adherent progenitor cells with no effect on the adherent layer cells (Bonnet et al., 1992a; Hong et al., 1995; Jackson et al., 1996). In addition AcSDKP protects cells that reconstitute long-term bone marrow stromal cultures from the effects of mafosfamide (Genevay et al., 1996). Studies using long term bone marrow cultures have demonstrated that addition of MIP 1β to cultures antagonises the inhibitory effect of AcSDKP on normal primitive cycling human progenitor cells (Cashman et al., 1994). Addition of > 50 ng/ml AcSDKP to long term cultures of human normal cells at the time of media change induces a selective and reversible decrease in the proportion of high proliferative potential

erythroid and granulopoietic progenitors in the adherent layer that are in S-phase (Cashman et al., 1994).

Using stromal lines, an increased rosette formation between stromal cells and haematopoietic cells was observed in the presence of AcSDKP (Aizawa et al., 1992; Lenfant et al., 1989a). Pre-incubation of stromal cells but not haematopoietic cells with AcSDKP increases haematopoietic cell binding to stromal cells (Aizawa et al., 1992). Moreover, stromal cell proliferation is inhibited but its production of growth factors is not affected (Lenfant et al., 1989).

Establishing LTBMCs in the presence of AcSDKP from bone marrow cells of dogs that have been treated with AcSDKP *in vivo*, showed that 7 days of AcSDKP treatment to LTBMCs had a marked effect on progenitor cell proliferation. When AcSDKP was added to cultures at weekly intervals, no effect was observed at 10^{-7} M AcSDKP with only 20 to 25% inhibition at 10^{-6} M (Hong et al., 1995).

1.7.4 *Quantification of AcSDKP in vivo*

Using an enzyme immunoassay, AcSDKP has been detected in lymphocytes, and in low concentrations in granulocytes (Pradelles et al., 1990). The mean value of AcSDKP in human serum is 1.5 ± 0.47 picomol./ml and there is no variation between age and sex (Liozon et al., 1993). There is an increase in the peptide levels in myeloproliferative disorders and in acute myelogenous leukaemia (Liozon et al., 1995). There is an early and sharp increase in the peptide during induction treatment in some patients with acute myeloid leukaemia (Liozon et al., 1995). The variations of the endogenous AcSDKP levels in patients are dependent on type, doses and schedule of chemotherapy following solid

tumour treatment (Comte et al., 1997b). In Xenopus laevis, the concentration of AcSDKP decreases during the final oocyte maturation and during the initial 15 hour period of exponential cell division of the embryo (Volkov et al., 1995). Interestingly AcSDKP has no clinical side effects in dogs when it is administered continuously through indwelling catheters and the serum chemistry remains normal (Hong et al., 1995). Surprisingly, some dogs show rebound thrombocytosis following discontinuation of AcSDKP (Hong et al., 1995).

1.7.5 *Comparison of the inhibitory effect of AcSDKP, TNFα, TGF-β, MIP 1α and pEECDK*

As demonstrated by Bonnet et al., (1995), AcSDKP, TNF α, TGF-β and MIP 1α all inhibit BFU-E and CFU-GM numbers in $CD34^+$ subpopulation. However TNFα reduces the number of CFU-GM present in $CD34^+DR^{high}$ cells more than other substances. AcSDKP and MIP 1α require daily addition to cell cultures to exert their inhibitory effect while TNFα and TGF-β inhibit cells regardless of the procedure used to administer them. AcSDKP is less inhibitory for $CD34^+$HLA-DR^{high} than for $CD34^{++}$HLA-DR^{low}. MIP 1α has the opposite effect to AcSDKP on the same cell population. TNFα and TGF-β inhibit both $CD34^+$ populations equally.

Just as with AcSDKP, pEEDCK decreases myeloid progenitor cells and CFU-S in a reversible manner and inhibits cell populations from entering DNA synthesis induced by chemotherapy (Paukovits et al., 1991; Aidoudi et al., 1996). Interestingly, both pEEDCK and AcSDKP inhibit non-haematopoietic cells (Volkov et al., 1996b; Reichelt et al., 1987; Elgjo et al., 1986). Both AcSDKP and

pEEDCK inhibit malignant cells (Paukovits et al., 1990; Defrad et al., 1997). However, the crucial difference between AcSDKP and other proliferation inhibitors is that AcSDKP inhibits leukaemic cells at very high concentrations that have no inhibitory effect on normal progenitor cell proliferation (Defard et al., 1997). AcSDKP has however no inhibitory effect on tumour cells at concentrations that affect normal cell cycling (Resnitzky et al., 1995; Bonnet et al., 1993).

From these studies it can be concluded that at present the only negative haematopoietic stem cell proliferation regulator known that shows differential effect on the proliferation of normal and leukaemic cells is AcSDKP. TGF-β and TNFα both inhibit the proliferation of normal and leukaemic cells (Sing et al., 1988; Broxmeyer et al., 1986). MIP 1α has a variable effect on the proliferation of myeloid leukaemia and in some cases it inhibits their proliferation (Broxmeyer et al., 1993).

1.8 AcSDKP AND TUMOUR THERAPY

The concept of using stem cell proliferation inhibitors to protect normal cells from cytotoxic effects of tumour cell cytotoxic agents is a feasible one. It is based on the ability of inhibiting normal cell proliferation without affecting the proliferation of neoplastic cells. In this way phase specific cytotoxic agents can kill proliferating neoplastic cells while sparing normal non-proliferating cells (Tubiana et al., 1993).

1.8.1 *Physical cytotoxic agents*

There are many ways by which physical cytotoxic agents can be used with AcSDKP to kill tumour cells while protecting normal haematopoietic cells. One way is by using phototherapy. Tumour cells selectively accumulate the hematoporphyrin derivative, photofrin 2, and merocyanine-540 which are very unstable to light of specific wave lengths (Bohmer & Morstyn, 1985; Atzpodien et al., 1987). Using this method it has been shown that the ability of AcSDKP to inhibit normal GM-CFC proliferation protects this cell population from the cytotoxic effects of photofrin-2 sensitiser and phototherapy. Leukaemic cells are neither inhibited nor protected from sensitiser and phototherapy (Coutton et al., 1994).

The other way of using physical agents and AcSDKP in protecting haematopoietic cells is to use heat treatment. Cycling haematopoietic cells are sensitive to heat treatment of about 43°C. This sensitivity is reduced in the presence of AcSDKP and normal progenitor cells are spared from hyperthermic damage while murine leukaemic cells are destroyed both in the presence and absence of the peptide (Wierenga & Konings, 1996; Wierenga et al., 1997).

Dogs given AcSDKP before and during 300cGy whole body irradiation are protected from the myelotoxic effects of irradiation (Deeg et al., 1997). AcSDKP however has no effect at higher doses of irradiation. Interestingly, AcSDKP has a protective effect on murine bone marrow myelotoxicity if given for 3 days beginning 24 hours before sublethal irradiation. Addition of G-CSF for 10 days after AcSDKP produces an increase in platelet numbers which is not observed in the presence of AcSDKP alone (Watanabe et al., 1996b).

1.8.2 *Chemical cytotoxic agents*

Administration of AcSDKP together with chemical cytotoxic agents may inhibit the proliferation of normal haematopoietic cells and give them a survival advantage over neoplastic cells. Indeed, giving mice low molecular weight extracts from foetal calf bone marrow 2 hrs before a lethal dose of Ara-C, protects animals with some surviving up to 8 months after treatment (Guigon et al., 1982). While a maximum number of animals survive Ara-C treatment when AcSDKP is given either simultaneously or 2 hrs after Ara-C, the same protective effect is noticed when AcSDKP and cyclophosphamide (CTX) are given 8 hrs apart (Bogden et al., 1991). The combination of AcSDKP with G-SCF induces an increase in the total numbers of leukocytes following Ara-C treatment without reducing the carboplatin-induced haematotoxicity (Li et al., 1998). Moreover AcSDKP and GM-CSF accelerate the recovery from leukopenia following three cycles of Ara-C with an increase in the white blood cells in general and granulocytes in particular (Bogden et al., 1998). AcSDKP also reduces doxorubicin-induced mortality in mice and protects cells responsible for long-term reconstitution, HPP-CFC and GM-CFC from doxorubicin-toxicity (Masse et al., 1998). Masse et al.,(1998), also found that the recovery of the GM-CFC population in the AcSDKP-doxorubicin treated mice was optimised by the subsequent administration of G-CSF. AcSDKP with platelet factor 4 (PF4) given to mice before 5 FU accelerated the recovery *in vivo* of HPP-CFC, CFU-GM and BFU-E (Aidoudi et al., 1996).

AZT an antiviral agent used in the treatment of Acquired Immunity Deficiency Syndrome (AIDS), induces anaemia due to its toxic effects on erythroid progenitor cells (Dainiak et al., 1988). Pretreatment of bone marrow

cells with AcSDKP *in vitro* induces a significant reduction in the toxicity of 3'-Azido-3-Deoxythymidine (AZT) to both human erythroid and myeloid progenitor cells (Grillon et al., 1993b). Moreover, AcSDKP prevents CFU-GM inhibition induced by HIV-infected cell-derived conditioned medium (Coutton & Chermann, 1997).

1.9 STABILITY OF AcSDKP IN SERUM AND PLASMA

1.9.1 *Angiotensin 1-converting enzyme degrades AcSDKP*

A comparative study of the stability of AcSDKP in human, horse, foetal calf serum and bone marrow cells revealed that the peptide had a very short t½ in serum. The t½ of AcSDKP in human serum is 45 minutes, in horse serum is 30 minutes and the peptide has a t½ of 80 minutes in human plasma. The degradation of AcSDKP in horse serum is 4 times higher than foetal calf serum. Addition of captopril, a metalloprotease and angiotensin 1-converting enzyme inhibitor, increases the t½ of AcSDKP *in vitro* (Grillon et al., 1993a). Interestingly, plasma from leukaemic mice degrades this tetrapeptide faster than plasma from normal mice, with leukaemic bone marrow cells degrading it only after 4 hours of *in vitro* incubation (Wdzieczak-Bakala et al., 1993).

The short t½ of AcSDKP in plasma / serum has been attributed to the ability of angiotensin 1-converting enzyme (ACE) to degrade this peptide (Rieger et al., 1993). ACE cleaves the peptide bond Asp-Lys to release the C-terminal dipeptide Lys-Pro that is further degraded to Lys. The second step in AcSDKP degradation is under the control of leupeptin and E-64, $HgCl_2$ insensitive enzyme. There is a marked inhibition of AcSDKP degradation in the presence of captopril

(10^{-8} M) while in the presence of the same concentration of lisinopril and enalaprilat, the activity of the enzyme on AcSDKP hydrolysis remains at 20% and 15% respectively (Rieger et al., 1993). Plasma that has been extensively dialysed degrades the peptide to a lesser extent (Rieger et al., 1993).

1.9.2 *Biology of ACE*

Angiotensin 1-converting enzyme is a zinc dipeptidyl carboxypeptidase with endopeptidase activity on certain substrates. It is also known as peptidyl dipeptidase A, kininase II and EC3.4.15.1. Its primary specific function is to cleave the C-terminal dipeptide from angiotensin 1 to produce angiotensin II a vasopotent octapeptide (Bakhle, 1968). In general the enzyme cleaves the C-terminal dipeptides from oligopeptide substrates with free C-terminus in the absence of a penultimate proline residue or a terminal dicarboxylic amino acid (Hooper & Turner, 1987). ACE also performs endopeptidase activity on substrates that are amidated at their C termini where it cleaves a C-terminal dipeptide amide and/or a C-terminal tripeptide (Dubreuil et al., 1989). As shown by the activity of ACE on luteinising hormone-releasing hormone, ACE has activity on both the COOH and NH_2 terminal peptides on some substrates (Skidgel & Erdos, 1985).

1.9.3 *Novel activity of ACE*

ACE hydrolyses several peptides with a differential effect on each peptide. In addition to the hydrolysis of angiotensin 1, ACE also hydrolyses substance P, bradykinin and luteinising hormone-releasing hormone (Skidgel & Erdos.,1985; Bakhle et al., 1968; Dorer et al., 1974; Hooper & Turner, 1987). The hydrolysis of

bradykinin and its higher homologues shows an inverse relationship of the hydrolysis rate with size and charge of the peptide (Dorer et al., 1974). ACE hydrolyses cholecystokinin and gastrin analogues by attacking the penultimate peptide bond that generates the C-terminal amidated dipeptide. It subsequently releases di-or-tri-peptides from the remaining N-terminal fragments (Dubreuil et al., 1989). As already stated, ACE is important in the first rate-limiting step in AcSDKP degradation (Rieger et al., 1993).

The activation of ACE to catalyse the hydrolysis of the peptides depends on the pH and presence of ions. It has been demonstrated using furanacryloyl-Phe-Gly-Gly that the hydrolysis of this molecule by ACE is activated by monovalent anions in the order $Cl^- > Br^- > F^- > NO_3^- > CH_3COO^-$. Anions are essential activator of ACE at both acidic and alkaline pHs (Bünning & Riodan, 1983).

1.9.4 *Characterisation of ACE*

Angiotensin 1-converting enzyme is membrane bound, but soluble ACE has been found in the body fluids such as lymph, plasma, amniotic fluid cerebrospinal fluid, seminal plasma, homogenates of prostate and epididymis (Erdos, 1990). Organs which contain ACE include kidney, intestine, placenta, choroid plexus (Erdos, 1990). ACE is bound to cell plasma membranes through its intracellular C terminus. The secreted form is derived from the membrane bound form by post-translation proteolytic cleavage of the C-terminal region (Wei et al., 1991; Soubrier et al., 1988).

ACE has a number of isoenzymes. For example there is an ACE isoenzyme found in the testes. This isoenzyme has a molecular weight of 90KDa while ACE from the rest of the tissues has a molecular weight of 140KDa. Both

ACEs have the capability of cleaving dipeptide residues from carboxylic termini of angiotensin 1, bradykinin, met-encephalin, and ACE inhibitors have identical effects on both isoenzyme activities (Lanzillo et al., 1985). Indeed ACE isoforms have also been found in the brain tissues. The two isoforms of ACE in the striatal part of the brain have molecular weights of 180KDa and 170KDa. The variation has been found to be due to differential glycosylation of the same enzyme in different cell types of the brain. Just as the isoenzyme from the testes, these brain isoforms also have similar properties with regards to enzymic activity and inhibitor sensitivity. They are both able to hydrolyse substance P (Hooper & Turner, 1987).

A single gene encodes for ACE in the human genome. The differences in the expression of isoenzymes are under the control of an internal promoter that is tissue specific. The study on the structure of the gene for the testicular ACE isoenzyme, using the λ phage human DNA library showed that the large ACE mRNA is transcribed from exon 1 to 26 excluding exon 13 from which the small testicular ACE is transcribed. The two isoenzymes have an identical sequence near their 3'-ends with unique sequences on the 5'-ends. The unique sequence of the large enzyme precedes that of the small enzyme which in turn precedes the sequence common to both enzymes by splicing introns from the primary mRNA (Hubert et al., 1991; Kumar et al., 1991).

1.9.5 *Two homologous domains of ACE*

Two putative active centres in human ACE have been deduced from the structure of cDNA. A highly hydrophobic sequence is located near the carboxylic terminal which anchors the enzyme to the cytoplasm of cells and the other region,

also displaying hydrophobicity, is classified as the signal peptide. Glutamic acid and histidine that are implicated in the catalytic processes of other enzymes are found in both domains (Soubrier et al., 1988). There is a high degree of homology between the two domains. The C-domain is located in the carboxylic terminal region and the N-domain is in the amino terminal region of ACE. Ileal-fluid ACE contains a single N-domain while germinal ACE contains a single C-domain (Deddish et al., 1994). The rest of ACE in other organs contains both domains.

These domains have individual high affinity inhibitory binding sites. At high chloride concentrations, the chloride effect is much greater on the C-domain than the N-domain. The inhibitory constants for captopril, enalaprilat and lisinopril are all in the nanomolar range on both domains (Wei et al., 1992). While some ACE inhibitors are site selective, a lot are not site selective. Lisinopril and delaprilat are C-site potent inhibitors while cilazapril, trandolaprilat ramiprilat, quinaprilat and enalaprilat are not site selective (Bevilacqua et al., 1996; Wei et al., 1992; Deddish et al., 1996; Rousseau-Plasse et al., 1996). On the other hand captopril and fosinopril are N-site potent inhibitors (Wei et al., 1992; Michaud et al., 1997). This site-specific action is influenced by the pH and chloride anion concentration. N-domain active site acts independently of chloride concentrations and loses it action at high chloride concentration (Wei et al., 1992, Deddish et al., 1996). Organ-specific glycosylation affects the binding characteristics of ACE inhibitors to N-or C-site of human tissue ACE (Bevilacqua et al., 1996)

Both the N and the C active sites of ACE exhibit dipeptidyl activity towards AcSDKP but the N-active site hydrolyses the peptide 50 times faster than the C-active site. Addition of monoclonal antibody directed towards the N-active site, results in inhibition of hydrolysis of AcSDKP by ACE. AcSDKP is therefore

a specific substrate for the N-domain of ACE (Rousseau et al., 1995; Deddish et al., 1996). Captopril a known N-site potent inhibitor of ACE increases the serum levels of AcSDKP both *in vivo* and *in vitro* (Azizi et al., 1996). However lisinopril and other known C-site potent inhibitor of ACE also inhibit AcSDKP degradation (Rousseau-Plasse et al., 1996; Rousseau et al., 1995; Azizi et al., 1997).

1.9.6 *ACE expression by pathological and haematopoietic cells*

Macrophages are some of the haematopoietic cells known to produce ACE (Costerousse et al., 1993). High levels of ACE are also found in alveolar macrophages of smokers (Hinman et al., 1979). Levels of ACE are elevated in the alveolar macrophages of people with sarcoidosis (Silverstein et al., 1976). Culturing human normal monocytes for 6 hours induces cells to produce high levels of ACE and enzyme levels are increased in the presence of autologous serum and dexamethasone (Friedland et al., 1978). Cells exhibiting lymphoblastic leukaemia antigen have an increased ACE activity with substantial ACE activity levels in the pre-B phenotype (Beaumont et al., 1989).

1.10 ACE INHIBITORS

1.10.1 *General mode of action of ACE inhibitors*

As reviewed by Erdo, (1990), ACE is an important enzyme involved in the regulation of blood pressure. There are two pathways in the regulation of blood pressure. These are the renin-angiotensin pathway and the kininogen-bradykinin pathway. Renin cleaves angiotensinogen to angiotensin I that is further cleaved to

angiotensin II. Angiotensin II is a vasoconstrictor and it also stimulates aldosterone release resulting in sodium retention and hence elevations of blood pressure. Kininogen is cleaved by kallikrein to bradykinin, which is a vasodilator in its own right. However ACE degrades bradykinin to inactive fragments. Bradykinin is a potent stimulator of endothelial prostaglandins and nitric oxide (Furchgott, 1983). Bradykinin also stimulates release of tachykinins and neurokinin A (Umemura et al., 1997). Therefore ACE inhibitor effects are mediated by increase in both bradykinin and its by products and also the suppression of angiotensin II production (Campbell et al., 1995). There is also evidence that ACE inhibitors may inhibit a kininase other than ACE (Campbell, 1995).

Angiotensin 1-converting enzyme inhibitors were first isolated from the venom of a snake called Borthrops jararaca (Ondetti et al., 1971). Subsequently a few have been synthesised. ACE inhibitors are valuable therapeutic agents for the management of hypertension, myocardial infarction, congestive cardiac failure and diabetic nephropathy (Campbell, 1995). All ACE inhibitors have a common 2-methyl propanalol-L-proline moiety (Barr & Cohen, 1992). The current clinically studied ACE inhibitors inhibit tissue located ACE. They differ in that some incorporate sulfhydryl group like captopril and zofenopril, the others have carboxyalkyl dipeptides like enalapril, lisinopril, ramipril, benazepril, cilazapril, peridopril, and quinapril, and others have the phosphinic acid for example fosinopril (Herman, 1992). The differences in the structure of ACE inhibitors may contribute to the differences in absorption, metabolism, distribution, and elimination of the drug *in vivo*. Moreover, it may also affect the affinity of the drug to ACE and therefore the observed effects and side effects of the drugs

(Herman, 1992). Some of ACE inhibitors are prodrugs (Opie, 1994). The binding of ACE inhibitors to ACE involves specific molecular interactions. For example captopril incorporates a sulfhydryl group that binds to a zinc atom in the enzyme (Opie, 1994).

1.10.2 *Side effects of ACE inhibitors*

Most common ACE inhibitor side effects are: First-dose hypotension, acute renal impairment, hyperkalemia, cough, skin rashes, dysgeusia, bone marrow suppression, hepatotoxicity, angio-oedema, and foetopathy (Alderman, 1996). Factors which predispose to first dose hypotension following ACE inhibitor therapy include sodium depletion, diuretics, vomiting, diarrhoea, old age, congestive cardiac failure and severe or complicated hypertension (Alderman, 1996). Hyperkalemia results from increased sodium excretion following ACE inhibitor therapy. Low levels of aldosterone following ACE inhibitor therapy can explain this side effect. ACE inhibitor therapy increases renal blood flow and glomerular filtration rate in normal kidney. However in renovascular disease there is a reduction in glomerular filtration rate and renal blood flow following ACE inhibitor therapy which makes kidney disease worse (Textor, 1997). Cough production has been attributed to inhibition of substance P degradation in the airways (Tomaki et al., 1996) and imbalance between thomboxane A_2 and prostaglandin I_2 (Umemura et al., 1997). Skin rashes may be explained by inhibition of kininase rather than ACE and autoimmune reactions to the sulphur moiety which forms part of captopril structure (Alderman, 1996). Hepatoxicity may also be attributed to the ability of ACE inhibitors to deplete liver glutathione levels. Reduced glutathione is an important substrate for the reductive

detoxification of reactive intermediates such as hydrogen peroxide or hydroperoxides produced by glutathione peroxidase (Golik et al., 1995). Bradykinin, substance P or angiotensin II reduction and autoimmunity to these drugs may mediate ACE inhibitor-induced angio-oedema. Since ACE is found in amniotic fluid and it regulates prostaglandin synthesis, it is not surprising that ACE inhibitors cause foetopathy (Shotan et al., 1994; Barr& Cohen, 1991).

Captopril administration to rats and dogs has been shown to induce haematological changes consistent with haemolytic anaemia (Imai et al., 1979). However, the most common haematological side effect of captopril is neutropenia with or without pancytopenia (aplastic anaemia) (Cooper at al., 1983, Israeli et al., 1985; van Brummelen et al., 1980). This side effect was first observed on hypertensive patients with systemic lupus erythematosis (Amann et al., 1980), renovascular hypertension (Staessen et al., 1980), chronic renal failure (Israeli et al., 1985), chronic obstructive uropathy (Strair et al., 1985) and on elderly patients (Kim et al., 1989). In hypertensive chronic haemodialysis patients, captopril induces anaemia necessitating frequent blood transfusions (Hachache et al., 1995). In some cases a high dose of captopril was given for the treatment of hypertension and this high dose induced anaemia (Amann et al., 1980; Staessen et al., 1980). However, pancytopenia induced by captopril has been observed in hypertensive patients without other complications (Kim et al., 1989). In addition to humans, pancytopenia has been observed in a dog with normal renal function (Holland et al., 1996). At times pancytopenia caused by captopril can be fatal as was observed in an elderly patient with diabetes mellitus and chronic renal failure receiving normal doses of captopril (Gavras et al., 1981).

All in all, the aim of this work is study the regulatory role of ACE on haematopoietic stem cell proliferation. By inhibiting ACE using captopril or lisinopril, the role of ACE on haematopoiesis will be evaluated. These investigations will indirectly analyse the role of the individual ACE domains on haematopoiesis. Therefore this work will emphasise the link between AcSDKP and ACE inhibitors in order to understand the underlying mechanism of ACE inhibitor induced bone marrow failure. The work will also concentrate on the beneficial effect of using ACE inhibitors with cytotoxic treatment with the aim of preserving stem cell integrity during neoplastic treatment. AcSDKP and its analogue made resistant to ACE degradation will also be studied. Studies will be done both *in vivo* and *in vitro* on mice. To understand the significance of ACE inhibitors and AcSDKP on haematopoiesis, long-term bone marrow cultures will also be studied with these substances.

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLING OF HAEMATOPOIETIC CELLS

2.1.1 *Mice*

Eight to twelve week old female CD1, CBA/H, and a cross breed between CBA/H and C57BL/6 mice (CBA/H x C57BL/6) F1 were used for these investigations. Some animals were purchased from Charles River, UK. In-house mice were bred and housed at the University of St. Andrews, Scotland, UK. They were kept in controlled conditions and fed on a diet of chlorinated water and RMI chow (Special Dietary Services, UK).

2.1.2 *In vivo recruitment of haematopoietic stem cells into cell cycle*

Mice were used in groups of three or four individuals per experiment unless otherwise stated. Haematopoietic stem cells were induced into cell cycle *in vivo* by 2 Gy whole body γ-irradiation (CIS BioInternational IBL 437C 137Cs γ-irradiation source. Dose rate 4.66 Gy/minute), 1-β-D-arabinofuranosylcytosine (cytosine arabinoside intraperitoneal injection (i.p) (Ara-C)) (Sigma, UK) at a dose of 100 mg/kg or 5 fluorouracil intravenous injection (i.v)(5 FU) (Sigma, UK) at a dose of 150 mg/kg. Ara-C and 5 FU are both S-phase specific cytostatic agents (Millard & Okell, 1975; Kerr, 1989). Mice were killed by cervical dislocation and the femoral marrow was harvested 24 hour after 2 Gy whole body irradiation and Ara-C administration. Following *in vivo* 5 FU treatment bone marrow cells were sampled on day 4.

2.1.3 *Medium*

The media used were made up from concentrates of either Dulbecco's 10x (Gibco BRL, UK) or Fischer's 10x (Gibco BRL, UK) supplemented with 50 iu/ml penicillin (Sigma, UK); 50μg/ml streptomycin sulphate (Sigma, UK); 2mM L-glutamine (Flow Lab, UK); $NaHCO_3$ (BDH, UK) according to manufacturer's recommendation. Dulbecco's medium routinely contained either 10% or 20% horse serum (Globepharm, UK) or 30% foetal calf serum (Globepharm, UK). These are referred to as either D10% or D 20% HS PS/G or D 30% FCS PS/G. In the case of Fischer's medium, 20% horse serum as well as hydrocortisone10^{-6}M (Sigma, UK) were included in the medium. This is referred to as F 20% HS PS/G Hydro. Fisher's medium that contained 10% horse serum without hydrocortisone was also used. This is referred to as F 10% HS PS/G. All media were made up with sterile distilled deionized water in a laminar horizontal flow hood (Bassaire, John Bass, UK).

2.1.4 *Bone marrow single cell suspension*

As soon as the mice were killed, the abdominal skin was swabbed with 70% alcohol and a lower abdomen transverse incision was made. The cut skin was reflected exposing the femurs and sometimes the tibias, which were removed with a pair of sterile scissors and put in 90-mm petri dishes (Sterilin, UK). Tissues were then carried into a sterile laminar horizontal flow hood where excess muscle tissue was scraped away using a sterile surgical blade (Swann-Morton, UK). The heads of the femurs were removed by cutting the neck of the femurs. The knee joints were disarticulated to reveal the soft cartilaginous articular surface. Using sterile forceps to hold the femurs, a 1 ml syringe (Becton Dickson, Ireland) with blue tip microlance needle (23G) (Becton Dickson, Ireland) containing 1 ml of appropriate medium was used to gently bore a hole through

the remaining cartilaginous part of the knee joint into the marrow cavity, and the medium was gently forced into the femur. This method was used to wash out bone marrow cells into a 5 ml plastic vial (Teklab, Sacristone, UK). The femur was flushed in the opposite direction using the same amount of medium by inserting the needle in the cut end of the neck of femur and the whole procedure was repeated as with the knee part of the bone. The femoral shaft was washed through in this manner twice. This process was carried out on all femurs. In cases where tibias were used, both ends of the tibias were cut and the proximal end only was used to flush cells out of the bones. A single cell suspension was made by progressively aspirating the bone marrow cell suspension through a 23G blue tipped microlance needle followed by a 25G orange tipped microlance needle (Becton Dickson, Ireland). Cells were then ready for assaying. Before assaying, cells were first counted.

2.1.5 *Cellularity determination*

Cell numbers in tubes were counted on a Coulter counter model ZM (Coulter Electronics, UK)' according to the manufacturer's recommendations. Pooled cells were routinely suspended in 2 mls of medium in a 30 ml universal tube (Sterilin, UK). The cellularity of the single cell suspension was determined by diluting 40 µl of cell suspension into 20 mls of Isoton II (Coulter Euro Diagnostics, UK) in a 20 ml clear plastic vial (Coulter Euro Dignostics, UK) with three drops of red cell lysing agent called Zaponin (an acetic acid based lysing agent) (Coulter electronics, UK). The Coulter counter setting was determined after calibration such that this dilution gave the number of nucleated cells per millilitre (see Table 2.1). The contents of each femur were routinely suspended in 1 ml of D20%HS PS/G. Therefore the femur cellularities were determined by diluting 20 µl from the cell suspension in half of the Isoton II solution used for pooled

cells. The average of three Coulter readings was determined as the number of cells per millilitre.

Table 2.1 Coulter Settings were:

Tissue	*Haematopoietic cells*	*Long term cultured bone marrow cells*
PARAMETER	0.5mls	0.5mls
current	130	700
Lower threshold	30	21
Full scale	10	1
polarity	+	+
attenuation	1	1
Pre set gain	2	2

2.2 Assays

2.2.1 *Colony assays*

a. Growth Factors

Conditioned medium was used as a source of growth factors in some experiments. Conditioned medium was derived from the WEHI-3B myelomonocytic leukaemic cell line and the L929 fibroblast cell line. WEHI-3B is a source of interleukin-3 (IL-3) (Ihle et al., 1982) and L929 cells produce macrophage colony stimulating factor (M-CSF) (Stanley & Heard, 1977).

Recombinant growth factors were also used. These were:

1. Recombinant murine granulocyte-macrophage colony stimulating factor (Immunex, U.S.A) at 40 Units/ml (r mu GM-CSF).
2. Recombinant murine interleukin-3 (R&D Systems, UK) at 10 ng/ml (r mu IL-3).
3. Recombinant human interleukin-1β (Immunex, U.S.A or R&D Systems, UK) at 10 units/ml (r hu IL-1β).

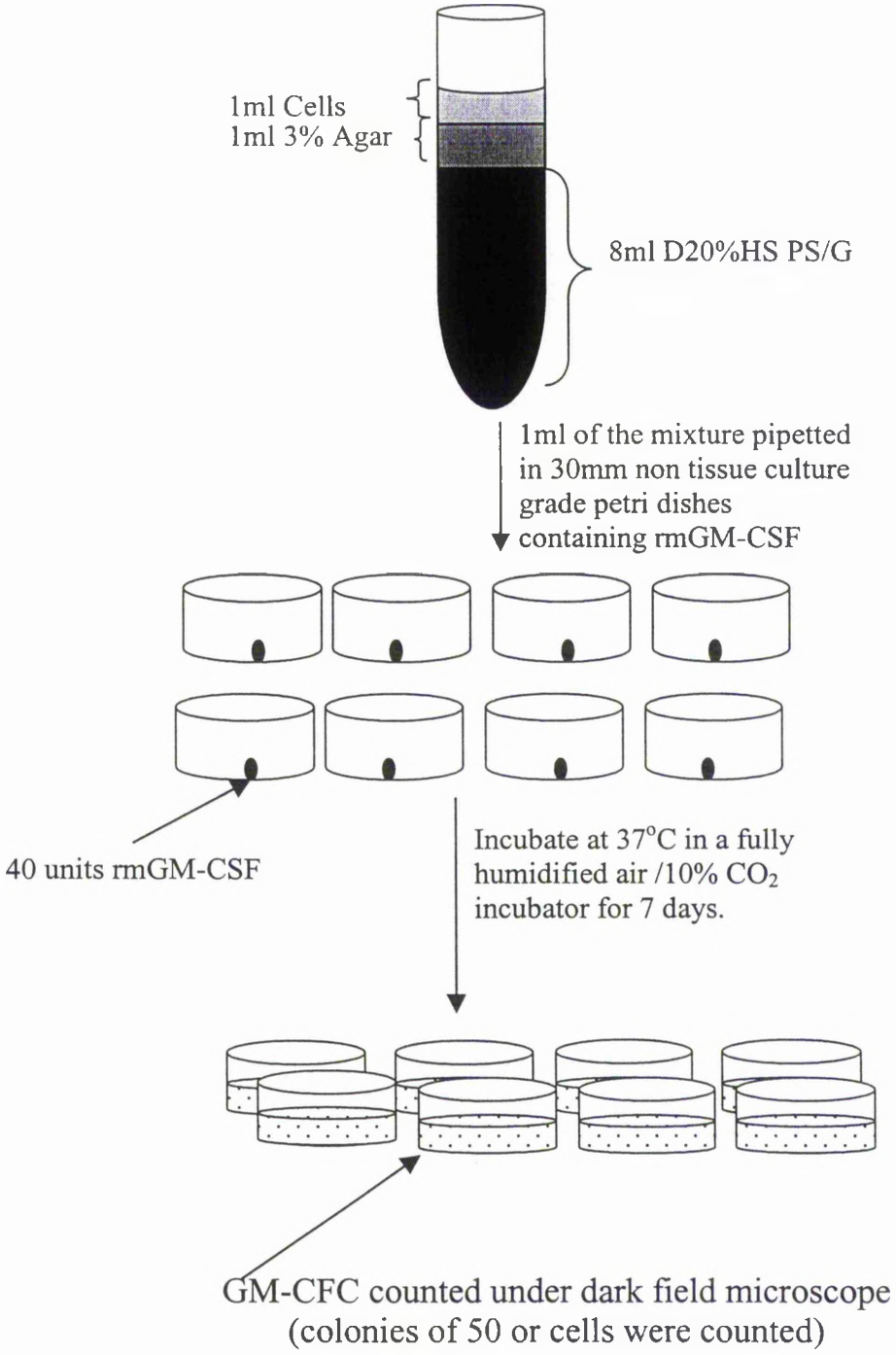
4. Recombinant murine macrophage colony stimulating factors (R&D Systems, UK) at 20 ng/ml (r mu M-CSF).

b. Granulocyte-Macrophage Colony Forming Cells Assay

Progenitor cells were investigated using the granulocyte-macrophage colony forming cell (GM-CFC) assay. Cells (5 x 10^5 cells/ml) were suspended in D20%HS PS/G in 30 ml universal tubes (Sterilin, UK) and kept in a water bath at 37°C. 8 mls of D20%HS PS/G were aliquoted into each of the 30 ml universal tubes and these tubes were placed in a water bath at 37°C. A bijoux bottle of 3% (v/v) agar (Bacto Agar; Difco Labs; Detroit, MI) was melted in boiling water in 100 ml glass beaker (Pyrex, UK). Eight 30 mm triple vent nontissue culture grade petri dishes (Sterilin, UK) per investigation were laid out in the hood and labelled. To each petri dish, 40-units r mu GM-CSF were added to give a final concentration of 40 units in the agar/cell mixture. The warmed universal tubes containing cells and the ones containing medium alone were removed from the water bath and placed into the laminar flow cabinet. 1 ml of melted agar was added to the universal tubes containing media followed by 1 ml of cell suspension and thoroughly mixed by gentle inversion. 1 ml of a mixture containing 5x10^4 cells/ml and 0.3% agar was pipetted into each of the eight petri dishes. Dishes were gently swirled around to spread the mixture out over the entire surface of the plate and allowed to gel for approximately five to ten minutes.

Semi-solid cultures were placed into cleaned plastic container that had been swabbed with 70% alcohol and placed in an incubator (Heraeus instruments, Germany). They were incubated at 37°C, in 10% CO_2 in a fully humidified atmosphere for 7 days.

Fig. 2.1 The GM-CFC assay



After 7 days dishes were removed from the incubator and colonies counted using a dark field microscope (Kyowa, Japan). Colonies containing 50 or more cells were counted. Different types of colonies were recorded. All colonies regardless of shape were considered. Fig. 2.1 summarises the general protocol for GM-CFC assay.

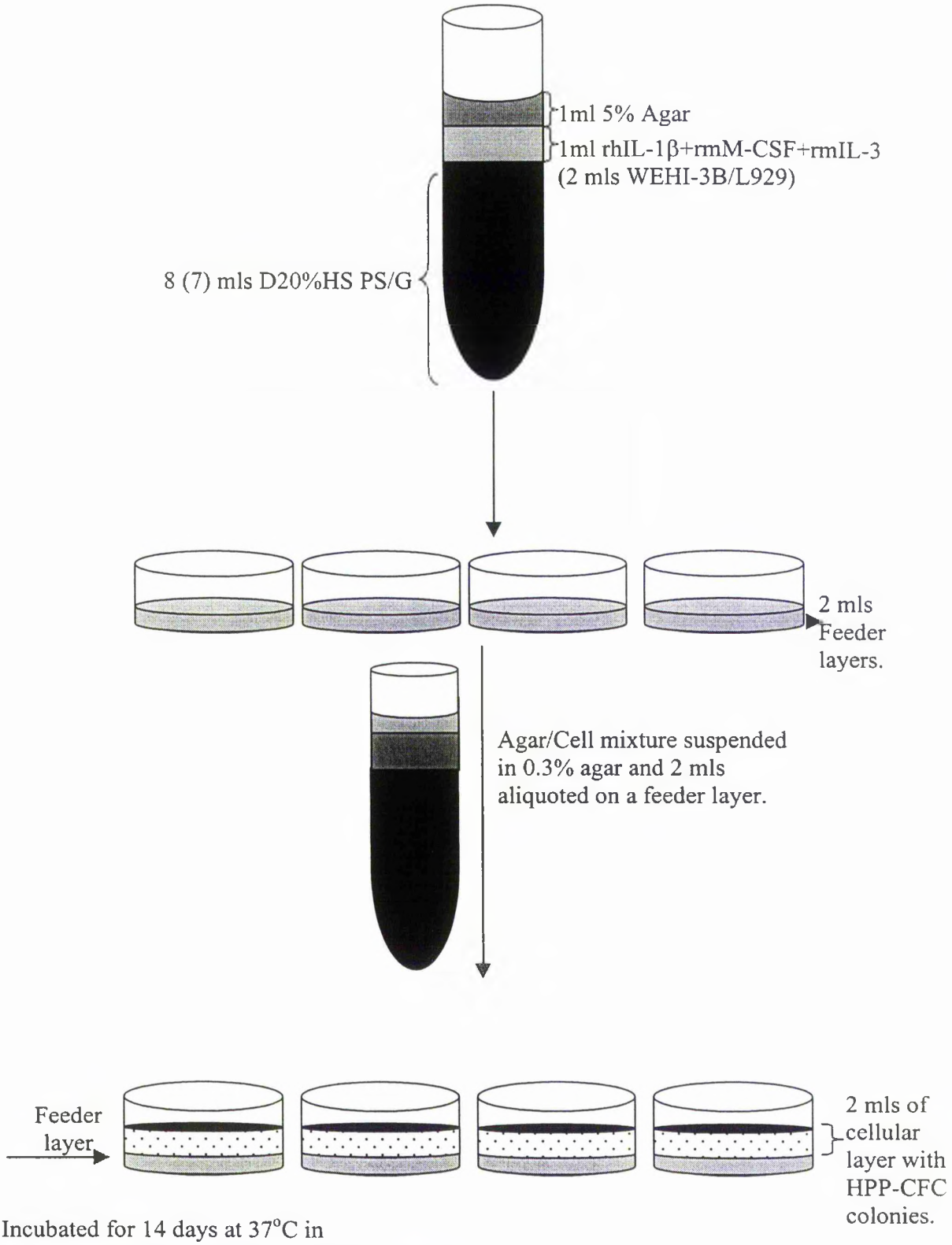
C. High Proliferative Potential- Colony Forming Cell Assay (HPP-CFC-1).

Two layers of semi-solid agar cultures are required for this assay. These layers are called the feeder and cellular layers. The basal layer is the feeder layer to which growth factors are added.

Feeder layers

8 mls of D20%HS PS/G were aliquoted into 30 ml universal tubes (Sterilin, UK). 1 ml of a mixture of r mu M-CSF at 200ng/ml, r mu IL-3 at 100ng/ml and r hu IL-1β at 100 units/ml suspended in D20%HS PS/G was added to each tube. When conditioned medium was used as a source of growth factors, 7 mls of D20%HS PS/G were aliquoted into 30 ml universal tubes and 1 ml of conditioned medium from WEHI-3B (10% v/v) and L929 (10% v/v) each separately was added to each tube. Tubes were then placed in a water bath at 37°C for approximately five minutes to be warmed. 5% (v/v) agar (Bacto Agar; Difco Labs; Detroit, MI) in a bijoux bottle was melted in boiling water in a 100 ml glass beaker (Pyrex, UK). Four 60 mm nontissue culture grade triple vent petri dishes per group were laid out in the horizontal laminar flow hood. Universal tubes were then removed from the water bath and 1 ml of 5% agar was added. Before the agar could gel, 2 mls of the agar-growth factors medium mixture, thoroughly mixed were pippetted into each of the petri dishes. Dishes were kept in the hood for approximately five minutes

Fig. 2.2 The HPP-CFC assay



Incubated for 14 days at 37°C in
fully humidified air/CO_2 and INT added on day 13.

until they gelled. To avoid pH changes they were then placed in the incubator until the cellular layer was ready to be added.

Cellular layers.

10 mls of single cell suspensions containing 2.5 x 10^5 cells/ml were suspended in D20% HS PS/G in 30 ml universal tubes. This was done under sterile conditions in the laminar flow hood. These universal tubes were then placed in a water bath at 37°C. 8 mls of D20%HS PS/G were pippetted in 30 ml universal tubes. 3% (v/v) agar was melted in boiling water in a 100 ml glass beaker. Feeder layer petri dishes were removed from the incubator and placed in the hood. Single cell suspensions and universal tubes containing media were removed from the water bath and taken to the hood. 1 ml of 3% (v/v) agar was then added to universal tubes containing media followed by 1 ml of single cell suspensions and the mixture was thoroughly mixed by gentle inversion of universal tubes. 2 mls of single cell suspension with 0.3% (v/v) agar mixture was then very gently pipetted over the feeder layer taking great care not to puncture the feeder layer. The cellular layer finally contained 0.3% (v/v) agar and $5x10^4$. Cultures were allowed to gel. Gelled cultures were stored in a 70% alcohol wiped plastic container and incubated at 37°C in 10% CO_2 in a fully humidified atmosphere for 14 days.

Staining and counting

On day 13 of incubation, cultures of HPP-CFC colonies were removed from the incubator and laid out in the laminar flow hood. 0.8 mls of an autoclaved solution of 1 mg 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)/ml 0.9% (w/v) NaCl (BDH, UK) was added to each petri dish (Bol et al., 1977). Petri dishes were then replaced in plastic containers and returned to the incubator for a final 24 hrs of

incubation. INT gets metabolised by viable cells from colourless to a red tetrazolium salt. This makes viable colonies appear dark red in colour thus making macroscopic counting possible. Colonies greater than 2 mm in diameter were counted when WEHI-3B and L929 were used as sources of growth factors. However, smaller colonies of 1 mm in diameter visible to the naked eye were counted when recombinant growth factors were used. Fig.2.2 summarises the way HPP-CFC were cultured.

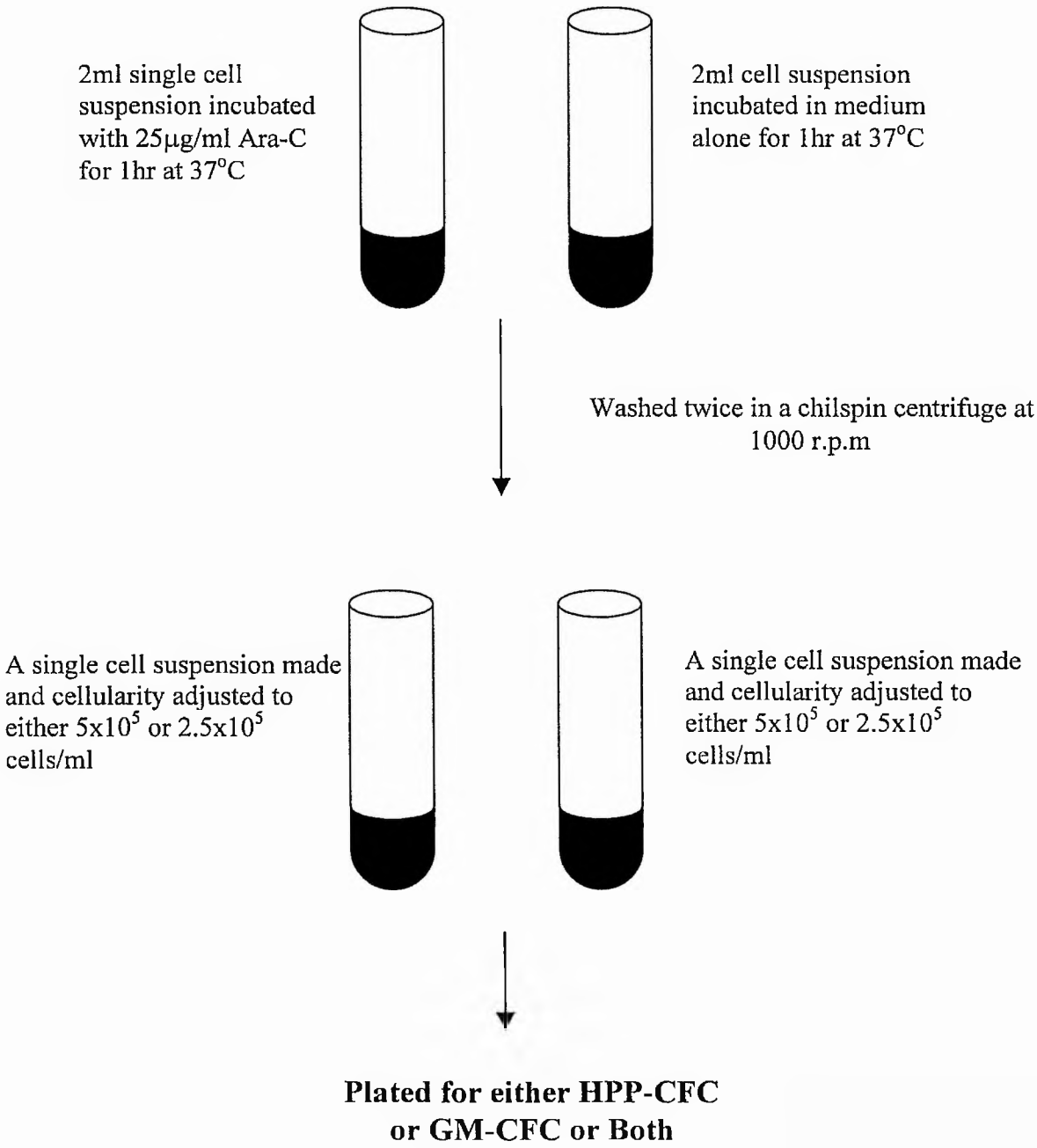
2.2.2 *S-phase suicide assay*

Suicide assay was performed to determine the proportion of haematopoietic cells in S-phase of the cell cycle. This was done on whole bone marrow cell suspension. This assay involved incubating half of the cell suspension with Ara-C (25 μg/ml) and the other half with media alone prior to clonogenic semi-solid agar assays. This killed cells that were in S-phase of cell cycle at the time of incubation. This is observed as a loss of colony forming potential when these incubated cells are plated in semi-solid agar cultures. Cell cycle specific cytotoxic agents either bind to enzymes required for DNA replication or get incorporated into DNA bases, stopping DNA replication and thus resulting in cell death. Therefore indirectly it was possible to assess the proportion of the tested haematopoietic cells that were in S-phase after cells were subjected to various treatments *in vivo* or *in vitro*. (Fig. 2.3 summarises the general protocol for the S-phase assay).

The general procedure for S-phase suicide assay involved two single cell suspensions in 30 ml universal tubes (Sterilin, UK). Ara-C was routinely dissolved in the medium used for cell incubation. To the first single cell suspension 25 μg/ml Ara-C was added with equal volume of incubation medium added to the other cell suspension. Cell suspensions were either incubated for 1 hr at 37^{O}C in a water bath or when Ara-C was

added at the end of incubation period, cell suspensions were kept for 1 hour in the incubator. At the end of any incubation period, cell suspensions were washed twice in 20 mls of D20%HS PS/G by chill spinning at 1000 r.p.m (approximately 140G) for 10 minutes in a centrifuge (Fisons-MSE, UK) to remove all Ara-C in the supernatant.

Fig. 2.3 The S-phase suicide assay.



Afterwards cells were resuspended as a single cell suspension by gently passing them through a 25G yellow needle a few times using a 1 ml syringe. They were then ready to be counted and plated for haematopoietic stem cell or progenitor cell assays.

Colony numbers derived from cells incubated with Ara-C prior to the GM-CFC or HPP-CFC-1 assays and colony numbers from control cell suspensions determined the approximate percentage of cell in S-phase. The computer program calculated the difference in colony numbers derived from Ara-C incubated cell suspensions and control cell suspension. The difference in colony numbers was divided by control cell suspension colony numbers multiplied by 100 to give the proportion of cells in S-phase.

2.2.3 *AcSDKP assay*

Single cell suspensions were incubated in D30%FCS PS/G for up to 24 hours. AcSDKP levels were assayed before *in vitro* incubation and at 24 hours following incubation. For the initial measurements of AcSDKP, 2 mls of $5x10^6$ cells/ml in D30%FCS PS/G were transferred to two pairs of 10 mls plastic tubes (Sterilin, UK). Tubes were immediately centrifuged at 1000 r.p.m for 10 minutes. From each tube, 0.5 ml of the supernatant was aliquoted into three 2 mls polypropylene cryotubes (Corning, Cambridge, Canada). All cryotubes were kept at $-20^{O}C$ until needed.

AcSDKP levels in regenerating bone marrow following 2 Gy and cytotoxic drug treatment were measured after incubation. Single cell suspensions in D30%FCS PS/G were transferred to each of the four 30 mm non tissue culture grade petri dishes. Petri dishes were then placed in the incubator at $37^{O}C$ in 10% CO_2 fully humidified atmosphere. After 24 hours cell suspensions were transferred to labelled 10 mls plastic tubes. As above cells were centrifuged and the supernatant was aliquoted into polypropylene cryotubes and stored at $-20^{O}C$ until needed for assay. Supernatants placed

on ice were transported to Institut de Chimie des Naturelles in France where AcSDKP assay was performed.

Assays were done by diluting samples 1 in 3 with methanol and incubated for 1 hour at 4°C. The samples were centrifuged at 5000G for 15 minutes. The protein pellet was discarded and the collected supernatants were submitted to an enzyme immunoassay after evaporation according to the procedure described by Pradelles et al., 1990. In brief the supernatant was aliquoted into microtitre plates washed with 10^{-2} M phosphate buffer pH 7.4 containing 0.05% Tween 20. The reagents were dispensed as follows: 50 µl of standard AcSDKP or biological samples, 50 µl of diluted enzymatic tracer (1.3 Ellman units/ml) and 50 µl of AcSDKP diluted antiserum (Lot TP13, 1/30,000). Plates were then covered with adhesive sheet and left for 18 hrs at 4°C. Afterwards, plates were washed again and 200 µl of enzymatic substrate (Ellman's reagent) was added to each well. After 90 minutes of gentle shaking at room temperature in half-light, the absorbance at 414 nm in each well was measured automatically.

2.3 ACETYL-SER-ASP-LYS-PRO (AcSDKP) AND ANGIOTENSIN 1-CONVERTING ENZYME INHIBITORS (ACEI).

Two types of AcSDKP were used in this work: the natural synthetic peptide and a further peptide that has been rendered resistant to degradation by proteases at the Asp-Lys bond by replacing it with an aminomethylene ψ bond (Gaudron et al., 1997). These are referred to as AcSDKP and AcSDψKP respectively. AcSDKP (Mw = 487) (IPSEN-Biotech; Paris, France) and AcSDψKP (CNRS, Gif-sur-Yvette, France) were supplied as lyophilised samples and stored at –20°C. The peptides were dissolved in sterile distilled water, aliquoted in polypropylene cryotubes (Corning; Corning Costar Corporation,

Cambridge, Canada), and stored at –20°C. Each peptide was used once at a final concentration of 10^{-9} M.

Two angiotensin 1-converting enzyme inhibitors (ACEI) were studied. These were captopril and lisinopril, both purchased from Sigma, UK. The drugs were dissolved in the same medium used for cell incubation and tissue culture. Both were used at a final concentration of 1μM (Grillon et al., 1993a). However for *in vivo* injection, they were dissolved in saline (0.9% NaCl) (Antigen Pharmaceuticals, Ireland) and injected intraperitoneally (i.p) at either 10 mg/kg or at 100 mg/kg mouse body weight.

2.4 METHOD FOR COLLECTING PLASMA AND SERUM

Mice were killed by schedule 1 method and the abdominal skin was swabbed with 70% alcohol. Chest wall was cut in V-neck using a pair of sterile scissors. The chest organs were pushed to one side and the tips of scissors were passed to the posterior thoracic wall. An incision was made through the aorta without searching for it. Following which blood filled the chest cavity. Using cooled pasteurpippettes, blood was transferred into 1.5 mls Eppendorf tubes (Nunc, Denmark) with or without 20 microlitres of heparin (100 units) (CP Pharmaceuticals Ltd., UK) which were placed on ice. Samples were transferred into a microcentrifuge (Denver, UK) which was kept at 4°C and centrifuged at 7000 rpm for 10 minutes. Plasma was sampled from blood collected in the presence of heparin. Serum was collected from blood sampled in the absence of heparin. These samples were taken off as supernatants with a pasteurpipette after centrifuging. Samples were placed into 30 ml universal tubes ready for *in vitro* incubation. Approximately 3 mice produced 1 ml of sample.

2.5 METHOD FOR ASSAYING ACE ACTIVITY

A sigma kit method was used for this assay. This is a spectrophotometric method which utilises the synthetic tripeptide substrate N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG). ACE catalyses FAPGG to form FAP and Glycylglycine. Hydrolysis of FAPGG results in a decrease in absorbance at 340 nm. ACE activity in the sample is determined by comparing the sample reaction rate to that obtained with the ACE calibrator. The procedure measures the catalytic rate of the COOH domain of ACE.

The Sigma kit used was kept at 4°C. ACE calibrator was reconstituted according to the instructions in its package insert. 200µl of ACE reagent solution was pipetted into each of two cuvettes (UV range, disposable; Merck, UK) labelled 'Test' and 'Calibrator'. 20µl of plasma or serum to be tested was added to the test cuvette and 20µl of ACE calibrator was added to the calibrator cuvette. Samples were mixed by inversion. Cuvettes were placed in a water bath at 37°C for approximately 5 minutes. Following this initial incubation, cuvettes were placed in a spectrophotometer (Cecil Instruments, CE 594 Double Beam, UK) and absorbance of test and calibrator were determined at 340nm. After the initial reading, samples were immediately transferred back to the water bath. Approximately 5 minutes later a second set of absorbances was recorded. ACE activity was determined by the difference in absorbance of the test sample over 5 minutes divided by the difference in absorbance of ACE calibrator multiplied by activity of calibrator. The assigned value of activity of calibrator at 37°C was 40 units/l.

2.6 *IN VITRO* INCUBATIONS

2.6.1 *The effect of AcSDKP and captopril on GM-CFC proliferation*

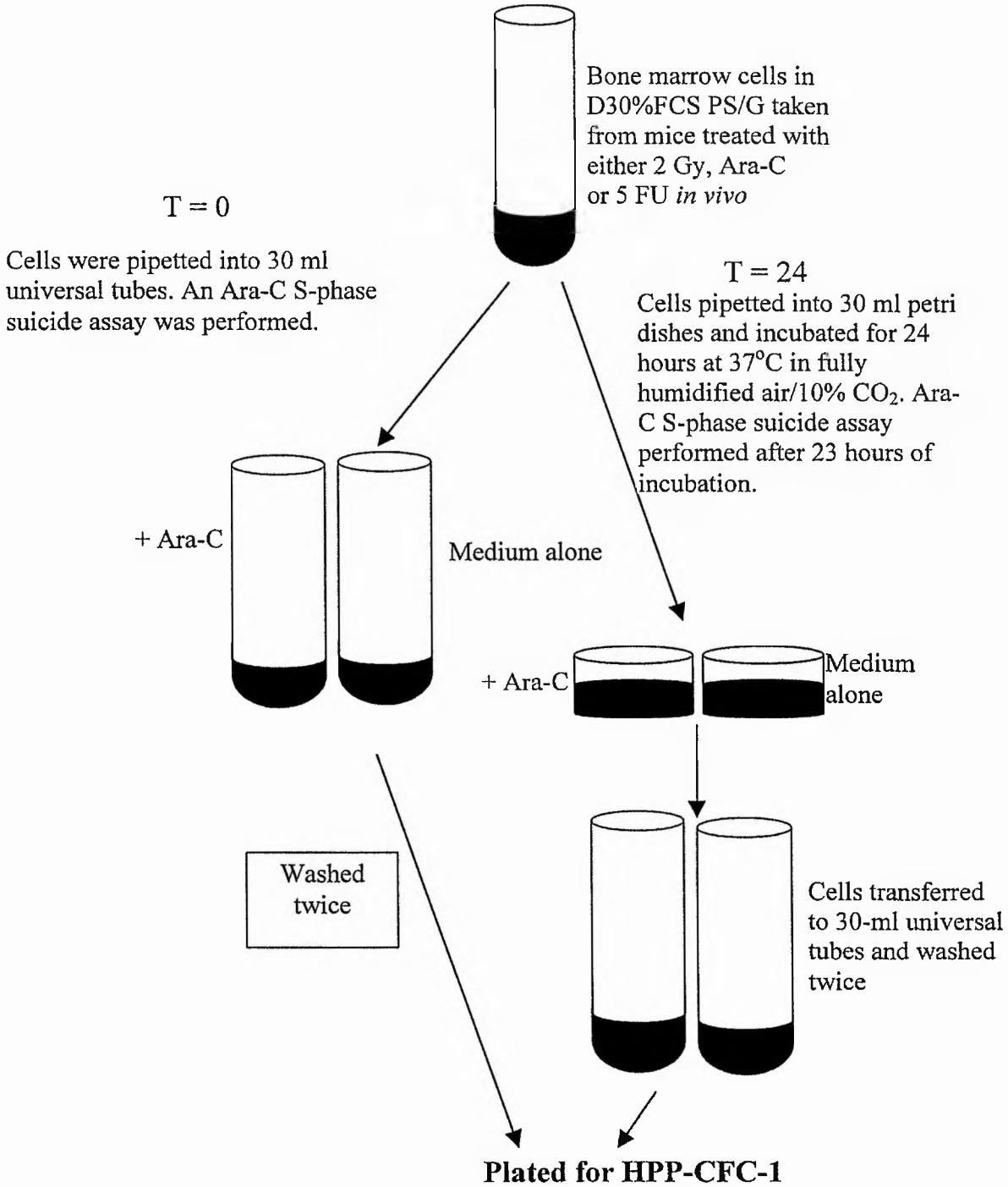
Bone marrow cells were flushed from femurs of CD1 mice using D30%FCS PS/G. A single cell suspension was suspended in the same medium and 2 mls was aliquoted into each of four 30 mm triple vent non tissue culture grade petri dishes. Dishes were then incubated at 37°C in 10% CO_2 in a fully humidified atmosphere for 7 hours. Ara-C suicide assay was carried out after 7 hours, making a total *in vitro* incubation time of 8 hours. At the end of the incubation period cells were transferred into 30 ml universal tubes and washed twice with D20%HS PS/G at 1000 r.p.m.(approximately 140G). A single cell suspension was made and the cellularity was adjusted to $5x10^5$ cells/ml. The single cell suspension was plated for GM-CFC.

2.6.2 *ACE inhibitors and AcSDKP on HPP-CFC-1 proliferation*

Following *in vivo* recruitment of haematopoietic stem cells into the cell cycle by 2 Gy-γ-irradiation, Ara-C (100mg/kg) i.p injection or 5 FU (150mg/kg) i.v injection, mice were killed and femoral bone marrow cells were sampled at appropriate times using D30%FCS PS/G. A single cell suspension was suspended in the same medium and samples containing $5x10^6$ cells/ml were incubated. Control bone marrow cells taken from mice that were not given cytotoxic insults were included.

A control short incubation period was initially done (T = 0). 2 mls of bone marrow cell suspensions in D30%FCS PS/G were transferred to each of the four universal tubes. An S-phase suicide assay was then performed. At the end of the suicide assay, cell suspensions were washed twice with D20%HS PS/G by centrifuging at 1000 r.p.m. (140G). A single cell suspension was made and cells were counted. After counting

Fig. 2.4 *In vitro* incubation of regenerating bone marrow cells with drugs.



the cellularity was adjusted to the number required for HPP-CFC-1 assay and cells were then plated.

The proportion of HPP-CFC-1 in S-phase was assessed after 24 hours of *in vitro* incubation (T = 24). 2 mls of bone marrow cell suspension in D30%FCS PS/G was transferred to each of four 30 mm triple vent nontissue culture grade petri dishes. Dishes were incubated at 37°C in a 10% CO_2 fully humidified atmosphere. After 23 hours of incubation, petri dishes were removed from the incubator and taken to a flow cabinet. Ara-C S-phase suicide assay was performed according to the general protocol. Dishes were then replaced in the incubator and incubated for a further 1 hour. At the end of the S-phase suicide assay, cell suspensions were transferred to 30 ml universal tubes and washed twice with D20%HS PS/G by centrifuging them. After washing a single cell suspension was made and cells were counted. The cellularity was adjusted for HPP-CFC-1 assay.

Supernatant from bone marrow incubated with drugs and their controls were assayed for AcSDKP concentration at the beginning and at the end of the incubation period. Cell suspensions were washed once at 1000 r.p.m and 0.5 mls from each of supernatants were aliquoted into 3 cryotubes and stored at –20°C and sent to France. The method used for *in vitro* incubation is summarised in Fig.2.4.

2.7 METHOD FOR INCUBATING SA2 JMB1 MYELOID LEUKAEMIC CELL LINE.

The myeloid leukaemic cell line (SA2 JMB1) was developed at the University of St. Andrews. Primary myeloid leukaemias were induced in 13 week old CBA/H male mice following exposure to whole-body doses of 3 Gy X-irradiation (250 kVp 14mA, 0.5 mm Cu at dose rate of 0.8 Gy/min). This is one of the clones developed after serial

passaging leukaemic cells in further groups of mice following i.p. injection of 1.5 x 10^6 cells (Hepburn et al., 1987). This cell clone is kept in liquid nitrogen at the University of St. Andrews.

SA2 JMB1 (SA2) at 10^5 cells/ml in cryotubes were thawed and drop by drop transferred into a 25-cm^2 tissue culture flask (Nunc, Denmark). 10 mls of F10%HS PS/G were slowly added to the flask. The flask was gassed with 5% CO_2/air to adjust pH and was incubated at 37°C for three days. This enabled cells to divide. After 3 days cells were counted on the Coulter counter. Due to the absence of red cells, no zaponin was added to Isoton-II. The Coulter settings were Current 700, Lower Threshold 30, Full Scale 1 and Attenuation 8.

SA2 were also incubated *in vitro* for (T = 0) and (T = 24). For control (T = 0), 2 ml of cell suspension (4 x 10^4 cells/ml) in D30%FCS PS/G were transferred to two pairs of universal tubes. 25μg/ml Ara-C for the S-phase assay was added to one tube of each pair. Cells were then incubated at 37°C in a water bath for 1 hour. After the incubation period cells were washed twice with D20%HS PS/G. Cells were counted and cellularity adjusted for the colony assay.

For *in vitro* incubation (T = 24), 2 mls of SA2 cell suspension (4 x 10^4 cells/ml) in D30%FCS PS/G were placed in each of four 30 mm nontissue culture petri dishes. Cells were incubated at 37°C /10% CO_2 in fully humidified air. After 23 hours of incubation, cultures were removed from the incubator and placed in a laminar flow hood. An Ara-C S-phase suicide assay was performed. After this, cell suspensions were transferred to labelled 30 ml universal tubes. They were washed twice with D20%HS PS/G and the cellularity was adjusted. They were then ready for plating in semi-solid agar cultures.

For agar cultures, cells were suspended in D20%HS PS/G (5 x 10^3 cells /ml) in 30-ml universal tubes. These were placed in a water bath at 37°C. 8 mls of D20%HS

PS/G was pipetted into each 30 ml universal tube and also placed in a water bath at 37°C. A bijoux bottle of 3 % (v/v) agar (Bacto Agar; Difco Labs; Detroit, MI) was placed in a 100 ml beaker with boiling water to melt the agar. 30 mm nontissue culture grade petri dishes (8 per group) were labelled and placed in the hood. Universal tubes containing medium alone were removed from the water bath and 1 ml of 3% agar was added to the universal tubes containing media followed by 1 ml of cell suspension. Cells and agar were thoroughly mixed by gentle inversion of the universals. 1 ml of the mixture was pipetted into each of the 30 mm nontissue culture grade petri dishes. Dishes were swirled around to spread the mixture equally over the base. No growth factors were used in this procedure. The 5 x 10^2 cells per dish with 0.3% agar were allowed to solidify for approximately 5 minutes. Incubation and counting procedure was identical to the one performed for GM-CFC assay in the general protocols.

2.8 *IN VIVO* EFFECTS OF ACE INHIBITORS ON HAEMATOPOEITIC STEM CELL PROLIFERATION

To test the effect of ACE inhibitors on haematopoietic stem cell cycling *in vivo*, two groups of animals were selected. Haematopoietic stem cell proliferation was induced in one group of mice by 2 Gy-γ-irradiation, Ara-C (100mg/kg) i.p injection or 5 FU (150mg/kg) i.v injection, the other group of mice did not receive cytotoxic stress. In general six animals were used per group. Three animals in each group received normal saline intraperitoneally (0.9% NaCl) at a dose proportional to the weight of the animal. The other three mice received ACE inhibitors that were dissolved in normal saline at the human therapeutic recommended dose for each ACE inhibitor. Therefore to test the effect of lisinopril or captopril on HPP-CFC-1 proliferation, mice received either a single dose at 10 mg/kg (Goa et al., 1996) or100mg/kg (Martin et al., 1984) 1-hour after irradiation. Animals were killed 24 hours following irradiation or Ara-C and at day 4 following 5 FU.

Femurs were removed and their contents flushed into 5 ml vials using D20%HS PS/G by the method described in the general protocol for sampling haematopoietic cells. Single cell suspensions were made in D20%HS PS/G and an S-phase suicide assay was performed. After 1 hour *in vitro* incubation, cells were washed twice in D20% HS PS/G, the cellularity adjusted to 2.5 x 10^5 cell/ml for HPP-CFC-1 assay.

2.9 CAPTOPRIL AND AcSDKP LEVELS *IN VIVO*

Captopril was administered 1 hour after 2 Gy γ-irradiation or Ara-C in the same way as performed in the HPP-CFC-1 *in vivo* assay. Plasma was sampled 1, 5, and 23 hours after captopril administration, which was 2, 6, 24 hours following cytotoxic insult. The method for plasma collection was the same as described for the *in vitro* ACEI studies. All plasma samples were collected on ice and stored at –20^{O}C in polypropylene cryotubes. They were then sent to France where AcSDKP concentrations were assayed. This assay was performed in the same way as the *in vitro* experiments described in the section for investigating the short and long-term effects of ACE1 on HPP-CFC-1 cycling following cytotoxic insults to the bone marrow.

2.10 HIGH OR LOW FRACTIONATED Ara-C DOSES

Six groups of mice each consisting of three mice per group were selected for this experiment. For low dose Ara-C fractionation, all animals received two doses of Ara-C at 200mg/kg body-weight 24 hours apart, making a total dose of 400mg/kg body-weight of Ara-C. Drugs were given intraperitoneally (i.p.). Three groups of mice received 100mg/kg body-weight captopril i.p. 1 hour after the first dose of Ara-C. The other three groups received normal saline (i.p.) at a dose equivalent to weight by volume. Animals

were killed at days 3, 7 and 10. The spleen from each mouse was excised after opening the abdomen in layers using sterile instruments and it was placed in a 5 ml tube and weighed on a scale (Sartorius, Germany). Afterwards, femurs were sampled. One femur from each mouse was initially made into a single cell suspension in D20%HS PS/G as described. Femur cellularities were determined for each femur as already described in section 2.1.5. Then all single cell suspensions were pooled from femur contents of each group of mice. Cells from each group were divided into two universal tubes. One tube was used for both GM-CFC and HPP-CFC-1 numbers and the other was used for determining the percentage of HPP-CFC-1 in S-phase for both GM-CFC and HPP-CFC-1 on a pooled cell suspension using methods already described in the colony assay section and the Ara-C S-phase suicide assay section.

To test the effect of a high-fractionated dose of Ara-C, Ara-C was administered in two doses of 400mg/kg (i.p) body-weight to make a total dose of 800mg/kg body weight Ara-C. The second dose was given 24 hours after the first dose to all six groups of mice. Captopril at 100mg/kg body-weight (i.p.) was given to three groups of mice 1 hour after the first dose. The three other groups received saline i.p. at the same time as the other mice received captopril. As in the previous investigation of low dose Ara-C, spleen weights, femur cellularity and the proportion of both HPP-CFC and GM-CFC in S-phase were assessed.

2.11 ESTABLISHMENT OF LONG-TERM BONE MARROW CULTURES

All haematopoietic cells for establishing long-term bone marrow cultures were taken from femurs of eight to twelve year old crossbred mice (CBA/H x C57BL) F1. Animals were killed by cervical dislocation and their femurs were dissected out. The

femur cellularity was approximately 1.2 x 10^7 nucleated cells per femur. One femur was used to set up one flask of LTBMC.

In a laminar flow hood with sterile instruments excess muscle was removed from femurs and the marrow of each femur flushed with F20%HS PS/G Hydro into 25-cm^2 conical flask, using a syringe with a 21 gauge blue tipped needle. No attempt was made to make a single cell suspension. Six flasks were set up for each protocol. To adjust pH, all flasks were gassed with 5% CO_2 for approximately 2 minutes and incubated at 33^{o}C. Total media change, addition of fresh drugs, and gassing was performed once a week. Establishment of the adherent layer was observed with an inverted microscope. Cells from each protocol were pooled and transferred into 100 ml conical glass beakers (Pyrex, UK) for analysis. The adherent layer cells were scraped from the bottom of the flasks by a disposable cell scraper (Greiner, UK). They were then suspended in 10 mls F20%HS PS/G hydro and transferred to a conical glass flask and analysed.

The procedures for determining flask cellularity in the non-adherent layers were the same as for counting bone marrow cells described in section 2.1.5. A sample of pooled cells from each group was put in 20 ml Isoton-II in plastic vials ready for Coulter counting. Coulter settings were different from those described with fresh bone marrow cell (see table 2.1) and no Zaponin was added in the 20 ml clear vials since there were no red cells to be destroyed. Adherent layer cells were counted in the same way as the non-adherent layer cells.

After counting, 2 mls taken from each pooled sample were transferred to each of the six universal tubes. Four universal tubes were used for HPP-CFC and GM-CFC suicide assay in the same way as described in the general protocol. One of the other two universal tubes was used for the HPP-CFC colony numbers and the other for GM-CFC colony numbers. The feeder layer for HPP-CFC was made up with conditioned medium

(10% v/v WEHI and L929 combined) and the GM-CFC were done in the same way as the rest of the investigations.

2.12 DATA ANALYSIS.

The data are presented in tables followed by bar graphs with standard errors of the means throughout. Each investigation was done in the presence of a control that was treated in the same way as the experimental sample. All *in vivo* experiments included animals that were not given any cytotoxic insults except for experiments where fractionated doses of Ara-C were used. In this group only 1 set of normal mice was used to compare all experiments. The testing of AcSDKP *in vivo* levels relative to cytotoxic insults and captopril entailed injecting control mice with saline before giving them captopril or saline. This controlled the effect of general stress on animals on AcSDKP before or after captopril.

Cell counting on a Coulter counter was performed three times from each sample. For Ara-C fractionated experiments three spleens per group were measured for weight. Four individual experiments were carried out for each investigation throughout all experiments.

For the GM-CFC Ara-C S-phase assay, eight individual petri dishes were plated from control cells and eight petri dishes from cells incubated in the presence of Ara-C. Individual colony numbers from each of eight petri dishes were entered into a computer programme. Control colony numbers were entered first followed by colony numbers obtained from Ara-C incubated bone marrow. The computer calculated the proportion of cells in S-phase from these colony numbers by dividing the difference in colony numbers by control colony numbers and multiplying the result by 100. Four individual repeats were done per investigation. Therefore, there were 64 petri dishes that were pooled from four different experiments for any GM-CFC S-phase investigation and about 32

individual petri dishes were pooled when GM-CFC numbers were evaluated. The same procedure was executed with SA2 leukaemic cells. However, no absolute numbers of SA2 cells were evaluated. GM-CFC numbers were calculated as follows: A = Y x $P/5x10^4$. Where A = GM-CFC numbers, Y = number of colonies and P = either mean femur cellularity or mean flask cellularity.

For the HPP-CFC Ara-C S-phase assay, four individual petri dishes were routinely set up from the control cells and a further four from the cells incubated with Ara-C. To assay HPP-CFC numbers, eight petri dishes were used. All individual experiments were repeated four times. Therefore 32 individual petri dishes were made from both control and Ara-C incubated cell suspension per investigation. In addition 32 dishes in all from four individual experiments were prepared where HPP-CFC numbers were assayed. The HPP-CFC numbers were calculated by the following formula. Z = Y x $P/5x10^4$. Where Z = HPP-CFC numbers, Y = number of colonies and P = either mean femur cellularity or mean flask cellularity.

Four independent repeat experiments were also carried out for the analysis of ACE activity following *in vitro* incubation of plasma or serum with ACEI. For *in vitro* haematopoietic cell incubation with ACEI or AcSDKP, AcSDKP levels were analysed in three individual experiments. In each experiment, samples were collected in three cryotubes. Three individual repeat experiments were carried out for *in vivo* regulation of AcSDKP levels following insults and captopril. Two cryotubes were routinely analysed for AcSDKP.

2.12.1 *Statistics*

For any S-phase assay, the values from each experiment were fed into a special statistical computer program which calculated the percentages of cells in S-phase. The

number of colonies from control petri dishes were initially fed into the computer followed by those from Ara-C treated petri dishes. The computer calculated the means of both sets of values ± standard errors. The percentages of cells in S-phase were also printed out. The programme assumed that values were normally distributed. The percentage in S-phase was calculated as a proportion of the difference between control (A) values and the values from Ara-C treated (B) values divided by the control values multiplied by 100 {(A-B)/A x 100}. In the result sections, the proportions in S-phase and numbers of both GM-CFC and HPP-CFC assays are presented together with standard errors. This final mean was calculated from the pooled means of all four experiments.

Two statistical tests were routinely performed in these investigations. These were the two-tailed Student t-test and Analysis of Variation (ANOVA). The significant p-value was set at less than 0.05. The two-tailed Student t-test was routinely used to determine the significance of the difference between the effect of a drug (i.e, ACEI or AcSDKP) relative to the controls. This test was used when 2 groups of samples needed to be compared. The student t-test was also used to analyse the changes in AcSDKP levels following *in vitro* incubation of regenerating bone marrow cells in the presence of captopril. The Student t-test was calculated with the aid of Excel for Windows 1995 Version 8.

In the long-term bone marrow cultures, AcSDKP, captopril and the combination of the two were investigated to see if each individual treatment had an effect relative to saline control and between different treatments. The test used to analyse these differences was ANOVA. If the means were found to be statistically significant after carrying out ANOVA, the Tukey test was done. ANOVA was also used to analyse the differences in AcSDKP levels following *in vivo* administration of captopril in mice after Ara-C and 2 Gy irradiation. ANOVA was carried out also with the aid of the Excel computer program.

CHAPTER 3

3.0 *IN VITRO* STUDIES ON THE EFFECT OF ACE ON HAEMATOPOIESIS

Serum and plasma from CBA/H mice was analysed to study the efficiency of ACE inhibitors in blocking ACE activity *in vitro*. This was done to establish a basis for studying their effects on haematopoietic cell proliferation. *In vitro* incubation with bone marrow cell suspension was performed in the presence of D30%FCS PS/G. This concentration of FCS was chosen because it reduced the *in vitro* cell loss following incubation. D30%FCS PS/G was not investigated for ACE activity as it was assumed that the levels of ACE activity would be modified by presence of macrophages in the bone marrow cell suspension. Macrophages are known to produce both ACE (Costerousse et al., 1993) and AcSDKP (Li et al., 1997).

It is well established that AcSDKP prevents the transition from G_0-G_1 into S-phase in proliferating cells (Mopezat & Frindel, 1989). It has been shown previously that AcSDKP has no effect on GM-CFC proliferation following a short *in vitro* incubation in serum free medium (Robinson et al., 1992). An investigation was carried out to determine if *in vitro* incubation with AcSDKP and captopril together in the presence of D30%FCS PS/G would affect the cycling status of GM-CFC from normal bone marrow. As AcSDKP is degraded by the N-domain ACE active site (Rousseau et al., 1995), captopril was used to prevent AcSDKP degradation. It has been shown previously that captopril on its own has no effect on the proliferation of GM-CFC after several days of incubation (Hammond et al., 1988). Therefore, a control incubation with captopril alone was not performed.

After studies on GM-CFC proliferation were performed a single bone marrow cell suspension was incubated from mice that had not received any insults and from

mice treated with 2 Gy-γ-irradiation, Ara-C (100 mg/kg i.p) and 5 FU (150 mg/kg i.v). 2 Gy-γ-irradiation is the standard dose used to recruit HPP-CFC-1 into cell cycle in our laboratory (Rousseau et al., 1998). Ara-C (100 mg/kg) produced least myelotoxicity and 5 FU (150 mg/kg) (Bartelmez et al., 1989) enriches for the HPP-CFC-1 population. ACE inhibitors were investigated on their ability to influence the proportion of HPP-CFC-1 in S-phase in normal bone marrow cells in order to determine if they could alter the proliferative status of this cell population. Cytotoxic insults increase the levels of stimulatory factors *in vivo* (Ali et al., 1989; Frindel et al., 1976). These stimulatory factors recruit HPP-CFC-1 into S-phase. By this method, inhibitors of stem cell proliferation can be studied. Therefore, the ability of ACE inhibitors to modify stem cell kinetics was studied following *in vivo* cytotoxic insults. The supernatant of bone marrow cell suspension incubated with captopril was analysed for endogenous AcSDKP concentration before and after incubation.

To determine whether captopril might have differential effects on normal and neoplastic cell populations, this drug was incubated with SA2 neoplastic cells. SA2 cells were chosen because they were easily accessible. This investigation was performed to assess the feasibility of using captopril to protect normal haematopoietic cells while making neoplastic cells vulnerable to cytotoxic agents during tumour therapy.

Because AcSDKP has been found to inhibit CFU-S proliferation only if given 2 hours following Ara-C, it has been suggested that AcSDKP given at this point blocks G0-G_1/S transition (Monpezat & Frindel, 1989). However, this has not been previously elucidated. Therefore an investigation was carried out to test the mode of action of AcSDKP *in vitro* and to confirm the fact that the peptide may be active at the G_1/S boundary. This hypothesis was tested using proliferating HPP-CFC-1.

3.0.1 *Ace activity in murine serum and plasma*

Procedure

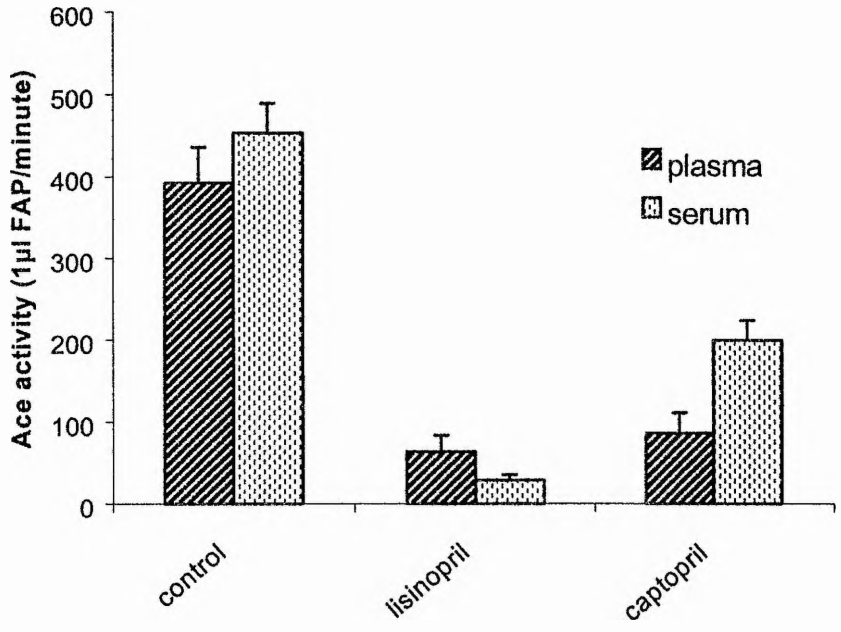
The ability of ACE inhibitors to inhibit background ACE activity was analysed before these drugs were studied for haematopoiesis. This was investigated *in vitro* by incubating plasma or serum with ACE inhibitors. Plasma or serum samples collected from CBA/H mice were incubated with captopril or lisinopril both at 1 μM final concentration for 2 hours in a water bath at 37°C. Control samples that were incubated with medium alone were included. After 2 hours samples were placed on ice to stop the enzymatic activity. Samples were then analysed for ACE activity using a kit from Sigma Diagnostics as described in materials and methods.

Results

As shown in Fig 3.1, ACE activity was high and identical in serum and plasma from normal mice after 2 hours of *in vitro* incubation ($p = 0.75$). Incubating both serum and plasma in the presence of ACE inhibitors resulted in a significant reduction in the activity of ACE ($p<0.00001$). However both ACE inhibitors equally inhibited ACE activity in plasma with no observable difference between captopril and lisinopril ($p = 0.34$). In contrast, there was a difference between captopril and lisinopril on ACE activity in serum. Lisinopril had a greater inhibitory effect on serum ACE than captopril. While only 28.6 ± 6.3 units of ACE activity was observed after 2 hours of serum incubation with lisinopril, 198.7 ± 24.3 units of ACE activity was observed when serum was incubated with captopril ($p = 0.00001$). While lisinopril had no differential inhibitory effect between serum or plasma ACE activity, captopril showed

FIG. 3.1 ACE activity in serum and plasma following incubation with ACE inhibitors

	Units (1μM FAP/minute)		
	plasma	**serum**	
control	392.9 ± 43.3	453.3 ± 36.0	P = 0.75, n = 4
lisinopril	63.2 ± 19.9	28.6 ± 6.6	
captopril	86.0 ± 24.5	198.7 ± 24.3	
	P = 0.34, n = 4	P = 0.00001, n = 4	



a reduced inhibitory effect on serum ACE when compared to plasma ACE. From these observations, AcSDKP levels were routinely analysed from plasma in all the *in vivo* experiments.

3.0.2 *AcSDKP and GM-CFC proliferation*

Procedure

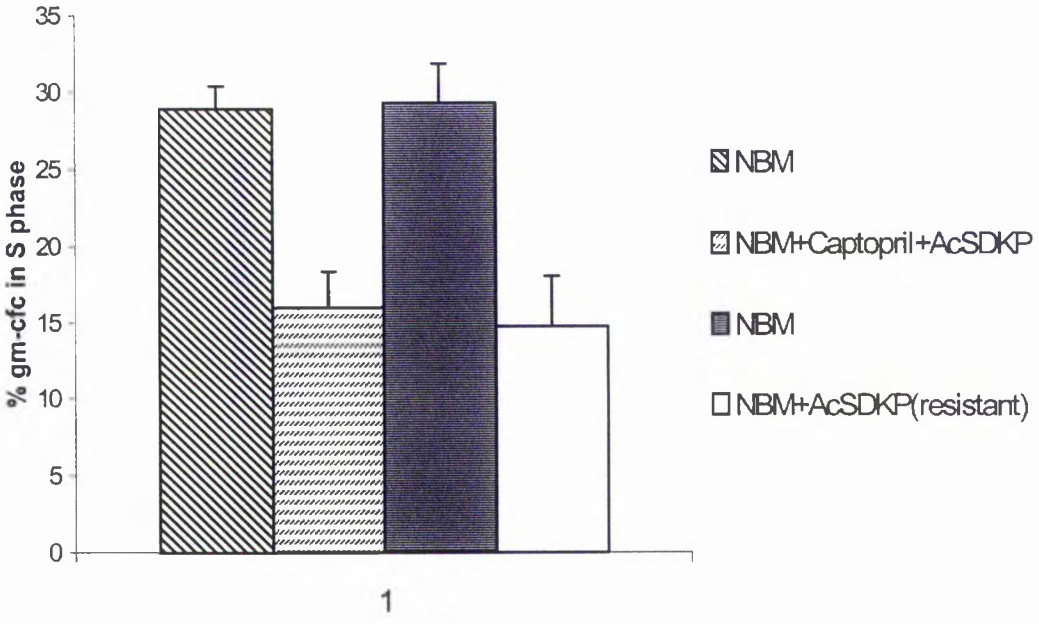
2 mls of bone marrow single cell suspensions suspended in D30%FCS PS/G from untreated CD1 mice were aliquoted into four universal tubes at 5 x 10^6 cells/ml. AcSDKP (10^{-9} M) together with captopril (1μM) was added to one pair and the other pair was used as a control. Cells were incubated at 37°C for 7 hours. After 7 hours of incubation an S-phase suicide assay was performed making a total incubation time of 8 hours. At the end of incubation period cells were washed and the cellularity was adjusted for GM-CFC assay. AcSDKP that has been made resistant to degradation by ACE by replacing the DK bond with an aminomethylene bond (AcSDψKP) (Gaudron et al., 1997) was also investigated without the addition of captopril.

Results

As shown in Fig 3.2, (28.9 ± 1.5)% of GM-CFC were in S-phase in the control groups and this percentage was reduced to (16.0 ± 2.4)% ($p = 0.0011$) when bone marrow cells were incubated with AcSDKP and captopril together after 8 hours. In the experiments where AcSDψKP was investigated, (29.3 ± 2.5)% of GM-CFC were in S-phase in the control group. This percentage was reduced to (14.7 ± 3.3)% ($p = 0.004$) in the presence of AcSDψKP after 8 hours. There was no difference between the percentage of GM-CFC in S-phase in the presence of AcSDKP together with captopril and with AcSDψKP alone.

FIG. 3.2 The proportion of GM-CFC in S-phase following incubation of bone marrow cells with captopril together with AcSDKP or AcSDψKP alone

Normal bone marrow	AcSDKP + Captopril	Resistant AcSDKP	
(28.9 ± 1.5)%	(16.0 ± 2.4)%		P = 0.0011, n = 4
(29.3 ± 2.5)%		(14.7 ± 3.3)%	P = 0.004, n = 4



3.0.3 Discussion

3.0.3.1 *Differential effects of captopril and lisinopril on serum and plasma ACE activity*

These investigations showed that both captopril and lisinopril were potent inhibitors of plasma ACE activity. Lisinopril had no differential effect on inhibiting ACE in both plasma and serum while captopril was less efficient at inhibiting serum ACE (see Fig 3.1). The superiority of lisinopril to captopril as an inhibitor of serum ACE activity from rats and humans has also been observed by others (Nguyen et al., 1994, Small Jr et al., 1997). This difference in potency between lisinopril and captopril in inhibiting ACE activity in serum and plasma is not well understood.

However, it is known that there is a difference between the relative capacity of ACE inhibitors to inhibit ACE in different organs (Bevilacqua et al., 1996). As reviewed by Herman (1992), the affinity of the ACE inhibitor for a particular enzyme is not only determined by the structure of the inhibitor, but also by the structure of the enzyme. ACE isoenzymes from different tissues may be slightly different, varying with regard to their molecular weight, amino- and carboxyl-terminal amino acids in their structure and also their degree of glycosylation.

Different active ACE isoenzymes in plasma and serum samples might explain the observed differential inhibitory effects of captopril and lisinopril on serum ACE. Heparin used in the collection of plasma may alter the glycosylation of this enzyme. Lisinopril and fosinopril have been found to have a more consistent effect on inhibiting ACE from all tissues (Herman, 1992). Lisinopril could be exhibiting an equal potency on isoenzymes in plasma and serum while captopril may affect these isoenzymes differently.

As reviewed by Beneteau-Brunat and Baudin (1991), ACE is mostly located on the luminal surface of the endothelial cells as an ectoglycoprotein. ACE activity measured in plasma is mostly of endothelial origin. Endothelial ACE may have the same affinity for both captopril and lisinopril. This may be one factor that brings about their equal potency in inhibiting plasma ACE. In addition ACE inhibitors have different tissue bioavailability making them show differential effects on tissue ACE in organs (Herman, 1992). There is also a difference in structure between captopril and lisinopril in that captopril uses its sulfhydryl group to bind to the zinc atom in the enzyme. Nevertheless, lisinopril has a greater affinity for ACE than captopril (Herman, 1992) resulting in equal inhibition of ACE activity in both serum and plasma in the present investigations.

There is also a possibility that lisinopril might exhibit a dual effect of inhibiting other enzymes that may be involved in FAPGG catabolism in addition to mouse ACE. This hypothesis is strengthened by the fact that lisinopril and peridopril inhibit a kininase that is neither ACE nor aminopeptidase P (Campbell et al., 1995). Captopril is a more potent inhibitor of the N-domain ACE active site while lisinopril favours the COOH domain ACE active site (Wei et al., 1992, Rousseau et al., 1995, Bevilacqua et al., 1996). It is possible that the physiological nature of serum means that the required electrolyte balance in the assay favours the inhibition of COOH domain ACE active site compared to the N-domain ACE active. Since bradykinin has an approximately 100 fold affinity for ACE compared to angiotensin-1 and is mainly cleaved by the COOH domain active site (Campbell, 1995, Dorer et al., 1974), lisinopril may be binding to the ACE active site that may be responsible for both FAPGG and bradykinin hydrolysis. This may reveal the strength of lisinopril as an inhibitor of ACE activity in plasma and serum.

3.0.3.2 *AcSDKP and GM-CFC proliferation*

In the present investigations AcSDKP reduced the proportion of GM-CFC in S-phase in bone marrow cells taken from untreated mice after 7 hours of *in vitro* incubation. In this investigation, the AcSDKP inhibitory effect on GM-CFC proliferation was shown not be related to captopril by the equal potency of GM-CFC inhibition in the presence of AcSDKP and captopril when compared to AcSDψKP alone. Therefore the reduction in the proportion of GM-CFC in S-phase in both cases was thought to be due to the effect of AcSDKP on GM-CFC proliferation. Others have shown that AcSDKP inhibits human progenitor cell proliferation *in vitro* (Guigon et al., 1990). It is not surprising that a short incubation period with AcSDKP

did not inhibit GM-CFC proliferation in previous investigations. The reason for this may be two fold. Firstly, the fact that AcSDKP acts exclusively at the G_0-G_1/S boundary (Mopezat & Frindel, 1989) means that its effect on cell proliferation would be noticed after proliferating cells have exited S-phase. Secondly the short $t_{1/2}$ of AcSDKP in serum prompted Robinson et al., (1992) to use serum free medium which may have retarded the progression of cells that were already in S-phase out of this phase.

Despite no observable effect of AcSDKP on the proliferation of GM-CFC both *in vitro* following a short incubation period and *in vivo* from murine bone marrow cells (Robinson et al., 1992, Monpezat & Frindel, 1989), some investigators observed an effect of the peptide on murine GM-CFC proliferation after cells were incubated for extended periods of time *in vitro* (Jackson et al., 1996, Wierenga et al., 1996). While Wierenga et al., (1996) used normal bone marrow cells without growth factors for a 24 hour-incubation period, Jackson et al., (1996) incubated normal bone marrow cells in the presence of growth factors for up to 72 hours. Wierenga et al., (1996) found a maximum inhibitory effect of AcSDKP and captopril (1 μM) after 8 hours of incubation. Jackson et al., (1996) found that addition of growth factors during incubation reduced the concentration of AcSDKP needed to inhibit GM-CFC proliferation from 10^{-12} M to 10^{-14} M.

30% of GM-CFC are in S-phase at any one time (Blackett et al., 1974; Wierenga et al., 1996). In addition, GM-CFCs are inhibited by a higher concentration of inhibitory factors than CFU-S *in vitro* (Tejero et al., 1984). From these observations it is clear that a long duration is needed for AcSDKP to inhibit GM-CFC proliferation. Moreover, the length of the cell cycle of GM-CFC will determine the time it takes for the peptide to show its effect. In these investigations the proportion of

GM-CFC in S-phase was reduced after 7 hrs. Wierenga et al., (1996), also observed a decrease in the proportion of GM-CFC in S-phase with AcSDKP to (9 ± 3)% at 8 hours. The proportion of GM-CFC in S-phase had increased to (18 ± 3)% after 16 hours incubation and remained so up to 24 hours of *in vitro* investigation. This suggests that the block of G_0-G_1/S transition on GM-CFC by AcSDKP is weak or reaches a peak. This means that any additional incubation time does not affect GM-CFC proliferation. Therefore the effect of AcSDKP on GM-CFC may be more complicated than previously thought.

In vivo studies on haematopoietic cell proliferation have shown that AcSDKP is a better inhibitor of CFU-S than GM-CFC when administered to mice following Ara-C treatment. Monpezat & Frindel, (1989) found that Ara-C recruits CFU-S into S-phase after 12 hours and administration of AcSDKP 6 hours after Ara-C prevents CFU-S recruitment into S-phase but not GM-CFC and no inhibitory effect was observed when AcSDKP was given 8 hours after Ara-C on both cell populations. In addition, Bogden et al., (1991), showed that AcSDKP protected mice when it was administered simultaneously with Ara-C or 2 hours after Ara-C treatment. The findings of Bogden et al., (1991) and Monpezat & Frindel, (1989), demonstrate the specificity of AcSDKP action at the G_0-G_1/S transition and the specific response of haematopoietic stem cells to AcSDKP inhibitory effects.

It is possible that AcSDKP may affect the G_0-G_1/S transition of haematopoietic cells by down regulating growth factor receptors or by blocking intracellular signalling pathways critical at the G_1/S transition (Yip & Levy, 1996). AcSDKP seems to function by interacting with glycoproteins on some responsive cells and the sequence SDK may be important in this interaction (Thierry et al., 1990; Robinson et al., 1993). Furthermore AcSDKP may inhibit

cells by influencing the release of inhibitory factors by accessory cells (Cashman et al., 1995). It has been found that AcSDKP enhances engraftment of intravenously transplanted haematopoietic stem cells into bone marrow of irradiated mice (Suzuki et al., 1998) an effect that involves accessory cells.

3.1 *IN VITRO* EFFECT OF AcSDKP AND ACE INHIBITORS ON HPP-CFC-1 PROLIFERATION

3.1.1 *Normal bone marrow*

Procedure

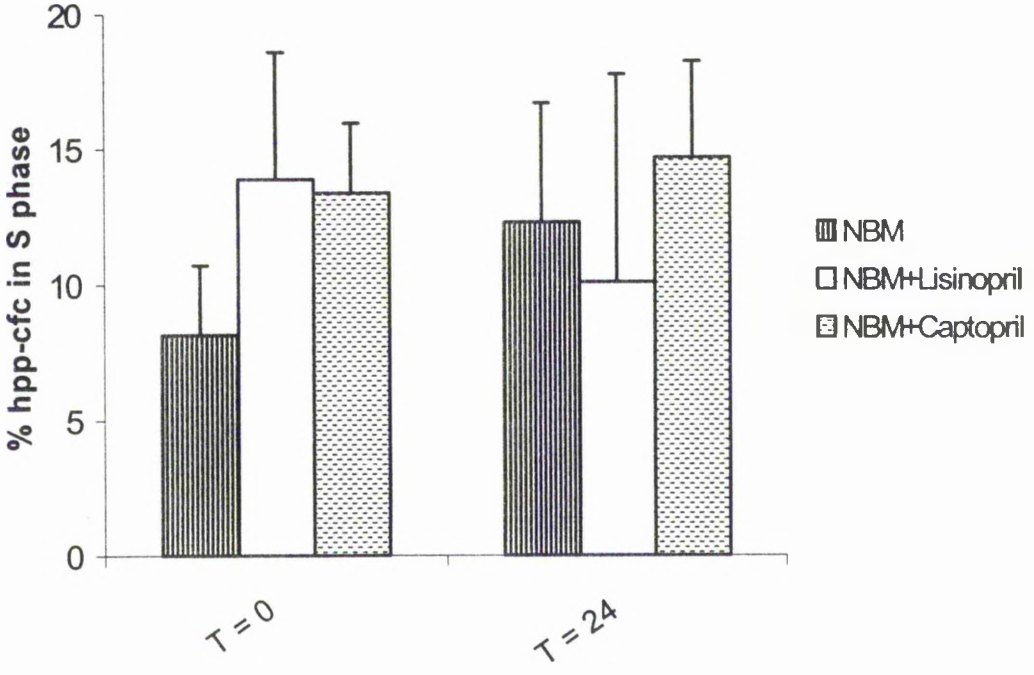
A bone marrow single cell suspension sampled from 3 untreated CD1 mice was suspended in D30%FCS PS/G. Some cells were immediately incubated with captopril or lisinopril both at 1μM separately for (T = 0) together with controls. The remaining cell suspension was aliquoted into each of the four petri dishes for (T = 24). Captopril or lisinopril were added to two petri dishes. The other dishes were used as controls. Control bone marrow cell suspensions were incubated with medium alone. An S-phase assay was performed according to the *in vitro* incubation methods already described. At the end of the incubation periods, the cellularity was adjusted and cells were plated for the HPP-CFC-1 assay as described.

Results

As shown in Fig 3.3, (8.1 ± 2.6)%, (13.6 ± 2.6)% and (13.9 ± 4.4)% of HPP-CFC were in S-phase in the control samples, in the presence of captopril and lisinopril respectively at the beginning of incubation period (T = 0). This proportion was not significantly altered after 24 hours (T = 24) of incubation. (12.3 ± 4.7)% of HPP-CFC-1 were in S-phase in control samples, (14.7 ± 3.5)% of HPP-CFC-1 were in S-

FIG. 3.3 The proportion of HPP-CFC-1 in S-phase following *in vitro* incubation of bone marrow cells from untreated mice with ACE inhibitors

Normal bone marrow	% HPP-CFC-1 in S-phase T = 0	T = 24	
control	(8.1 ± 2.6)%	(12.2 ± 4.7)%	P = 0.58, n = 4
lisinopril	(13.9 ± 4.4)%	(10.1 ± 7.7)%	P = 0.72, n = 4
captopril	(13.4 ± 2.6)%	(14.7 ± 3.5)%	P = 0.84, n = 4



phase in the presence of captopril and (10.1 ± 7.7)% of HPP-CFC-1 were in S- phase in the presence of lisinopril ($p>0.5$) after 24 hours of incubation. Therefore *in vitro* incubation of normal bone marrow cells in the presence of ACE inhibitors showed no influence on the proliferation of HPP-CFC-1 in normal bone marrow cells.

3.1.2 *Regenerating bone marrow HPP-CFC-1 proliferation*

Procedure

Bone marrow cells from mice that had received 2 Gy γ-irradiation, Ara-C (100mg/kg-body weight i.p injection.) and 5 FU (150mg/kg-body weight i.v injection.) *in vivo* were sampled with D30%FCS PS/G at appropriate times. A single cell suspension was made with the same medium and an S-phase suicide assay was immediately performed on half of the cells in the presence or absence of ACE inhibitors with or without AcSDKP. The other half were aliquoted into four 30-mm petri dishes. Following 2 Gy γ-irradiation, bone marrow cells were incubated with AcSDKP (10^{-9}M) alone or in combination with one of the ACE inhibitors (1μM), captopril or lisinopril alone both at 1μM individually were added to one pair. The other pair was used as a control to which medium alone was added. Bone marrow cells taken from mice treated with Ara-C or 5 FU were incubated with ACE inhibitors alone. Cells were incubated for (T = 0) and (T = 24) according to the described method. At the end of the incubation period, an S-phase was carried out and cells were washed and plated for HPP-CFC-1.

Results

i. 2 Gy regenerating bone marrow

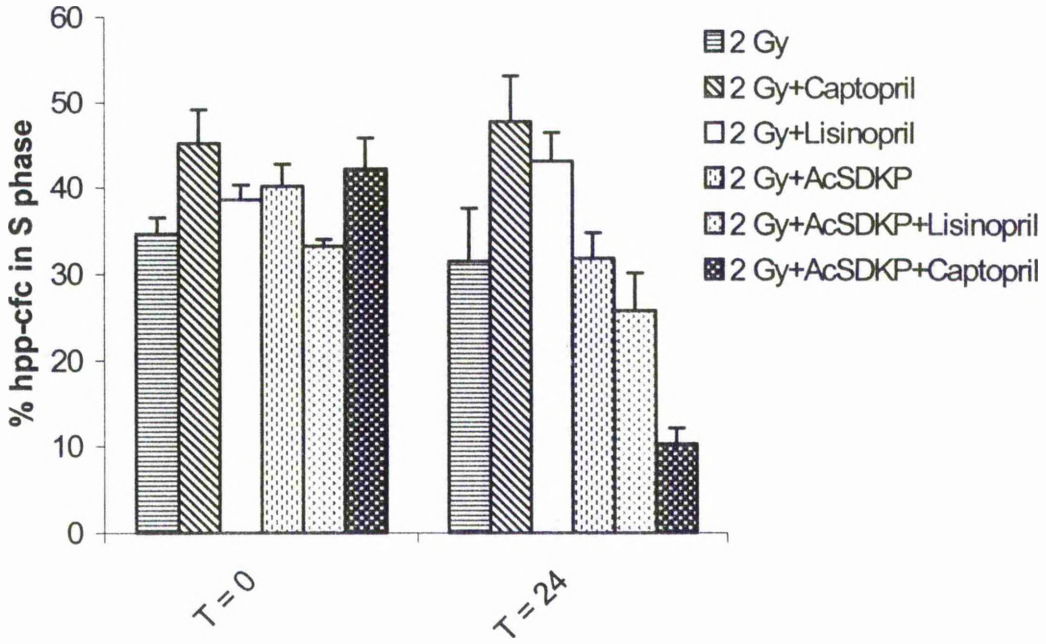
As shown in Fig 3.4, (34.6 ± 1.9)% HPP-CFC-1 were in S-phase in 2 Gy bone marrow at beginning of incubation (T = 0). In the presence of lisinopril and captopril, (38.6 ± 1.7)% and (45.2 ± 6.2)% of HPP-CFC-1 were in S-phase at the same time respectively. In the presence of AcSDKP alone, (40.2 ± 2.6)% of HPP-CFC-1 were in S-phase at the beginning of the incubation period. After 24 hours of incubation

(T = 24), (31.5 ± 4.0)% of HPP-CFC-1 were in S-phase in 2 Gy regenerating bone marrow cell suspension (p = 0.34) and (43.1 ± 3.4)% of HPP-CFC-1 were in S-phase in the presence of lisinopril (p = 0.28). (47.7 ± 5.4)% and (31.8 ± 3.0)% of HPP-CFC-1 were in S-phase in the presence of captopril (p = 0.46) and AcSDKP (p = 0.09)

FIG. 3.4 The proportion of HPP-CFC-1 in S-phase in bone marrow cells taken from mice treated with 2 Gy following *in vitro* incubation with ACE inhibitor and AcSDKP

2 Gy regenerating bone marrow	% HPP-CFC-1 in S-phase		
	T = 0	T = 24	
Medium alone	(34.6 ± 1.9)%	(31.5 ± 4.0)%	P = 0.34, n = 4
+Captopril	(45.2 ± 6.2)%	(47.7 ± 5.4)%	P = 0.46, n = 4
+Lisinopril	(38.6 ± 1.7)%	(43.1 ± 3.4)%	P = 0.28, n = 4
+AcSDKP	(40.2 ± 2.6)%	(31.9 ± 3.0)%	P = 0.09, n = 4
+AcSDKP&Lis.	(33.2 ± 0.8)%	(25.7 ± 4.4)%	P = 0.06, n = 4
+AcSDKP&Cap.	(42.1 ± 3.7)%	(10.3 ± 1.9)%	P = 0.005, n = 4

NB: Lis. = lisinopril, Cap. = captopril



respectively at the end of the incubation period (T = 24). Thus no significant inhibitory effect was observed on 2 Gy regenerating bone marrow HPP-CFC-1 when AcSDKP or ACE inhibitors were investigated individually. Therefore both AcSDKP and ACE inhibitors showed no direct inhibitory effect on 2 Gy irradiation when they were used separately.

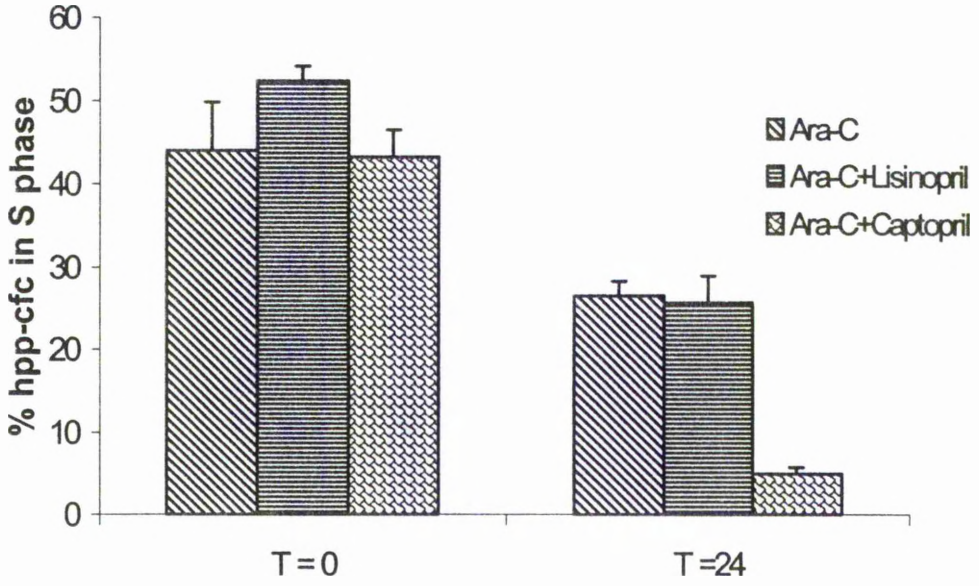
Both ACE inhibitors were incubated in combination with AcSDKP for 24 hours. As shown in Fig. 3.4, there was no significant reduction in proportion of HPP-CFC-1 in S-phase at the end of incubation period when lisinopril and AcSDKP were combined. The initial proportion of HPP-CFC-1 in S-phase was (33.2 ± 0.8)%, and it was (25.7 ± 4.4)% after 24 hours of incubation ($p = 0.06$). In the presence of captopril and AcSDKP, the initial proportion of HPP-CFC-1 in S-phase was (42.1 ± 3.7)%. This proportion was significantly reduced after 24 hours of incubation to (10.3 ± 1.9)% ($p = 0.005$). Therefore AcSDKP was crucial in inhibiting HPP-CFC-1 in 2 Gy regenerating bone marrow cells. Captopril was able to restore the AcSDKP inhibitory activity.

ii. Ara-C regenerating bone marrow

(44.0 ± 5.9)% of HPP-CFC-1 were initially in S-phase from bone marrow cell suspension of Ara-C treated mice. In the presence of lisinopril the proportion of HPP-CFC-1 in S-phase was (52.3 ± 1.8)% at time 0. In the presence of captopril, (43.2 ±3.2)% were in S-phase at the same time (see Fig.3.5). At the end of 24 hour incubation period the proportion of HPP-CFC-1 in S-phase in Ara-C regenerating bone marrow control cell suspension was reduced to (26.5 ± 1.8)% ($p = 0.0009$) and to (25.6 ± 3.3)% ($p = 0.00012$) in the presence of lisinopril. While incubation alone reduced the cycling proportion of HPP-CFC-1 in both controls and lisinopril petri

FIG. 3.5 The proportion of HPP-CFC-1 in S-phase in bone marrow cells taken from mice treated with Ara-C following *in vitro* incubation with ACE inhibitors

Ara-C regenerating bone marrow	% HPP-CFC-1 in S-phase		
	T = 0	T = 24	
Medium alone	(44.0 ± 5.9)%	(26.5 ± 1.8)%	P = 0.0009, n = 4
+Lisinopril	(52.3 ± 1.8)%	(25.6 ± 3.3)%	P = 0.00012, n = 4
+Captopril	(43.2 ± 3.2)%	(4.9 ± 0.8)%	P = 0.000003, n = 4
Control & Lisinopril			P = 0.17
Control & Captopril			P = 0.00034
Lisinopril & Captopril			P = 0.00004



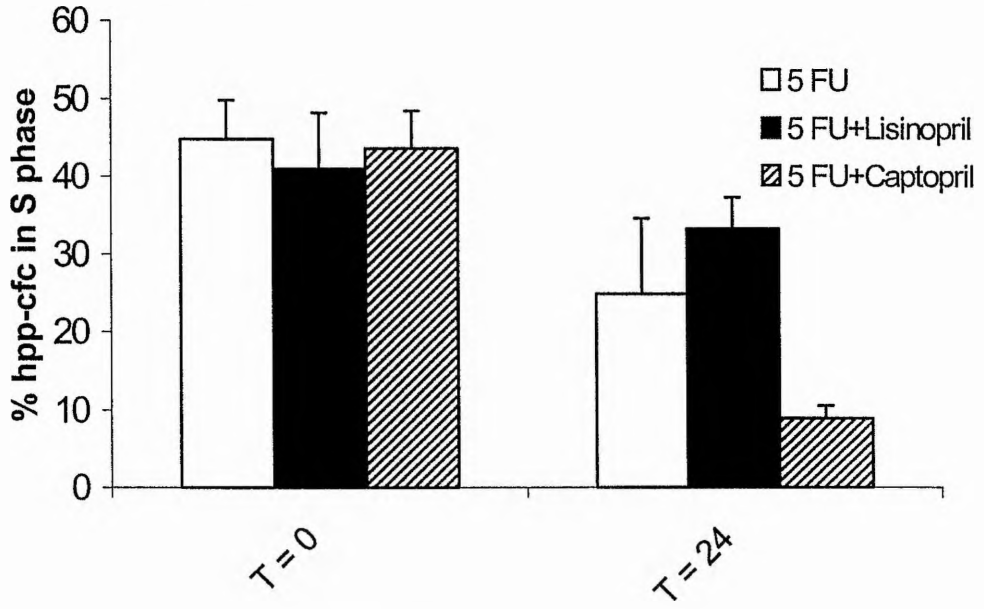
dishes, no significant reduction was observed between Ara-C controls and Ara-C lisinopril incubated petri dishes after 24 hours (p = 0.17). Ara-C regenerating bone marrow cell suspension incubated in the presence of captopril had only (4.9 ± 0.8)% of HPP-CFC-1 in S-phase after 24 hours of incubation (p = 0.000003). This indicated that captopril reduced the proportion of proliferating HPP-CFC-1 when compared to both Ara-C controls (p = 0.00034) and Ara-C lisinopril incubated bone marrow cells (p = 0.00004). In addition captopril prevented HPP-CFC-1 entry into S-phase as shown by the low proportion of HPP-CFC-1 in S-phase.

iii. 5 FU regenerating bone marrow

At the beginning of the incubation period, (44.9 ± 5.0)% of HPP-CFC-1 were in S-phase in 5 FU regenerating bone marrow control cell suspension. The proportion

FIG. 3.6 The proportion of HPP-CFC-1in S-phase in bone marrow cells from mice treated with 5 FU following *in vitro* incubation with ACE inhibitors

5 FU Regenerating bone marrow.	% HPP-CFC-1 in S-phase T = 0	T = 24		
Medium alone	(44.9 ± 5.0)%	(24.9 ± 7.2)%	P = 0.004,	n = 4
+Lisinopril	(41.0 ± 9.6)%	(33.1 ± 4.1)%	P = 0.21,	n = 4
+Captopril	(43.6 ± 4.8)%	(8.9 ± 1.6)%	P = 0.00008,	n = 4
Control & Lisinopril			P = 0.14	
Control & Captopril			P = 0.0005	
Captopril & Lisinopril			P = 0.0015	



of HPP-CFC-1 in S-phase with lisinopril was (41.0 ± 9.6)% at time 0. (43.6 ± 4.8)% of HPP-CFC-1 were in S-phase with captopril initially. The proportion of HPP-CFC-1 in S-phase in the 5 FU control regenerating bone marrow cell suspension was reduced to (24.9 ± 7.2)% (p = 0.004) after a 24 hour incubation and to (33.1 ± 4) (p = 0.21)

when 5 FU regenerating bone marrow cell suspension was incubated with lisinopril. In contrast 5 FU regenerating bone marrow cell suspension incubated with captopril had only (8.9 ± 1.6)% ($p = 0.00008$) of HPP-CFC-1 in S-phase after 24 hours of incubation. Captopril significantly reduced the proportion of HPP-CFC-1 in 5 FU regenerating bone marrow cell suspension when compared to 5 FU controls ($p = 0.0005$) and to 5 FU lisinopril treated bone marrow cell suspensions ($p = 0.002$). Captopril also inhibited HPP-CFC-1 S-phase entry. Hence the effect of captopril was the same on the proliferation of HPP-CFC-1 following the two drugs studied. There was no difference in the proportion of HPP-CFC-1 in S-phase between 5 FU controls and bone marrow cell suspension incubated with lisinopril ($p = 0.14$) after 24 hours of incubation (see Fig 3.6).

3.2 *The effect of Captopril on AcSDKP concentration after in vitro incubation with normal bone marrow, 2 Gy and Ara-C regenerating bone marrow cells*

Procedure

Supernatants from normal and regenerating bone marrow treated with 2 Gy γ-irradiation, Ara-C (100mg/kg-body weight i.p.) and 5 FU (150mg/kg-body weigh i.v) were analysed for AcSDKP concentrations before incubation and after 24 hrs of incubation. No supernatant from lisinopril incubated bone marrow was analysed. After sampling control bone marrow cells and regenerating bone marrow cells from CD1 mice treated with each of the three studied insults at appropriate times, a single cell suspension was made. Cells were suspended in D30%FCS PS/G. Half of the cell suspensions were immediately analysed for AcSDKP in the presence of captopril (T = 0) and the other half was aliquoted into 30 mm-petri dishes. Captopril (1μM) alone was added to cell suspensions. All experiments were performed with controls. For T =

0, cell suspensions were immediately centrifuged after sampling and supernatants were aliquoted into cryotubes. For T = 24, Petri dishes were incubated for 24 hour in 10% CO_2/fully humidified air at 37°C. At the end of the incubation period cell suspensions were centrifuged and the supernatant was aliquoted into cryotubes. The general method of cell sampling, storage of samples and AcSDKP assay was carried out as already described.

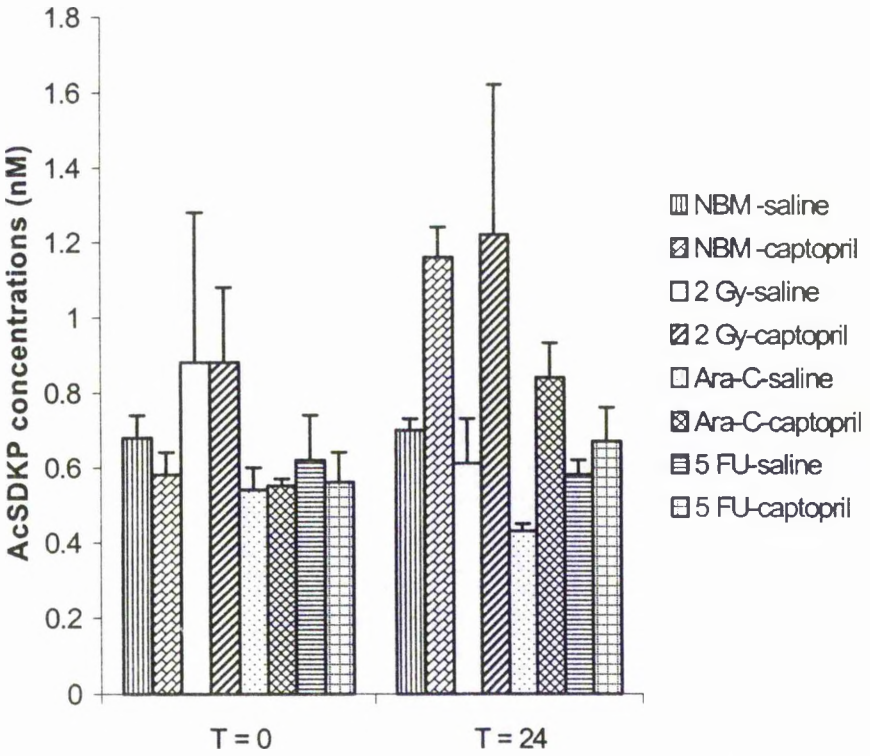
Results

As shown in Fig.3.7, the concentration of AcSDKP in the supernatants of normal bone marrow cell suspensions incubated with saline at the beginning of incubation period (T = 0) was 0.68 ± 0.06 nM. The concentration of AcSDKP remained unchanged at 0.70 ± 0.03 nM ($p = 0.77$) after 24 hours. On the other hand 0.58 ± 0.06 nM of AcSDKP was obtained in the supernatant of normal bone marrow cell suspension incubated with captopril at the beginning of the incubation period (T = 0). This concentration was significantly increased to 1.16 ± 0.08 nM ($p = 0.000014$) after 24 hours in the presence of captopril. Neither saline ($p = 0.3$) nor captopril ($p = 0.19$) influenced the production of AcSDKP following incubation with 2 Gy regenerating bone marrow cell suspension after 24 hours. On the contrary, captopril had a marked effect on AcSDKP concentration when it was incubated with Ara-C regenerating bone marrow cell suspension. AcSDKP levels significantly increased from 0.55 ± 0.02 nM at the beginning of the incubation period (T = 0) when captopril was added to Ara-C regenerating bone marrow cell suspension to 0.84 ± 0.09 nM ($p = 0.00064$) after 24 hours of incubation. Interestingly in the absence of captopril there was a significant reduction in AcSDKP levels in Ara-C regenerating bone marrow cell

suspension. At time 0, Ara-C regenerating bone marrow had 0.54 ± 0.06 nM AcSDKP in the presence of saline. This value was significantly reduced to 0.43 ± 0.02 nM (p =

FIG. 3.7. AcSDKP concentration following *in vitro* incubation of bone marrow cells with captopril

	AcSDKP concentration (nM) T = 0	T = 24		
NBM + Saline	0.68 ± 0.06	0.70 ± 0.03	P = 0.77,	n = 3
NBM + Captopril	0.58 ± 0.06	1.16 ± 0.08	P = 0.000014,	n = 3
2 Gy + Saline	0.88 ± 0.40	0.61 ± 0.12	P = 0.3,	n = 3
2 Gy + Captopril	0.88 ± 0.20	1.22 ± 0.40	P = 0.19,	n = 3
Ara-C + Saline	0.54 ± 0.06	0.43 ± 0.02	P = 0.02,	n = 3
Ara-C + Captopril	0.55 ± 0.02	0.84 ± 0.09	P = 0.00064,	n = 3
5 FU + Saline	0.62 ± 0.12	0.58 ± 0.04	P = 0.59,	n = 3
5 FU + Captopril	0.56 ± 0.08	0.66 ± 0.09	P = 0.15,	n = 3



0.02). Neither saline (p = 0.59) nor captopril (p = 0.15) significantly influenced AcSDKP production in 5 FU regenerating bone marrow cell suspension after 24 hours of incubation.

3.3 *The response of SA2 leukaemic cell line to in vitro incubation with captopril.*

Procedure.

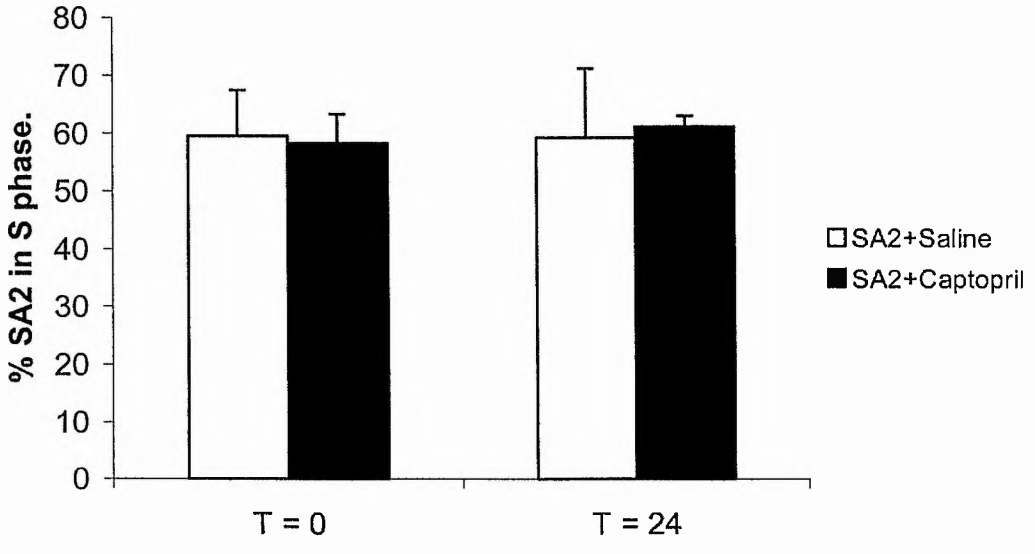
SA2 were suspended in D30%FCS PS/G at 4 x 10^4 cells/ml. Captopril was added to cell suspensions and they were incubated for (T = 0) and (T = 24) according to the general method used in these investigations. However saline was added to the control cell suspensions. After incubation and an S-phase assay, cells were washed and the cellularity was adjusted. They were plated in semi solid agar cultures according to the described methods for SA2 semi-solid agar cultures.

Results

(59.5 ± 8.0)% and (58.2 ± 12.0)% of SA2 leukaemic cells were in S-phase in the presence of saline and captopril at the beginning (T = 0) of incubation

FIG. 3.8 The proportion of SA2 myeloid leukaemia cell line following *in vitro* incubation with captopril

SA2 cells	% SA2 in S-phase T = 0	T = 24	
+Saline	(59.5 ± 8.0)%	(59.1 ± 5.1)%	P = 0.97, n = 4
+Captopril	(58.2 ± 12.0)%	(61.1 ± 1.9)%	P = 0.77, n = 4
	P = 0.87, n = 4	P = 0.69, n = 4	



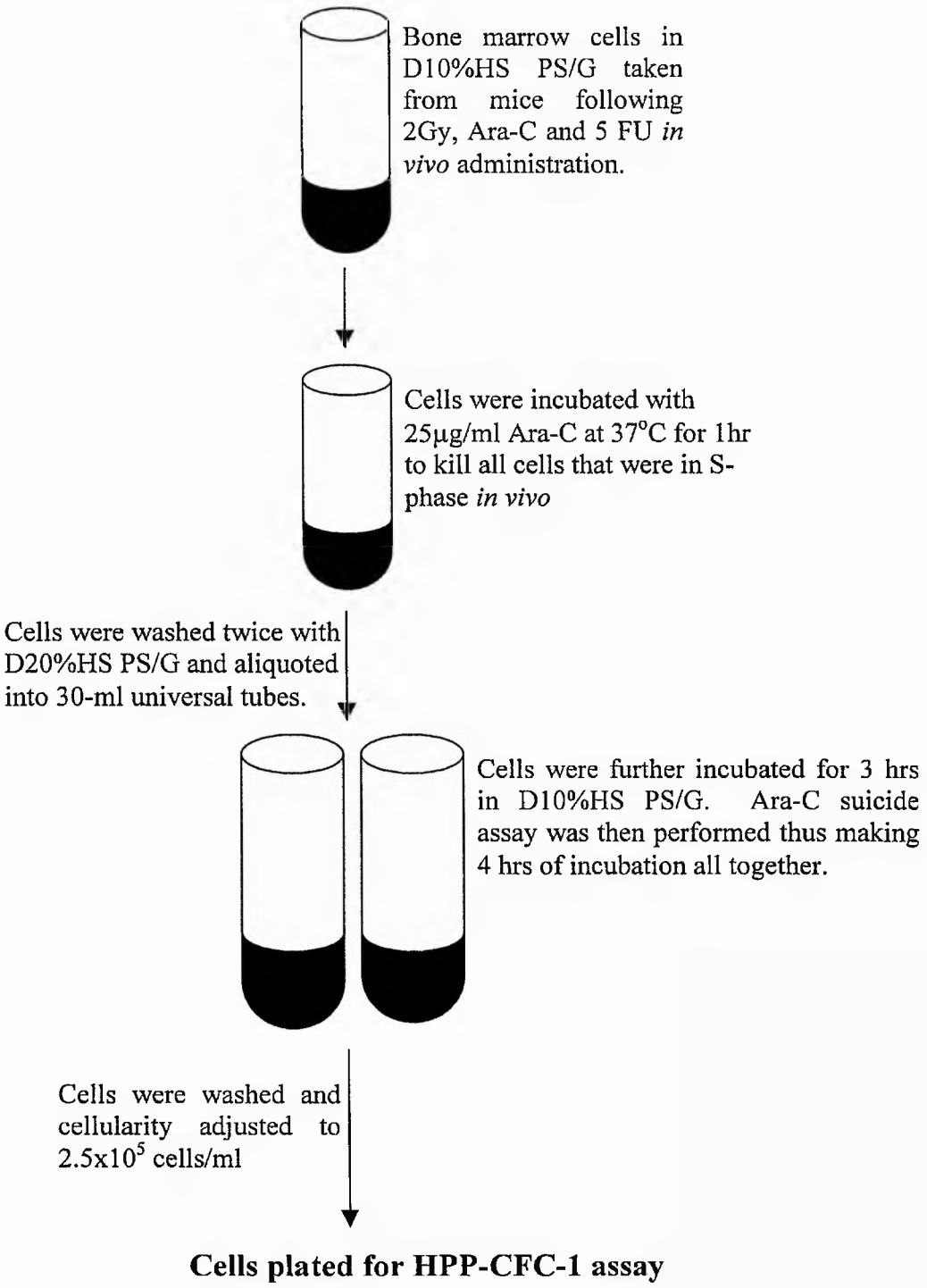
respectively. The proportion of SA2 leukaemic cells in S-phase was not affected by either incubation alone or with captopril. As shown in Fig 3.8, (59.1 ± 5.1)% and (61.1 ± 1.9)% were still in S-phase in the presence of saline ($p = 0.97$) and captopril ($p = 0.77$) respectively after 24 hours of incubation. Therefore captopril's inhibitory effect was observed to be specific to normal HPP-CFC-1 at the doses used.

3.4 *The in vitro effect of AcSDKP on the HPP-CFC-1 proliferation*

Procedure

Bone marrow cell suspension in D10%HS PS/G prepared from CD1 mice 24 hours after 2 Gy γ-irradiation and Ara-C (100mg/kg) i.p. injection and 4 days following 5 FU (150mg/kg) i.v injection were initially incubated with Ara-C (25μg/ml) for 1 hour to deplete all cells that were in S-phase *in vivo*. Following this initial incubation period, cells were washed twice with D20%HS PS/G to remove Ara-C. Clots that formed after centrifuging were dispersed with a 25G orange needle to make a single cell suspension. This single cell suspension was resuspended in D10%HS PS/G. 2 mls of the same cell suspension was aliquoted into each of the four 30 ml universal tubes. Captopril (1μM) and AcSDKP (10^{-9}M) together were added to one pair of universal tubes and the other pair had medium alone and placed in a water bath at 37°C. They were incubated for 3 hours and an Ara-C S-phase suicide was performed making a total incubation period of 4 hours. At the end of the second incubation period cells were again washed twice with D20%HS PS/G. A single cell suspension was made again and cells were counted. The cellularity was adjusted to $2.5x10^5$ cells/ml and cells were then plated for the HPP-CFC-1 assay. Fig. 3.9 summarises the experimental procedure.

Fig. 3.9 AcSDKP mode of action

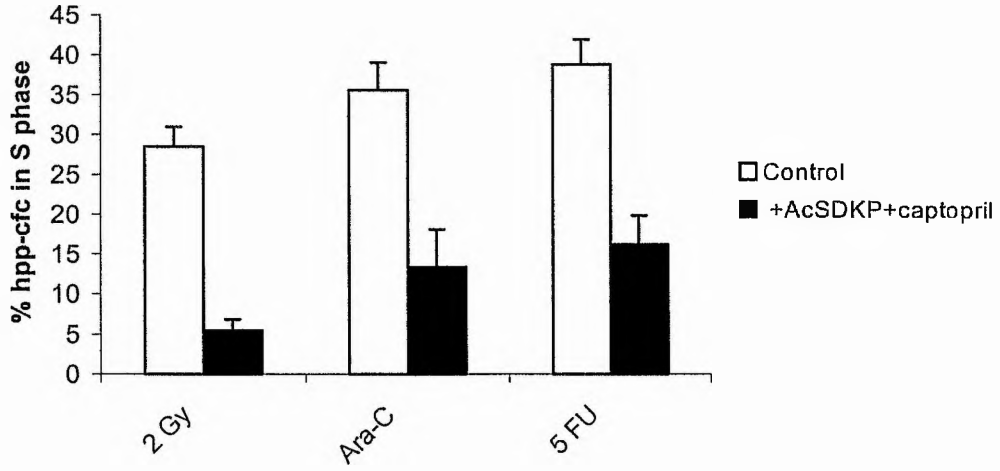


Results

As shown in Fig 3.10, (28.4 ± 2.5)%, (35.6 ± 3.5)% and (38.8 ± 3.1)% of HPP-CFC-1 had entered into S-phase after 3 hrs in the 2 Gy, Ara-C and 5 FU control

FIG. 3.10 The proportion of HPP-CFC-1 in S-phase following Ara-C depletion of the *in vivo* S-phase cell population from regenerating bone marrow cells incubated *in vitro* with AcSDKP and captopril

Regenerating bone marrow cells.	% HPP-CFC-1 in S-phase		
	Control	AcSDKP + Captopril	
2 Gy	(28.4 ± 2.5)%	(5.4 ± 1.4)%	P = 0.00019, n = 4
Ara-C	(35.6 ± 3.5)%	(13.3 ± 4.8)%	P = 0.009, n = 4
5 FU	(38.8 ± 3.1)%	(16.1 ± 3.7)%	P = 0.0033, n = 4



regenerating bone marrow cell suspensions respectively. However when AcSDKP and captopril were incubated with these bone marrow cell suspensions, (5.4 ± 1.4)% (p = 0.00019), (13.3 ± 4.8)% (p = 0.009) and (16.1 ± 3.7)% (p = 0.0033) of HPP-CFC-1 had entered into S-phase from 2 Gy, Ara-C and 5 FU regenerating bone marrow cell suspension respectively. Therefore AcSDKP and captopril prevented G_1/S transition thus confirming the previous observation that AcSDKP acts at the G_1/S boundary in CFU-S (stem cells).

3.5 Discussion

3.5.1 *In vitro effect of ACE inhibitors on haematopoietic cell proliferation*

The HPP-CFC-1 cell population from normal bone marrow cells is quiescent with only a small proportion of cells in S-phase. A normal bone marrow cell suspension was incubated for up to 24 hour in the presence of captopril, lisinopril and without ACE inhibitors. As expected ACE inhibitors had no effect on the proliferation of normal quiescent haematopoietic stem cells (see Fig.3.3). This is due to the fact that haematopoietic stem cell inhibitory factor influence is already at its peak in quiescent HPP-CFC-1. Moreover, inhibitory factors are thought to act by antagonising the production of stimulatory factors by stromal cells this maintains a balance on the proportion of HPP-CFC-1 in cell cycle (Lord et al., 1979). To increase the levels of stimulatory factors, mice were given 2 Gy, Ara-C and 5 FU cytotoxic insults. Following *in vivo* recruitment of bone marrow HPP-CFC-1 into S-phase, cells were sampled and these proliferating haematopoietic stem cells were incubated *in vitro* in the presence of AcSDKP, ACE inhibitors with or without AcSDKP. This was performed in order to investigate whether prevention of AcSDKP degradation would inhibit HPP-CFC-1 proliferation. In addition, the investigations were also carried out in order to observe the differences between the two ACE inhibitors on HPP-CFC-1 cell proliferation. A short incubation time was used as a control and a 24 hour *in vitro* incubation time was used to study these effects.

i. 2 Gy γ-irradiation

Bone marrow cells prepared from mice that were given 2 Gy irradiation were incubated with either captopril, lisinopril, AcSDKP or ACE inhibitors combined with

AcSDKP. This was done to evaluate a direct or indirect inhibitory effect of AcSDKP on 2 Gy regenerating HPP-CFC-1. No inhibitory effect on cell proliferation was observed when all three substances were used separately (see Fig 3.4). It is not surprising that AcSDKP was unable to inhibit HPP-CFC-1 proliferation because it has a very short half-life in serum and plasma (Grillon et al., 1993a). Moreover, the peptide shows its inhibitory effect on haematopoietic progenitor cell proliferation at optimum concentrations. Thus doses higher than 10^{-7}M or lower than 10^{-14}M have no effect on the proportion of these cells in S-phase from both mice and human bone marrow cells (Jackson et al., 1996; Bonnet et al., 1995). The fact that the peptide is thought to act at only one crucial specific point in the cell cycle (Monpezat & Frindel, 1989), also means that it may only affect a small number of proliferating cells at any one time. Therefore, the length of the peptide bioavailability directly relates to the potency of the inhibitory action. Others have shown that ACE inhibitors increase plasma concentration of AcSDKP both *in vivo* and *in vitro* (Azizi et al., 1996, Azizi et al., 1997). It can therefore be hypothesised that haematopoietic stem cell proliferation can be inhibited in the presence of ACE by up regulating AcSDKP levels.

The fact that captopril and lisinopril have no individual inhibitory effect on HPP-CFC-1 proliferation from 2 Gy regenerating bone marrow cell suspension rules out the involvement of angiotensin-II as a contributor to 2 Gy regenerating HPP-CFC-1 proliferation in this assay. AcSDKP when incubated together with captopril reduced the proportion of HPP-CFC-1 in S-phase. The proportion of HPP-CFC-1 in S-phase was not reduced when 2 Gy regenerating bone marrow cells were incubated with AcSDKP and lisinopril together. Therefore it can be assumed that captopril was able to synergise with AcSDKP to reduce the proportion of HPP-CFC-1 in S-phase in 2 Gy regenerating bone marrow cell suspensions. This observation possibly suggests

that captopril prevents AcSDKP degradation. It also demonstrates an indirect mechanism of captopril in bringing about its inhibitory effect on cell proliferation.

Since macrophages are responsible for AcSDKP production *in vitro* (Li et al., 1996), the incubation medium of bone marrow cell suspension was sampled after incubation in the presence of captopril alone. The incubation medium was evaluated for any changes in AcSDKP concentration following incubation to analyse the balance between production and degradation of the peptide. This study was also performed to verify that AcSDKP levels influenced the proportion of HPP-CFC-1 in S-phase following incubation. Secondly this investigation was performed in order to show whether the inability of ACE inhibitors alone to reduce the proportion of HPP-CFC-1 in S-phase in 2 Gy regenerating bone marrow cell suspension was due to lack of endogenous AcSDKP production in the incubation dishes. 2 Gy regenerating bone marrow cells had very high levels of AcSDKP at the beginning of the incubation with no significant increase in the peptide levels in the presence of captopril after 24 hours of incubation (see Fig 3.7). The reason behind this finding is unknown. However, there are several possibilities that might lead to initial high AcSDKP levels following 2 Gy irradiation. One reason may be cell death. AcSDKP may leak from the cytoplasm into the serum following irradiation injury since there are high levels of AcSDKP in the cell cytoplasm (Volkov et al., 1995; Volkov et al., 1997). An alternative explanation may rest on the cytotoxic nature of irradiation. Since irradiation kills only radiosensitive cells, there may be an imbalance between the number of cells that are responsible for AcSDKP production and those that are responsible for its degradation. Cells responsible for the production of stimulatory factors may be spared from irradiation injury because these cells are thought to be radioresistant(Wright & Lorimore, 1987). These cells may exert an increase in

stimulator production. An increase in stimulator production may activate a negative feed back loop that may increase the production of AcSDKP by some viable cells responsible for AcSDKP production. These high levels of AcSDKP have no inhibitory effect on the proliferation of HPP-CFC-1 in this cell suspension probably due to a high concentration of stimulators. The inability of captopril to increase levels of AcSDKP after 24 hours of incubation in 2 Gy bone marrow cell suspension might also mean that the system responsible for AcSDKP production is working at a maximum capacity.

Since HPP-CFC-1 in 2 Gy regenerating bone marrow were already cycling in the presence of high AcSDKP levels might suggest that these high levels of AcSDKP were achieved by a feed back loop *in vivo* which could not be maintained *in vitro*. Higher concentrations of AcSDKP may be required to inhibit HPP-CFC-1 proliferation following 2 Gy irradiation probably due to the increased levels of stimulatory factors. The inhibitory concentration of AcSDKP on cell proliferation is achieved by exogenous addition of the peptide to incubation dishes. Assuming that ACE activity is not at its saturation point, addition of exogenous AcSDKP would activate ACE thereby increasing the degradation of AcSDKP. This might explain the inability of lisinopril and AcSDKP to inhibit the proliferation of HPP-CFC-1 following 2 Gy irradiation. This hypothesis is strengthened by the fact that lisinopril is a weak inhibitor of the N-domain ACE active site (Wei et al., 1992, Rousseau et al., 1995).

Therefore, irradiation either disrupts the integrity of molecules involved in AcSDKP production and inhibitory mechanisms or it increases the concentrations of stimulators of stem cell proliferation to high levels that can not be opposed by the initial observed levels of AcSDKP *in vitro*. Hence no inhibitory effect on HPP-CFC-1

proliferation following 2 Gy irradiation is observed in the presence of captopril alone but in the presence of captopril together with AcSDKP. The ability of captopril to prevent the degradation of AcSDKP *in vitro* ensures high levels of AcSDKP that can directly inhibit HPP-CFC-1 proliferation. Moreover, these results suggests that captopril on its own is not an inhibitor of cell proliferation following 2 Gy irradiation.

ii. Ara-C regenerating bone marrow

The effect of AcSDKP on Ara-C regenerating bone marrow cell suspension was investigated previously by Robinson et al., 1992. AcSDKP alone was unable to reduce the proportion of HPP-CFC-1 in S-phase in Ara-C regenerating bone marrow cell suspension in their investigations. Therefore AcSDKP alone was not investigated with regenerating bone marrow cell suspensions from Ara-C drug treated mice. In contrast to 2 Gy regenerating bone marrow HPP-CFC-1, the HPP-CFC-1 population is switched out of cycle in the presence of captopril in Ara-C regenerating bone marrow cell suspension after 24 hours of incubation (see Fig. 3.5). Surprisingly, incubation alone also reduced the proportion of HPP-CFC-1 in S-phase following Ara-C treatment. Lisinopril reduced the proportion of HPP-CFC-1 in S-phase to the same levels as control bone marrow cells. This demonstrates that there was an endogenous production of inhibitors of cell proliferation following incubation. Captopril had a synergistic effect with the endogenously produced inhibitors of cell proliferation, while lisinopril was unable to synergise with these endogenously produced inhibitory factors. Since Ara-C kills cells that are specifically in S-phase, differentiated cells responsible for inhibitor production are not compromised following Ara-C treatment. This is in contrast to irradiation, which affects differentiated as well as proliferating cells and it therefore compromises the functional

integrity of cells that may be involved in the production of factors that regulate cell proliferation. Indeed, levels of AcSDKP were significantly increased when captopril was added to Ara-C regenerating bone marrow cell suspensions after 24 hours of incubation (see Fig. 3.7). Interestingly, incubation alone significantly decreased the levels of AcSDKP in Ara-C treated bone marrow cell suspension. This may contradict the inhibitory effect observed on HPP-CFC-1 proliferation by incubation alone. However, the presence of uninjured bone marrow macrophages in the cell suspension may lead to the production of other stem cell proliferation inhibitors such as MIP 1α and TGF-β (Maltman et al., 1993) that may reduce AcSDKP levels.

Incubation alone may also increase the endogenous production of prostaglandin-E_2 (PG-E_2) (Goodwin et al., 1978). Prostaglandins are produced by the action of the enzyme cyclooxygenase (COX) on arachidonic acid liberated from membrane phospholipids. There are two forms of COX called COX-1 and COX-2. COX-2 is produced following lipopolysacchride (LPS) stimulation on alveolar macrophages while COX-1 is present only in resisting macrophages and is not affected by LPS (Lee et al., 1992). Therefore an increase in prostaglandins in LPS-stimulated macrophages (Humes et al., 1977) results from selective expression of COX-2. PG-E_2 stimulates production of cyclic-3',5'adenosine monophosphate (cAMP) which decreases the production of IL-2 and IFN-γ by T cells (Phipps et al., 1991). The increase in intracellular cAMP may interfere with signal transduction cascades involving MAP kinase because cAMP stops the physical association of Ras and Raf-1 (Wu et al., 1993). IFN-γ induces Ia (class II major histocompatibility complex (MHC)) expression on macrophages by rapid exchange of Na^+ and H^+ by means of the Na^+/H^+ antiporter (Prpic et al., 1989). Therefore PG-E_2 is an indirect potent inhibitor of the expression of Ia-antigens on macrophages (Snyder et al., 1982;

Figueiredo et al., 1990). Macrophages expressing Ia-antigens are essential in the production of stimulatory factors (Ali et al., 1982). In addition to its effect on IL-2 and IFN-γ, PG-E_2 is potent inhibitor of IL-12 production and a potent stimulator of IL-10 production (van der Pouw Kraan et al., 1995; Strassman et al., 1994). PG-E_2 also inhibits lipopolysaccharide (LPS)-production of TNF-α and IL-6 by an autocrine feedback mechanism involving up regulating the levels of IL-10 (Strassmann et al., 1994). IL-1 and TNF-α in combination increase PG-E_2 production (Topley et al., 1989). TNF alone stimulates the production of IL-1 and PG-E_2 in resting macrophages (Bachwich et al., 1986). Interestingly anti-inflammatory cytokines (IL-4 and IL-10) both decrease levels of TNFα, IL-1, and PG-E_2 (Hart et al., 1989; Niho et al., 1998).

Therefore PG-E_2 may bring about its suppressive effect by up regulating the levels of IL-4 and IL-10. Indeed both IL-4 and IL-10 inhibit LPS-induced COX-2 expression thus leading to down-regulation of PG-E_2 production (Niiro et al., 1995). Therefore PG-E_2 regulates a feed back loop on cytokine production which in turn regulates its own production. It is sensible to suggest that an increase in PG-E_2 production results in a reduction in cytokines that may have some stimulatory activity on the proliferation of stem cells. The net result is inhibition of stem cell proliferation.

Interestingly, dose dependently captopril but not lisinopril suppresses IL-1β-induced synthesis of TNF and IL-1α (Schindler et al., 1995). However both captopril and lisinopril suppress production of interleukin-12 by human peripheral blood mononuclear cells stimulated by LPS (Constantinescu et al., 1998). Excitingly, lisinopril suppresses production of IL-12 at 10^{-3}M with little effect at low concentrations, while captopril suppresses IL-12 at concentrations as a low as 10^{-7}M. In these investigations we used 10^{-6}M for all investigations since this concentration

was shown to inhibit ACE activity. Therefore these results are in agreement with the hypothesis that reduced levels of stimulatory factors may be behind the effects of ACE inhibitors on HPP-CFC-1 proliferation. The high concentration of lisinopril required to suppress IL-12 production may also suggest that AcSDKP levels may influence IL-12 production. Captopril is 50 times better at inhibiting the N-domain ACE active site and preventing AcSDKP degradation (Rousseau et al., 1995) than lisinopril. Therefore higher levels of lisinopril are needed to inhibit the N-domain ACE active site than captopril. However, in addition to an increase in AcSDKP concentration due to reduced degradation as observed in Ara-C generating bone marrow cell suspension incubated with captopril, captopril also increases PG-E_2 production (Swartz et al., 1980). The inhibitory effect of captopril on T lymphocyte proliferation has been shown to involve an increase in PG-E_2 production (Johnsen et al., 1997). Therefore, it can be concluded that captopril might be working in concert with most of the mechanisms involved in the inhibition of haematopoietic stem cell proliferation to bring about its inhibitory effect on Ara-C proliferating HPP-CFC-1.

Since the mode of action of AcSDKP is unknown it can not be ruled out that AcSDKP may utilise the same pathway in its inhibitory effect on HPP-CFC-1 proliferation as captopril. It is plausible to suggest that captopril may influence cell proliferation by increasing the endogenous levels of AcSDKP which may in turn increase the endogenous levels of PG-E_2, IL-10 or IL-4 resulting in the reduction in the production of stimulatory factors. The fundamental difference between the inhibitory effects observed in the presence of Ara-C and 2 Gy can therefore be attributed to cell damage that is incurred by these two cytotoxic agents. The absence of damage to differentiated cells in the presence of Ara-C results in the normal feed back loops which are involved in the regulation of stem cell proliferation. This is not

observed following 2 Gy because cells may be experiencing the late effect of irradiation on cell function. The above hypothesis suggests that the ability of captopril to influence HPP-CFC-1 from Ara-C regenerating bone marrow is likely to be indirect. This mechanism requires the involvement of accessory cells. AcSDKP also shows the need for accessory cells for its cell proliferation inhibitory effect (Lauret et al., 1989). Therefore the observed effect of captopril may not due to the drug alone.

iii. 5 FU regenerating bone marrow

Regenerating bone marrow cell suspensions from 5 FU treated mice were also incubated with or without ACE inhibitors. This was done for three reasons, firstly to evaluate whether the captopril effect on Ara-C regenerating HPP-CFC-1 cycling was unique to Ara-C. Secondly, to investigate the response of very primitive HPP-CFC-1 which responds to the three recombinant growth factors used in these investigations following 5 FU treatment *in vivo*. Thirdly to evaluate whether cytotoxic drug induced HPP-CFC-1 proliferation would respond in the same way to ACE inhibitors regardless of the type of drug used to induce insult.

While incubation alone reduced the proportion of HPP-CFC-1 in S-phase in control bone marrow after 24 hours, no such effect was observed with lisinopril. In contrast, captopril significantly reduced the proportion of HPP-CFC-1 in S-phase when compared to controls (see Fig. 3.6). Furthermore, no increase in the levels of AcSDKP was observed in the presence of captopril after 24 hours of incubation of 5 FU regenerating bone marrow cells (see Fig. 3.7). This is rather surprising. However, 5 FU enriches for HPP-CFC-1 after 4 days *in vivo* (Bartelmez et al., 1989). The HPP-CFC population needs low concentrations of inhibitory factors to be inhibited (Pragnell et al., 1988). The fact that 5 FU metabolites also kill cells (Hodgson et al.,

1982; Donowitz & Quesenberry, 1986) means that there is a marked cell death following 5 FU treatment with reduction in cells that may be involved in inhibitor production. Therefore, it is not surprising that the levels of AcSDKP do not increase in the presence of captopril after 24 hours. Lisinopril may antagonise inhibitor production as shown by its inability to inhibit IL-1β production of IL-1α and TNF (Schindler et al., 1995). Moreover, endogenous PG-E_2 also greatly enhances the synthesis of GM-CSF and it may upregulate the expression of IL-1 receptors on monocytes (Phipps et al., 1991). It follows then that the concentrations of stimulators are not significantly reduced in the presence of lisinopril. The small amount of inhibitor produced manages to slightly inhibit HPP-CFC-1 proliferation in the absence of ACE inhibitors. Despite no change in AcSDKP levels, captopril significantly reduced the proportion of HPP-CFC-1 in S-phase. This can be attributed to the inherent property of captopril in addition to its effect on cytokine production.

Captopril's inhibitory effect may involve the scavenging of free radicals (Bagchi et al., 1989). For example, TNF stimulates superoxide (O_2^-) release directly in human granulocytes in a dose-dependent manner (You et al., 1989). This granulocyte activation may also activate other cells with a net increase in the production of cytokines. Therefore, the ability of captopril to scavenge free radicals induced by TNF may inhibit the production of cytokines that utilise this mechanism. As discussed by Wang et al., 1995, antioxidants and free radical scavengers inhibit NFκB activation. Therefore captopril by its ability to scavenge free radicals, may inhibit the production of stimulatory factors by inhibiting NFκB. In addition there is evidence that captopril inhibits GM-CFC proliferation in the presence of copper, a mechanism that has been attributed to free radical scavenging (Hammond et al., 1988). Moreover, captopril has marked anti-inflammatory activity as shown by its ability to inhibit delayed

hypersensitivity reactions in rodents (Wang et al., 1996). Since inflammatory processes are regulated by cytokines, captopril is therefore a potent inhibitor of cytokine production. To conclude, captopril may in general inhibit cell proliferation by mechanisms that may involve ACE inhibition, inhibition of cytokine production and the free radical scavenging mechanism. All these mechanisms may be involved in captopril inhibitory effect following cytotoxic drugs.

From the *in vitro* investigation, it seems that angiotensin-II is not involved in HPP-CFC-1 proliferation *in vitro*. This is shown by the fact that lisinopril had no effect on HPP-CFC-1 proliferation in all the three models tested. Moreover, high levels of foetal calf serum were used in all these investigations and lisinopril was demonstrated to be more effective on ACE inhibition in serum. Therefore, if the inhibitory effect of ACE inhibitors was mediated by reduction in angiotensin-II levels, then lisinopril should have preferentially inhibited HPP-CFC-1 proliferation.

While AcSDKP is a strong candidate in the inhibitory effect imposed by captopril, angiotensin-II mechanism can not be ruled out. Indeed AcSDKP and angiotensin-II may share some common characteristic in that they both inhibit immunological reactions brought about by cells that may be involved in immune regulatory mechanisms and reactions (Dezso & Foris, 1981; Foris et al., 1983; Thierry et al., 1990, Frindel et al., 1992a; Lavignac et al., 1992; Aizawa et al., 1992). Therefore by inhibiting one molecule captopril may up regulate the other hence revealing the common pathways of AcSDKP and angiotensin-II. Interestingly, angiotensin-II has both stimulatory and inhibitory effects on cell proliferation. However, stimulatory effects of angiotensin-II are more marked than its inhibitory effects on cell proliferation. For example, angiotensin-II is a growth factor with receptors on haematopoietic (Dezso & Foris, 1991; Foris et al., 1983; Shimada &

Yazaki, 1978) and on non haematopietic cells (Crabos et al., 1994; Sadoshima & Izumo, 1993). On the other hand, the response of peripheral blood T-lymphocyte stimulation to lectin is impaired in people with essential hypertension and is increased after captopril treatment (Shasha et al., 1991) suggesting that angiotensin-II may be an inhibitory factor on T-cell proliferation in these patients. Captopril enhances *in vitro* human lymphocyte thymidine incorporation stimulated by phytohaemagglutinin-P (PHA) and concovalin-A (Con-A) but not pokeweed mitogen (PWM) (Simon et al., 1984). Simon et al, 1984 argue that this stimulatory effect is mediated by ACE inhibition since both PHA and Con-A are mitogens that are macrophage dependent and macrophages produce ACE (Costerousse et al., 1993). In addition, angiotensin-II inhibits active, and passive IgG2a-erythrocyte-antibody (EA) rosette formation on monolayers of rat peritoneal macrophages at a dose range of 10^{-5} to 5 x 10^{-7} M and stimulates them in the dose range of 10^{-7} to 10^{-8} M (Dezso & Foris, 1981, Foris et al., 1983). Therefore this dual effect of angiotensin-II on cell proliferation makes it difficult to interpret the effects angiotensin-II may have *in vitro* in the present investigation. If angiotensin-II is acting as an inhibitor of cell proliferation, its reduction should recruit HPP-CFC-1 into cell cycle. Therefore, ACE inhibitors should have increased the proportion of HPP-CFC-1 into S-phase rather than reduce it. However, if it was acting as a stimulatory factor, its reduction following ACE inhibitors should have been shown by both captopril and lisinopril.

3.5.2 *The effect of captopril on SA2 cell proliferation*

The observation that captopril inhibited HPP-CFC-1 proliferation in regenerating bone marrow cell suspensions with or without AcSDKP was investigated further. The specificity of the captopril inhibitory action on normal haematopoietic

cells was evaluated using the SA2 myeloid leukaemia cell line. The main point was to find out whether captopril inhibits all cycling cells regardless of being normal or neoplastic. In these investigations captopril failed to inhibit the proliferation of neoplastic cells. This is in agreement with what has been observed by others before. The underlying cause of this effect is largely unknown. Interestingly, captopril inhibits glioma cell invasion without affecting the motility or proliferation of these cells (Nakagawa et al., 1995). In addition, captopril *in vivo* retards the expansion of kidney tumours without affecting its cell proliferation (Hii et al., 1998). Therefore, the effect of captopril on tumour cell proliferation is a complex one. Some possible mechanisms involved in captopril's ability to influence tumour cell proliferation will be discussed further in chapter 6.

3.5.3 *The effect of AcSDKP on HPP-CFC-1 cell cycle*

This was investigated by an initial incubation of regenerating bone marrow cells with Ara-C followed by a second incubation of bone marrow cells with AcSDKP and captopril together. The proportion of cells in S-phase following the second incubation time is an indication of cell entry into cell cycle following the second *in vitro* incubation. Moreover, this method synchronises cells in the cell cycle. The fact that AcSDKP inhibits CFU-S cycling following Ara-C is well-established (Monpezat & Frindel, 1989). It is also known that AcSDKP has an inhibitory effect on cycling HPP-CFC that is mediated by opposing the effect of stimulatory factors on cell proliferation (Robinson et al., 1992). Since Ara-C is an S-phase specific agent and AcSDKP only inhibits CFU-S cycling if given 2 hours following Ara-C, it has been suggested that AcSDKP blocks G_0-G_1/S transition. However, this has not been directly shown and the *in vitro* cell cycle mode of action of AcSDKP has not been

elucidated. Therefore an investigation was carried out to test the mode of action of AcSDKP *in vitro* and to confirm the fact that the peptide may be active at the G_1/S boundary. This hypothesis was tested using cycling HPP-CFC-1. HPP-CFC-1 S-phase entry was inhibited in the presence of AcSDKP and captopril from all cytotoxic insults (see Fig. 3.8). Bone marrow cells prepared from mice following 2 Gy irradiation showed the greatest reduction in S-phase entry, and bone marrow cells prepared from mice following 5 FU treatment showed the least potency in S-phase entry reduction. Moreover, the same results were demonstrated after only 1 hour of incubation following the initial Ara-C suicide (results not shown). From these results, it is confirmed that AcSDKP inhibits S-phase entry of stem cells regardless of the assay used. Moreover, it also confirms that the peptide has little or no effect on other phases of the cell cycle. This is so because 4 hours is not enough time for the completion of the cell cycle progression. Considering that there were no cells in S-phase during the second incubation, the proportion of HPP-CFC-1 in S-phase in control samples is very high thus suggesting that this pool of cells might have just been recruited into S-phase from G_1/S boundary. A high proportion of HPP-CFC-1 in S-phase would have been observed in AcSDKP and captopril incubated bone marrow cells if the peptide was acting at a different point in the cell cycle rather than the G_1/S boundary. This is due to specificity of Ara-C in killing cells that are in S-phase of the cell cycle. From the results presented in these investigation and the argument put forward, it is likely that AcSDKP and captopril inhibit S-phase entry in proliferating cells.

The molecular mechanisms employed by AcSDKP in its inhibitory effect are unknown. There are several pathways by which inhibitors of cell proliferation can achieve their effect. An increase in the levels of inhibitory cytokines may be

responsible for AcSDKP action. Other mechanisms may involve competitive displacement of growth factors from their receptors; down-regulating growth factor receptor expression; counter-acting one or more steps involved in the signal transduction pathways; disruption of cell communication by affecting the integrity of gap junctions, imbalance between cytoplasmic ions, like Ca^{2+} and small molecules like cyclic adenosine monophosphate (cAMP). Very little information is available on the molecular mode of action of the most commonly known inhibitors of haematopoietic stem cell proliferation. However, there is evidence that AcSDKP causes an incomplete competitive inhibition of calmodulin-dependent phosphodiesterase (Voelter et al., 1995). It is thus possible that the AcSDKP inhibitory effect may involve cyclic nucleotide metabolism. In addition the findings of Voelter et al., 1995 suggest that Ca^{2+} may be crucial in the AcSDKP induced cellular proliferation inhibitory effect. In addition, AcSDKP has no effect on neoplastic cell proliferation at concentrations that affect normal cell proliferation (Bonnet et al., 1992a). This suggests that AcSDKP can only function if the cellular molecular integrity is preserved. The crucial step in the induction of S-phase progression in the cell cycle is the passage of proliferating cells at the restriction point, which is in the late G_1 phase before they can progress into the S-phase. Neoplastic cells may by-pass this stage while normal cells can not. It therefore follows that the genetic integrity of the restriction point in the late G_1 phase of cell cycle will have a tremendous influence on normal cell cycling. AcSDKP may exert its influence indirectly at this point by inhibiting the phosphorylation of the retinoblastoma gene protein. The molecular mechanisms involved in the AcSDKP inhibitory effect require further investigation.

CHAPTER 4

4.0 *IN VIVO* EFFECT OF ACE INHIBITORS ON HPP-CFC-1 PROLIFERATION

Having established the inhibitory effect of captopril on HPP-CFC-1 proliferation *in vitro*, investigations were conducted to see whether ACE inhibitors might also reduce the proportion of HPP-CFC-1 in S-phase *in vivo* following cytotoxic treatments. The doses of insults given to mice were identical to the ones given for *in vitro* investigations. Since it was difficult to give a dose of ACE that would produce 1μM drug concentration *in vivo* without pharmacokinetic studies, the recommended human therapeutic dose of each ACE inhibitor was chosen as the dose to be investigated for *in vivo* experiments. Thereafter the ability of captopril to regulate AcSDKP concentrations *in vivo* was studied. Captopril was further investigated on its potential ability to protect the haematopoietic system from myelotoxicity following cytotoxic agents.

Procedure

CD1 mice were exposed to 2 Gy irradiation or injected with Ara-C (100mg/kg i.p) or 5 FU (150mg/kg i.v). 1 hour after the insult they were injected i.p with saline, lisinopril or captopril. The doses chosen were 10 mg/kg-body weight for the lisinopril (Goa et al., 1996) investigation and 100 mg/kg-body weight for the captopril (Martin et al., 1984) investigation. Mice were killed after 24 hours following 2 Gy or Ara-C and after 4 days following 5 FU treatment. Bone marrow cells were pooled from mice and a single cell suspension produced in D20%HS PS/G. The proportion of HPP-CFC-1 in S-phase was estimated using an S-phase suicide assay. All experiments were performed with control mice that did not receive insults but received saline or

ACE inhibitors. After an S-phase suicide assay, cells were washed twice and plated for HPP-CFC-1 assay according to the general methods.

Results

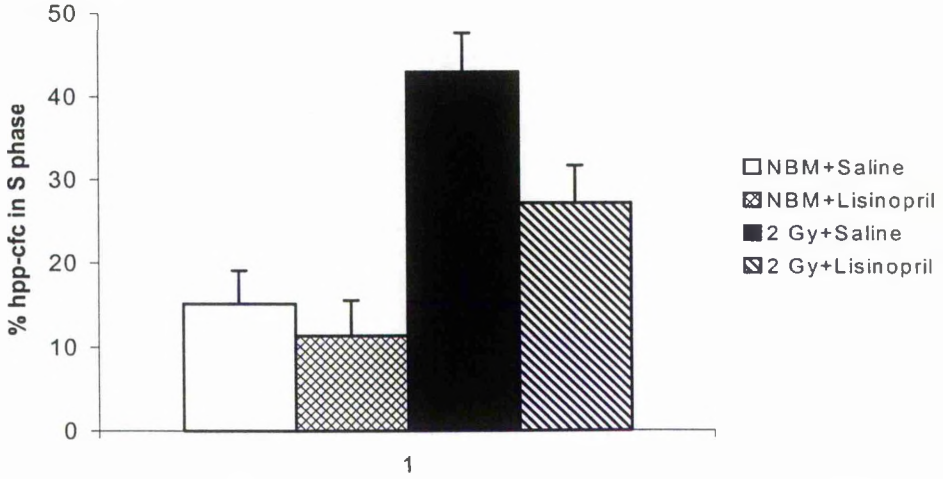
4.0.1 *The effect of 10 mg/kg lisinopril following 2 Gy*

As shown in Fig 4.1, HPP-CFC-1 in animals that did not receive irradiation were not in cell cycle. Lisinopril had no effect on the proliferation of this HPP-CFC-1

FIG. 4.1 Proportion of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with 10mg/kg lisinopril

10mg/kg ACE ACE Inhibitor	**% HPP-CFC-1 in S-phase**	
NBM + Saline	(15.1 ± 3.9)%	
NBM + Lisinopril	(11.4 ± 4.2)%	P = 0.63, n = 4
2 Gy + Saline	(42.9 ± 4.7)%	
2 Gy + Lisinopril	(27.2 ± 4.0)%	P = 0.05, n = 4
NS & 2 GyS		P = 0.002
NS & 2 GyL		P = 0.03

NB: NS = Normal bone marrow + saline, 2 Gy S = 2 Gy bone marrow + saline, 2 GyL = 2 Gy bone marrow + lisinopril.



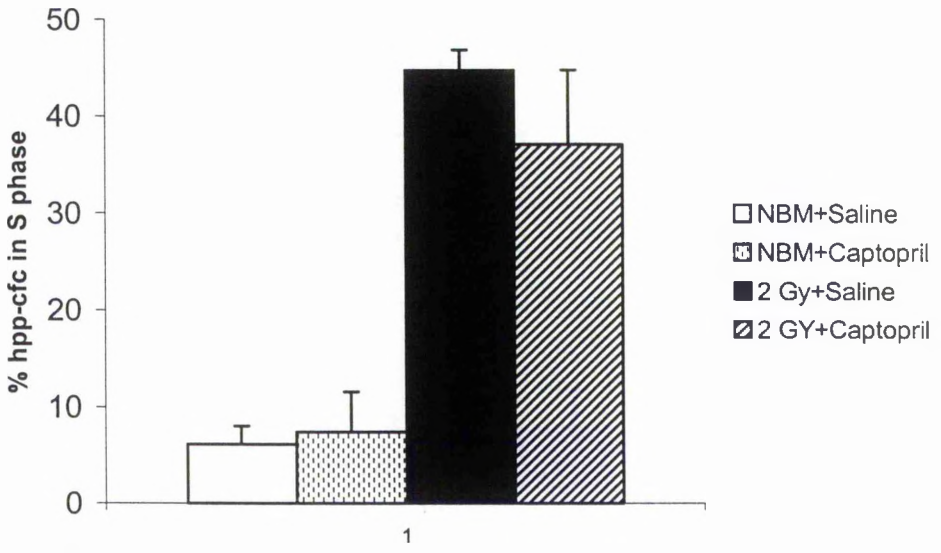
cell population. Mice that received 2 Gy irradiation and saline had (42.9 ± 4.7)% HPP-CFC-1 in S-phase and the proportion was slightly reduced to (27.2 ± 4.5)% in mice that were given lisinopril following 2 Gy (p = 0.05). Therefore *in vivo* lisinopril was unable to strongly reduce the proportion of HPP-CFC-1 in S-phase following 2

Gy γ–irradiation. Having established that lisinopril had a borderline effect on HPP-CFC-1 proliferation following 2 Gy, the same dose of captopril as lisinopril was investigated. Just as with the lisinopril experiments, normal bone marrow HPP-CFC-1 proliferation was not affected by captopril (see Fig 4.2). Furthermore, captopril had no effect on HPP-CFC-1 proliferation at 10 mg/kg ($p = 0.42$). Hence, low doses of captopril did not affect HPP-CFC-1 proliferation following 2 Gy irradiation *in vivo*.

FIG. 4.2 The proportion of HPP-CFC-1 in S-phase following 2 Gy *in vivo* with captopril (10mg/kg)

10mg/kg ACE ACE Inhibitors	**% HPP-CFC-1 in S-phase**		
NBM + Saline	(6.1 ± 1.9)%		
NBM + Captopril	(7.5 ± 4.1)%	P = 0.74,	n = 4
2 Gy + Saline	(44.7 ± 2.1)%		
2 Gy + Captopril	(37.1 ± 7.6)%	P = 0.42,	n = 4
NS & 2 GyS		P = 0.00002	
NC & 2 GyC		P = 0.0004	

NB: NC = normal bone marrow + captopril, 2 GyS= 2 Gy bone marrow + saline, 2 GyC = 2 Gy bone marrow + captopril



4.0.2 *The effect of 10mg/kg ACE inhibitors following Ara-C*

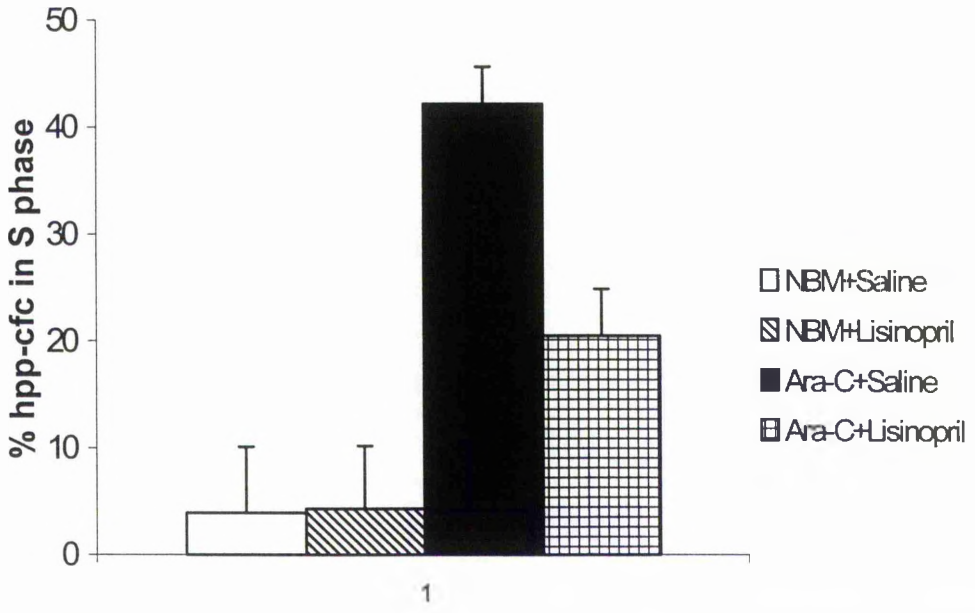
ACE inhibitors did not have an effect on HPP-CFC-1 proliferation from bone marrow cells of untreated mice (Fig 4.3). However (42.1 ± 3.5)% of HPP-CFC-1 were in S-phase in Ara-C treated mice followed by saline injection whereas lisinopril

significantly reduced this proportion to (20.5 ± 4.4)% (p = 0.0084). Interestingly, captopril at a dose of 10mg/kg was unable to reduce the proportion of HPP-CFC-1 in S phase following Ara-C treatment. Ara-C treated mice that were given saline had (38.4 ± 2.1)% of HPP-CFC-1 in S-phase while mice that were given captopril had

FIG. 4.3 The proportion of HPP-CFC-1 in S-phase following Ara-C treatment *in vivo* with lisinopril (10mg/kg)

10mg/kg ACE ACE Inhibitors	**% HPP-CFC-1 in S-phase**	
NBM + Saline	(3.9 ± 6.2)%	
NBM + Lisinopril	(4.3 ± 5.9)%	P = 0.85, n = 4
Ara-C + Saline	(42.1 ± 3.5)%	
Ara-C + Lisinopril	(20.5 ± 4.4)%	P = 0.0084, n = 4
NS& 2 GyS		P = 0.00007
NL & 2 GyL		P = 0.0012

NB: NL = Normal bone marrow with lisinopril, 2 GyL = 2 Gy bone marrow with lisinopril.



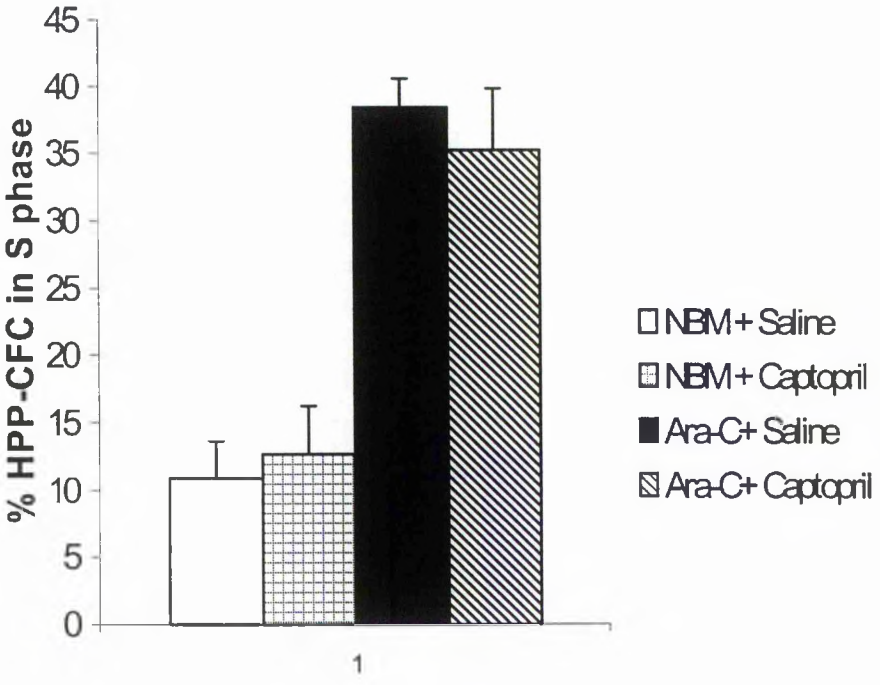
(35.2 ± 4.6)% of HPP-CFC-1 in S-phase (p = 0.55) (Fig 4.4). Therefore lisinopril reduced the proportion of HPP-CFC-1 in S-phase in Ara-C regenerating bone marrow

at these low doses without affecting the proportion of HPP-CFC-1 in S-phase following 2 Gy. Lisinopril inhibitory effect on HPP-CFC-1 proliferation was incomplete in that a large proportion of HPP-CFC-1 was still in S-phase.

FIG 4.4 The proportion of HPP-CFC-1 in S-phase following Ara-C treatment *in vivo* with captopril (10mg/kg)

10mg/kg ACE ACE Inhibitors	**% HPP-CFC-1 in S-phase**	
NBM + Saline	(11.0 ± 2.8)%	
NBM + Captopril	(12.7 ± 3.5)%	P = 0.75, n = 4
Ara-C + Saline	(38.4 ± 2.1)%	
Ara-C + Captopril	(35.2 ± 4.6)%	P = 0.55, n = 4
NS & AS		P = 0.002
NC & AC		P = 0.005

NB: AS = Ara-C bone marrow + saline, AC = Ara-C bone marrow + captopril

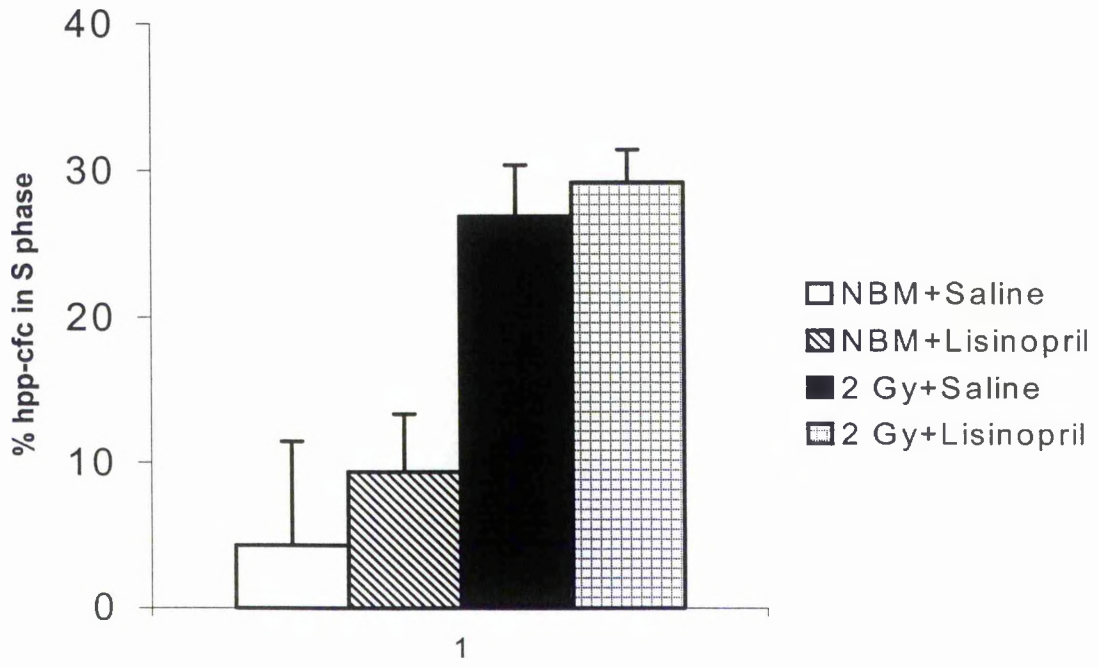


4.0.3 *The effect of 100mg/kg ACE inhibitors on 2 Gy regenerating bone marrow HPP-CFC-1*

ACE inhibitors did not affect the proportion of HPP-CFC-1 in S-phase in untreated mice. 2 Gy followed by saline treated mice had (26.9 ± 3.5)% of HPP-CFC-1 in S-phase, this proportion was unchanged when 2 Gy was followed by lisinopril (100mg/kg) at (29.2 ± 2.2)% (p = 0.6) (see Fig 4.5). However, when mice that were exposed to 2 Gy γ-irradiation were injected with captopril (100mg/kg), there was a significant reduction in the proportion of HPP-CFC-1 in S-phase. (35.9 ± 3.1)% of HPP-CFC-1 were in S-phase following 2 Gy γ-irradiation and saline. This proportion

FIG. 4.5 The proportion of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with lisinopril

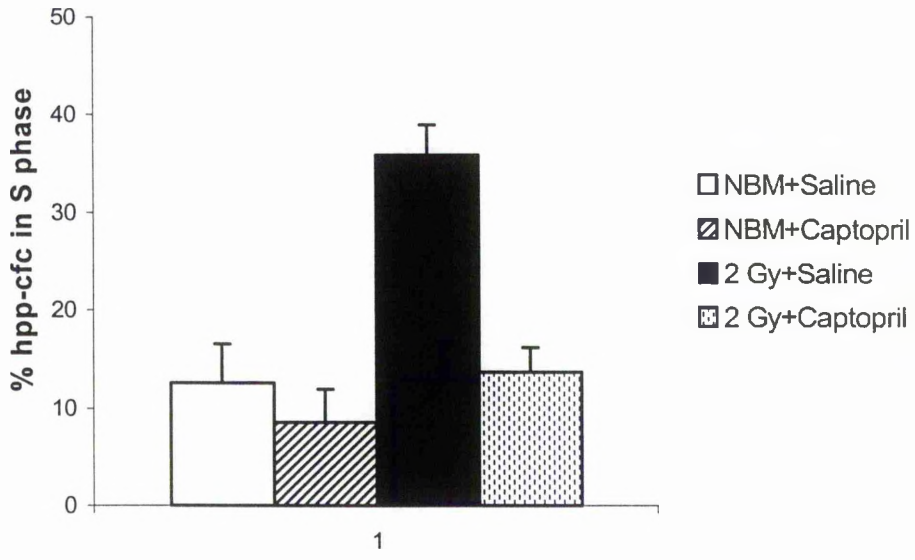
100mg/kg ACE ACE Inhibitors	**% HPP-CFC-1 in S-phase**	
NBM + Saline	(4.4 ± 7.1)%	
NBM + Lisinopril	(9.4 ± 4.0)%	P = 0.87, n = 4
2 Gy + Saline	(26.9 ± 3.5)%	
2 Gy + Lisinopril	(29.2 ± 2.2)%	P = 0.6, n = 4
NS & 2 GyS		P = 0.003
NL & 2 GyL		P = 0.006



was reduced to (13.7 ± 2.5)% (p = 0.0013) when 2 Gy exposed mice were given captopril (Fig 4.6). Despite having no effect at low doses captopril reduced the proportion of HPP-CFC-1 in S-phase following 2 Gy irradiation at therapeutic doses used in man in these mice.

FIG. 4.6 The proportion of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with captopril (100mg/kg)

100mg/kg ACE ACE Inhibitors	% HPP-CFC-1 in S-phase	
NBM + Saline	(12.6 ± 4.0)%	
NBM + Captopril	(8.6 ± 3.4)%	P = 0.65, n = 4
2 Gy + Saline	(35.9 ± 3.1)%	
2 Gy + Captopril	(13.7 ± 2.5)%	P = 0.0013, n = 4
NS & 2 GyS		P = 0.002
NC & 2 GyC		P = 0.42



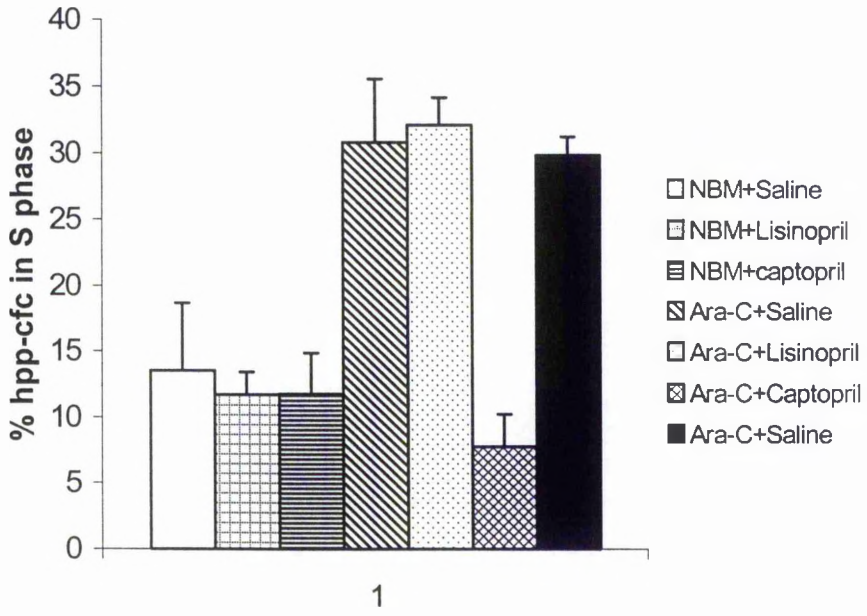
4.0.4 *The effect of 100mg/kg ACE inhibitors on Ara-C regenerating bone marrow HPP-CFC-1*

No difference in the proportion of HPP-CFC-1 was observed in the presence of ACE inhibitors in untreated control mice. (30.8 ± 4.6)% of HPP-CFC-1 were in S-

phase following Ara-C and saline which remained unchanged at (32.1 ± 2.0)% when mice received lisinopril (100mg/kg) following Ara-C treatment (p = 0.79) (see fig 4.7). However captopril showed a significant reduction in the proportion of HPP-CFC-1 in S-phase after

FIG 4.7 The proportion of HPP-CFC-1 in S-phase following Ara-C *in vivo* following ACE inhibitors (100mg/kg)

100mg/kg ACE ACE Inhibitors	**% HPP-CFC-1 in S-phase**		
NBM + Saline	(13.6 ± 5.1)%		
NBM + Lisinopril	(11.7 ± 1.7)%	P = 0.81,	n = 4
NBM + Captopril	(11.8 ± 3.1)%	P = 0.73,	n = 4
Ara-C + Saline-1	(30.8 ± 4.8)%		
Ara-C + Lisinopril	(32.1 ± 2.0)%	P = 0.79,	n = 4
Ara-C + Saline-2	(29.8 ± 1.5)%		
Ara-C + Captopril	(7.8 ± 2.5)%	P = 0.00024,	n = 4
NS & AS-1		P = 0.003	
NS & AC		P = 0.06	
AC & AL		P = 0.00014	



Ara-C treatment. (29.8 ± 1.4)% of HPP-CFC-1 were in S-phase when Ara-C and saline were administered and this proportion was significantly reduced to (7.8 ± 2.5)% (p = 0.00024)(Fig 4.7) when Ara-C and captopril (100mg/kg) were administered. The proportion of HPP-CFC-1 in S-phase observed after captopril was given to Ara-C treated mice was the same as the one observed in the control mice receiving only saline or ACE inhibitors.

4.0.5 *The effect of 100mg/kg ACE inhibitors on 5 FU regenerating bone marrow HPP-CFC-1 cycling*

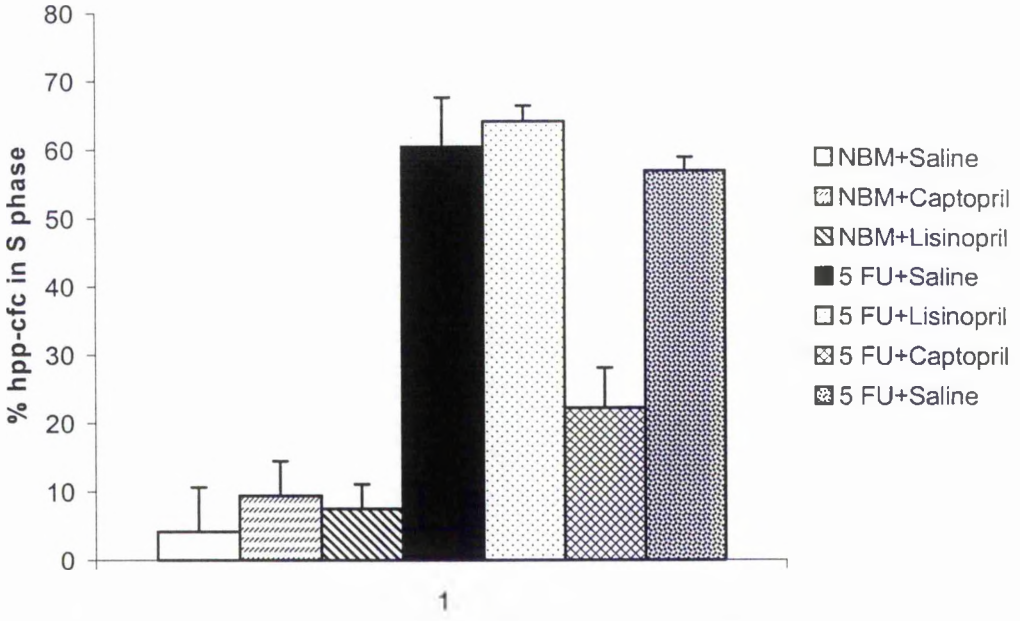
As shown in Fig 3.22, (60.5 ± 7.2)% of HPP-CFC-1 were in S-phase in 5 FU treated mice given saline and the proportion remained unchanged with lisinopril at

FIG. 4.9 The proportion of HPP-CFC-1 in S-phase following 5 FU treatment *in vivo* with ACE inhibitors (100mg/kg)

100mg/kg ACE ACE Inhibitors	% HPP-CFC-1 in S-phase	
NBM+ Saline	(4.1 ± 6.5)%	
NBM+ Lisinopril	(9.4 ± 5.0)%	P = 0.74, n = 4
NBM+ Captopril	(7.5 ± 3.6)%	P = 0.83, n = 4
5 FU + Saline	(60.9 ± 7.2)%	
5 FU + Lisinopril	(64.2 ± 2.4)%	P = 0.64, n = 4
5 FU + Saline	(57.0 ± 2.0)%	
5 FU + Captopril	(22.3 ± 5.8)%	P = 0.0013, n = 4
NS & 5 FUS		P < 0.001
NS & 5 FUC		P = 0.032
5 FUC & 5 FUL		P = 0.0004

NB: 5 FUS = 5 FU bone marrow + saline, 5 FUC = 5 FU bone marrow + captopril, 5 FUL = 5 FU bone marrow + lisinopril.

(64.2 ± 2.4)% (p = 0.64). On the other hand (57.0 ± 2.0)% of HPP-CFC-1 were in S-



phase in mice given saline after 5 FU treatment and captopril reduced this proportion to (22.3 ± 5.8)% (p = 0.0013)(see Fig 4.9). Thus high levels of captopril inhibited HPP-CFC-1 proliferation from 5 FU treated mice. However the inhibitory effect of captopril on HPP-CFC-1 proliferation following 5 FU treatment was incomplete. Captopril at its recommended therapeutic dose clearly had a suppressive effect on HPP-CFC-1 proliferation *in vivo* regardless of the length of time following its *in vivo* metabolism.

4.0.6 *Effect of Captopril on AcSDKP concentration in vivo following 2 Gy and Ara-C administration*

Procedure

3 groups of CD1 mice were given captopril 100 mg / kg-body weight an hour after saline, 2 Gy-γ-irradiation or Ara-C (100mg/kg-body weight) treatments. The procedure included control mice that received saline instead of captopril. Mice were

killed at 2, 6, and 24 hours following insult *in vivo*. Plasma was collected and samples were assayed for AcSDKP concentrations according to the methods already described.

Results

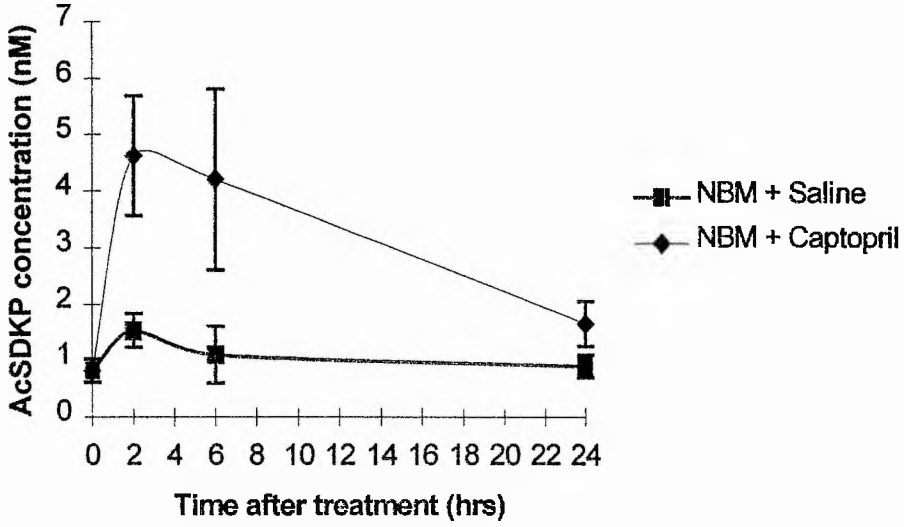
As shown in fig 4.8 plasma levels of AcSDKP are highest at 2 hours and they dropped to normal levels after 24 hours in captopril treated mice regardless of whether they received cytotoxic insults or not. The concentration of AcSDKP in the plasma of untreated mice was 0.82 ± 0.20 nM of AcSDKP. When captopril was administered to mice that had received saline, AcSDKP concentration increased to

FIG. 4.8 The *in vivo* effect of captopril on AcSDKP levels

100mg/kg Captopril	**AcSDKP concentration (nM)** T = 2	T = 6	T = 24		
N-plasma	0.82 ± 0.20				
NBM+ saline	1.53 ± 0.31	1.10 ± 0.50	0.90 ± 0.20	P = 0.126,	n = 3
NBM + Captopril	4.62 ± 1.06	4.20 ± 1.60	1.65 ± 0.40	P = 0.023,	n = 3
2 Gy + saline	0.92 ± 0.32	1.10 ± 0.50	1.00 ± 0.66	P = 0.886,	n = 3
2 Gy + captopril	9.70 ± 1.70	4.20 ± 0.60	1.95 ± 0.70	P = 0.000003,	n = 3
Ara-C + saline	1.10 ± 0.64	1.10 ± 0.60	1.00 ± 0.30	P = 0.97,	n =3
Ara-C + captopril	7.85 ± 2.20	4.14 ± 1.00	1.63 ± 0.35	P = 0.00027,	n = 3
N-plasma & NS	P = 0.03	P = 0.5	P = 0.77		
N-plasma & NC	P = 0.001	P = 0.03	P = 0.41		
N-plasma & GyS	P = 0.698	P = 0.41	P = 0.7		
N-plasma &GyC	P = 0.00006	P = 0.00003	P = 0.054		
N-plasma & AS	P = 0.56	P = 0.49	P = 0.48		
N-plasma & AC	P = 0.001	P = 0.001	P = 0.03		

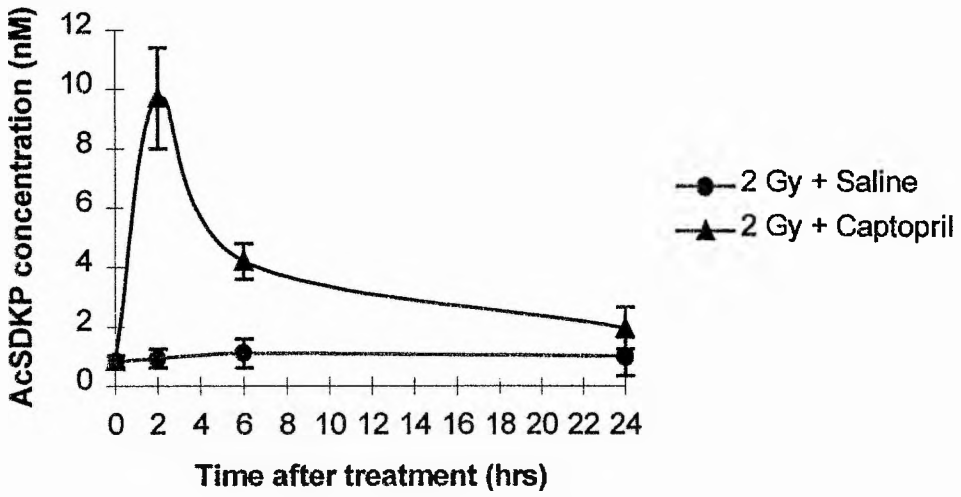
NB: N-plasma = Normal bone marrow plasma.

a. In vivo effect of captopril on AcSDKP concentration following saline treatment



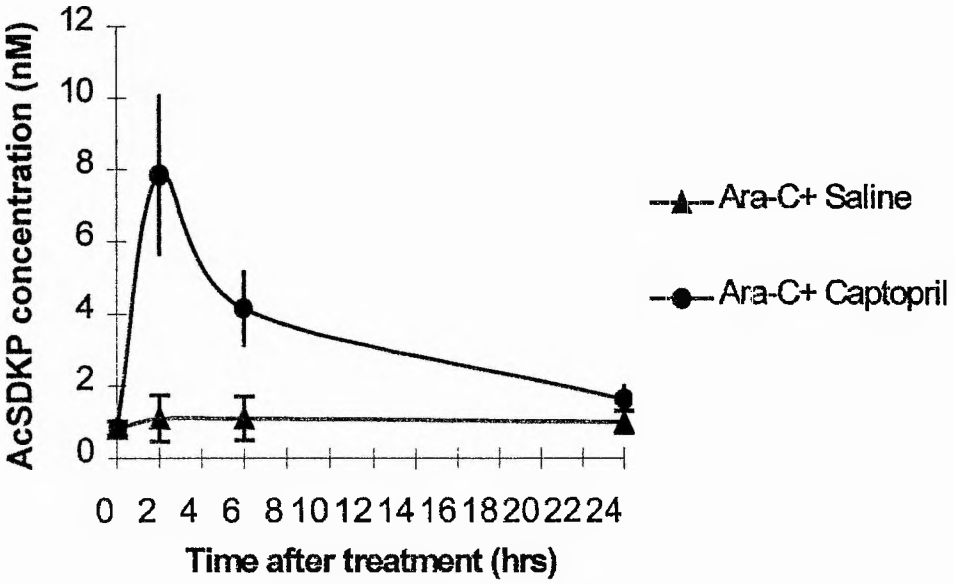
4.62 ± 1.06 nM after 2 hours, and were still high at 4.20 ± 1.60 nM after 6 hours. However after 24 hours the levels had decreased to 1.65 ± 0.40 nM ($p = 0.023$). No significant increase in AcSDKP levels was observed when saline treated mice received a second injection of saline. At 2 hours saline treated mice given saline had 1.53 ± 0.31 nM of AcSDKP and 1.10 ± 0.50 nM of AcSDKP were obtained at 6 hours. The levels of AcSDKP remained unchanged after 24 hours at 0.90 ± 0.02 nM ($p = 0.12$) in these mice. Thus saline did not influence AcSDKP production (see Fig 4.8a).

b. In vivo effect of captopril on AcSDKP concentration following 2 Gy γ-irradiation



Following 2 Gy irradiation captopril significantly elevated AcSDKP levels after 2 hours. 9.70 ± 1.70 nM of AcSDKP was obtained after 2 hours following 2 Gy and captopril and 4.20 ± 0.60 nM of AcSDKP was obtained after 6 hours. The levels of AcSDKP had dropped to 1.95 ± 0.70 nM after 24 hours ($p = 0.0000034$). On the other hand no such effect was observed with 2 Gy irradiated mice that received saline. The values of AcSDKP were 0.92 ± 0.32 nM, 1.10 ± 0.50 nM and 1.00 ± 0.66 nM at 2, 6, and 24 hours respectively ($p = 0.88$) (see Fig. 4.8b).

c. In vivo effect of captopril on AcSDKP concentration following Ara-C treatment



Mice that were given captopril after Ara-C treatment had an increase *in vivo* AcSDKP production. The values of AcSDKP were 7.85 ± 2.20 nM, 4.14 ± 1.00 nM and 1.63 ± 0.35nM at 2, 6, and 24 hours following Ara-C and captopril respectively ($p = 0.0003$). Therefore captopril had the same trend of AcSDKP production in Ara-C treated mice and 2 Gy irradiated mice. Just as with 2 Gy irradiation, no variation in AcSDKP endogenous production was observed when Ara-C treated mice were given saline. The values were 1.10 ± 0.64 nM, 1.10 ± 0.60 nM and 1.00 ± 0.3 nM at 2, 6, and 24 hours when saline was administered following Ara-C (see Fig.4.8c). These results

clearly showed that the ability of captopril to inhibit HPP-CFC-1 proliferation *in vivo* might partly involve the up-regulation of the natural levels of AcSDKP.

4.1 THE EFFECT OF CAPTOPRIL ON THE FRACTIONATED DOSE OF Ara-C

Procedure

Mice were given two doses of Ara-C 24 hours apart and 1 dose of captopril an hour after the first dose. Studies were done in two parts, the first part was to investigate the effect of 200mg/kg Ara-C low dose fractionation. The second part was a fractionation of a higher dose of Ara-C at 400mg/kg Ara-C dose. Four parameters were investigated after 3, 7 and 10 days following the first dose. These parameters were femur cellularity, spleen weights, GM-CFC and HPP-CFC-1 numbers per femur, and finally the proportion of GM-CFC and HPP-CFC-1 in S-phase at these times. These parameters were also studied on normal mice. Parameters from mice that did not receive any cytotoxic drug are included with each investigation. Femur cellularity, spleen weights, GM-CFC and HPP-CFC-1 measurements were carried out according to the already described methods.

Results

4.1.1 The effect of two doses 200mg/kg Ara-C fractionation by captopril at 100mg/kg or saline

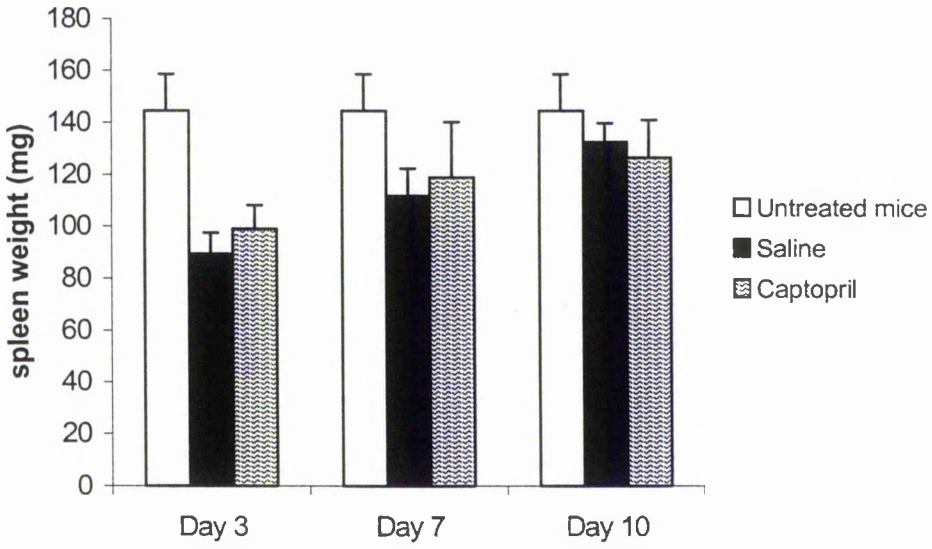
As shown in Fig 4.10, there was no spleen weight difference between mice that received saline and those that received captopril in between the two Ara-C doses at days 3 ($p = 0.09$), 7 ($p = 0.46$) and 10 ($p = 0.4$). However two doses of Ara-C markedly reduced the spleen weight at day 3 compared to control mice regardless of whether mice received saline or captopril in between the two doses of Ara-C. The

spleen size had recovered to normal weight on day 7. No difference in femur cellularity was observed between saline or captopril treatment in between the two Ara-C dose treatments at day 3 ($p = 0.48$), day 7 ($p = 0.74$) and at day 10 ($p = 0.54$). However at day 3 there was a marked reduction in femur cellularity between mice that received two doses of Ara-C compared to normal untreated mice (see Fig. 4.11). At day 7, however the difference in femur cellularity had disappeared between all groups. On the other hand, at day 10, the femur cellularity was higher in the Ara-C treated mice compared to normal control mice. GM-CFC numbers were also the same

FIG. 4.10 Mean spleen weights following 2 doses of Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**Mean Spleen weights**			
	(Control)	Saline	Captopril	
N-spleen	(144.5 ± 14)			
Day 3		89.5 ± 8.2	99.0 ± 9.3	P = 0.09, n = 4
Day 7		111.9 ± 10.7	119.0 ± 21.2	P = 0.46, n = 4
Day 10		132.6 ± 7.2	126.5 ± 14.3	P = 0.4, n = 4
N-spleen & Day 3 (S +C)				P < 0.05

NB: N-spleen = average normal spleen weight of 3 mice, S = saline, C = captopril

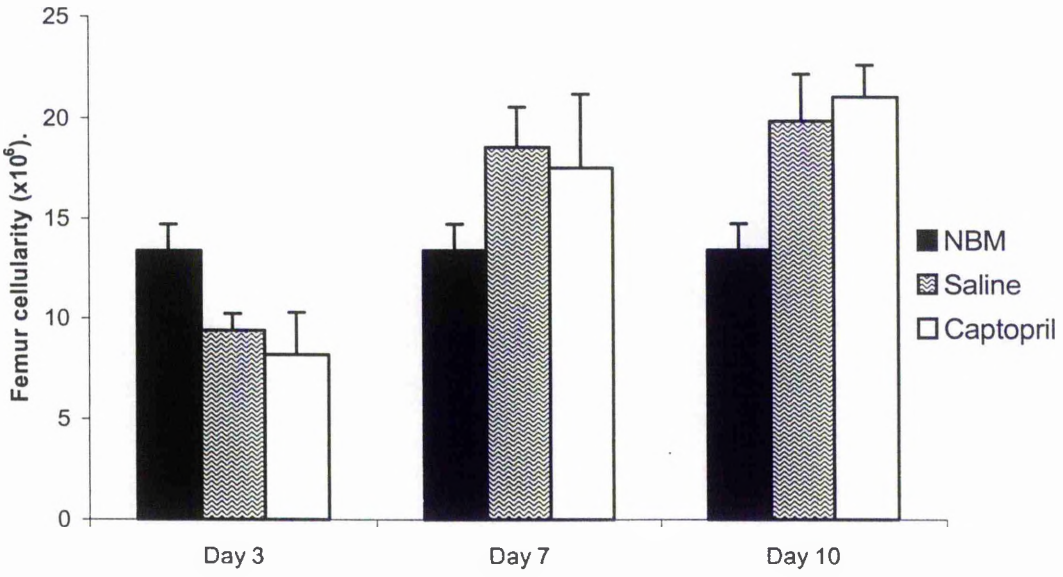


between Ara-C saline and Ara-C captopril treated mice at day 3 (p = 0.86) day 7 (p = 0.56) and at day 10 (p = 0.81). The absolute numbers of GM-CFC were markedly reduced following Ara-C treatment when compared to normal bone marrow control GM-CFC at day 3. GM-CFC numbers however returned to the levels found in normal mice at day 7 and remained at this level at day 10 (see Fig.4.12). There was a difference in the proportion of GM-CFC in S-phase in Ara-C treated mice between mice that were given saline and those that were given captopril in between the two Ara-C doses. At days 3 and 7 significantly less GM-CFC were in S-phase in the

FIG. 4.11 Femur cellularity following 2 doses of Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**Mean Femur cellularity (n ± SEM) x 10^6**		
	(Control) Saline	Captopril	
NFc	(13.4 ± 1.2)		
Day 3	9.4 ± 0.8	8.2 ± 2.1	P = 0.48, n = 4
Day 7	18.5 ± 2.0	17.5 ± 3.7	P = 0.74, n = 4
Day 10	19.8 ± 2.3	21.0 ± 1.5	P = 0.58, n = 4
NFc& day 3 (S+C)			P < 0.05
NFc & day 7 (S+C)			P > 0.05
NFc & day 10 (S +C)			P > 0.05

NB: NFc = Normal femur cellularity.

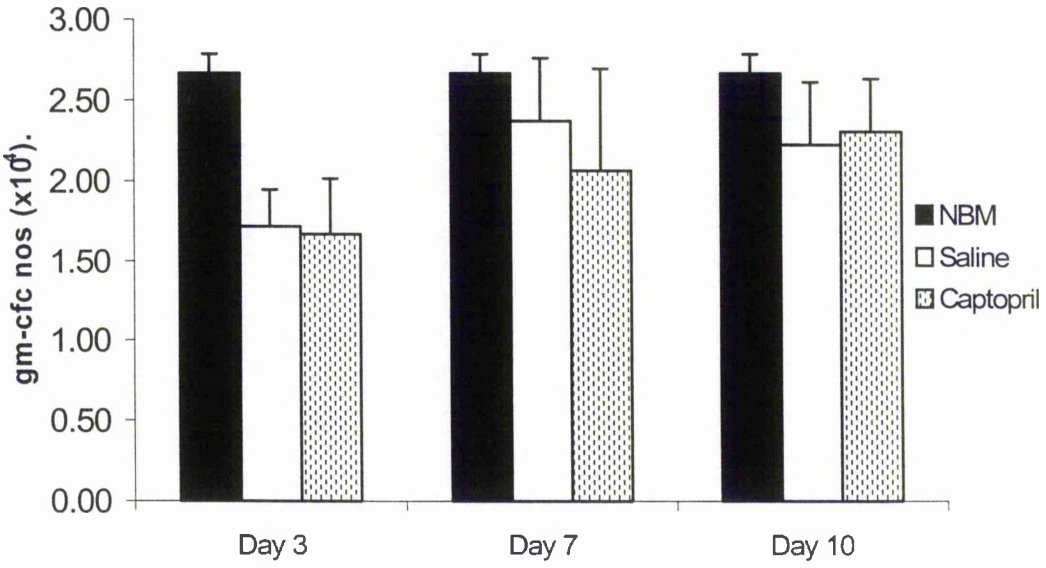


captopril group compared to the saline controls (Fig 4.13). At day 3, (29.6 ± 2.1)% of GM-CFC were in S-phase in mice given Ara-C and saline. This proportion was significantly reduced to (10.1 ± 1.7)% (p = 0.00008) in mice that were given captopril. At day 7, (32.3 ± 2.4)% of GM-CFC were in S-phase with saline. A significantly reduced proportion was observed with captopril and Ara-C at (5.8 ± 2.1)% (p = 0.00003). However, this difference was lost after 10 days. While saline

FIG. 4.12 GM-CFC numbers following 2 doses Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**Mean GM-CFC nos (per femur)(n ± SEM) x 10^4**			
	(Control)	Saline	Captopril	
NGM	(2.7 ± 1.2)			
Day 3		1.7 ± 0.2	1.7 ± 0.4	P = 0.48, n = 4
Day 7		2.4 ± 0.4	2.1 ± 0.6	P = 0.74, n = 4
Day 10		2.2 ± 0.4	2.3 ± 0.3	P = 0.58, n = 4
NGM & day 3 (S) (C)				P = 0.03, 0.03
NGM & day 7 (S) (C)				P = 0.62, 0.34
NGM & day 10 (S) (C)				P = 0.38, 0.47

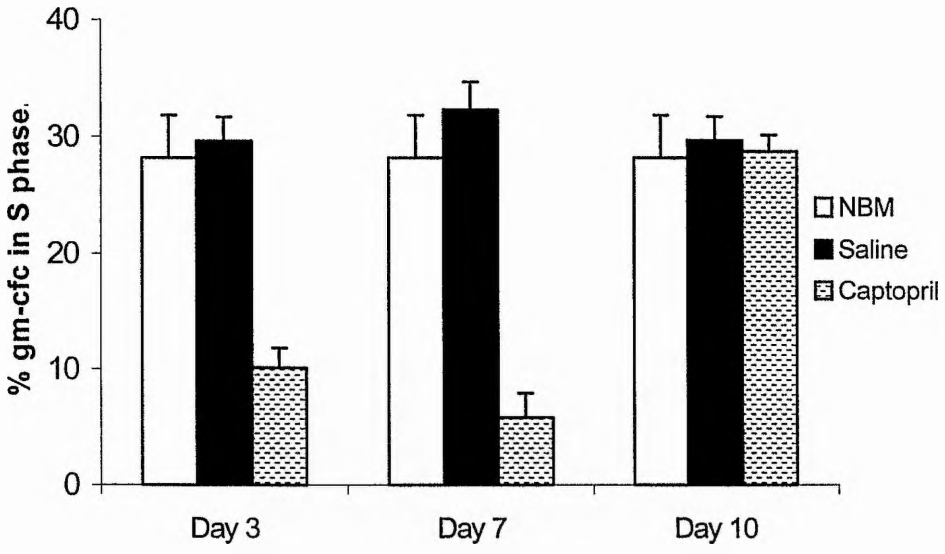
NB: NGM = Normal GM-CFC numbers per femur, S = Saline, C = Captopril.



treated mice had (29.7 ± 2.1)%, captopril treated mice had (28.7 ±1.4)% of GM-CFC in S-phase (p = 0.68). There was no difference between the proportion of unstressed bone marrow GM-CFC in S-phase and those treated with two doses of Ara-C (see Fig 4.13).

FIG. 4.13 The proportion of GM-CFC in S-phase following 2 doses of Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	% GM-CFC in S-phase			
	(Control)	Saline	Captopril	
NGM	(28.2 ± 3.6)%			
Day 3		(29.6 ± 2.1)%	(10.1 ± 1.7)%	P = 0.00008, n = 4
Day 7		(32.3 ± 2.4)%	(5.8 ± 2.1)%	P = 0.00003, n = 4
Day 10		(29.7 ± 2.1)%	(28.7 ± 1.4)%	P = 0.68, n = 4
NGM & day 3 (S)				P > 0.5
NGM & day 7 (S)				P > 0.5
NGM & day10 (S +C)				P > 0.5



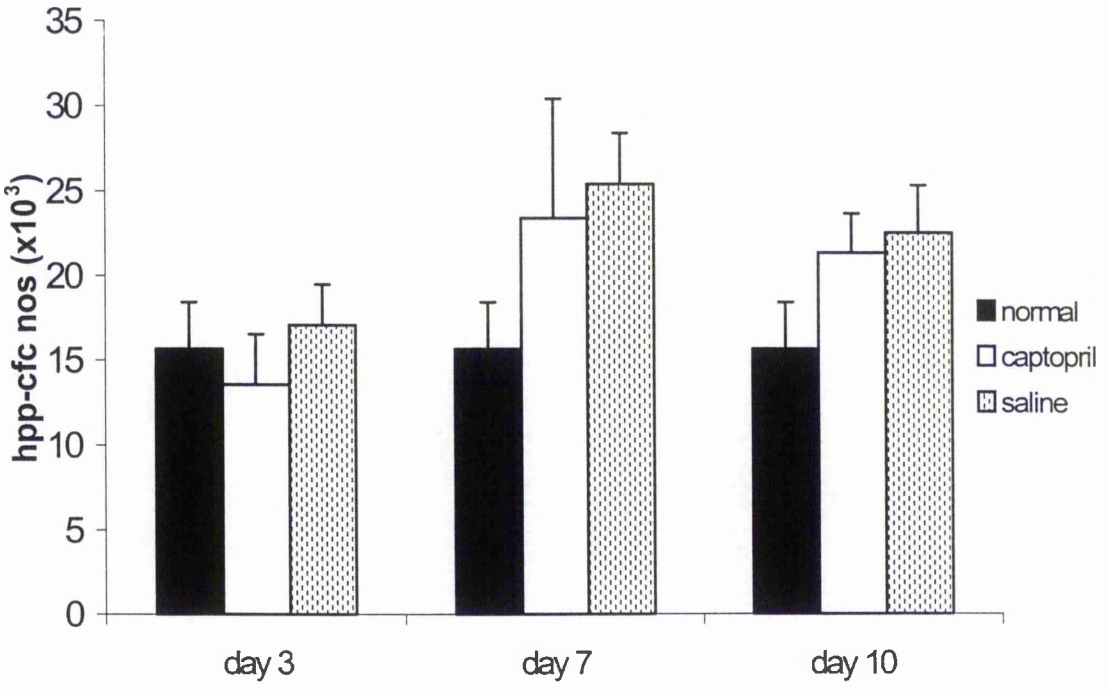
HPP-CFC-1 numbers were same between Ara-C treated mice regardless of whether they were given saline or captopril in between the two Ara-C doses at days 3, 7, and 10 (p = 0.24, 0.71, 0.66 respectively). HPP-CFC-1 numbers were not

influenced by the two Ara-C doses unlike GM-CFCs whose numbers were reduced at day 3. (see Fig. 4.14). However fractionating 2 doses of Ara-C with captopril markedly reduced the proportion of HPP-CFC-1 in S-phase at all times investigated. Indeed HPP-CFC-1 S-phase entry was blocked at day 7 with captopril given in

FIG. 4.14 HPP-CFC-1 numbers following 2 doses of Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**Mean HPP-CFC-1nos (per femur)(n ± SEM) x 10^3**			
	(control)	Saline	Captopril	
NHPP	(15.7 ± 2.7)			
Day 3		17.0 ± 2.5	13.6 ± 3.0	P = 0.24, n = 4
Day 7		23.4 ± 3.0	23.4 ± 7.1	P = 0.71, n = 4
Day 10		22.5 ± 2.8	21.4 ± 2.3	P = 0.66, n = 4
NHPP & day 3 (S+C)				P > 0.05
NHPP & day 7 (S+C)				P > 0.05
NHPP & day 10 (S +C)				P > 0.05

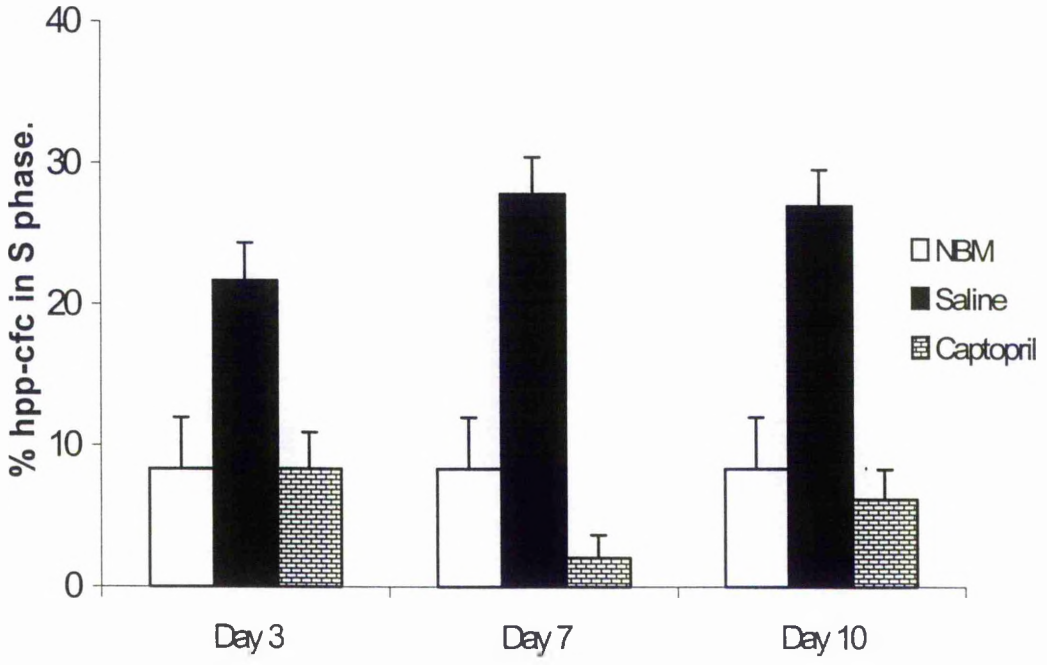
NB: NHPP = Normal HPP-CFC-1 numbers per femur.



between the two doses of Ara-C (see Fig 4.15). At day 3, (21.7 ± 2.7)% of HPP-CFC-1 were in S-phase with two Ara-C doses given saline and captopril reduced this proportion to (8.3 ± 2.7)% (p = 0.0027). At day 7, (27.8 ± 2.6)% of HPP-CFC-1 were in S-phase in the presence of saline. This was reduced to (2.1 ± 1.6)% with captopril (p = 0.00003). And finally at day 10, while (26.9 ± 2.6)% of HPP-CFC-1 were in S-phase in the presence of saline, only (6.2 ± 2.1)% of them were in S-phase with the two Ara-C doses separated by captopril (p = 0.00023).

FIG. 4.15 The proportion of HPP-CFC-1 in S-phase following 2 doses of Ara-C (200mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**% HPP-CFC-1 in S-phase**			
	(Control)	Saline	Captopril	
NHPP	(8.3 ± 3.6)%			
Day 3		(21.7 ± 2.7)%	(8.3 ± 2.7)%	P = 0.0027, n = 4
Day 7		(27.8 ± 2.6)%	(2.1 ± 1.6)%	P = 0.00003, n = 4
Day 10		(26.9 ± 2.6)%	(6.2 ± 2.1)%	P = 0.00023, n = 4
NHPP & day 3 (Cap.)				P > 0.5
NHPP & day 7 (Cap.)				P < 0.05
NHPP & day 10 (Cap.)				P > 0.5

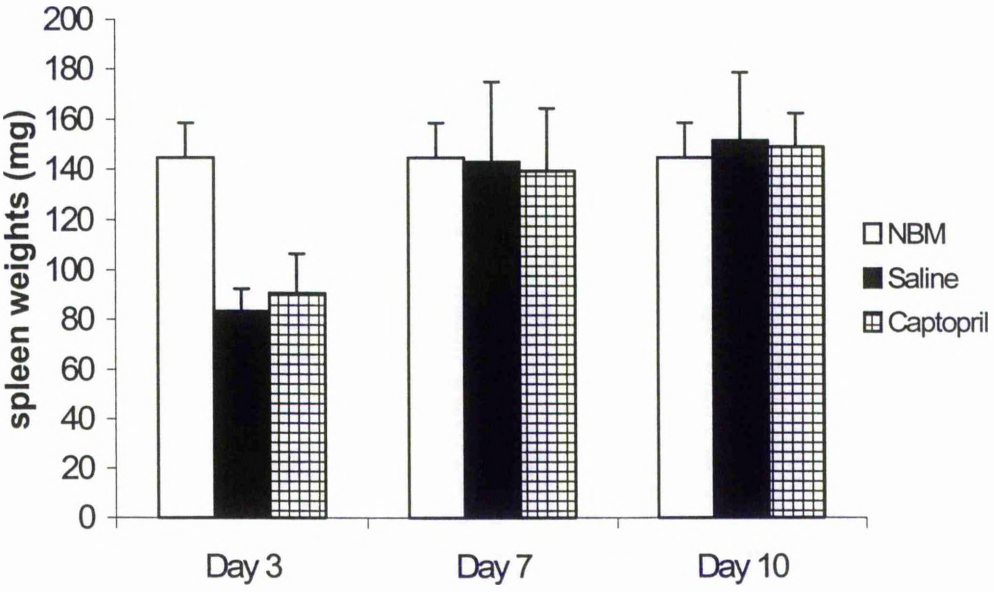


4.1.2 *The effect of two doses of 400mg/kg Ara-C fractionation by captopril at 100mg/kg or saline*

There was also no difference in the spleen weight between Ara-C fractionated with saline mice and those fractionated with captopril at days 3 ($p = 0.05$), 7 ($p = 0.87$) and 10 ($p = 0.9$) (see Fig 4.16). A marked reduction in spleen weight was

FIG. 4.16 Mean spleen weights following 2 doses of Ara-C (400mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (400mg/kg)	Mean Spleen weights (gms) (Control)	Saline	Captopril	
N-spleen	(144.6 ± 14.0)			
Day 3		83.5 ± 9.00	90.8 ± 15.8	P = 0.052, n = 4
Day 7		143.0 ± 31.9	139.3 ± 25.1	P = 0.87, n = 4
Day 10		151.5 ± 27.1	149.1 ± 13.3	P = 0.9, n = 4
N-spleen & day 3 (S+C)				P < 0.05
N-spleen & day 7 (S+C)				P > 0.5
N-spleen & day 10(S +C)				P > 0.5



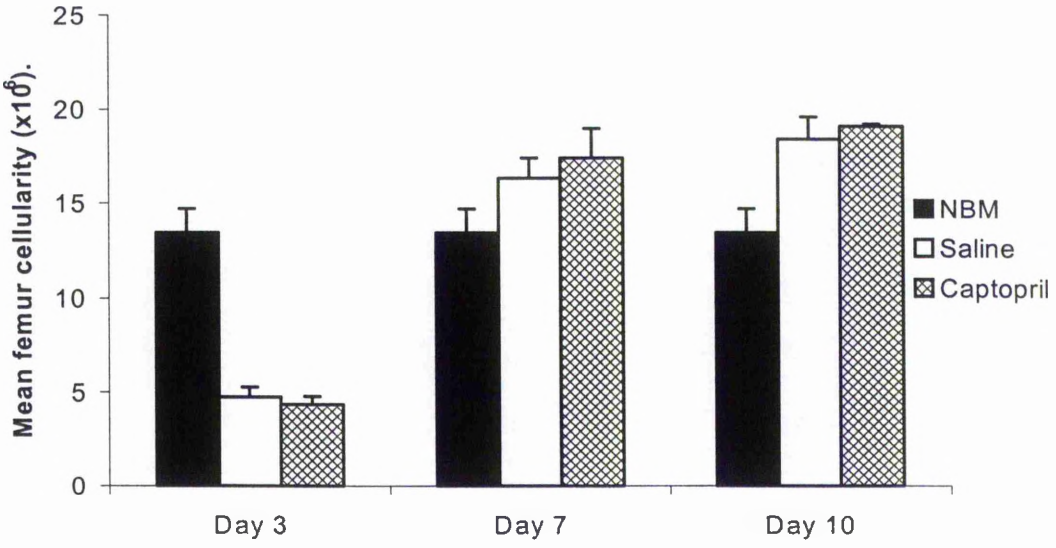
observed between normal spleen control weights and mice treated with two dose of Ara-C regardless of whether they were given captopril or saline at day 3. Spleen

weights returned to levels equivalent to the weights found in control untreated mice at day 7.

The femur cellularities were the same at all times between Ara-C fractionated with saline or captopril at days 3 (p = 0.4), 7 (p = 0.45) and 10 (p = 0.37) (see Fig 4.17). However 2 high doses of Ara-C reduced the femur cellularity to less than half of the values found in femurs from untreated mice. The reduction in femur cellularity

FIG. 4.17 Mean femur cellularity following 2 doses of Ara-C (400mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (400mg/kg)	**Mean Femur cellularity (n ± SEM) x 10^6**			
	(Control)	Saline	Captopril	
NFc	(134.0 ± 12.9)			
Day 3		4.8 ± 0.5	4.4 ± 0.4	P = 0.4, n = 4
Day 7		16.3 ± 1.1	17.4 ± 1.6	P = 0.45, n = 4
Day 10		18.4 ± 1.2	19.1 ± 0.1	P = 0.37, n = 4
NFc & day 3 (S+C)				P < 0.001
NFc & day 7 (S+C)				P < 0.05
NFc & day 10 (S +C)				P < 0.05

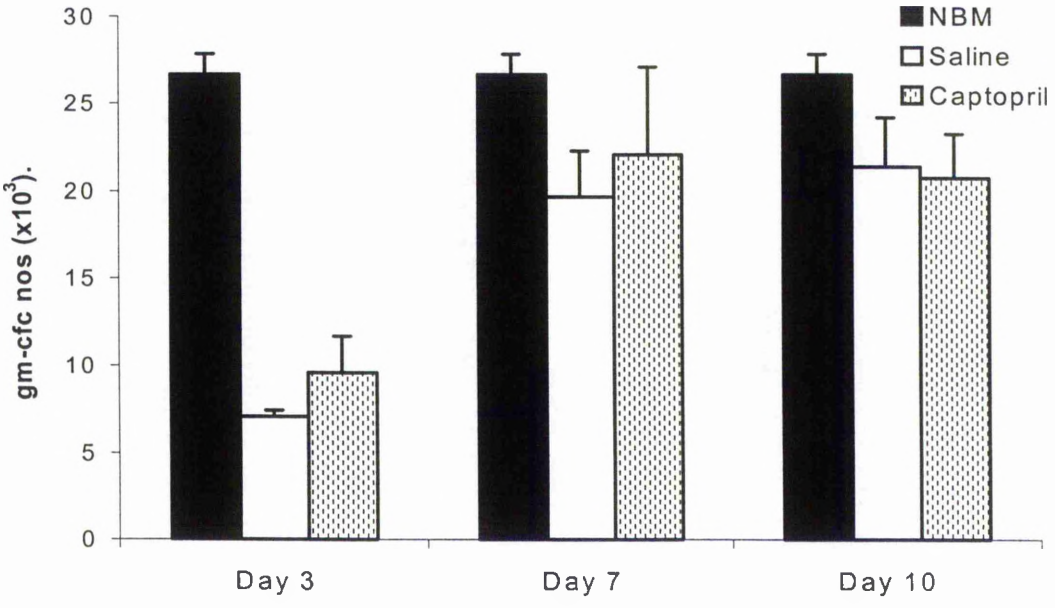


was the same in Ara-C treated mice regardless of whether saline or captopril was used for fractionation. The femur cellularity returned to higher levels than normal femur cellularities at day 7 and remained high for the duration of the investigation. When the mean femur cellularities of low and high dose Ara-C are compared, high doses show a greater decrease in the number of nucleated cells per femur (see Figs 4.11 & 4.17).

As with low doses Ara-C treatments, GM-CFC numbers were not affected by captopril fractionation after high dose Ara-C treatment at day 3 ($p = 0.13$) day 7 ($p =$

FIG. 4.18 GM-CFC numbers following 2 doses of Ara-C (400mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (400mg/kg)	**Mean GM-CFC nos (per femur)(n ± SEM) x 10^3**			
	(Control)	Saline	Captopril	
NGM-CFC	2.7 ± 1.2			
Day 3		7.0 ± 0.4	9.6 ± 2.1	P = 0.13, n = 4
Day 7		19.7 ± 2.6	22.1 ± 5.0	P = 0.57, n = 4
Day 10		21.4 ± 2.8	20.8 ± 2.2	P = 0.82, n = 4
NGM & day 3 (S) (C)				P = 0.00025, 0.0012
NGM & day 7 (S) (C)				P = 0.097, 0.44
NGM & day 10 (S) (C)				P = 0.22, 0.15

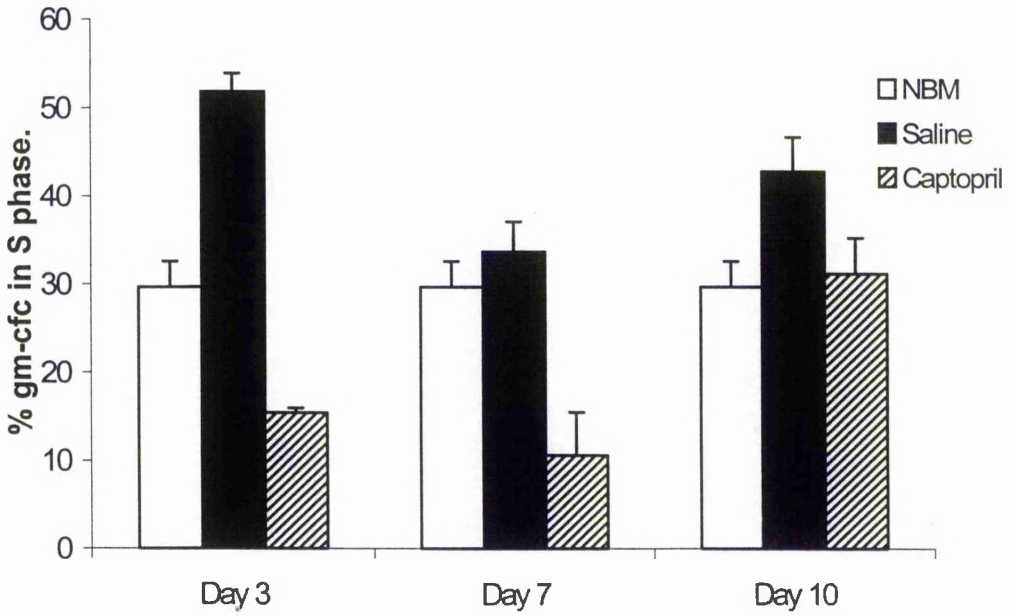


0.57) and at day 10 (p = 0.82) (see Fig. 4.18). However there was a far greater reduction in GM-CFC numbers following two Ara-C high dose treatments when compared GM-CFC numbers found in mice that did not receive any Ara-C at day 3. GM-CFC numbers returned to levels equivalent to those found in femurs of mice that were not subjected to any treatment at day 7 and remained so at day 10.

However, high doses of Ara-C had a marked effect on the proportion of GM-CFC in S-phase when fractionated with saline at day 3. (51.9 ± 2.1)% were in S-phase at this time. At day 7, (33.7 ± 3.4)% of GM-CFC were in S-phase with saline treated and at 10, (42.8 ± 3.9)% of GM-CFC were in S-phase in the same saline group.

FIG. 4.19 The proportion of GM-CFC in S-phase following 2 doses of Ara-C (400mg/kg)

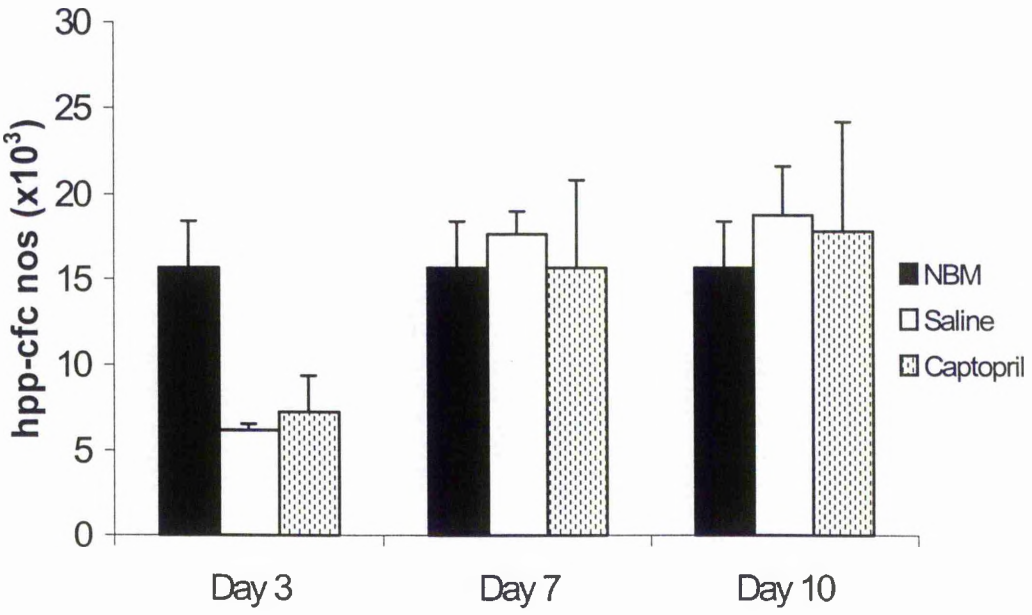
100mg/kg Captopril with 2 doses of Ara-C (400mg/kg)	% GM-CFC in S-phase			
	(Control)	Saline	Captopril	
NGM	(29.7 ± 2.8)%			
Day 3		(51.9 ± 2.1)%	(15.7 ± 0.6)%	P = 0.0000026, n = 4
Day 7		(33.7 ± 3.4)%	(10.7 ± 4.8)%	P = 0.0079, n = 4
Day 10		(42.8 ± 3.9)%	(31.2 ± 4.0)%	P = 0.084, n = 4
NGM & day 3 (S)				P < 0.05
NGM & day 7 (S)				P > 0.05
NGM & day 10 (S +C)				P > 0.05



Captopril significantly reduced the proportion of GM-CFC in S-phase at day 3 to (15.4 ± 0.6)% ($p = 0.0000026$) and to (10.7 ± 4.8)% at day 7 ($p = 0.0079$). However, only a slight non-significant reduction in the proportion of GM-CFC in S-phase was observed in the presence of captopril at (31.2 ± 4.0)% ($p = 0.084$) after 10 days. Interestingly, the proportion of GM-CFC in S-phase after 10 days in captopril treated mice was similar to the proportion of GM-CFC in S-phase observed from mice that did not receive any insult (see Fig 4.19).

FIG. 4.20 HPP-CFC-1 numbers following 2 doses of Ara-C (400mg/kg)

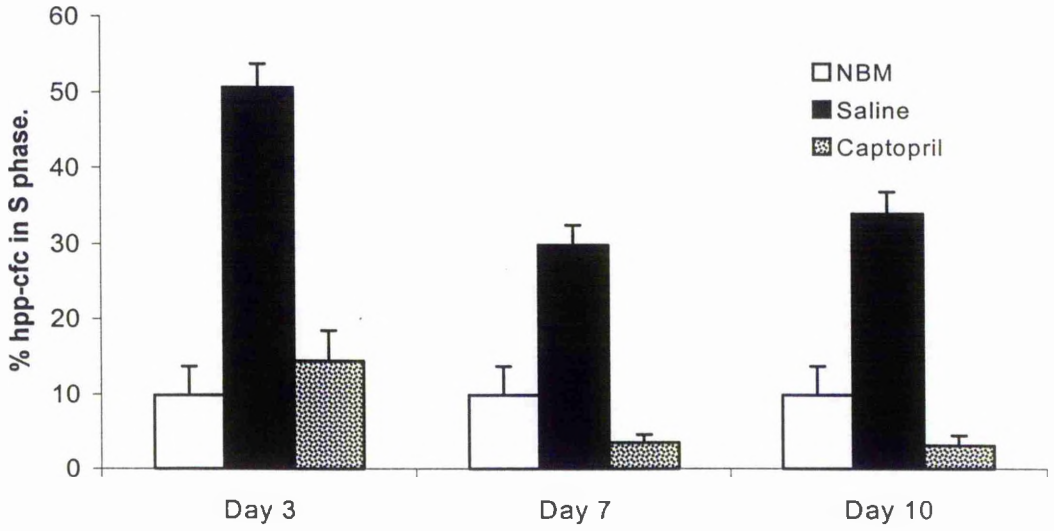
100mg/kg Captopril with 2 doses of Ara-C (200mg/kg)	**Mean HPP-CFC-1 nos (per femur)(n ± SEM) x 10^3**			
	control	Saline	Captopril	
NHPP-CFC-1	15.7 ± 2.7			
Day 3		6.2 ± 0.4	7.2 ± 2.5	P = 0.52, n = 4
Day 7		17.6 ± 1.3	15.7 ± 5.2	P = 0.58, n = 4
Day 10		18.8 ± 2.9	17.8 ± 6.4	P = 0.84, n = 4
NHPP & day 3 (S+C)				P < 0.05
NHPP & day 7 (S+C)				P > 0.05
NHPP & day 10 (S +C)				P > 0.05



Interestingly, high doses of Ara-C reduced the numbers of HPP-CFC-1 at day 3 while this was not the case with low doses of Ara-C (see fig.4.14). However high Ara-C fractionation showed no difference in HPP-CFC-1 numbers between saline and captopril at all times investigated. There was no difference in HPP-CFC-1 numbers at days 3, 7 and 10 ($p = 0.52, 0.58, 0.84$ respectively) between captopril and saline fractionations (see Fig 4.20). The reduction in HPP-CFC-1 numbers was observed at day 3 between control mice and mice that received Ara-C. This difference was resolved at day 7 and the numbers remained at the same levels as those found in

FIG 4.21 The proportion of HPP-CFC-1 in S-phase following 2 doses of Ara-C (400mg/kg)

100mg/kg Captopril with 2 doses of Ara-C (400mg/kg)	**% HPP-CFC-1 in S-phase**			
	(Control)	Saline	Captopril	
NHPP-CFC-1	(9.9 ± 3.9)%			
Day 3		(50.6 ± 3.1)%	(14.7 ± 4.0)%	P = 0.00038, n = 4
Day 7		(29.8 ± 2.7)%	(3.5 ± 1.1)%	P = 0.000094, n = 4
Day 10		(33.9 ± 2.8)%	(3.1 ± 1.3)%	P = 0.000065, n = 4
NHPP & day 3 (Cap.)				P > 0.05
NHPP & day 7 (Cap.)				P < 0.05
NHPP & day10 (Cap.)				P < 0.05



untreated mice for the duration of the investigations. High doses of Ara-C had a marked effect on the proportion of HPP-CFC-1 in S-phase. The proportion of HPP-CFC-1 in S-phase markedly increased to (50.6 ± 3.1)% at day 3 (see Fig 4.21) in Ara-C treated mice that were fractionated with the saline. With low doses at day 3, the proportion of HPP-CFC-1 in S-phase with Ara-C saline fractionation was only (21.7 ± 2.7)% (see Fig 4.15). After 7 days (29.8 ± 2.7)% of HPP-CFC-1 were in S-phase in saline fractionated mice and after 10 days these mice had (33.9 ± 2.8)% of HPP-CFC-1 in S-phase. However at all these times captopril fractionation reduced the proportion of HPP-CFC-1 in S-phase following high dose Ara-C dose treatment (see Fig 4.21). At day 3, (14.4 ± 4.0)% of HPP-CFC-1 were in S-phase in Ara-C fractionated with captopril group ($p = 0.00038$) and only (3.5 ± 1.1)% ($p = 0.000094$) of HPP-CFC-1 were in S-phase at day 7 in the presence of captopril. After 10 days, captopril still inhibited HPP-CFC-1 S-phase transition. The proportion of HPP-CFC-1 in S-phase at this time was (3.1 ± 1.3)% ($p = 0.000065$). Therefore regardless of the doses of Ara-C used, captopril was able to inhibit HPP-CFC-1 S-phase entry at all times but was unable to influence GM-CFC proliferation after 7 days.

4.2 Discussion

The normal clinical therapeutic dose of lisinopril is 10mg/kg (Goa et al., 1996) and that of captopril is from 50mg/kg to 150mg/kg (Martin et al., 1984). Therefore an average dose of 100mg/kg for captopril was chosen as a dose used to investigate the effect of captopril on HPP-CFC-1 proliferation *in vivo*. The chosen dose of captopril to be investigated was a single dose given to mice 1 hour after the various insults.

HPP-CFC-1 recruited in S-phase by 5 FU are assayed after 4 days. The effects of ACE inhibitors were investigated after 4 days following 5 FU treatment. Animals

were given insults and ACE inhibitors in the same way as with 2 Gy or Ara-C. Due to a long lag period before HPP-CFC-1 suicide assays are performed following 5 FU, it was difficult to investigate the correlation between AcSDKP and ACE inhibitors following 5 FU treatment. Low doses of lisinopril were not investigated because they showed a discrepancy for their inhibition of HPP-CFC-1 proliferation between 2 Gy and Ara-C regenerating bone marrow cells. Due to a short $t_{½}$ of ACE inhibitors in general, it may be difficult to interpret the effect of low dose lisinopril on HPP-CFC-1 proliferation after 4 days. Therefore, it was decided to investigate the effects of captopril on HPP-CFC-1 proliferation using only high doses of ACE inhibitors following 5 FU treatment.

Plasma from animals which received 2 Gy or Ara-C with 100mg/kg captopril was assayed for AcSDKP *in vivo* levels at 2 hrs, 6 hrs and 24 hours following the insult. No AcSDKP plasma levels from mice receiving lisinopril after insult was assayed because AcSDKP levels following 2 Gy and lisinopril have been investigated before (Rousseau et al., 1998). AcSDKP levels *in vivo* were not assayed following 5 FU treatment because 5 FU bone marrow cells were sampled after 4 days. In addition, both 2 Gy and Ara-C treated mice showed normal levels of AcSDKP following captopril after 24 hour.

4.2.1 *In vivo ACE inhibitor effect on stem cell proliferation*

ACE inhibitors were given to mice following *in vivo* administration of 2 Gy γ-irradiation, Ara-C and 5 FU. At therapeutic doses lisinopril slightly inhibited HPP-CFC-1 proliferation following Ara-C (see Fig. 3.12). Lisinopril had no effect on HPP-CFC-1 proliferation when it was administered at high doses. At therapeutic doses lisinopril had a boarder line inhibitory effect on HPP-CFC-1 proliferation following 2

Gy and it was unable to influence HPP-CFC-1 proliferation following 5 FU *in vivo* treatment at high doses. Both captopril and lisinopril did not affect the proliferation of HPP-CFC-1 in mice that did not receive insults. On the other hand, captopril at therapeutic doses inhibited HPP-CFC-1 proliferation regardless of the insult used. This is in agreement with the findings of Boyd et al., (1982). Boyd et al., (1982) found that captopril failed to produce myelotoxicity in normal Swiss mice but the same dose of captopril given to mice with busulfan-induced stem cell damage produced myelosuppression. They suggested that the suppressive action of captopril on neutrophilic progenitor cell proliferation *in vivo* might be caused by the suppression of an earlier myeloid progenitor cell rather than the GM-CFC population (Boyd et al., 1984). The fact that captopril inhibited HPP-CFC-1 proliferation following 5 FU *in vivo* also suggests an indirect mechanism of captopril induced inhibition of cell proliferation. 5 FU bone marrow was sampled after 4 days and the short duration of captopril *in vivo* (Duchin et al., 1982), suggests involvement of accessory factors in captopril's inhibitory mechanism. The fact that captopril inhibits HPP-CFC-1 proliferation following 5 FU treatment after 4 days, suggests the strength of captopril as an inhibitor of haematopoietic cell proliferation

The differential effects of captopril and lisinopril on cell proliferation in the present investigations are unknown. These drugs may have a direct effect on cell proliferation or they may act indirectly by regulating endogenous production of inhibitors of cell proliferation. Captopril and lisinopril both down-regulate angiotensin-II levels *in vivo*. The fact that captopril was not able to inhibit cell proliferation at low doses and lisinopril inhibited Ara-C regenerating bone marrow HPP-CFC-1 at its therapeutic dose might however suggest that the degree of ACE inhibition may be crucial to the cellular inhibitory effects imposed by these drugs.

However, if this was the only mechanism, an increase in the inhibitory influence should have been observed by lisinopril at high doses. Captopril is a weaker inhibitor of ACE and is normally given at least twice a day (Martin et al., 1984). *In vivo* captopril inhibitory effect on cell proliferation may involve other mechanisms other than an increase in AcSDKP levels. While partial involvement of angiotensin-II is a possibility, the effects of captopril on haematopoietic cell proliferation both *in vivo* and *in vitro* may also involve the up-regulation of some other natural inhibitors of stem cell proliferation. Lisinopril may not be able to influence the endogenous production of these unknown inhibitors. The present results *in vivo*, just as with *in vitro* results cast doubts on a direct involvement of angiotensin-II on the captopril inhibitory effect on HPP-CFC-1 proliferation. Captopril has the ability to inhibit the proliferation of a number of cells both *in vivo* and *in vitro* as will be discussed in chapter 6.

The noticeable effect of high doses of captopril to influence the proliferation of regenerating HPP-CFC-1 from 2 Gy and Ara-C regenerating bone marrow cells *in vivo* was investigated further to show whether captopril's inhibitory mechanism was indirectly influenced by the endogenous production of AcSDKP. The correlation between the cycling status of haematopoietic stem cells and AcSDKP levels following captopril administration was studied. This was carried out using plasma but not on serum since captopril was a shown to be a weak inhibitor of ACE activity in serum. The control group of animals that received saline followed by captopril was also included. In the present investigations, the correlation between captopril and AcSDKP levels showed that captopril increased levels of AcSDKP to a peak after 2 hours following 2 Gy and Ara-C *in vivo* treatments (see Fig. 4.8a,b.c).

4.2.2 *The effect of captopril on Ara-C fractionated doses*

Having established that captopril has inhibitory properties on the proliferation of HPP-CFC-1, an investigation was carried out to evaluate whether this effect would be employed to protect haematopoietic cells from cytotoxic insults *in vivo*. Cytotoxic drugs are generally given in fractionated doses in the treatment of neoplastic disease. In addition, more than one cytotoxic drug is administered at any one time in some treatment regimes. An investigation was carried out to find out whether captopril given with cytotoxic drugs would reduce the proportion of HPP-CFC-1 in S-phase following fractionated doses of cytotoxic drugs. This would protect HPP-CFC-1 from the subsequent doses of cytotoxic drugs. The study was conducted to mimic the therapeutic regimes in the treatment of neoplastic diseases. Since Ara-C has a short duration of action and is cytotoxic to cells in S-phase, it was ideal for this investigation. The parameters studied were spleen weights, femur cellularity, GM-CFC and HPP-CFC numbers with their kinetics.

Since spleens are active haematopoietic organs in mice, spleen weights are a good indication of haematopoietic cell recovery in mice. The spleen weights were reduced in size following two doses of Ara-C but returned to normal levels within 1 week regardless of the doses of Ara-C used both in captopril treated mice and saline control mice. The same trend was observed with femur cellularities and GM-CFC numbers. However only high doses of Ara-C were able to reduce HPP-CFC-1 numbers with no observable effect on HPP-CFC-1 numbers with low dose Ara-C fractionation. This signifies that mild insults may not affect the haematopoietic system while larger insults may injure the haematopoietic system. Regardless of the insult to the haematopoietic system, the system has a remarkable ability to recover. The fact that Ara-C is an S-phase cytotoxic specific drug (Crisp et al., 1996), means that only a

small pool of haematopoietic stem cells is killed with the first Ara-C dose while about 30 % of GM-CFC are killed. Since captopril inhibits HPP-CFC-1 proliferation, the second dose of Ara-C should have no effect on the stem cell pool. However, the number of GM-CFC affected by the second Ara-C dose will be greater because GM-CFC are actively cycling and a high concentration of inhibitory factors is needed to inhibit GM-CFC proliferation (Wright et al., 1985; Tejero et al., 1984). The reduction in haematological parameters observed at day 3 can be explained by this mechanism.

In these investigations captopril was not able to protect the numbers of both GM-CFC and HPP-CFC-1 per femur following high dose Ara-C treatments as shown by their reduced numbers on day 3. However, it was able to significantly reduce the proportion of both HPP-CFC-1 and GM-CFC in S-phase. Despite the inhibitory effect it imposed on GM-CFC proliferation on days 3 to 7 and HPP-CFC-1 proliferation for the duration of the investigations, femur cellularity and HPP-CFC-1 numbers completely recovered at day 7. Captopril did not stop the regeneration of GM-CFC per femur since both captopril and saline had the same GM-CFC numbers for the duration of the investigation. In addition, the femur cellularity which was low at day 3 had recovered by day 7 and an over-shoot from the control levels was observed at day 10 in both captopril and saline treated animals following Ara-C. Thus the progenitor cell generation was not inhibited in the presence of captopril. The ability of a small percentage of HPP-CFC-1 to recover haematopoietic cell numbers can mostly be explained by the quality of these stem cells. Stem cells following Ara-C and captopril have undergone few cell divisions and therefore, have a greater capacity of self-renewal and cell division before they can differentiate to committed progenitor cells (Schofield et al., 1983). The net result is that a few cells are undergoing a lot of cell divisions. Moreover, the length of the both HPP-CFC-1 and GM-CFC cell cycle will

also determine the total femur cellularity. It is possible the time it takes for these cells to complete the cell cycle is reduced following captopril. This might also explain the discrepancy between the femur cellularity and the number of both HPP-CFC-1 and GM-CFC in S-phase. The fact that cell recovery is the same in the presence of captopril and saline can be attributed to the feed back loop. There is a minimum number of haematopoietic cells required for the system to function and a lot of HPP-CFC-1 are recruited in the presence of saline to achieve this number while only a few HPP-CFC-1 in captopril treated mice can achieve the same numbers. Therefore, in this model captopril not only protected HPP-CFC-1 from cytotoxic insults but it also assured a long term recovery of the haematopoietic system. However, it is still intriguing that HPP-CFC-1 that were not cycling were able to rescue the haematopoietic system regardless of the doses of Ara-C used. In theory, mice treated with saline can easily develop long term bone marrow failure should they receive another high cytotoxic insult to the system because a lot of their stem cells have already undergone cell division (Mauch et al., 1988).

It is very unlikely that captopril was directly inhibiting both GM-CFC and HPP-CFC-1 proliferation after 1 week since it is known that one dose of captopril has a very short t ½ *in vivo* (Duchin et al., 1982). Therefore, an indirect mechanism is likely to be responsible for this inhibitory effect of captopril on haematopoietic progenitor cells. In this investigation captopril inhibited the G_1/S transition as shown by the low levels of HPP-CFC-1 in S-phase at all times investigated and GM-CFC in S-phase at days 3 to 7. This inhibition is consistent with the one observed with AcSDKP both *in vivo* and *in vitro* (Wierenga et al., 1996; Monpezat & Frindel, 1989; Jackson et al., 1996). It also confirms the observation that the effects of inhibitors of stem cell proliferation are not lost, so that once they have become effective, stem cells

remain in a quiescent state until such time as they are switched on again by an increase in proliferation stimulators (Lord et al., 1979). It seems that the second dose of Ara-C was not able to produce enough stimulators of stem cell proliferation to oppose the effect of captopril induced inhibitory factors. This can be explained by the lack of the autologous effects of the second dose of Ara-C in the presence of captopril. However, in the absence of captopril, the second dose of Ara-C produces a marked increase in stimulator production as shown by the high proportions of HPP-CFC-1 in S-phase. Moreover, a high dose of Ara-C induced about 50% of HPP-CFC-1 in S-phase on day 3 (see Fig. 4.21) while low doses induced only about 20-30% of the same cell population in S-phase (see Fig. 4.15). On the other hand, low doses of Ara-C had the same proportion of GM-CFC in S-phase as control mice at day 3 (see Fig. 4.13) and high doses induced an increase to 50% of GM-CFC in S-phase (see Fig. 4.19). These results indicate that the loss of a small numbers of stem cells has a mild effect on the production of stimulators of stem cell proliferation, the small concentration of stimulatory factors produced might not affect the proliferation of committed progenitor cells. However, a greater stem cell loss will induce a marked increase in stimulator production that might effect progenitor cell proliferation (Lord, 1986). The intriguing thing about these findings is the ability of haematopoietic HPP-CFC-1 to achieve a quiescent state at Ara-C high doses from 7 days onwards in the presence of captopril, while GM-CFC are induced back into cell cycle at day 10. An increase in cellularity to normal levels is achieved within 1 week despite a low level of proliferating HPP-CFC-1 and GM-CFC in captopril treated mice.

The low levels of HPP-CFC-1 in S-phase in mice that did not receive any insult could explain the reason for the rare observation of pancytopenia with ACE inhibitors following hypertensive treatment. However at times of proliferative stress

the demand for GM-CFC is increased since most of GM-CFC differentiate into effector cells to combat infection, moreover, there is a massive death of mature haematopoietic cells. This induces the production of stimulators of stem cell proliferation in order to induce stem cells into a proliferative state so that destroyed progenitor and precursor cells can be replaced. ACE inhibitors may prevent the feed back loop on stem cell proliferation by opposing the stimulator effect or by up-regulating inhibitors of stem cell proliferation. The net result is no haematopoietic progenitor and precursor cell production. Therefore, the observed result is granulocytopenia or pancytopenia. Indeed, most reported cases of pancytopenia presented with infection that might have been primary or secondary after captopril treatment (Gravras et al., 1981; Israeli et al., 1985; Holland et al., 1996).

Captopril has been used in the treatment of myeloproliferative disorders (Nomura et al., 1996). Thus a possibility exists that administration of captopril *in vivo* antagonises the stimulatory effects on cell proliferation. Moreover, the ability of captopril to reduce LTC_4 and LTB_4 concentrations which synergies with GM-CSF on GM-CFC proliferation may account for the antagonism of stimulator effect on haematopoietic cell proliferation by captopril (Shindo et al., 1994; Stenke et al., 1994). The most important characteristic of captopril induced-pancytopenia is its reversibility. This not only rules out permanent haematopoietic damage by captopril but it also provides a tool that can be used to temporarily suppress haematopoietic cell proliferation during cytotoxic therapy for the treatment of neoplastic disease. This investigation has shown that indeed captopril can be used as an adjuvant in chemotherapy, mainly in the treatment of leukaemia in which Ara-C is the main drug of choice (Crisp et al., 1996).

Whatever the underlying mechanism of captopril induced-pancytopenia is, this side effect can be turned into a beneficial effect in the treatment of neoplastic disease. These results show that the inhibitory effect of captopril is reversible on GM-CFC and permanent on HPP-CFC-1, thus rendering them resistant to at least S-phase specific cytotoxic drugs and possibly to non specific cycle phase cytotoxic drugs too. The fact that GM-CFC can return to their normal proliferative state in the presence of captopril within 10 days, suggests that granulocytopenia may be prevented following chemotherapy. Moreover, only a short stay in the hospital may be necessary. However, administration of growth factors may accelerate this recovery with stem cell integrity intact as observed by the ability of AcSDKP to synergise with G-CSF (Masse et al., 1998) and GM-CSF (Bogden et al., 1998) in recovering mice from leucopenia following chemotherapeutic agents. Therefore, the use of captopril may prove very beneficial in the treatment of neoplastic disease. All in all, captopril may reduce the need for blood transfusions and reduce the incidences of long term bone marrow failure following fractionated doses chemotherapy. The use of captopril not only provides a cheap adjuvant to the treatment of neoplastic disease, but it is also safe as ACE inhibitors rarely cause side effects in individuals without any other underlying disease, apart from hypertension (Alderman, 1996). Patients in countries with poor health care facilities might also benefit from this therapy.

CHAPTER 5

5.0 THE EFFECT OF AcSDψKP, AcSDKP AND CAPTOPRIL IN LONG-TERM BONE MARROW CULTURES

Long-term bone marrow cultures are similar to the *in vivo* haematopoiesis requirements in that haematopoiesis is only achieved in the presence of stromal cells. The long-term bone marrow culture assay allows haematopoiesis to be maintained for many weeks. Captopril's effect on LTBMCs was investigated for three reasons. Firstly, to see if captopril has any effect on the proliferation of haematopoietic progenitor cells in LTBMCs. Secondly, to investigate whether captopril could be toxic to the LTBMCs. Lastly to investigate whether captopril's action on the LTBMC was similar to AcSDKP action in the same assay. To this purpose LTBMCs were cultured for 6 weeks in the presence of saline, AcSDψKP, captopril, and captopril plus physiological AcSDKP. AcSDψKP has been rendered resistant to proteases in serum by replacing the DK bond with the aminomethylene bond (Gaudron et al., 1997).

Procedure

Bone marrow cells (1.2×10^7 cells/ml) prepared from femurs of cross bred mice ((CBA/H x C57BL) F1) were suspended in F20%HS PS/G Hydro and put in 24 100-cm^2 flasks. Six flasks were used per protocol and four protocols were established in all. In the first protocol that was used as a control, 1 ml of saline was added to 9 mls of cell suspension in each of the six flasks. In the second protocol, 1 ml of captopril dissolved in F20%HS PS/G Hydro was added to 9 mls of cell suspension in each of the six flasks making a final concentration of 1 μM captopril. In the third

protocol resistant AcSDψKP was added to 10 mls of cell suspension in each of the six flasks making a final concentration of 10^{-9} M AcSDψKP. In the last protocol captopril at 1 μM and conventional AcSDKP at 10^{-9}M were added to each of the six flasks. LTBMC cultures were maintained for 6 weeks according to the already described method. Non adherent layer cells were sampled at the ends of week 2, 3 and 5. Adherent layer cells were sampled at the end of week 6. Flasks were analysed for cellularity, GM-CFC numbers with their kinetics and finally HPP-CFC numbers also with their kinetics in both layers.

Results

5.0.1 *AcSDΨKP and Captopril do not affect cellularity in the non-adherent layer*

The cellularity of the LTBMCs was determined over 5 weeks of culture. On week 6 the cellularity of the adherent layers was also determined. There was no statistical significant difference between the cellularity of non-adherent layer cells per week in the flasks of each treatment group. As shown in Table 5.1, at week 2 the number of cells per ml in the flasks treated with saline was (5.8 ± 1.0) x 10^5. Flasks treated with captopril, AcSDψDK, and captopril together with AcSDKP had (7.5 ± 1.5) x10^5, (7.8 ± 2.1) x10^5, and (6.5 ± 2.4) x 10^5 (p = 0.79) cells per ml respectively. At week 3, the cellularity varied between (7.2 ± 0.6) x 10^5 cells per ml in the flasks where captopril was combined with AcSDKP to (9.9 ± 2.3) x 10^5 cells per ml in the saline flasks (p = 0.87). The greatest increase in cellularity was noticed at week 5 of culture. At this time the cellularity ranged from (10.0 ±1.6) x 10^5 cells per ml in flasks where captopril and AcSDKP were combined to (13.9 ± 1.1) x 10^5 cells per ml in the

control flasks (p = 0.053). In general there was an increase in cellularity in each set as the weeks progressed.

Table 5.1 Total flask cellularities (x10^5cells/ml) in the non-adherent layers following different treatments

Time of LTBC	Saline (±SE)	Captopril 1μM (±SE)	AcSDψKP 10^{-9} M	AcSDKP (10^{-9} M) plus Captopril 1μM
Week2	5.8 ± 1.0	7.5 ± 1.5	7.9 ± 2.1	6.5 ± 2.5
Week3	9.9 ± 2.3	8.3 ± 1.3	8.0 ± 0.8	7.2 ± 0.6
Week5	13.9 ±1.1	12.0 ± 1.7	11.1 ± 2.0	10.0 ± 1.6

There is no significant difference between each treatment group (p>0.05)

5.0.2 *AcSDψKP and Captopril increase the number of cells in the adherent layer (week 6)*

After 6 weeks in culture the cellularity of the adherent layer was analysed. There was a statistically significant difference between cultures (p = 0.042) (see Table 5.2). This significant difference was observed between cultures established in the presence of AcSDψKP or captopril separately when compared to control saline flasks.

Table 5.2 Total flask cellularities (x10^6 cell/flak) in the adherent layers (week 6) following different treatments

Saline (±SE)	23.8 ± 2.7
Captopril (1μM) (±SE)	33.0 ± 2.1 (p<0.05)
AcSDψKP (10^{-9} M) (±SE)	31.8 ± 3.4 (p<0.05)
Captopril (1μM) plus AcSDKP (10^{-9}M) (±SE)	25.0 ± 1.5 (p>0.05)

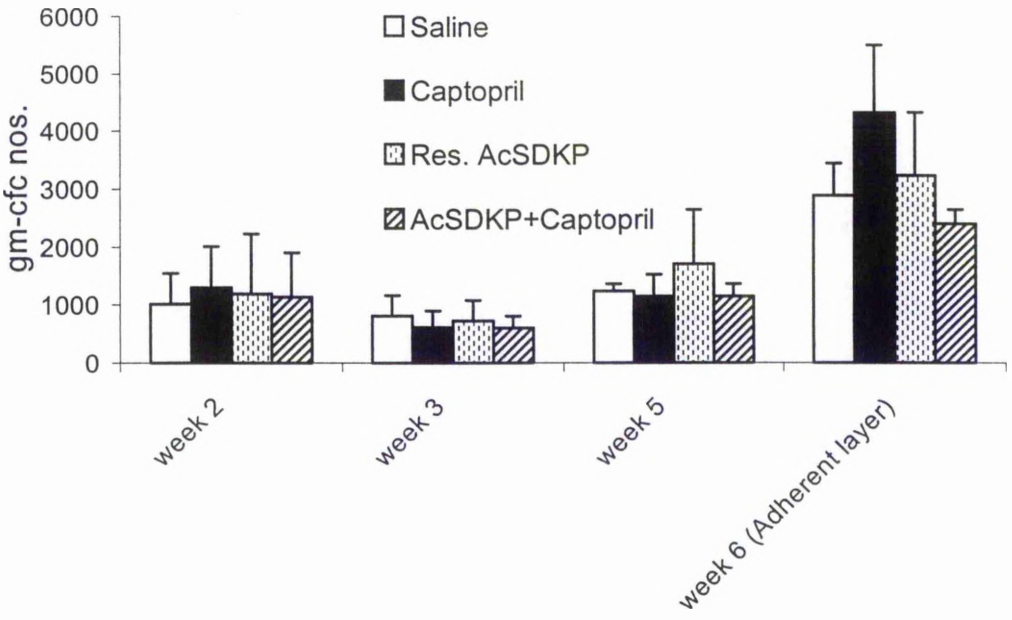
Flasks established in the presence of captopril together with AcSDKP had cell numbers equivalent to control saline treated cultures showing fewer cells when compared to captopril or AcSDψKP treated cultures. As shown in table 5.2, the number of cells per ml in the saline control in the adherent layers was (23.8 ± 2.7) x 10^6 cells per flask. This number was increased to (33.0 ± 2.1) x 10^6 cells per flask in flasks treated with captopril ($p<0.05$). The flasks treated with AcSDψKP had (31.8 ± 3.4) x 10^6 cells per flask. While there was no statistical difference in flask cellularity between flasks treated with captopril and those treated with AcSDψKP ($p>0.05$), there was a difference between saline controls and AcSDψKP treated flasks ($p<0.05$). There was also no difference in cellularity between saline control flasks and flasks treated with captopril and AcSDKP together which had (25.0 ±1.5) x 10^6 cells per flask ($p>0.05$).

5.0.3 *The effect of Captopril, AcSDψKP and captopril plus AcSDKP on GM-CFC*

The numbers of GM-CFC were also determined during the whole period of long term cultures. As shown in Fig.5.1, the numbers of GM-CFC were not statistically different in each group at the times sampled. At week 2, the numbers ranged from 1011.9 ± 528 GM-CFC per flask in the saline controls to 1296.9 ± 706 GM-CFC per flask in flasks treated with captopril ($p = 0.98$). The values at week 3 ranged from 605.7 ± 197 in flasks treated by a combination of captopril and AcSDKP to 809.8 ± 356 in the flasks treated with saline ($p = 0.29$). At week 5, the numbers of GM-CFC ranged from 1240.0 ± 119 in captopril treated flasks to 1698.9 ± 943 in the AcSDψKP treated group ($p = 0.55$). There was no difference in the numbers of

FIG. 5.1 Absolute GM-CFC numbers of LTBMCs

LTBMCs	Absolute nos of GM-CFC (per flask) Week 2	Week 3	Week 5	Week 6 (Adherent layer)
Saline	1011.8 ± 258	809.8 ± 356	1240.0 ± 119	2888.4 ± 558
Captopril	1296.8 ± 706	618.6 ± 279	1146.9 ± 373	4316.0 ± 1180
AcSDψKP	1192.9 ± 1027	730.7 ± 348	1698.9 ± 943	3238.9 ± 1087
AcSDKP+ Captopril	1136.3 ± 767	605.7 ± 197	1148.9 ± 215	2404.0 ± 248
	P = 0.98, n = 4	P = 0.29, n = 4	P = 0.55, n = 4	P = 0.14



GM-CFC in the adherent layers of long-term bone culture established in the presence of any of the treatment groups. As shown in Fig 5.1, the values ranged from 2888.4 ± 558 in the saline controls to 2404.0 ± 248 in flasks treated with captopril and AcSDKP together for GM-CFC per flask (p = 0.14).

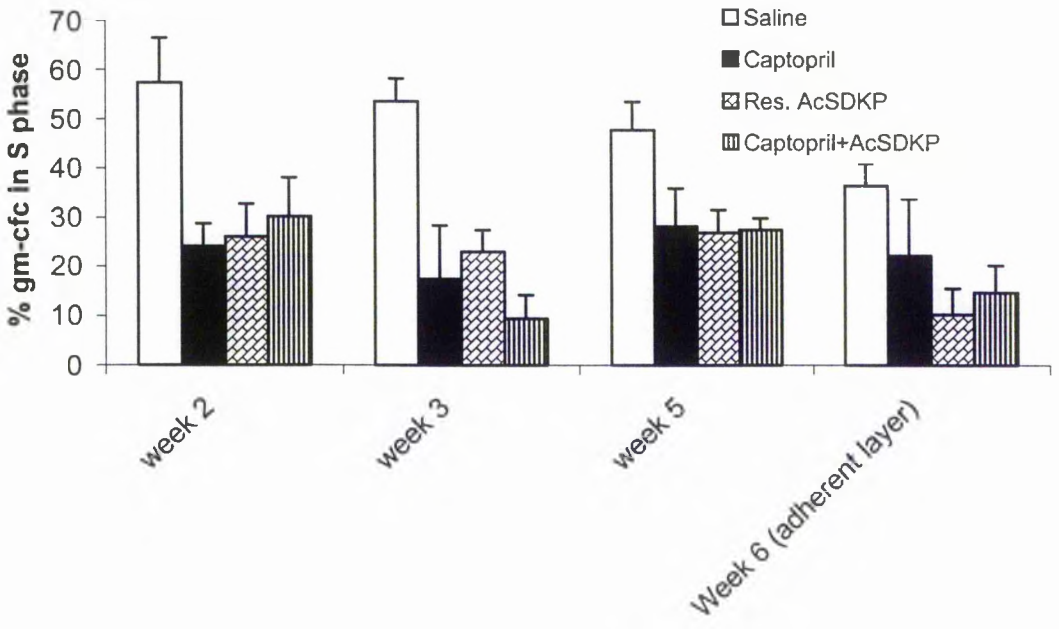
There was a statistically significant difference in the proportion of GM-CFC in S-phase within treatment groups at weeks 2 (p = 0.02) and 3 (p = 0.003). As shown in Fig 5.2, less GM-CFC were in S-phase in the treatment groups compared to the saline controls at week 2. However no significant difference was observed between individual treatment groups. (57.5 ± 9.0)% GM-CFC were in S-phase in the saline

control flasks at week 2. The values in other flasks ranged from (24.1 ± 4.6)% in flasks treated with captopril to (30.1 ± 7.9)% in flasks treated with AcSDKP together

FIG. 5.2 The proportion of GM-CFC in S-phase in LTBMCS

LTBMCs	% GM-CFC in S-phase			
	Week 2	Week 3	Week 5	Week 6 (Adherent layer)
Saline	(57.5 ± 9.0)%	(53.6 ± 4.6)%	(47.6 ± 5.8)%	(36.4 ± 4.5)%
Captopril	(24.1 ± 4.6)%	(17.4 ± 11.0)%	(28.1 ± 7.8)%	(22.1 ± 11.6)%
AcSDψKP	(26.0 ± 6.7)%	(23.0 ± 4.6)%	(26.9 ± 4.6)%	(10.2 ± 5.2)%
AcSDKP + Captopril	(30.1 ± 7.9)%	(9.3 ± 4.5)%	(27.5 ± 2.3)%	(14.6 ± 5.6)%
	P = 0.02, n = 4	P = 0.003, n = 4	P = 0.05, n = 4	P = 0.12
S with Cap	P < 0.05	P < 0.05	P < 0.05	
S with Res. AcSDKP	P < 0.05	P < 0.05	P < 0.05	
S with AcSDKP+Cap	P< 0.05	P <0.05	P < 0.05	

NB: S = saline, Cap = Captopril, Res. AcSDKP = AcSDψKP,



with captopril (p<0.05). At week 3, there were also differences in the proportions of GM-CFC in S-phase between saline controls relative to other treatments (p<0.05).

The proportion of GM-CFC in S-phase in the saline control was (53.6 ± 4.6)%. This was reduced to only (17.4 ± 11.0)% in the presence of captopril. AcSDψKP reduced the proportion of GM-CFC in S-phase to (23.0 ± 4.6)%. Captopril and AcSDψKP separately reduced the proportion of GM-CFC in S-phase by the same margin hence showed the same inhibitory effect on GM-CFC proliferation ($p>0.05$). However the proportion of GM-CFC in S-phase in cultures treated with AcSDKP and captopril together was reduced further to (9.3 ± 4.5)%. Therefore cultures treated with AcSDKP and captopril together showed the least proportion of GM-CFC in S-phase ($p<0.05$). At week 5, there was a significant difference between individual sets of investigations ($p = 0.05$). While (47.6 ± 5.8)% GM-CFC were in S-phase in saline control, the values between treatment flask groups were (26.9 ± 4.6)% with AcSDψKP treatment and (28.1 ± 7.8)% with captopril. AcSDKP in combination with captopril had (27.5 ± 2.3)% of GM-CFC in S-phase. As shown in Fig.5.2, there was no difference in the proportion of GM-CFC in S-phase between all the treatment groups ($p = 0.12$) in adherent layer. (36.4 ± 4.5)% of GM-CFC were in S-phase in saline control flasks. The proportion GM-CFC in S-phase in the presence of captopril were (22.1 ± 11.6)% ($p>0.05$), (10.2 ± 5.2)% were in S-phase in the presence of AcSDψKP ($p > 0.05$) and (14.6 ± 5.6)% were in S-phase in the presence captopril plus AcSDKP ($p > 0.05$).

5.0.4 *The effect of Captopril, AcSDψKP and AcSDKP plus Captopril on HPP-CFC*

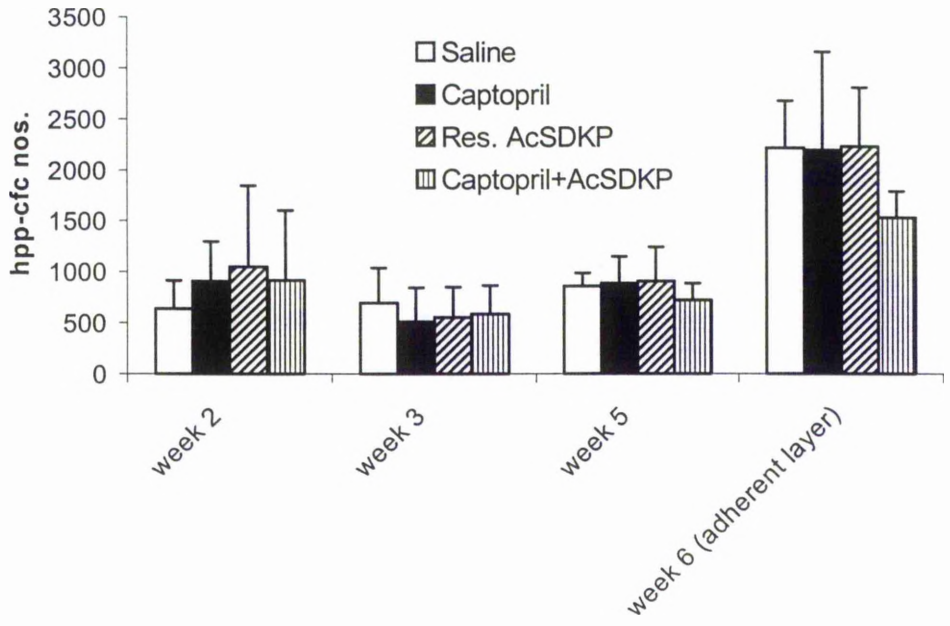
There was a lower number of HPP-CFC per treatment flask compared to GM-CFC. The highest and lowest numbers of HPP-CFC were both in week 2 (see Fig 5.3). These were 1051.0 ± 794 HPP-CFC in flasks treated with AcSDψKP and 637.5 ± 272

HPP-CFC in the control flasks in the non-adherent layers ($p>0.05$). In the adherent layer, the values for HPP-CFC per flasks ranged from 2212.3 ± 467 in saline controls to 1534.5 ± 258 HPP-CFC ($p = 0.86$) in flasks that were treated with captopril combined with AcSDKP (see Fig 5.3). In general there was an increase the numbers of both HPP-CFC and GM-CFC in the adherent layer compared to the non-adherent layer.

There was no difference between all four-treatment groups in the proportion of HPP-CFC in S-phase at week 2 ($p = 0.06$) (see Fig 5.4). There was however a statistical significant difference between treatment groups at weeks 3 ($p = 0.01$) and 5 ($p = 0.03$). At week 3, (24.1 ± 3.4)% of HPP-CFC were in S-phase in saline control flasks. This proportion was reduced to only (6.6 ± 3.8)% of HPP-CFC in S-phase with captopril and to (6.5 ± 2.1)% of HPP-CFC in S-phase with AcSDψKP. Captopril or AcSDψKP had the lowest proportion of HPP-CFC in S-phase relative to both saline controls and the combination of AcSDKP and captopril together ($p > 0.05$). No statistical significant difference in the proportion of HPP-CFC in S-phase between captopril and AcSDψKP ($p > 0.05$) was observed. On the other hand (15.6 ± 7.6)% of HPP-CFC were in S-phase in flasks treated with captopril and AcSDKP together. Therefore, there was no significant difference between the proportion of HPP-CFC in S-phase in flasks treated with captopril and AcSDKP together when they were compared to control saline treated flasks ($p>0.05$). At week 5, saline controls had (47.0 ± 9.8)% of HPP-CFC in S-phase. Captopril slightly reduced this proportion to (25.2 ± 10.1)% ($p<0.05$). AcSDψKP had the same influence. It reduced the proportion of HPP-CFC in S-phase to (17.9 ± 7.3)% ($p<0.05$). While both captopril and

FIG. 5.3 Absolute HPP-CFC numbers in LTBMCs

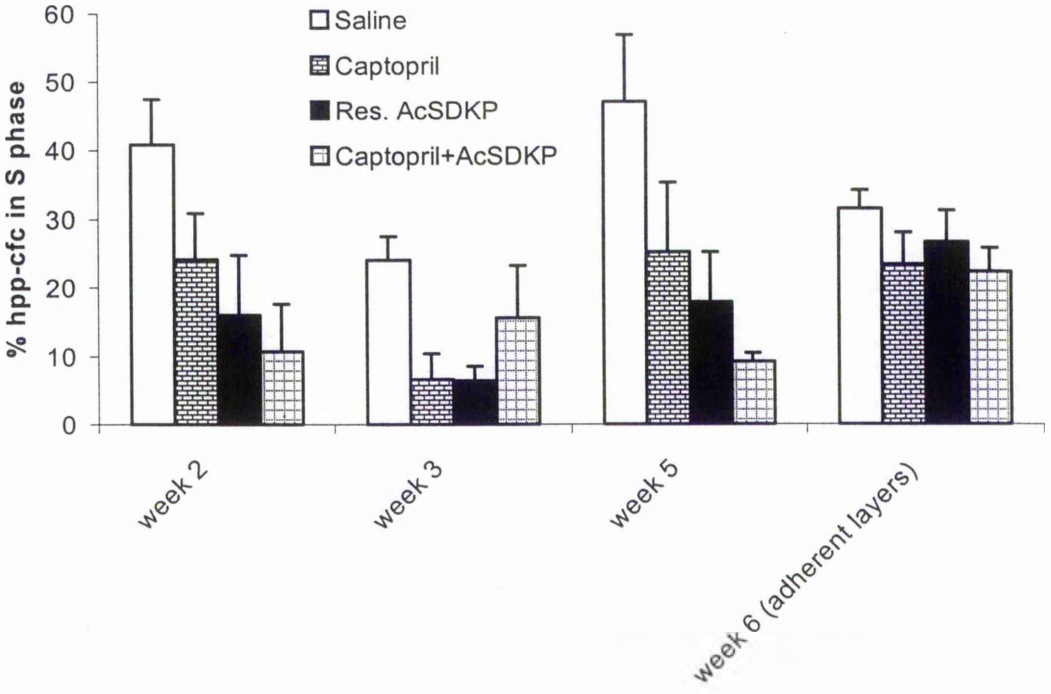
LTBMCs	Absolute nos of HPP-CFC (per flask)			
	Week 2	Week 3	Week 5	Adherent layer
Saline	637.5 ± 272	694.7 ± 342	856.7 ± 129	2212.3 ± 467
Captopril	907.0 ± 388	512.5 ± 330	887.1 ± 264	2190.3 ± 968
AcSDψKP	1051.3 ± 794	553.6 ± 296	905.0 ± 341	2229.2 ± 580
AcSDKP + Captopril	916.9 ± 688	584.0 ± 278	724.8 ± 160	1534.5 ± 258
	P = 0.86, n = 4	P = 0.54, n = 4	P = 0.14, n = 4	P = 0.86, n = 4



AcSDψKP reduced the proportion of HPP-CFC in S-phase relative to saline controls, there was no observed statistically significant difference between them ($p > 0.05$). In contrast AcSDKP combined with captopril reduced the proportion of HPP-CFC in S-phase to (9.8 ± 1.3)% ($p<0.05$). Therefore a statistically significant difference was observed by combining AcSDKP and captopril when it was compared to saline controls and to both captopril and AcSDψKP ($p < 0.05$). There was also no significant difference in the proportion of HPP-CFC in S-phase between all the treatment groups

FIG. 5.4 The proportion of HPP-CFC in S-phase in LTBMCs

LTBMCs	% HPP-CFC in S-phase			
	Week 2	Week 3	Week 5	Week 6 (Adherent layer)
Saline	(40.8 ± 6.6)%	(24.1 ± 3.5)%	(47.0 ± 9.8)%	(31.5 ± 2.6)%
Captopril	(24.1 ± 6.8)%	(6.6 ± 3.8)%	(25.2 ± 10.1)%	(23.3 ± 4.7)%
AcSDψKP	(16.0 ± 8.8)%	(6.5 ± 2.1)%	(17.9 ± 7.3)%	(26.6 ± 4.6)%
AcSDKP + Captopril	(10.7 ± 6.9)%	(15.6 ± 7.6)%	(9.8 ± 1.3)%	(22.3 ± 3.5)%
	P = 0.06, n = 4	P = 0.01, n = 4	P = 0.03, n = 4	P = 0.38
S with Cap	P > 0.05	P < 0.05	P < 0.05	
S with Res. AcSDKP	P > 0.05	P < 0.05	P < 0.05	
S with AcSDKP+Cap	P> 0.05	P > 0.05	P < 0.05	



in the adherent layer (p = 0.38). The proportion of HPP-CFC in S-phase in saline control flasks was (31.5 ± 2.6)%, in captopril flasks was (23.3 ± 4.6)%, in AcSDψKP flasks was (26.6 ± 4.6)% and in captopril with AcSDKP flasks was (22.3 ± 3.5)% showing no statistical significant difference between the treatment groups.

5.1 Discussion

The requirement of accessory cells for haematopoiesis to be accomplished in LTBMCs, makes this model ideal for studying haematopoiesis *in vitro*. Inhibition of stem cells and committed progenitors growth in LTBMCs treated with AcSDKP is well documented (Hong et al., 1995; Bonnet et al., 1992; Jackson et al., 1996). The observation that AcSDKP catabolism is under the control of ACE (Rousseau et al., 1995) means that LTBMC is an ideal model for studying the role of ACE on haematopoiesis. Moreover, one of the adverse effects of chronic treatment with ACE inhibitors is haematological toxicity (Gravras et al. 1981), this means that LTBMC can resolve some of the underlying mechanism of ACE inhibitor induced myelotoxicity. These hypotheses prompted the evaluation of the effects of captopril on the growth of hematopoietic cells in LTBMCs. The similar tendency for AcSDKP accumulation in the supernatants of bone marrow cells treated with captopril for 24 hours has been shown in these investigations (see Fig. 3.7). This raises the possibility that the effect of captopril on the proliferation of stem cells is mediated via inhibition of AcSDKP degradation. These investigations have shown that captopril with AcSDKP can inhibit *in vitro* the proliferation of HPP-CFC-1 triggered into S-phase by irradiation. On the other hand captopril on its own inhibits HPP-CFC-1 proliferation *in vivo* following all insults tested and *in vitro* following cytotoxic drug treatment. This suggests that captopril and AcSDKP separately or combined may affect the proliferation of stem cells.

In the LTBMCs shown by these investigations Captopril or AcSDψKP had no effect on the cellularity of the non-adherent layers in LTBMCs. However, captopril combined with AcSDKP slightly reduced cell numbers in the non-adherent layers after 5 weeks in culture (see Table 5.1). On the other hand,

captopril or AcSDψKP significantly increased the cellularity of the adherent layers of LTBMCs after 6 weeks in culture. In contrast, Jackson et al., 1996 did not find an increase in the adherent layer cell numbers. Their method of establishing LTBMCs was different from the present experiments. They used a single cell suspension and they had to re-feed their cultures with new bone marrow cells before assaying for AcSDKP. This increase in cellularity of adherent layers of LTBMCs may be attributable to the ability of captopril to reduce intracellular Ca^{2+} levels thereby inhibiting cell proliferation (Tepel et al., 1997). This would stop cell differentiation and death. Moreover, AcSDψKP also inhibits GM-CFC cell proliferation (see Fig. 3.2). The increase in adherent layer cellularity might also be explained by an imbalance between the production of stimulatory factors and inhibitory factors following captopril or AcSDψKP treatment. For example, stromal cells may be resistant to the influence of these drugs. Stromal cells may keep on producing stimulatory factors in their presence.

Assuming that serum used for tissue culture has angiotension-1 as well as ACE, the presence of ACE inhibitors in culture will inhibit ACE activity. This will result in reduced levels of angiotensin-II. Since angiotensin-II is known to increase production of TGF-β (Sadoshima & Izumo, 1993; Brooks et al., 1997; Guh et al., 1996), the presence of captopril in LTBMCs may lead to a reduction in TGF-β levels. TGF-β has been shown to be continuously produced in the adherent layers of LTBMCs and is responsible for the quiescent nature of HPP-CFC in LTBMCs (Eaves et al., 1991). Therefore, the presence of captopril will enhance cell division by reducing the levels of TGF-β. The reduction in TGF-β may also result in an enhanced endogenous production and activity of MIP 1α, since in the presence of TGF-β, MIP-1α is at best a weak contributor to the overall

physiological inhibition of stem cell proliferation (Maltman et al., 1993). MIP 1α synergises with M-CSF or GM-CSF to stimulate development of colonies from enriched murine GM-CFC (Lord et al., 1993). Adherent layer cells produce GM-CSF, this means that progenitor cell production can be enhanced in the presence of MIP 1α and in the absence of TGF-β (Lord et al., 1993; Eaves et al., 1991). The involvement of AcSDKP on increased cell production may be mediated by an increase in MIP 1α production as suggested by Cashman et al., (1994). Interestingly MIP 1α has been shown to increase intracellular cAMP levels (Mantel et al., 1995). Therefore, there is a strong possibility that AcSDKP may mediate the effects of captopril on cell proliferation. The possible involvement of MIP 1α in the cellular response by both captopril and AcSDψKP strengthens this hypothesis.

AcSDKP is known to enhance the adherence of haematopoietic cells to stromal cells (Lenfant et al., 1989a; Aizawa et al., 1992; Suzuki et al., 1998), therefore, the observed increase in cellularity can be due in part to redistribution of cells from the non-adherent layers to the adherent layers. However, this is unlikely because there is no difference in non-adherent layer cellularity numbers between saline, AcSDψKP and captopril. In addition, the combination of AcSDKP with captopril show reduced non-adherent layer cell numbers despite having the same cellularity with saline treated cultures in the adherent layers. The increase in cell numbers of the adherent layers with captopril or AcSDψKP may be exploited in the expansion of adherent layer cell numbers for establishment of LTBMCs. The increase in cell numbers includes stromal cells as well as haematopoietic cells. Therefore, the levels of ground substances like fibronectin may also be regulated resulting in enhanced haematopoiesis. It has to be underlined that both captopril and

AcSDψKP are not toxic to haematopoietic cells as shown by the increase in cellularity in the adherent layers of LTBMCs and similar numbers of GM-CFC and HPP-CFC in each set of investigations.

There was a marked inhibition of HPP-CFC proliferation in LTBMCs treated for 3 weeks with either AcSDψKP or captopril. Captopril and AcSDψKP had identical suppressive effects on the proliferation of GM-CFC. However, captopril combined with AcSDKP showed a more pronounced inhibitory effect on GM-CFC proliferation measured at week 3 and on HPP-CFC proliferation measured at week 5. In addition, the effect of captopril combined with AcSDKP compared to the inhibitory effect of each drug alone was shown to be different in respect to time and responding cell population. The explanation for these differences is unclear. However, it may be attributed to the varying concentrations of the active molecule employed by these drugs in their modulatory role in LTBMCs haematopoiesis. It is thus likely that captopril protects AcSDKP from degradation. Since there is a steady state production of AcSDKP *in vivo* and in long-term bone marrow cultures (Wdzieczak-Bakala et al., 1990), a possibility exists that captopril may increase the levels of AcSDKP. This is plausible because AcSDKP is endogenously produced by macrophages in long-term bone marrow cultures (Li et al., 1997). It may also arise from the degradation of thymosin β4 and other macromolecules *in vivo* under steady state conditions (Grillon et al., 1990). These results show that captopril modulates haematopoiesis in LTBMCs with the same potency as AcSDKP, thus demonstrating a common pathway in their action.

AcSDKP inhibits haematopoietic progenitor cells only at optimal concentrations, high or low concentrations of AcSDKP have no effect on the proliferation of haematopoietic stem or progenitor cells in long-term cultures (Jackson

et al., 1996; Bonnet et al., 1992). This may be the reason for the varying effects on HPP-CFC proliferation observed when the combination of captopril and AcSDKP was used. It could also explain the observed difference of inhibitory effect imposed by the two substances together on HPP-CFC and GM-CFC proliferation. The increase in the number of macrophages as a result of the increase in adherent cell numbers may lead to higher production of both ACE and AcSDKP (Costerousse et al., 1993; Li et al., 1997). The net result is unstable levels of active AcSDKP causing variable effects on cell proliferation. This is a plausible explanation because AcSDKP is only active at the G_1/S boundary with no effect on cells beyond the S-phase of the cell cycle (Monpezat & Frindel, 1989). Therefore, sufficient peak levels of AcSDKP are required at this critical period of the cell cycle to allow an inhibitory effect of this peptide. There was no effect on the proliferation of both HPP-CFC and GM-CFC in the adherent layers. These findings are in agreement with the findings of other people who have shown no inhibitory effect of AcSDKP on the proliferation of HPP-CFC in the adherent layers of murine long term bone marrow cultures (Jackson et al., 1996).

The mechanism underlying captopril-induced inhibition of hematopoietic cell proliferation remains unknown. However, the general discussion chapter will point out a few possible mechanisms that may need further investigations to be confirmed. It is well documented that AcSDKP prevents primitive hematopoietic cell entry into S-phase. Taking into consideration a steady state production of AcSDKP *in vivo* and LTBMCs (Wdzieczak-Bakala et al., 1990; Li et al., 1997), a possibility exists that captopril indirectly controls the proliferation of hematopoietic stem cells via the regulation of endogenous AcSDKP levels as already pointed out. The close correlation between the enhanced plasma AcSDKP levels and the inhibition of HPP-CFC proliferation reported for irradiated mice given one dose of lisinopril (Rousseau

et al., 1998) stays in agreement with such hypothesis. Therefore captopril and AcSDKP can be exploited in the study of haematopoiesis both *in vivo* and *in vitro*. Moreover, their use in myeloprotection is strongly strengthened by their shown ability on LTBMCs.

CHAPTER 6

General Discussion

6.1 *Captopril and cell proliferation*

Captopril suppresses interstitial fibrin deposition in coxsackievirus B3 myocarditis by inhibiting inflammatory fibrin deposition, postmyocarditic myocyte hypertrophy and ventricular remodelling during inflammatory phase but not during fibrotic phase (Takada et al., 1997). This drug also inhibits the proliferation of cardiomyocytes and inhibits c-myc, c-fos mRNA expression in these cells (Lei & Guo, 1998). Captopril decreases granuloma size in the liver, colon, and ileum in mice infected with schistosoma mansoni eggs (Weinstock et al., 1981). It has shown beneficial effects in alleviating symptoms of rheumatoid arthritis (Martin et al., 1984). This ACE inhibitor inhibits proliferation of aortic smooth muscle cells caused by hyperlipidemic serum (Xiong & Zhao, 1996). This effect is mediated by an increase in 6-keto-PGF1α and cAMP with a decrease in malondialdehyde and plasminogen activator inhibitor. High concentrations of captopril produce a dose dependent inhibition of platelet-derived growth factor (PDGF)-stimulated mitogenesis and can potentiate the inhibitory effects mediated by kinins (Dixon & Dennis, 1997). Captopril inhibits mitosis in human lung fibroblasts, bovine aorta and pulmonary artery endothelial cells, and of hamster pancreatic duct carcinoma cells (Nguyen et al., 1992). Fibroblasts stimulated to proliferate by basic fibroblast growth factor are more sensitive to the antimitotic effect of captopril than unstimulated cells (Nguyen et al., 1994). Moreover, the ability of captopril to inhibit human lung fibroblast proliferation is neither affected by exogenous angiotensin-II nor mimicked by lisinopril (Nguyen et al., 1994). Captopril and enalapril both reduce neuroblastoma cell growth rate but

they have differential effects on the proliferation of these cells. Captopril at doses higher than 0.1 mg *in vitro* suppresses neuroblastoma cell proliferation while enalapril showing effect only at doses of 5.0 mg (Chen et al., 1991). Captopril modulates hormone receptor concentrations and at high doses inhibits proliferation of human mammary ductal carcinoma cells in culture. Both lisinopril and penicillamine, a thiol compound, have no such effect on these carcinoma cells (Small Jr. et al., 1997). When captopril is incubated with hamster pancreatic duct cancer cells, it inhibits cell proliferation by increasing K-ras expression and lowering proliferation associated cell nuclear antigen (Reddy et al., 1995). In addition, captopril has been shown to inhibit Tenon's fibroblast proliferation (Singh et al., 1997), a property it shares with Ca^{2+} channel blockers and other substances known to inhibit protein synthesis.

In general the proliferation of tumour cells is not affected by captopril at low doses (Chen et al., 1991; Hii et al., 1998; Volpert et al., 1996; Nakagawa et al., 1995). Most of the cell proliferation inhibitory effects of captopril show that captopril has an indirect mode of action. Moreover, as shown in this investigation and by others, captopril has a universal inhibitory effect on normal cell proliferation while it has a little or no effect on inhibiting neoplastic cell proliferation at doses that inhibit normal cell proliferation (see Fig. 3.8). The capacity of captopril to inhibit normal cell proliferation is unique to this drug and is not mimicked by other ACE inhibitors. The effect of captopril on neoplastic cell proliferation is complicated and it requires further investigations.

The possibility that captopril may have a direct effect on cell proliferation can not be excluded. However, it is difficult to envisage how captopril can bring about all its cellular actions directly unless cells take it up into the intracellular space. It is tempting to suggest that captopril influences cell proliferation by increasing AcSDKP

concentrations *in vivo* and in the incubation medium *in vitro*. This speculation is strengthened by the observation that high levels of AcSDKP are found in the cell cytoplasm (Volkov et al., 1995). Indeed it is suggested that a co-ordinated decrease of intracellular and extracellular concentrations of AcSDKP could be necessary to allow the G_0-G_1 to S transition of quiescent cells (Volkov et al., 1997). Since AcSDKP is a natural peptide, AcSDKP may have receptors on cell surfaces and cells may take it up by endocytosis or other mechanisms.

6.2 *Captopril and matrix metalloproteinase*

The most intriguing finding of captopril is its ability to reduce tumour size without affecting cell proliferation (Hii et al., 1998; Volpert et al., 1996). This effect of captopril may be mediated by inhibiting the activity of enzymes that are involved in tumour cell invasion. One group of such enzymes is the matrix metalloproteinases (MMPs). MMPs expression plays a critical role in cell invasion, migration and the process of angiogenesis. Indeed captopril has shown a beneficial effect by inhibiting angiogenesis in Kaposi's sarcoma (Vogt & Frey, 1997). There is ample evidence that ACE inhibitors in general can inhibit the activity of MMPs. For example, both captopril and fosinoprilat inhibit leukotriene (LT) A_4 hydrolyse, a metalloproteinase, resulting in the inhibition of LTB_4 synthesis with captopril having a greater potency than fosinopril (Shindo et al., 1994) on enzyme inhibition. Moreover, captopril at low doses increases the levels of a 72-KDa type IV gelatinase in a human melanoma cell line while it decreases it at high doses (Prontera et al., 1997). Captopril also inhibits capillary endothelial cell migration by inhibiting a zinc-dependent endothelial cell-derived 72 and 92-KDa MMPs known to be essential for angiogenesis (Volpert et al., 1996). In addition captopril inhibits gelatinolytic activities in the culture media of

T98G glioma cell line (Nakagawa et al., 1995). The inability of ACE inhibitors to inhibit neuroblastoma cell growth may also be attributed to inhibition of MPPs since both captopril and enalapril are weak inhibitors of neuroblastoma cell proliferation (Chen et al., 1991). The differential effects of captopril, fosinopril and enalapril on MPPs inhibition may be the reasons for the observed differences in their potency on inhibiting neoplastic cell proliferation.

The processing of TNF α precursor to the active TNF α is dependent on a matrix metalloproteinase-like enzyme (Gearing et al., 1994). Despite the ability of captopril to inhibit the production of other cytokines such as IL-1β, IL-2, or IL-6, the production of these cytokines is not mediated by metalloproteinases (McGeehan et al., 1994). However, captopril inhibits IL-1α and TNF α synthesis at a post-transcriptional level, a factor that may be mediated by the inhibition of a metalloproteinase (Schindler et al., 1995). Therefore captopril is utilising another mechanism in the negative regulation of IL-1β production. While the ability of captopril to inhibit tumour cell invasion and an increase in tumour size may depend on its ability to inhibit MMPs, its inhibition of normal cell proliferation may depend on its ability to reduce levels of stimulatory cytokines during culture. The capacity of captopril to reduce the levels of inflammatory cytokines may have no effect on tumour cell proliferation since it is known that tumour cells may produce their own cytokines in an autocrine loop. Therefore in addition to inhibiting ACE, captopril may interact with other pathways involved in cell activation and proliferation *in vivo*. This shows the complexity of captopril in its possible role in the regulation of cell proliferation. As discussed already lack of inhibitory effect on neoplastic cell proliferation makes captopril a suitable drug to be used as adjuvant for neoplastic treatment. This then means that the inhibition of stem cell proliferation augmented

with MMPs inhibition may stop tumour cell invasion and protect stem cells during therapy.

6.3 *Captopril and irradiation*

Captopril reduces the incidence, growth and mitotic activity of radiation-induced fibrosarcomas in rats (Ward et al., 1994). It also ameliorates radiation-induced pulmonary endothelial dysfunction in rats sacrificed 2 months post irradiation (Ward et al., 1988). In addition, this drug reduces the severity of moist desquamation reaction after 30 Gy irradiation in addition to reducing the malignant skin reactions in the radiation treatment field (Ward et al., 1990). Animals receiving total body irradiation before bone marrow transplants develop bone marrow transplant nephropathy after 3 to 6 months (Moulder et al., 1993). However, at 6 months after total body irradiation, captopril-treated animals have a lower systolic blood pressure and a well-preserved renal function (Moulder et al., 1993), while captopril has no effect on bone marrow ablation. Captopril therapy started 25 days post bone marrow transplant is as effective as therapy started prior to bone marrow transplant in preventing nephropathy. However, bone marrow transplant nephropathy progresses very quickly when captopril treatment is stopped (Moulder et al., 1997). Moreover, radiation nephropathy can be significantly attenuated by the use of captopril from 3.5 to 9.5 weeks after irradiation in young animals following total body irradiation (Cohen et al., 1997). Captopril therefore has a complex mode of action when administered to irradiated animals.

While captopril has no effect on 2 Gy HPP-CFC-1 proliferation *in vitro* in the present investigations, it reduces the proportion of HPP-CFC-1 in S-phase when administered *in vivo*. In addition, from this literature review, captopril has various

properties on cell proliferation when administered *in vivo* following irradiation. It can be concluded that captopril has an indirect effect on the modification of irradiation induced cytotoxic effects. Captopril may impose its action through its effect on the renin-angiotensin system, AcSDKP enhancing property or on its ability to scavenge free radicals. The observed effects of captopril on irradiation are consistent with the ability of captopril to inhibit cell proliferation *in vivo*. It is difficult to understand how captopril may affect radiation induced cell proliferation in general partly due to the complexity of irradiation induced cell damage.

6.4 *Captopril as a scavenger of free radicals*

There is a possibility that the effect of captopril on cell proliferation may be mediated by the ion scavenging properties of this drug. Captopril is an extremely potent free radical scavenger with equivalent potency when compared to superoxide dismutase, catalase and allopurinol against polymorphonuclear-derived free radicals (Bagchi et al., 1989). Enalapril induces a progressive decline of red blood cell reduced glutathione, an important substrate for the reductive detoxification of free radicals, while captopril has no such effect (Golik et al., 1995). This suggests that captopril is able to scavenge free radicals without involving glutathione. However, captopril may also act as a pro-oxidant in the presence of ferritin *in vivo* as shown by the fact that captopril induces both oxidative stress and releases iron from ferritin (Lapenna et al., 1995). The sulfhydryl group has been implicated in the free radical scavenging ability of captopril by many authors quoted in this work. It has also been implicated in the inhibition of dopamine β-hydroxylase by captopril (Palatin et al., 1989) and prostacylin synthesis by the same drug (Guivernau et al., 1991). This free radical scavenging property of captopril has been implicated in some of the cellular

proliferation inhibitory effects of captopril (Hammond et al., 1988). Therefore, it is possible that some of the observed inhibitory effects of captopril may be mediated by its free radical scavanging properties.

AcSDKP may also have free radical scavenging properties. Coutton & Chermann, 1997 speculate that the effect of AcSDKP on HIV conditioned medium may involve free radical scavenging mechanism. Even though there is no concrete evidence to back up this hypothesis, the ability of AcSDKP to inhibit haematopoietic cell proliferation *in vivo* following irradiation (Watanabe et al., 1996) and cytotoxic drugs (Bogden et al., 1991), may involve the ability of AcSDKP to scavenge free radicals.

6.5 *ACE inhibitors and granulocytopenia*

Captopril is the most studied ACE inhibitor with regards to ACE inhibitor induced granulocytopenia. While most cases of granulocytopenia do not affect erythropoiesis or megakaryocytosis (Amann et al., 1980; DeSilva et al., 1995), in some cases of agranulocytosis, a bone marrow aspirate revealed hypocellularity of the erythropoietic and granulopoietic cells with a normal population of megakaryocytes (Staessen et al., 1981). In such cases a granulopoietic cell that is regenerating and consisting of myeloblasts, myelocytes with few other myeloid cells was observed.

More often than not in captopril induced granulocytopenia, no maturation of granulocytes is observed beyond the promyelocytic stage (DeSilva et al., 1995). In cases of captopril induced pancytopenia bone marrow biopsy shows empty fatty spaces and stromal elements without haematopoietic tissue (Kim et al., 1989). In the dog captopril induced severe hypoplasia of erythrocytic, granulocytic, and megakaryocytic cells with a relative increase in early granulocytic cells in the bone

marrow (Holland et al., 1996). In some cases of reversible captopril-associated bone marrow aplasia, bone marrow aspirate shows lymphoid cells, plasma cells and histiocytes without haematopoietic elements (Strair et al., 1985).

Almost all cases of captopril induced pancytopenia are reversible after drug discontinuation. Moreover, administration of G-CSF after captopril withdrawal accelerates the recovery of haematopoietic cell numbers (DeSilva et al., 1995; Holland et al., 1996). Other ACE inhibitors have been reported to cause pancytopenias. Lisinopril causes pancytopenia in elderly patients (Schratzlseer et al., 1994). A case of fatal aplastic anaemia associated with lisinopril treatment has also been reported (Harrison et al., 1995), however in this case the patient was first treated with captopril before lisinopril was started. Enalapril too is associated with anaemia in renal transplant recipients treated for hypertension (Vlahakos et al., 1991). Patients treated with either captopril or enalapril combined with interferon, develop severe granulocytopenia a few days after starting treatment (Casato et al., 1995). Therefore, haematological toxicity caused by ACE inhibitors is not only drugs related but might also involve ACE inhibition. Lack of literature on the haematological toxicity of other ACE inhibitors apart from captopril, lisinopril and enalapril, makes it difficult to generalise this property of ACE inhibitors. An individual drug effect on haematological toxicity cannot be ruled out because captopril has been the only drug that has been extensively studied in relation to haematological toxicity. This is due to historical reasons as well as the molecular design of captopril. However the ability of captopril to suppress haematopoietic cell proliferation may be beneficial as shown by the fact that captopril ameliorates haematological toxicities induced by adriamycim (Al-Shabanah et al., 1998).

The observation of a pancytopenia suggests an inhibition of the proliferation of cells with stem cell potential. Moreover, the histology of bone marrow aspirations following ACE inhibitor induced pancytopenia is consistent with what is expected following the inhibition of haematopoietic stem cell proliferation. The observation of lymphoid cells without megakaryocytic, granulocytic cell lines (Strair et al., 1985) adds weight to this hypothesis. Lymphoid cells in the bone marrow have been shown to represent cells with stem cell properties (Jones et al., 1990). In addition, the absence of cell maturation to promyelocytic stages in the presence of captopril (DeSilva et al., 1995), also demonstrates the multipotential nature of cells being inhibited.

Stem cell inhibition can result from either an increase in the concentration of inhibitors of stem cell proliferation or a decrease in stimulators of stem cell proliferation. G-CSF can act as both a maturation growth factor as well as a synergistic growth factor involved in the recruitment of primitive haematopoietic cells into cell cycle (Ogawa, 1993). The fact that G-CSF can rescue ACE inhibitor induced pancytopenia, suggests that stem cells may mediate this effect. Therefore, it is reasonable to speculate that G-CSF not only helps with maturation of granulocytes following captopril treatment but it also helps in the induction of haematopoietic stem cells into cell cycle. If G-CSF was acting only on committed progenitor cells, the recovery in other cell lines would not be observed and anaemia resulting from lack of red blood cells as well as platelets would be observed. Thus, inhibitors of stem cell proliferation may be involved in ACE inhibitor induced pancytopenia. Moreover, the complete recovery of haematopoietic cells after discontinuation of captopril suggests the non-toxic nature of captopril on haematopoietic cells. It also shows the ability of captopril to preserve the stem cell pool. This later effect might be achieved by the

inhibition of stem cell proliferation. Therefore, captopril is either a stem cell inhibitor or it induces the production of inhibitors of stem cell proliferation *in vivo*. The later mechanism is likely to be the case as shown by cited literature in the earlier part of this discussion. Moreover, the rarity of captopril induced pancytopenia agrees with the second assumption.

6.6 *Haematopoietic stem cell inhibitors may mediate ACE inhibitor induced pancytopenia.*

The observation that ACE inhibitors cause severe granulocytopenia in combination with interferon (Casato et al., 1995), suggests that inhibitors of stem cell proliferation may be involved in this mechanism. Interferon is an inhibitor of haematopoietic stem cell proliferation (Coutinho et al., 1986; Snoeck et al., 1994). ACE inhibitors have been shown to increase plasma levels of AcSDKP (Azizi et al., 1996; Azizi et al., 1997; Comte et al., 1997a). In addition both human burst forming units-erythroid (BFU-E) and CFU-GM are significantly reduced *in vivo* in the presence of increased levels of AcSDKP (Comte et al., 1997a). Since AcSDKP is an inhibitor of stem cell proliferation with inhibitory effect on progenitor cells, both pancytopenia and anaemia observed after ACE inhibitors can be explained by this mechanism.

However, the effect of AcSDKP on haematopoietic cell proliferation may be mediated by accessory cells (Lauret et al., 1989). In addition to increasing AcSDKP levels, captopril may be increasing the levels of active substances produced by these accessory cells involved in the AcSDKP inhibitory mechanism. In theory, there is a differential effect by ACE inhibitors in the magnitude by which they can increase AcSDKP levels due to their differential effects on the two domains of ACE (Wei et al., 1992). However, since the mode of action of both ACE inhibitors and AcSDKP is

largely unknown, some of the underlying mechanisms that may be involved in inhibitory effect of AcSDKP and ACE inhibitors in cell proliferation, will be speculated.

6.7 *Angiotensin-II is a growth factor*

There are two types of angiotensin-II receptors designated AT_1 and AT_2 (Crabos et al., 1994). The AT_1 receptor mediates most of the known functions of angiotensin-II. It shows characteristic features of a G-protein coupled, seven transmembrane receptors with three cytosolic loops (Ohyama et al., 1992). Angiotensin-II induces c-fos gene expression (Lei & Guo, 1998) via activation of phospholipases A, C and D, and protein kinase C with Ca^{2+} playing a permissive role (Sadoshima & Izumo, 1993). Therefore, angiotensin-II receptor activation causes an increase in cAMP, inositol triphosphate, diacylglycerol, phosphatidic acid and arachidonic acids. Moreover, angiotensin-II causes an induction of many immediate-early genes such as c-jun, jun B, Erg-1, c-myc in addition to c-fos (Sadoshima & Izumo, 1993). In addition, angiotensin-II AT_1 receptor activation induces a rapid phosphorylation of tyrosine in the intracellular kinases Jak2 and Tyk2 which phosphorylate STAT1 and STAT2 (Marrero et al., 1995) and activates the STAT91-nuclear signaling pathway (Bhat et al., 1994). Angiotensin-II AT_1 receptor is tyrosine and serine phosphorylated and can serve as a substrate for the Src family of tyrosine kinases, a factor that may be mediated by the SH2 domain of PLC-γ (Paxton et al., 1994; Liao et al., 1993). Indeed, angiotensin-II activates $pp60^{c\text{-}src}$ in vascular smooth muscle cells (Ishida et al., 1995). However angiotensin-II is a weak mitogen and it induces the expression of endogenous growth factors including TGF-β1 and PDGF and this effect can be inhibited by the growth factor inhibitor drug suramin (Weber et

al., 1994). Angiotensin-II increases synthesis of a 230-KDa tenascin and fibronectin without stimulating laminin production or general increase in the synthesis of secreted proteins (Sharifi et al., 1992). In addition, angiotensin-II induces collagen type 1, α1 chain transcript expression in cardiac fibroblasts as well as the synthesis and secretion of collagen by these cells (Crabos et al., 1994). ACE inhibitors antagonise some of the effects of angiotensin-II. For example, in cultured monocytic and vascular smooth muscle cells, angiotensin-II elicits an increase in nuclear factor-κB activation and monocyte chemo-attractant protein-1 while quinapril inhibits these effects of angiotensin-II (Hernandez-Presa et al., 1997). In addition, captopril decreases TGF-β receptor types 1 and type II protein expression without affecting TGF-β production (Guh et al., 1996).

Interestingly, captopril inhibits c-fos expression induced by angiotensin-II in neurones (Pastuskovas & Vivas, 1997) and modifies gene expression in hypertrophied and failing hearts of aged spontaneously hypertensive rats without affecting plasma levels of angiotensin-II (Brooks et al., 1997). Surprisingly, bradykinin also stimulates c-fos expression, AP-1-DNA binding activity and proliferation of mesangial cells (El-Dahr et al., 1996), while bradykinin B2 receptor antagonist icatibant blocks the ability of captopril to reduce c-fos and c-myc expression of cultured neonatal rat cardiomyocytes (Lei & Guo, 1998). This makes it difficult to understand the role bradykinin might have on gene expression. *In vivo* inhibition of ACE results in an increase in bradykinin levels and a reduction in angiotensin-II. Therefore the inhibition of c-fos expression by ACE inhibitors is difficult to interpret. Assuming that bradykinin increases c-fos expression, ACE inhibitors should not be expected to reduce c-fos expression. Since ACE inhibitors inhibit c-fos expression, it can be assumed that this mechanism is partly regulated by

bradykinin. Therefore, in addition to affecting the renin-angiotensin system, the effect of ACE inhibitors on cell proliferation may be mediated by other mechanisms that may involve ACE independent of the renin-angiotensin system. This is also confirmed by the fact that captopril inhibits pp60 $^{c\text{-}src}$ tyrosine phosphorylation in cultured human mesangial cells with a greater effect than lisinopril (Ruiz-Gines et al., 1997).

6.8 *ACE inhibitor induced pancytopenia may be mediated by angiotensin-II reduction*

High plasma renin activity is associated with high erythropoietin (Epo) levels in chronic haemodialysis patients. However, in the presence of captopril high renin activity is not associated with high Epo levels (Vlahakos et al., 1995) in these patients. Moreover, captopril reduces erythrocytosis in post renal transplant patients with normal renal function allografts (Suh et al., 1996). It has been shown that this effect may be mediated by reduction in Epo levels following ACE inhibitor therapy. This results in a significant reduction in haematocrit, haemoglobin and red blood cell count (Torregrosa et al., 1994). Therefore, ACE inhibitor related anaemia in renal transplant recipients seems to be due to the erythropoietin-lowering effect of this group of drugs (Gossmann et al., 1996). Indeed, there is need for increased recombinant human Epo maintenance doses in haemodialysis patients who are receiving angiotensin-converting enzyme inhibitors (Matsumura et al., 1997). The inability of captopril to reduce Epo levels in the presence of angiotensin-II in subsuppressor amounts has also been observed (Gould et al., 1980). Thus a direct involvement of angiotensin-II in Epo production has been confirmed. From these observations it is clear that ACE inhibitors may decrease angiotensin-II, which stimulates erythropoietin production (Nomura et al., 1996). Surprisingly losartan,

angiotensin-II AT_1 receptor antagonist does not alter Epo levels (Shand et al., 1995). Since there are two potential receptors for angiotensin-II, it is possible that AT_2 angiotensin-II receptors or other unknown mechanisms may mediate the Epo increasing effect of angiotensin-II. While the above observations explain the cause of anaemia by ACE inhibitors in the post renal transplant patients and haemodialysis patients, they do not explain the underlying mechanism in aplastic anaemia caused by ACE inhibitors in other patients. Epo is a growth factor which is committed to the mature erythroid progenitors (Ogawa, 1993) therefore, its absence should not cause a dramatic effect on cells of the granulocytic series. This observation also suggests that ACE inhibitors induce anaemia in haemodialysis patients by both the Epo mechanism and indirect of it. It also shows that the mechanism involved in anaemia in haemodialysis patients may be different from the ACE inhibitor induced granulocytopenia in individuals without renal transplants. There are pathways shared by angiotensin-II and haematopoietic cell growth factors that can be utilised by ACE inhibitors in bringing about myelosuppression. It is possible that one of these pathways may be significant in ACE inhibitor induced pancytopenia both in normal individuals and in individuals with underlying disease. However, there may also be independent pathways that may affect cell proliferation by ACE inhibitors. One of these pathways may involve interleukin-10 (IL-10).

6.9 *Interleukin-10 may mediate ACE inhibitor induced pancytopenia*

Interleukin-10 (IL-10) has important regulatory effects on immunological and inflammatory responses because of its capacity to downregulate class II MHC expression and to inhibit the production of proinflammatory cytokines by monocytes. As reviewed by Moore et al., 1993, IL-10 also known as cytokine synthesis inhibitory

factor (CSIF), was discovered by searching for a product of T helper 2 (Th2) that inhibited proliferation, effector function and development of T helper 1 (Th1) cells. In the presence of monocyte/macrophage activated peripheral blood cells, IL-10 inhibits T-cell proliferation by reducing IL-2 production. IL-10 also inhibits monocyte/macrophage-dependent synthesis of IFN and TNF by human natural killer cells stimulated by IL-2. In addition IL-10 inhibits both proliferation and cytokine production in a primary mixed lymphocyte reaction (MLR) with allogenic human monocyte. This characteristic of IL-10 is shared by AcSDKP (Lavignac et al., 1992). IL-10 inhibits LPS-induced PG-E_2 production in monocytes and PG-E_2 augments the production of IL-10 by LPS (Niho et al., 1998). This mechanism may involve the ability of IL-10 to reduce the expression of COX-2 (Niiro et al., 1995). In addition IL-10 also suppresses the ability of mouse peritoneal macrophages to release TNF-α and reactive oxygen intermediates (Bogdan et al., 1991). Moreover, IL-10 inhibits the expression of I antigens (MHC class II) on lymphocytes without influencing the capacity of tumour cells to induce cytotoxic T lymphocyte (CTL)-mediated lysis by cold target inhibition (Matsuda et al., 1994; de Waal Malefyt et al., 1991). As observed by de Waal Malefyt et al., (1991), IL-10 is produced following LPS-stimulation of monocyte *in vitro* after 7 hours and maximum production is only observed after 24-48 hours. However, high levels of IL-1α, IL-1β, IL-6, IL-8 GM-CSF and G-CSF are produced between 4-8 hours after activation. de Waal Malefyt et al., 1991 also demonstrated that addition of IL-10 to monocytes activated by IFN-γ, LPS, or a combination of LPS and IFN at the on set of cultures strongly inhibited the production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, G-CSF and IL-10 mRNA synthesis. Indeed, IL-10 may bring about the reduction in cytokine production by its ability to inhibit NFκB activation in monocytes without affecting NF-IL-6, AP-1, AP-

2, GR, CREB, Oct-1 and Sp-1 (Wang et al., 1995). NFκB is involved in the expression of inflammatory cytokine genes.

It is possible that the inhibitory effect of captopril on cell proliferation may be mediated by IL-10. Captopril may bring about IL-10 production by its ability to induce PG-E_2 production (Swartz et al., 1980; Johnsen et al., 1997). The increased in IL-10 levels may contribute to the ability of captopril to inhibit MHC class-II expression on macrophages indirectly by inhibiting IFN-γ production (Snyder et al., 1982; Figueiredo et al., 1990). Captopril also delays hypersensitivity reactions in rodents (Wang et al., 1996). This effect may involve the loop cascade that involves a number of cytokines with an ultimate involvement of IL-10. Interestingly captopril induces pancytopenia in patients with systemic lupus erythematosis (SLE) (Amann et al., 1980). In SLE patients, a positive correlation is observed between IL-6 and TNF-α levels (Mongan et al., 1997). While IL-10 causes a concentration-dependent suppression of IL-6 production in normal B cells and monocytes, this suppression is deficient in B cells and monocytes from lupus patients (Mongan et al., 1997). Captopril also reduces the granuloma size following Schistosoma mansoni infestation (Weinstock et al., 1981). The proliferative response of peripheral blood monocytes (PBMC) following stimulation with Schistosoma mansoni soluble eggs and adult warm antigens is increased by blockage of IL-10 production with specific IL-10 monoclonal antibodies (Malaquias et al., 1997). Moreover, immune complexes isolated from sera of Schistosoma infected individuals reduce the granulomatous hypersensitivity by decreasing production of TNF-α and increasing production of IL-10 by PBMC treated with immune complexes (Rezende et al., 1997). From the present investigations it can be suggested that administration of captopril in mice following cytotoxic treatment produced high levels of IL-10 that caused the inhibition

of cytokine production with a net result of inhibiting HPP-CFC-1 proliferation. Indeed the ability of captopril to alleviate symptoms of autoimmune diseases such as rheumatoid arthritis, may also be mediated by the ability of captopril to alter the pattern of cytokines produced in these diseases (Martin et al., 1984).

AcSDKP inhibitory effect on HPP-CFC-1 may involve IL-10 as well. Captopril increases both AcSDKP *in vivo* and *in vitro* levels (Grillon et al., 1993; Azizi et al., 1996; Azizi et al., 1997; Rousseau et al., 1995), and it increases PG-E_2 levels which may induce IL-10 production. It follows that AcSDKP and IL-10 may augment each other in bringing about the inhibitory effects of captopril. Moreover, the inhibitory effect of AcSDKP on cell proliferation will be enhanced in the presence of captopril due to IL-10 production.

The ability of AcSDKP to inhibit the inhibitory factors involved in the suppression of GM-CFC proliferation following HIV infection (Coutton & Chermann, 1997), may involve the ability of AcSDKP to oppose the effect of PG-E_2 on GM-CFC proliferation. Increased concentrations of PG-E_2 are found in ultrafiltrates from HIV-infected monocyte (Foley et al., 1992). Since the mode of AcSDKP action is unknown, it is possible that AcSDKP may have an individual effect on IL-10 production which in turn may lead to inhibition of PG-E_2 production.

6.10 *Captopril and AcSDKP may inhibit phosphodiesterase*

The observation that TNF-α production may be under the control of a metalloproteinase while production of IL-1β is not (McGeehan et al., 1994), suggests that another mechanism is involved in the observed inhibitory action of captopril on the production of both TNF-α and IL-1β (Schindler et al., 1995). This other mechanism may involve inhibition of phosphodiesterases. Phosphodiesterases

degrade cyclic AMP and cyclic GMP. Phosphodiesterase inhibitors cause a concentration dependent inhibition of TNF-α production by lipopolysaccharide (LPS) stimulated human and rat blood (Foster et al., 1993). TNF-α suppression is mediated by an increase in cyclic AMP while IL-1 suppression is mediated by an increase in cyclic GMP following LPS stimulation of mouse macrophages (Endres et al., 1991). However, the expression of both TNF and IL-1 following LPS activation of murine peritoneal macrophages may be selectively suppressed by agents that only elevate intracellular cAMP (Tannenbaum & Hamilton, 1989). The specific types III and IV phosphodiesterase inhibitors suppress the formation of TNF by macrophages. This effect is synergised with adenylate cyclase activator PG-E_2 (Schade & Schudt, 1993). Moreover, type IV phosphodiesterase inhibitors inhibit LPS-induced TNF-α with 500-fold greater potency than non-specific phosphodiesterase inhibitors (Angel et al., 1995). From these observations it is possible to see that immunological effects of captopril may be mediated by inhibiting phosphodiesterases. While captopril, enalapril, cilazapril and delapril all inhibit cytokine production following LPS human mononuclear cell activation, ramipril, lisinopril, peridopril and spirapril do not have this effect (Fukuzawa et al., 1997; Schindler et al., 1995). This mechanism may or may not be mediated by angiotensin-II inhibition. The effects of angiotensin-II on phosphodiesterase activity are not well known, therefore, it is difficult to rule out the effects of angiotensin-II on the phosphodiesterase activity. The ability of captopril to inhibit granuloma formation following injections of schistosoma mansoni eggs (Weinstock et al., 1981), may also be mediated by phosphodiesterase inhibition by this drug. Granuloma formation following injections of schistosoma mansoni eggs in mice is mediated by early recruitment of IL-1 with TNF taking part in the later stages of hypersensitivity (Chensue et al., 1989). Captopril has also been shown to inhibit

neutrophil synthesis of Leukotriene B4 (LTB_4) *in vitro* and *in vivo* (Shindo et al., 1994). Phosphodiesterase inhibitors block the formation of LTC_4 (Schade & Schudt, 1993).

If the hypothesis that captopril inhibits phosphodiesterases is correct, an increase in AcSDKP might be one of mechanisms of this effect. This may be mediated by the ability of captopril to differentially inhibit the N-domain ACE active site thereby increasing the levels of AcSDKP. Since the mode of action of AcSDKP is still unknown, it is reasonable to speculate that the peptide may be responsible for the inhibition of phosphodiesterases. This is plausible because AcSDKP levels are markedly elevated *in vivo* and *in vitro* in the presence of captopril in mice that have not received cytotoxic insults (see Figs. 3.7 & 4.8). This is also confirmed by the fact that AcSDKP inhibits phosphodiesterase (Voelter et al., 1995) and the observation that AcSDKP prevents CFU-GM inhibition induced by HIV-infected cell-derived conditioned medium (Coutton & Chermann, 1997). As discussed by Coutton & Chermann, 1997, HIV infected cells produce a number of cytokines that may be involved in the inhibition of CFU-GM proliferation. Moreover, the exclusive potentiation by TNFα of the metabolic events triggered by an increase in intracellular Ca^{2+} in human granulocytes is inhibited by cAMP agonists (Yuo et al., 1989). There is enough evidence therefore, to suggest that the phosphodiesterase inhibitory effect of captopril is crucial in the cellular proliferation effects of this drug. Captopril does not seem to be a selective inhibitor of phosphodiesterase isoforms. This is confirmed by the fact that captopril is a potent inhibitor of LTB_4 synthesis in intact neutrophils rather than in cytosol (Shindo et al., 1994). Therefore the captopril inhibitory effect on cell proliferation requires cytoplasmic molecules for the effect of captopril to be shown. In addition type IV phosphodiesterase inhibitors are weak inhibitors of LTC_4

and there is no additive effect between adenylate cyclase activators and type III or type IV phosphodiesterase inhibitors on the inhibition of LTC_4 synthesis (Schade & Schudt, 1993).

However, as stated already LTC_4 inhibition is mainly mediated by the ability of captopril to inhibit a matrix metalloproteinase in addition to this observed phosphodiesterase mechanism. Moreover, both LTB_4 and LTC_4 induce a two to three fold increase in GM-CSF-stimulated myeloid progenitor cells (GM-CFC) proliferation and GM-CSF enhances *in vitro* synthesis of LTB_4 and LTC_4 by neutrophils and eosinophils (Stenke et al., 1994). Thus, the ability of captopril to inhibit the production of these leukotrienes may have a direct effect on cell proliferation by reducing the levels of stimulatory factors. This mechanism may require accessory cells to be accomplished. The involvement of phosphodiesterases in the ability of captopril to influence cell proliferation may be responsible for its immune regulation. Indeed this effect may link to IL-10 production. For example, $PG-E_2$ any increase cAMP, which in turn may play a role on the production of IL-10, TNFα, IL-1 and other cytokines, as already described. The involvement of phosphodiesterases, may also explain the differential effect of captopril on HPP-CFC-1 proliferation following 2 Gy irradiation and cytotoxic drugs. Captopril suppresses the primary antibody response of B-lymphocytes only in the presence of both adherent cells and radiosensitive T_8 suppressor effector lymphocytes (Delfraissy et al., 1984).

6.11 *Captopril and intracellular ion concentrations*

Signal transduction pathways can only be achieved in the presence of appropriate intracellular pH and ionic balance. ACE inhibiting drugs in general

increase the Na^+-K^+ pump current of isolated cardiac myocytes when intracellular Na^+ is at near-physiological levels, an effect that is opposed by angiotensin-II (Hool et al., 1996). The study of captopril on potassium influx in a kidney epithelial cell line showed that the frusemide sensitive $Na^+/K^+/2Cl^-$ co-transport was significantly decreased and the Na^+/K^+-ATPase activity was only lowered when high amounts of captopril were used. Moreover, low doses of captopril strongly inhibited cell proliferation and higher amounts did not further suppress cell proliferation (Bauer et al., 1990). This suggests an indirect mechanism in the action of captopril. Captopril decreases the concentrations of zinc in blood, of copper and calcium in the epididymis and of magnesium in the testes (Kohler-Samouilidis et al., 1997). The decrease in blood zinc ion concentration affects the activity of both zinc dependent metalloproteinases and ACE. In addition captopril has been shown to reduce human sperm binding to the plasma membrane of zona-free hamster oocytes (Kohn et al., 1997). In contrast, AcSDKP has been shown to enhance binding of sperm to interstitial cells of the testes (Wdzieczak-Bakala personal communication). It can therefore be speculated that changes in ionic concentrations imposed by AcSDKP and captopril (dependent or independent of AcSDKP) may increase the affinity of sperm to testicular tissue while reducing its affinity to eggs. Captopril reduces the arginine vasopressin and PDGF-induced increase of cytosolic free calcium concentration in vascular smooth muscle cells and cultured glomerular mesangial cells. In the presence but not absence of extracellular Ca^{2+}, captopril reduces the angiotensin-II induced intracellular Ca^{2+} increase (Tepel et al., 1997). This mechanism may lead to an inhibition of the molecular mechanism involved in one or more key steps in signal transduction pathways. The involvement of extracellular Ca^{2+} ions in captopril's effect on angiotensin-II ionic exchange may involve the integrity of gap junction or

Ca^{2+} channels. The integrity of gap junctions is regulated by connexins. Connexin 43 is phosphorylated by phosphorylated C-src (Swenson et al., 1990; Crow et al., 1990). C-src is phosphorylated by angiotensin-II (Ishida et al., 1995). Therefore by this mechanism captopril may affect the integrity of gap junctions. The ability of captopril to affect Ca^{2+} channels is suggested by the fact that an increase in intracellular calcium by angiotensin-II has been shown to be blocked or reversed by addition of verapamil, a Ca^{2+} channel blocker (Baker et al., 1989).

It is possible that the inhibition of Ca^{2+} intracellular influx may be responsible for captopril induced inhibition of cell proliferation. If this hypothesis is true then the intracellular Ca^{2+} concentration following both captopril and AcSDKP should be regulated by Ca^{2+} concentrations from the extracellular space as shown by the effect of captopril on angiotensin-II mediated intracellular Ca^{2+} increase. Captopril inhibits an increase of the intracellular free Ca^{2+} in thymocytes in spontaneously hypertensive rats (Xie et al., 1996). In addition to being a co-factor to many enzymes involved in signal transduction, Ca^{2+} is involved in cell cycle progression into S-phase. This is shown by the fact that elevation of calmodulin at the G_1-S is independent of the G_1, and the increase in calmodulin appears to be related to progression into S-phase (Chafouleas, et al., 1982). Therefore, the absence of Ca^{2+} at the G_1/S boundary may be responsible for AcSDKP G_1/S block during cell proliferation. This points strongly to the involvement of pp60 $^{c\text{-}src}$ and gap junctions. Certainly in the absence of calmodulin, AcSDKP reduces the basal activity of phosphodiesterase 2- to 3-fold. However, AcSDKP causes an incomplete competitive inhibition of hypothalamic calmodulin dependent phosphodiesterase in the presence of calmodulin (Voelter et al., 1995). Therefore the absence of Ca^{2+} enhances the inhibitory activity of AcSDKP on cell proliferation. The ability of captopril to reduce intracellular free Ca^{2+} and to

increase AcSDKP levels *in vivo* and *in vitro* may synergise to bring about the effects of AcSDKP on cell proliferation.

6.12 *Rous sarcoma virus proto-oncogene ($pp60^{c\text{-}src}$) as a mediator of AcSDKP and Captopril induced cellular proliferation inhibition*

As reviewed by Bolen in 1993, the C-src kinase family belongs to a group of the non-receptor tyrosine protein kinases. The function of C-src kinases appears to be to phosphorylate members of the src family of tyrosine protein kinases at a common tyrosine residue corresponding to the C-src tyrosine 527. There are nine members of src gene family group. The src-family protein tyrosine kinases are widely expressed in cells of haematopoietic origin (Liao et al., 1993). Other transmembrane proteins including cytokine receptors, integrins, glycophosphatidylinositol-linked proteins, and multichain immuno-recognition receptors have been found associated with one or more C-src family kinases (Cooper & Howell, 1993). The viral oncogene of $pp60^{src}$ is not an integral membrane protein, but rather associates peripherally with the cytoplasmic face of several cytoplasmic membranes including plasma membrane and perinuclear membranes (Hunter & Cooper, 1985). Phosphorylated C-src can activate STAT3 in the Jak/STAT signaling pathways (Yu et al., 1995). Keeping src inactive requires phosphorylation of tyrosine residue 527 and dephosphorylation of tyrosine residue 416 (Cooper & Howell, 1993). Activation of C-src requires phosphorylation of SHP-1 tyrosine phosphatase that dephosphorylates C-src by dephosphorylating the COOH-terminal regulatory tyrosine (Somani et al., 1997).

Captopril has been shown to inhibit $pp60^{c\text{-}src}$ tyrosine phosphorylation (Ruiz-Gines et al., 1997). The net result of this may be an indirect inhibition of PLC (Liao et al., 1993). Since activated $pp60^{v\text{-}src}$ phosphorylates connexin 43, a protein involved in the formation of gap junctions, with a net result of loss of gap junctional

communication (Swenson et al., 1990; Crow et al., 1990), inhibition of $pp60^{c\text{-}src}$ tyrosine phosphorylation will restore gap junction function with inhibition of cell proliferation. Moreover, inhibition of C-src tyrosine phosphorylation will reduce the levels of diacylgycerol through inhibition of PLC phosphorylation. Diacylgycerol inhibits gap junction communication in cultured epidermal cells (Gainer & Murry, 1985). Therefore, inhibition of C-src by captopril may directly or indirectly influence cell proliferation. The fact that captopril is shown to inhibit haematopoietic cell proliferation in these investigations and tyrosine phosphorylated C-src is involved in the cytokine mediated signal transduction strongly supports the involvement of $pp60^{c\text{-}src}$ in the inhibitory effect imposed by captopril in the inhibition of HPP-CFC-1 proliferation. Moreover, a slight inhibition of $pp60^{c\text{-}src}$ tyrosine phosphorylation was also observed in the presence of lisinopril but not tocopheral, a pure antioxidant (Ruiz-Guines et al., 1997), suggesting that the inhibition of $pp60^{c\text{-}src}$ is slightly mediated by angiotensin-II with no involvement of antioxidant mechanism of captopril. Interferon-γ and TNF-α enhance $pp60^{c\text{-}src}$ expression in human macrophages and myelomonocytic cell lines (Sorio et al., 1993). Therefore, in the presence of captopril which inhibits TNF-α production, $pp60^{c\text{-}src}$ expression will be inhibited with a loss of some signal transduction amplifications.

Captopril has been shown to alleviate the symptoms of polycythaemia rubra vera (PV) (Nomura et al., 1996). Polycythaemia rubra vera is a clonal myeloproliferative disorder that leads to trilineage (erythroid, myeloid, and megakaryocytic) hyperplasia in the bone marrow with a principal clinical manifestation of erythrocytosis and plethora. People with this condition have high levels of normal $CD34^+$ cells in peripheral blood (Andreasson et al., 1997). The levels of AcSDKP in serum of people with PV are significantly increased (Liozon et al.,

1993). An increase in membrane or membrane associated protein tyrosine phosphatase activity is shown to be responsible for this disease (Sui et al., 1997). In addition, increased PKC activity is also thought to be responsible for the increased cell proliferation in PV patients (Kawada et al., 1995). The involvement of membrane associated protein tyrosine phosphatase activity may suggest the involvement of C-src in the pathogenesis of PV. Moreover, high levels of AcSDKP in serum of people with PV may suggest a feed loop mechanism that increases the levels of stem cell inhibitory factors. This disease may set the levels of AcSDKP at a higher threshold than normal. It can be speculated that addition of captopril inhibits C-src activity directly or indirectly to levels that will stop the transduction pathway involved in the proliferation of progenitors responsible for red blood cell production. The net effect is inhibiting $CD34^+$ proliferation directly or by gap junctional mechanisms. Apart from a direct effect of captopril on cell proliferation and indirect effect mediated by angiotensin-II, the only other mechanism known by which captopril can influence cell proliferation is the AcSDKP inhibitory mechanism.

As already discussed, either inhibition of phosphodiesterases or an increase in cAMP activity may be a common pathway between captopril and AcSDKP proliferation inhibitory mechanisms. Moreover, both lisinopril and captopril can increase levels of AcSDKP (Rousseau et al., 1995; Azizi et al., 1997). Therefore, captopril may be acting to increase AcSDKP levels that counteract the C-src dependent phosphatase activity or the PLC activity mediated by C-src which in turn affects the PKC activity. This is confirmed by the fact that in normal cells the retinoblastoma gene protein and C-src gene proteins are both associated with phosphatases (Coats & Roberts, 1996; Somani et al., 1997). Moreover, a 60 KDa fragment of hypophosphorylated retinoblastoma gene has been found in the nucleolus

(Rogalsky et al., 1993). It is speculated by Rogalsky et al., (1993) that this fragment of the retinoblastoma gene protein may have some effect on the ribosomal RNA production in the nucleolus. While low levels of AcSDKP have no effect on tumour cell cycling, high levels inhibit the proliferation of human leukaemic cells (Defard et al., 1997). This characteristic is also shown by captopril. Therefore, derangement of cell proliferation in PV patients can be explained simply by an increase in C-src activity and by up regulating levels of AcSDKP. However, it is difficult to rule out a direct involvement of captopril on this effect since a direct correlation between captopril and AcSDKP levels has not been shown *in vivo* in PV patients.

The wide distribution of C-src on cells of the haematopoietic system suggests that the observed inhibition of HPP-CFC-1 proliferation *in vivo* and *in vitro* in this investigation by captopril may be mediated by the phosphorylation status of C-src. The involvement of angiotensin-II on C-src phosphorylation (Ishida et al., 1995) as already stated suggests that angiotensin-II inhibition, while not the main mode of action of ACE inhibitors on cell proliferation, may play a small part in the ACE inhibitor inhibitory effect on cell proliferation. Since AcSDKP is haematopoietic stem cell specific inhibitor, C-src phosphorylation may also play a major part.

6.13 CONCLUSION.

These investigations have demonstrated unique properties of ACE inhibitors on haematopoiesis. Not only have they shown the differential effects of captopril and lisinopril on reducing the proportion of HPP-CFC-1 in S-phase both *in vitro* and *in vivo*, they have also shown that captopril has a unique property as an inhibitor of haematopoietic cell proliferation. Captopril has been shown to inhibit haematopoietic cell proliferation by mechanisms specific to captopril and ones common to all ACE

inhibitors. In addition, the signal transduction pathways employed by captopril and its cellular inhibitory effects have also been discussed even though mostly by speculation. The ability of captopril to inhibit cell proliferation may depend on the endogenous production of cytokines. The involvement of PG-E_2 and IL-10 has been discussed in relation to the ability of captopril to induce an inhibition of cell proliferation. The ability of captopril to inhibit cell proliferation strongly points to the involvement of C-src pp 60. It seems that cells expressing this gene are likely to be inhibited by captopril. Moreover, the effect of captopril on cell proliferation may also involve phosphodiesterases. The uniqueness of captopril to increase endogenous levels of AcSDKP seems to be a strong mechanism in captopril's ability to inhibit cell proliferation. Apart from hepatocyte proliferation *in vivo* (Lombard et al., 1990), the effect of AcSDKP on non-haematopoietic cell proliferation has not been extensively studied. It is possible that AcSDKP may also inhibit cells that are inhibited by captopril. The similarities between the captopril and the AcSDKP effect on neoplastic cell proliferation strongly suggests a common mode of action between AcSDKP and captopril in their proliferation inhibitory effects. Moreover, both substances inhibit cell proliferation better in the presence of accessory cells. In addition, involvement of cytokines in the cellular inhibitory effects of both substances has been shown. Both captopril and AcSDKP seem to inhibit G_1/S transition in the proliferating cells and have no effect on quiescent cells. In the long-term bone marrow presented in this investigation, captopril and AcSDKP had the same effect on the integrity of LTBMCs. While it is not possible to rule out the inherent effect of captopril on cell proliferation, these results and the literature reviewed strongly suggest that AcSDKP mediate the effects of captopril.

The use of AcSDKP instead of captopril may prove beneficial in the immune regulatory mechanisms due to the blood lowering effects of captopril. The treatment of shock, Asthma, HIV and other hypersensitivity reactions may be envisaged with AcSDKP. Moreover, captopril may be employed for the treatment of neoplastic diseases as an adjuvant to chemotherapy. Not only will captopril protect haematopoietic stem cell damage, but it will also stop tumour cell invasion by inhibiting matrix metalloproteinases. Therefore, the net result may be a reduction in the severity of pancytopenia following chemotherapy and cure of the disease. Captopril may also be employed in the purging of neoplastic cells from bone marrow transplants. All in all, this thesis has analysed the underlying mechanism of ACE inhibitor induced pancytopenia. It has also suggested the possible use of this mechanism in the protection of haematopoietic cells during therapy. The use of ACE inhibitors in haematoprotection would help to enhance the therapy of neoplastic disease by reducing the severity of bone marrow suppression.

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APPENDIX

ACE activity means used to calculate the final means presented in the text (U/L)

Experiment	Plasma Control	Serum Control	Plasma with Lisinopril	Plasma with Captopril	Serum with Lisinopril	Serum with Captopril
1	520	420	52	62	30	169
2	353.3	355	34.25	202	35.7	229
3	483.8	417	102.6	51	29	185
4	214.3	621.2	64	28.8	20.4	211.9

The Proportion (%) of Normal Bone Marrow GM-CFC in S-phase after incubation with AcSDKP + Captopril

Experiment	% S-phase Control	% S-phase AcSDKP + Captopril
1	33.0 ± 3.4	17.9 ± 2.1
2	26.3 ± 2.2	14.3 ± 2.8
3	29.5 ± 2.4	13.7 ± 3.2
4	27.0 ± 2.3	20.1 ± 4.3

The proportion (%) of Normal Bone Marrow GM-CFC in S-phase after incubation with AcSDψKP

Experiment	% S-phase Control	% S-phase AcSDKP + Captopril
1	26.6 ± 2.4	9.3 ± 2.7
2	34.9 ± 2.2	13.3 ± 3.1
3	26.2 ± 3.7	16.8 ± 1.8
4	30.6 ± 2.1	20.9 ± 1.7

Proportion (%) of Normal Bone Marrow HPP-CFC-1 in S-phase Following *in vitro* incubation

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	12.2 ± 2.1	16.8 ± 2.5
2	4.2 ± 2.3	15.6 ± 2.8
3	7.1 ± 3.2	2.7 ± 3.8
4	9.0 ± 3.7	13.7 ± 4.3

The proportion (%) of Normal Bone Marrow HPP-CFC-1 in S-phase following *in vitro* incubation with Lisinopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	16.9 ± 2.1	17.9 ± 5.2
2	19.4 ±10.2	16.3 ± 3.8
3	13.5 ± 4.4	2.7 ± 3.2
4	5.8 ± 3.6	3.5 ± 4.3

The proportion (%) of Normal Bone Marrow HPP-CFC-1 in S-phase following *in vitro* incubation with Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	17.0 ± 7.9	18.5 ± 10.2
2	10.6 ± 3.6	17.9 ± 3.8
3	11.2 ± 5.5	13.3 ± 3.2
4	14.8 ± 2.3	9.1 ± 6.3

The proportion (%) of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with Medium

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	37.4 ± 2.2	29.2 ± 3.1
2	34.6 ± 3.2	43.1 ± 3.9
3	29.0 ± 2.7	29.0 ± 3.0
4	35.4 ± 1.2	24.7 ± 2.2

The proportion (%) of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	40.2 ± 5.8	35.2 ± 14.7
2	62.3 ± 5.4	42.6 ± 5.7
3	43.8 ± 4.0	59.0 ± 6.7
4	32.9 ± 4.0	54.2 ± 10.6

The proportion (%)of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with Lisinopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	41.9 ± 2.9	41.5 ± 3.2
2	38.9 ± 2.4	51.8 ± 2.3
3	34.0 ± 2.8	43.6 ± 3.5
4	39.7 ± 1.3	35.4 ± 5.9

The proportion (%) of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with AcSDKP

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	46.6 ± 5.6	35.9 ± 8.7
2	34.6 ± 3.3	24.5 ± 6.4
3	41.6 ± 4.5	37.2 ± 5.4
4	38.0 ± 6.8	29.7 ± 8.0

The proportion (%) of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with AcSDKP & Lisinopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	33.5 ± 2.8	34.6 ± 3.2
2	35.3 ± 2.2	21.0 ± 5.9
3	34.0 ± 2.5	21.5 ± 3.6
4	30.8 ± 0.8	25.7 ± 3.1

The proportion (%) of HPP-CFC-1 in S-phase from 2 Gy irradiated mice following *in vitro* incubation with AcSDKP & Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	33.2 ± 5.0	9.7 ± 3.7
2	42.6 ± 3.4	8.1 ± 5.6
3	32.8 ± 3.0	11.9 ± 5.7
4	59.8 ± 2.6	11.5 ± 3.0

The proportion (%) of HPP-CFC-1 in S-phase from Ara-C mice following *in vitro* incubation with Medium

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	51.4 ± 1.8	29.0 ± 2.7
2	43.2 ± 2.1	25.8 ± 5.6
3	35.7 ± 2.3	19.9 ± 3.1
4	45.7 ± 3.4	31.3 ± 1.5

The proportion (%) of HPP-CFC-1 in S-phase from Ara-C treated mice following *in vitro* incubation with Lisinopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	53.7 ± 5.4	24.5 ± 2.8
2	48.7 ± 3.9	33.7 ± 2.2
3	56.6 ± 2.5	29.5 ± 2.8
4	50.2 ± 2.5	26.3 ± 2.6

The proportion (%) of HPP-CFC-1 in S-phase from Ara-C treated mice following *in vitro* incubation with Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	52.6 ± 3.2	6.7 ± 2.2
2	40.6 ± 5.2	3.0 ± 2.8
3	37.8 ± 3.3	4.6 ± 4.2
4	41.9 ± 3.2	5.3 ± 2.9

The proportion (%) of HPP-CFC-1 in S-phase from 5 FU treated mice following *in vitro* incubation with Medium

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	40.0 ± 1.7	26.1 ± 3.3
2	46.5 ± 2.0	20.8 ± 4.1
3	40.7 ± 1.9	25.5 ± 4.8
4	57.4 ± 2.5	29.8 ± 7.2

The proportion (%) of HPP-CFC-1 in S-phase from 5 FU treated mice following *in vitro* incubation with Lisinopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	44.3 ± 3.2	43.7 ± 7.0
2	45.4 ± 1.9	24.0 ± 2.9
3	44.5 ± 3.3	31.1 ± 2.4
4	29.6 ± 1.8	33.8 ± 4.6

The proportion (%) of HPP-CFC-1 in S-phase from 5 FU treated mice following *in vitro* incubation with Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	34.9 ± 2.3	7.5 ± 3.2
2	47.8 ± 2.9	5.6 ± 3.3
3	49.8 ± 2.5	9.4 ± 4.4
4	49.2 ± 6.7	13.0 ± 12.0

The proportion (%) of SA2 cells in S-phase following *in vitro* incubation with Saline

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	44.7 ± 2.3	67.9 ± 4.7
2	51.3 ± 5.0	55.7 ± 4.8
3	60.9 ± 2.0	46.3 ± 4.0
4	81.0 ± 1.2	66.5 ± 2.6

In Vitro AcSDKP concentration following incubation

	AcSDKP (nM)				
	Supernatant	Captopril		Medium (Placebo)	
Normal Bone Marrow		T = 0	T = 24	T = 0	T = 24
	Experiment 1	0.5	1.14	0.65	0.71
	Experiment 1	0.62	1.19	0.71	1.41
	Experiment 2	0.52	1.01	0.75	0.66
	Experiment 2	0.58	1.33	0.75	0.75
	Experiment 3	0.68	1.12	0.66	0.68
	Experiment 3	0.75	0.92	0.88	0.68
Ara-C Bone Marrow					
	Experiment 1	0.55	0.93	0.46	0.44
	Experiment 1	0.55	0.94	0.57	0.41
	Experiment 2	0.59	0.79	0.52	0.45
	Experiment 2	0.52	0.66	0.48	0.44
	Experiment 3	0.54	0.9	0.72	0.39
	Experiment 3	0.6	0.62	0.67	0.4
5 FU Bone Marrow					
	Experiment 1	0.68	0.79	0.49	0.51
	Experiment 1	0.5	0.77	0.45	1.05
	Experiment 2	1.08	0.6	0.62	0.58
	Experiment 2	0.43	0.53	0.77	0.67
	Experiment 3	0.59	0.56	0.78	0.53
	Experiment 3	0.6	0.6	0.81	0.61
2 Gy Bone Marrow					
	Experiment 1	0.79	0.82	0.58	0.54
	Experiment 1	1.75	0.93	0.83	0.52
	Experiment 2	1.1	1.61	2.68	0.74
	Experiment 2	1.16	1.88	1.82	0.74
	Experiment 3	0.74	0.83	0.5	0.36
	Experiment 3	0.62	0.87	0.67	0.49

The proportion (%) of SA2 cells in S-phase following *in vitro* incubation with Captopril

Experiment	% S-phase (T = 0)	% S-phase (T = 24)
1	37.8 ± 6.5	62.1 ± 2.9
2	75.9 ± 2.2	55.6 ± 5.8
3	37.2 ± 3.3	63.3 ± 2.3
4	82.0 ± 0.7	63.4 ± 3.1

The proportion (%) of HPP-CFC-1 in S-phase following a 4 hr *in vitro* incubation with Captopril & AcSDKP after depleting all cells that were in S-phase *in vivo*.
2 Gy Bone Marrow

Experiment	% S-phase (Control)	% S-phase (Captopril & AcSDKP)
1	23.3 ± 2.9	5.0 ± 4.7
2	25.6 ± 4.5	7.4 ± 3.1
3	34.5 ± 2.5	7.6 ± 2.1
4	30.4 ± 3.0	1.7 ± 3.8

The proportion (%) of HPP-CFC-1 in S-phase following a 4 hr *in vitro* incubation with Captopril & AcSDKP after depleting all cells that were in S-phase *in vivo*.
Ara-C Bone Marrow

Experiment	% S-phase (Control)	% S-phase (Captopril & AcSDKP)
1	45.2 ± 2.1	19.8 ± 4.4
2	29.0 ± 3.8	1.5 ± 4.9
3	32.5 ± 7.4	9.8 ± 7.7
4	35.7 ± 5.0	22.0 ± 3.1

The proportion (%) of HPP-CFC-1 in S-phase following a 4 hr *in vitro* incubation with Captopril & AcSDKP after depleting all cells that were in S-phase *in vivo*.
5 FU

Experiment	% S-phase (Control)	% S-phase (Captopril & AcSDKP)
1	40.9 ± 5.3	22.8 ± 5.9
2	45.8 ± 7.9	18.9 ± 7.9
3	31.0 ± 11.2	5.6 ± 7.2
4	37.5 ± 13.8	17.2 ± 10.4

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 10 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	10.2 ± 3.7	18.9 ± 3.7
2	20.1 ± 11.8	12.8 ± 3.6
3	17.2 ± 3.2	7.4 ± 4.5
4	12.9 ± 4.7	6.5 ± 2.7

The proportion (%) of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with 10 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	54.1 ± 4.0	26.1 ± 5.2
2	42.4 ± 9.4	38.2 ± 5.2
3	43.0 ± 4.2	16.4 ± 4.2
4	31.3 ± 10.7	28.1 ± 4.7

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 10 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	4.2 ± 3.5	15.1 ± 3.2
2	4.4 ± 6.4	8.2 ± 4.2
3	7.0 ± 2.2	4.4 ± 4.8
4	8.8 ± 3.7	2.3 ± 4.3

The proportion (%) of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with 10 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	50.8 ± 5.8	47.1 ± 2.7
2	43.3 ± 3.1	50.0 ± 7.2
3	43.8 ± 4.5	16.5 ± 9.4
4	40.7 ± 10.0	34.9 ± 4.0

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 10 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	16.4 ± 6.3	-4.4 ± 10.7
2	1.4 ± 9.6	14.7 ± 4.6
3	-2.4 ± 6.2	5.4 ± 3.7
4	0.0 ± 4.8	1.2 ± 5.9

The proportion (%) of HPP-CFC-1 in S-phase following Ara-C (100 mg/kg) *in vivo* with 10 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	31.8 ± 17.6	19.4 ± 3.8
2	45.7 ± 5.1	32.3 ± 6.6
3	43.7 ± 2.9	19.1 ± 4.5
4	47.4 ± 3.8	11.2 ± 6.6

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 10 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	7.5 ± 2.3	8.6 ± 2.1
2	13.8 ± 3.8	17.8 ± 4.3
3	13.6 ± 2.7	14.3 ± 2.5
4	9.1 ± 3.9	10.1 ± 2.3

The proportion (%) of HPP-CFC-1 in S-phase following Ara-C (100 mg/kg) *in vivo* with 10 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	37.7 ± 3.8	27.0 ± 5.6
2	33.1 ± 4.4	30.0 ± 4.1
3	43.4 ± 4.8	47.7 ± 2.1
4	39.5 ± 3.1	36.2 ± 4.1

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 100 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	-1.5 ± 8.9	12.5 ± 1.8
2	19.4 ± 6.8	14.3 ± 3.5
3	0.8 ± 5.6	5.6 ± 4.8
4	-1.3 ± 7.7	5.2 ± 3.1

The proportion (%) of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with 100 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	32.9 ± 2.0	29.8 ± 3.5
2	19.4 ± 5.1	25.2 ± 3.3
3	22.2 ± 5.4	35.3 ± 7.7
4	32.9 ± 4.3	26.5 ± 5.3

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 100 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	13.4 ± 2.9	14.9 ± 1.9
2	19.8 ± 3.5	9.9 ± 2.3
3	6.6 ± 4.4	2.6 ± 4.8
4	10.5 ± 2.8	7.1 ± 5.2

The proportion (%) of HPP-CFC-1 in S-phase following 2 Gy irradiation *in vivo* with 100 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	29.7 ± 5.4	14.3 ± 2.7
2	41.5 ± 3.8	10.2 ± 4.8
3	31.5 ± 2.7	10.0 ± 3.2
4	40.9 ± 4.9	20.4 ± 8.1

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 100 mg/kg lisinopril

Experiment		% S-phase (Lisinopril)
1		10.8 ± 4.5
2		9.1 ± 3.9
3		12.5 ± 3.5
4		14.4 ± 5.2

The proportion (%) of HPP-CFC-1 in S-phase following Ara-C (100 mg/kg) *in vivo* with 100 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	34.0 ± 4.4	36.2 ± 4.4
2	33.8 ± 3.8	26.5 ± 4.0
3	17.3 ± 3.2	32.7 ± 3.0
4	38.0 ± 2.3	33.0 ± 4.6

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration with 100 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	17.1 ± 5.9	15.4 ± 3.2
2	-0.9 ± 5.2	3.4 ± 4.1
3	14.9 ± 7.7	17.3 ± 5.1
4	23.1 ± 5.7	10.8 ± 5.2

The proportion (%) of HPP-CFC-1 in S-phase following Ara-C (100 mg/kg) *in vivo* with 100 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	33.3 ± 3.5	5.0 ± 6.3
2	27.2 ± 4.5	2.4 ± 4.8
3	27.9 ± 5.6	10.2 ± 4.2
4	30.7 ± 3.4	13.3 ± 2.6

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 100 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	17.2 ± 6.8	19.9 ± 7.7
2	-1.3 ± 9.4	7.4 ± 8.9
3	2.4 ± 5.9	6.4 ± 11.2
4	-1.8 ± 4.8	3.3 ± 7.4

The proportion (%) of HPP-CFC-1 in S-phase following 5 FU (150 mg/kg) *in vivo* with 100 mg/kg lisinopril

Experiment	% S-phase (Saline control)	% S-phase (Lisinopril)
1	70.6 ± 2.5	60.2 ± 2.5
2	64.1 ± 3.1	71.0 ± 3.3
3	39.1 ± 4.2	63.1 ± 2.2
4	68.1 ± 3.1	62.5 ± 2.7

The proportion (%) of HPP-CFC-1 in S-phase in mice following *in vivo* administration of 100 mg/kg captopril

Experiment		% S-phase (Captopril)
1		3.2 ± 5.5
2		14.1 ± 3.6
3		7.2 ± 4.9
4		5.6 ± 4.1

The proportion (%) of HPP-CFC-1 in S-phase following 5 FU (150 mg/kg) *in vivo* with 100 mg/kg captopril

Experiment	% S-phase (Saline control)	% S-phase (Captopril)
1	53.3 ± 5.2	36.4 ± 8.6
2	60.4 ± 7.0	21.9 ± 12.1
3	60.3 ± 4.4	22.9 ± 9.1
4	53.8 ± 6.1	7.9 ± 18.8

In Vivo AcSDKP concentration in plasma following Captopril (100 mg/kg)

		AcSDKP (nM)						
	Plasma	Captopril			Saline			
		2 hrs	6 hrs	24 hrs	0 hrs	2 hrs	6 hrs	24 hrs
Saline	Experiment 1	7.06	7.38	2.66		2.3	2.08	1.22
	Experiment 2	3.85	1.9	1.05	0.94	1.41	1.02	0.9
	Experiment 2	4.85		1.28	1.12	1.54	1.21	1.05
	Experiment 3	3.58	3.55	1.62	0.69	1.3	0.46	0.64
	Experiment 3	3.78	3.95	1.64	0.52	1.12	0.56	0.56
Ara-C	Experiment 1	10.94	5.36	2.48		2.72	2.52	1.74
	Experiment 2	7.1	4.4	1.55		0.47	0.93	0.51
	Experiment 2	10.15	5.2	1.65		0.58	0.86	0.67

	Experiment 3	5.25	2.75	1.35		1.0	0.7	1.05
	Experiment 3	5.8	3.0	1.1		0.80	0.60	1.10
2 Gy	Experiment 1	11.42	4.54	3.58		1.72	2.28	2.66
	Experiment 2	12.30	4.85	1.85		0.94	1.13	0.61
	Experiment 2	9.50	4.75	1.60		0.72	1.23	0.49
	Experiment 3	8.0	3.8	1.25		0.69	0.57	0.65
	Experiment 3	7.65	3.25	1.45		0.55	0.53	0.63

RECOVERY EXPERIMENTS

Femur cellularities & Spleen weights in untreated mice

Mouse	Femur Cellularities (x 10^7)	Spleen Weights (gms)
1	1.1	138.7
2	1.35	128.9
3	1.56	166.1
4	1.35	144.3

GM-CFC & HPP-CFC-1 numbers in untreated mice

Experiment	GM-CFC Nos (x10^4)	HPP-CFC-1 Nos (10^3)
1	2.7	12.1
2	2.4	16.1
3	2.8	13.7
4	2.7	20.7

The Proportion (%) of GM-CFC & HPP-CFC-1 in S-phase in untreated mice

Experiment	GM-CFC	HPP-CFC-1
1	26.4 ± 3.7	5.9 ± 4.1
2	32.7 ± 1.9	9.1 ± 5.2
3	32.9 ± 4.2	14.3 ± 2.2
4	21.7 ± 3.3	4.1 ± 3.7

Femur cellularities (10^6) in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	9.6	14.5	18.9	7.6	13.2	23.1
2	10.8	20.9	24.5	9.2	24.5	22.2
3	9.0	18.6	18.0	11.4	14.4	18.6
4	8.1	20.0	20.3	4.6	17.4	17.9

Spleen weights (gms) in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	100	143.5	119.5	92.8	124.7	145.3
2	108.3	103.4	116.9	85.2	117.9	116.6
3	89.6	127.5	143.3	159.6	99.0	106.7
4	94	123.5	115.2	93.1	152.4	146.8

GM-CFC numbers ($x10^4$) in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	1.8	1.6	1.8	2.0	1.4	2.6
2	1.7	2.4	3.0	1.6	3.1	2.6
3	2.1	2.6	1.8	2.0	1.5	1.7
4	1.3	2.8	2.3	1.1	2.2	2.3

HPP-CFC-1 numbers ($x10^3$) in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	21.9	19.4	21.5	16.5	15.4	25.8
2	15.6	28.5	27.2	13.5	37.5	21.0
3	16.0	26.4	17.9	16.5	21.1	17.8
4	14.5	27.3	23.4	7.7	19.4	20.7

The Proportion (%) of GM-CFC in S-phase in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	27.1 ± 3.9	23.6 ± 3.7	33.1 ± 3.5	12.6 ± 2.7	12.8 ± 3.3	25.7 ± 2.9
2	31.6 ± 2.3	35.0 ± 2.9	33.0 ± 2.9	14.6 ± 1.8	2.2 ± 3.2	32.0 ± 1.6
3	34.1 ± 3.3	33.9 ± 3.6	26.4 ± 2.6	5.0 ± 4.4	1.5 ± 4.4	27.1 ± 3.2
4	22.8 ± 2.0	31.6 ± 2.4	28.9 ± 3.8	10.0 ± 4.6	4.2 ± 2.9	33.9 ± 3.1

The Proportion (%) of HPP-CFC-1 in S-phase in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	17.0 ± 4.9	26.1 ± 4.0	32.0 ±2.7	5.0 ± 3.6	6.6 ± 2.9	5.3 ± 3.6
2	24.1 ± 2.7	27.8 ± 2.4	17.6 ± 2.2	4.0 ± 6.4	5.0 ± 4.0	3.7 ± 4.0
3	16.1 ± 4.5	25.1 ± 5.1	28.5 ± 5.3	11.7 ± 3.2	0.34 ± 7.3	5.2 ± 6.2
4	20.6 ± 6.6	22.4 ± 2.6	26.1 ± 5.6	12.2 ± 2.7	-2.3 ± 6.9	14.2 ± 3.8

Femur cellularities (10^6) in mice given 2 doses of Ara-C (400 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	4.0	14.5	19.3	4.0	14.8	17.7
2	3.7	18.2	18.3	5.2	18.4	19.2
3	4.4	19.8	19.2	4.5	16.4	16.8
4	5.1	17.3	19.1	5.3	15.7	19.9

Spleen weights (gms) in mice given 2 doses of Ara-C (400 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	75.2	122.2	110.7	66.4	89.2	122.5
2	73.7	101.4	138.1	83.6	141.9	154.3
3	91.6	176.6	169.5	105.7	175.6	151.1
4	93.5	174.6	187.7	107.6	150.6	168.6

GM-CFC numbers ($x10^3$) in mice given 2 doses of Ara-C (400 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	6.8	15.1	24.0	8.1	32.1	25.8
2	7.6	19.0	15.8	13.8	19.3	20.8
3	6.5	24.0	24.3	7.5	21.3	17.4
4	7.1	20.6	21.5	8.8	15.6	19.1

HPP-CFC-1 numbers ($x10^3$) in mice given 2 doses of Ara-C (200 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	5.4	16.1	24.0	4.5	24.2	21.2
2	6.8	20.3	13.8	11.5	17.4	27.2
3	6.3	16.8	19.3	6.7	10.4	10.9
4	6.1	17.3	17.8	6.2	10.6	12.0

The Proportion (%) of GM-CFC in S-phase in mice given 2 doses of Ara-C (400 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	56.7 ± 1.9	40.5 ± 5.0	48.2 ± 2.5	16.8 ± 3.1	21.6 ± 4.1	43.0 ± 2.7
2	53.6 ± 2.5	38.1 ± 2.9	50.8 ± 2.0	15.0 ± 3.0	15.6 ± 2.8	29.0 ± 3.5
3	50.0 ± 2.2	30.4 ± 2.4	36.5 ± 2.3	15.5 ± 2.3	0.8 ± 2.9	25.0 ± 3.0
4	47.2 ± 2.1	25.7 ± 3.4	35.9 ± 2.1	14.1 ± 2.1	4.8 ± 4.8	27.9 ± 4.0

The Proportion (%) of HPP-CFC-1 in S-phase in mice given 2 doses of Ara-C (400 mg/kg) fractionated by captopril (100 mg/kg)

	Saline			Captopril		
Experiment No.	Day 3	Day 7	Day 10	Day 3	Day 7	Day 10
1	57.2 ± 3.6	30.0 ± 3.7	33.3 ± 3.6	25.0 ± 3.3	1.5 ± 3.6	7.1 ± 5.0
2	52.7 ± 2.4	37.2 ± 5.4	38.4 ± 2.7	15.7 ± 2.9	6.4 ± 5.8	1.4 ± 3.3
3	42.3 ± 3.4	25.9 ± 5.3	37.8 ± 2.7	6.3 ± 6.4	2.4 ± 3.4	1.5 ± 4.9
4	50.2 ± 2.1	26.0 ± 4.0	26.1 ± 3.8	10.8 ± 6.4	3.7 ± 4.2	2.4 ± 7.2

Femur Cellularities in LTBMC (x10^5 Cells/ml)

Week / Experiment No.	Saline	Captopril (10^{-6}M)	AcSDψKP (10-9M)	AcSDKP (10^{-9}M) + Captopril (10^{-6}M)
Week 2				
1	3.2	3.5	2.9	2.1
2	7.6	10.0	8.8	10.0
3	6.8	9.6	13.1	11.4
4	3.0	6.4	4.8	5.4
Week 3				
1	5.2	7.4	7.3	6.6
2	15.8	12.0	8.9	8.0
3	10.8	8.2	6.1	8.3
4	7.7	9.1	12.8	9.8
Week 5				
1	15.9	16.3	17.0	12.8
2	12.1	10.0	10.3	8.4
3	11.9	8.7	8.3	12.6
4	9.5	5.8	16.3	10.6
Week 6 (Adherent layer) (x10^6 Cells/ ml)				
1	22.8	35.7	22.5	22.2
2	31.6	27.0	38.3	22.5
3	21.7	36.1	31.5	27.7
4	18.8	43.7	39.9	27.2

Absolute GM-CFC numbers in LTBMC

Week / Experiment No.	Saline	Captopril (10^{-6}M)	AcSDψKP (10-9M)	AcSDKP (10^{-9}M) + Captopril (10^{-6}M)
Week 2				
1	422.9	521.0	305.3	180.3
2	690.6	701.2	614.2	324.5
3	1600	2057.8	1713.5	1960.0
4	1334.0	1908.4	2138.7	2080.5
Week 3				
1	327.6	356.5	678.9	433.6
2	202.1	262.7	423.4	277.5
3	951.8	540.0	708.8	448.2
4	1757.6	1315.0	1111.7	1263.6
Week 5				
1	1450.8	1540.3	2588.1	1235.2
2	1085.0	1524.0	2639.8	1344.0
3	1255.2	730.0	996.4	1297.8
4	1169.1	793.3	571.9	718.5
Week 6 (Adherent layer)				
1	2587.8	4506.7	2756.3	2475.2
2	2485.2	3168.0	2829.2	2278.9
3	3333.8	2835.0	4298.8	2390.4
4	3146.5	6754.4	3071.3	2472.0

Absolute HPP-CFC numbers in LTBMC ($x10^5$ Cells/ml)

Week / Experiment No.	Saline	Captopril (10^{-6}M)	AcSDψKP (10-9M)	AcSDKP (10^{-9}M) + Captopril (10^{-6}M)
Week 2				
1	410.2	564.1	305.6	247.2
2	322.0	473.5	207.4	210.7
3	1088.6	1535.2	1546.2	1291.4
4	729.3	1054.9	2145.0	1918.2
Week 3				
1	616.2	428.9	513.9	336.6
2	793.8	213.7	224.1	520.7
3	467.4	235.2	330.2	339.4
4	901.3	1172.3	1146.1	1139.4
Week 5				
1	1240.2	884.1	977.5	841.5
2	772.2	1304.0	1113.5	614.4
3	768.4	777.4	782.8	872.4

4	645.9	582.9	746.1	571.0
Week 6 (Adherent layer)				
1	1540.7	2284.2	2240.5	1136.3
2	2670.2	2301.5	2031.8	1385.0
3	1491.7	2420.0	3188.0	1884.0
4	3146.4	4034.0	1456.7	1732.8

The Proportion (%) of GM-CFC in S-phase in LTBMC

Week / Experiment No.	Saline	Captopril (10^{-6}M)	AcSDψKP (10-9M)	AcSDKP (10^{-9}M) + Captopril (10^{-6}M)
Week 2				
1	73.0 ± 1.4	33.8 ± 4.6	40.6 ± 4.4	53.7 ± 4.6
2	50.8 ± 1.9	25.6 ± 2.6	26.6 ± 2.8	23.9 ± 4.2
3	34.9 ± 4.7	11.6 ± 3.7	8.0 ± 2.6	19.5 ± 2.1
4	70.9 ± 2.3	25.5 ± 3.9	28.7 ± 4.6	23.6 ± 5.4
Week 3				
1	63.1 ± 2.9	30.8 ± 2.9	32.7 ± 3.7	8.6 ± 3.1
2	51.9 ± 2.7	-1.7 ± 5.6	23.1 ± 3.8	20.9 ± 3.7
3	41.5 ± 3.2	-0.8 ± 13.7	11.0 ± 9.3	10.2 ± 7.8
4	57.8 ± 2.7	41.2 ± 4.5	25.0 ± 4.7	-2.3 ± 6.8
Week 5				
1	30.8 ± 5.8	28.6 ± 3.9	24.0 ± 4.5	28.3 ± 4.4
2	55.8 ± 3.5	30.1 ± 3.2	16.7 ± 6.1	27.9 ± 5.0
3	50.0 ± 2.6	7.8 ± 7.1	27.9 ± 4.6	21.2 ± 2.6
4	54.0 ± 4.0	45.6 ± 4.7	38.9 ± 2.7	32.4 ± 7.2
Week 6 (Adherent layer)				
1	37.8 ± 1.9	31.3 ± 3.1	9.1 ± 3.7	0.0 ± 6.4
2	42.4 ± 2.5	2.7 ± 3.4	0.3 ± 3.2	22.4 ± 3.3
3	41.9 ± 2.3	9.3 ± 3.7	8.9 ± 3.3	12.0 ± 3.9
4	23.3 ± 4.2	54.9 ± 3.9	25.4 ± 5.3	24.0 ± 5.1

The Proportion (%) of HPP-CFC in S-phase in LTBMC

Week / Experiment No.	Saline	Captopril (10^{-6}M)	AcSDψKP (10-9M)	AcSDKP (10^{-9}M) + Captopril (10^{-6}M)
Week 2				
1	55.6 ± 2.6	43.2 ± 2.7	16.9 ± 4.2	9.1 ± 6.0
2	27.5 ± 3.3	17.6 ± 2.7	6.4 ± 3.7	5.5 ± 3.1
3	32.0 ± 6.6	12.1 ± 4.3	0.5 ± 4.0	0.0 ± 4.6
4	48.1 ± 5.8	23.6 ± 3.6	40.3 ± 4.5	30.3 ± 4.1
Week 3				
1	24.4 ± 2.8	17.6 ± 2.5	9.0 ± 4.6	0.0 ± 3.7
2	25.0 ± 4.1	2.7 ± 8.3	7.2 ± 7.7	36.0 ± 4.7
3	31.9 ± 3.7	0.8 ± 12.1	9.4 ± 6.3	9.5 ± 5.4
4	15.0 ± 4.7	5.2 ± 5.1	0.3 ± 4.7	17.1 ± 3.0
Week 5				
1	19.0 ± 8.0	12.6 ± 6.3	17.0 ± 4.7	11.7 ± 4.5
2	48.2 ± 2.6	23.9 ± 5.1	5.1 ± 5.8	8.8 ± 7.1
3	61.9 ± 1.2	10.2 ± 2.3	10.9 ± 4.7	5.7 ± 4.9
4	59.1 ± 4.1	54.0 ± 9.8	38.5 ± 8.3	10.4 ± 5.6
Week 6 (Adherent layer)				
1	34.8 ± 3.9	9.5 ± 4.7	19.8 ± 4.1	14.2 ± 4.9
2	28.4 ± 4.5	27.2 ± 3.5	29.3 ± 4.2	31.4 ± 6.2
3	25.7 ± 5.1	29.6 ± 2.5	19.0 ± 4.0	20.0 ± 6.1
4	37.0 ± 4.6	27.0 ± 6.6	38.4 ± 8.2	22.9 ± 5.6

Inhibitory Action of the Peptide AcSDKP on the Proliferative State of Hematopoietic Stem Cells in the Presence of Captopril but not Lisinopril

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Key Words. *AcSDKP · Irradiation · Angiotensin I-converting enzyme (ACE) · Captopril · Lisinopril · Hematopoietic stem cells*

Abstract

The effect of Angiotensin I-converting enzyme (ACE) inhibitors on their own and in combination with the peptide AcSDKP on the proliferation of hematopoietic stem cells has been investigated. Hematopoietic stem cells from murine bone marrow induced into cell cycle following exposure to 2 Gy γ-irradiation were incubated in vitro for up to 24 h in the presence of medium, captopril/lisinopril, AcSDKP, and AcSDKP with either ACE inhibitor. Hematopoietic stem cells were monitored using the high proliferative potential-colony forming cell-1 (HPP-CFC-1) population cloned in the presence of human IL-1β, murine IL-3, and murine M-CSF. No significant inhibitory effect was observed in the presence of AcSDKP on its own and AcSDKP in combination with lisinopril. However, there was a significant inhibition of stem cell cycling when AcSDKP and captopril were combined. This suggests that captopril inhibits AcSDKP breakdown better than lisinopril. The combination of AcSDKP and captopril also had an inhibitory effect on cell recruitment into S phase. The fact that a combination of AcSDKP and captopril switches cycling hematopoietic stem cells out of cycle indicates the importance of the N-active catalytic site of ACE in AcSDKP hydrolysis in vitro. Thus, AcSDKP in combination with appropriate ACE inhibitors may be of use in regulating the proliferation of hematopoietic stem cells in vitro. *Stem Cells 1997;15:455-460*

Introduction

Hematopoietic stem cell proliferation is controlled by a series of positive and negative regulatory factors. Stimulatory factors are produced following insult to the hematopoietic system [1]. This damage induces stem cells to enter into cell cycle. If the insult persists when stem cells are cycling, there is a possibility of stem cell depletion and therefore bone marrow failure [2]. To prevent this depletion, it is suggested that a balance between inhibitory and stimulatory factors be maintained during cytotoxic insults to the hematopoietic system by increasing the concentrations of inhibitors [3]. A number of inhibitory factors have been identified, but some lack specificity while others can be toxic. Some of these inhibitors also suppress tumor cells from cycling [4, 5].

A tetrapeptide, Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP), was first identified in extracts from fetal calf bone marrow [6]. It has been shown to exhibit no effect on the cycling status of tumor cells at a concentration which inhibits normal hematopoietic progenitor cell cycling [7]. It is synthesized in mice under steady-state conditions and is secreted by the bone marrow in long-term culture [8]. AcSDKP opposes the action of stimulators which recruit cells from G_0 or early G_1 to enter into S phase [9, 10]. Since AcSDKP enhances the adherence of hematopoietic cells to stromal cells [11], it has been suggested that the peptide may influence inhibitory factor production by the stroma. AcSDKP protects normal hematopoietic cells from cytotoxic effects of drugs [12], hyperthermia [13], and phototherapy [14]. This is of clinical significance because these agents are used to purge neoplastic cells from autologous bone marrow transplants. AcSDKP with granulocyte colony-stimulating factor rescues stem cells from the cytotoxic effects of irradiation in vivo [15]. Cells responsible for AcSDKP production in vivo have not yet been identified. However, it has been recently reported that macrophages could be responsible for AcSDKP synthesis [16]. Certain endogenous macromolecules contain

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the SDKP sequence [17]. There have been suggestions that thymosin β4, one of these macromolecules, may be the source of AcSDKP. It has also been shown that thymosin β4 inhibits hematopoietic stem cell proliferation [18].

AcSDKP is degraded by Angiotensin I-converting enzyme (ACE), a ubiquitous dipeptidyl carboxypeptidase. This enzyme is involved in the first rate-limiting step of AcSDKP degradation in human plasma [19]. Human ACE has NH_2 and CO_2H active catalytic domains which act independently with ACE inhibitors [20]. ACE hydrolyses AcSDKP by the N-active site [21]. Inhibition of ACE activity by captopril in vivo in humans has been shown to increase the plasma concentration of AcSDKP [22].

We have studied the effects of two ACE inhibitors: captopril, which is more potent at the N-active site, and lisinopril, which is more active at the C-active site in combination with AcSDKP on the kinetics of hematopoietic stem cell. The stem cells were studied using the in vitro high proliferative potential colony-forming cells-1 (HPP-CFC-1) assay [23].

Materials and Methods

Mice

All experiments were carried out using female 8- to 12-week-old CD1 mice. Animals were bred and housed at the University of St. Andrews, Scotland, UK. Animals in groups of threes were given 2 Gy whole body γ-irradiation (CIS BioInternational IBL 437C ^{137}Cs γ-irradiation source. Dose rate 4.66 Gy/minute).

AcSDKP and Ace Inhibitors

AcSDKP (M_r = 487) (IPSEN-Biotech; Paris, France) was supplied as a lyophilized sample and stored at –20°C. The peptide was dissolved in sterile distilled water, and aliquoted in polypropylene cryotubes (Nunc; Denmark), and stored at –20°C. For use, each sample was diluted in Dulbecco's medium (GIBCO BRL; Paisley, Scotland, UK) supplemented with 2% fetal calf serum (FCS) (Globepharm; Esher, Surrey, UK). A final concentration of 10^{-9} M was added to a pair of petri dishes or universal tubes containing irradiated bone marrow cell suspensions with 1 mM captopril (Sigma; UK) or 1mM lisinopril (Sigma) [24].

Stem Cell Assay: HPP-CFC-1

A feeder layer containing two ml of Dulbecco's medium, 20% horse serum (Globepharm) penicillin 50 UI/ml (Sigma)/streptomycin 50 μg/ml (Sigma) and 2 mM L-glutamine (D20% HS PS/G) with 0.5% (v/v) melted agar (Bacto Agar; Difco Labs; Detroit, MI) and recombinant murine interleukin-3 (IL-3) at 100 ng/ml (R&D; Abingdon, Oxon, UK), recombinant human interleukin-1β (IL-1β) at 100 U/ml and recombinant murine macrophage colony-stimulating factor (M-CSF) at 200 ng/ml (R&D) were plated in a 60 mm non-tissue-culture petri dish (Nunc).

Cells were sampled 24 h post-irradiation. Cytosine arabinoside (Ara-C) suicide assay was carried out before plating to analyze the percentage of cells in S-phase [25]. Cells were washed twice after incubation, suspended in D20% HS PS/G with 0.3% melted agar (Bacto Agar; Difco Labs) and plated on the feeder layer at a final dose of 5×10^4 cells per petri dish in four separate petri dishes. Petri dishes were incubated for 14 days at 37°C in 10% CO_2 in a fully humidified atmosphere. A day before assaying, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (BDH Chemicals, Ltd.; Poole, UK) was added to all culture dishes [26] and colonies were scored macroscopically. For comparison, normal bone marrow cells were incubated and treated in the same way as irradiated cells. Statistical comparison of the proportion of HPP-CFC-1 population in S phase among the cell populations was determined by using the unpaired Student's *t*-test.

Effect of Ace Inhibitors and AcSDKP on Stem Cell Cycling

The proportion of HPP-CFC-1 in S phase was measured in the presence or absence of ACE inhibitors. It was also measured in the presence of ACE inhibitors with or without AcSDKP and in the presence of AcSDKP on its own. All these protocols were measured using a one-h incubation with Ara-C. The bone marrow cell suspension (2 ml) was transferred to two pairs of 20 ml universal tubes (Sterilin, Stone; Staffordshire, UK). Captopril or lisinopril was added to one pair. 25 μg/ml Ara-C (Sigma) in Dulbecco's medium, 30% FCS, penicillin 50 IU/ml/streptomycin 50 μg/ml and 2 mM L-glutamine (D30% FCS PS/G) was added to one tube of each pair and plain medium (D30% FCS PS/G) to the other. All tubes were incubated for one h at 37°C. To test the effect of AcSDKP, ACE inhibitors plus AcSDKP (10^{-9} M) or AcSDKP (10^{-9} M) only were incubated in the same way as above. At the end of the incubation period, cells were washed twice with D20% HS PS/G. Cellularity was adjusted to 2.5×10^4 cells/ml and plated for HPP-CFC-1 assay. The proportion of HPP-CFC-1 in S phase was assessed 24 h after in vitro incubation to test the response of cells to ACE inhibitors and AcSDKP. Bone marrow cell suspension (2 ml) was transferred to each of four 30 mm triple-vent non-tissue-culture grade petri dishes (Sterilin). To one pair, captopril or lisinopril was added. The other pair of dishes had no ACE inhibitors added. For the AcSDKP assay, ACE inhibitors plus AcSDKP (10^{-9} M) or AcSDKP (10^{-9} M) alone were added to petri dishes. Dishes were incubated at 37°C in a 10% CO_2 fully humidified atmosphere. After 23 h of incubation, an Ara-C suicide assay was performed. Cells were transferred to labeled 20 ml universal tubes, washed, the

cellularity adjusted, and plated for HPP-CFC-1 assay. Eight independent experiments were performed in the assay where captopril was incubated in the presence of AcSDKP. Four independent experiments were performed in the rest.

The Effect of AcSDKP and Captopril on the Cell Cycle

Irradiated bone marrow cells were flushed from femurs and tibias and incubated in the presence of D10% HS PS/G and 25 µg/ml Ara-C for one h. Cells were immediately washed twice with D20% HS PS/G. They were suspended in D10% HS PS/G then transferred to two pairs of universal tubes each containing 2 ml cell suspension. They were incubated with or without 1 mM captopril and AcSDKP (10^{-9} M) per pair for three h, after which another S phase suicide assay was performed. At the end of the incubation period, cells were washed twice and the cellularity adjusted for HPP-CFC-1 assay.

Results

In Vitro Incubation with Captopril Has No Effect on Normal Hematopoietic Stem Cell Cycling

As shown in Figure 1, in the absence of captopril, 8.13% ± 2.57% of HPP-CFC-1 were in S phase at time 0. The proportion in S phase did not significantly change after 24 h of incubation and was found to be 12.28% ± 4.72% ($p > 0.05$). Incubation of normal bone marrow cells with captopril in vitro did not significantly change the proportion of stem cells in S phase. While 13.58% ± 2.58% were in S phase at time 0, the proportion in S phase after 24 h of incubation in the presence of captopril was 14.67% ± 3.54% ($p > 0.05$).

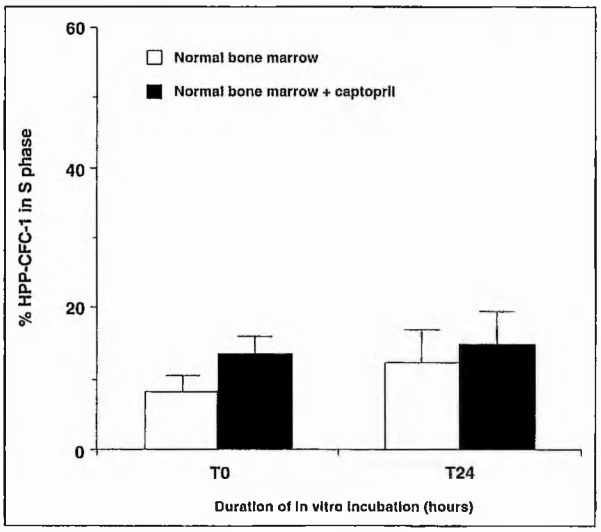

Figure 1. Normal bone marrow cells were incubated for 24 h in vitro with and without captopril (1 mM). *The percentage of HPP-CFC-1 in S phase was not significantly reduced after 24 h incubation in the absence of captopril (T = 0 and T = 24; p > 0.05). Captopril had no effect on the proportion of HPP-CFC-1 in S phase after 24 h incubation (T = 0 and T =24; p > 0.05) (n = 4, error bars = ± SE).*

Effects of ACE Inhibitors and AcSDKP on the Proliferation of Hematopoietic Stem Cells

As shown in Figures 2 and 3, when irradiated bone marrow cells were incubated for up to 24 h in the absence of ACE inhibitors, they remained in cell cycle. With lisinopril, the proportion in S phase was 38.61% ± 1.68% at time 0 and 43.08% ± 3.39% ($p > 0.05$) after 24 h of incubation (Fig. 2A). There was no significant reduction in the proportion of stem cells in S phase after the addition of AcSDKP (10^{-9} M) in the presence of lisinopril. The initial proportion in S phase was 33.19% ± 0.83%, and it was 25.69% ± 4.44% after 24 h of incubation ($p > 0.05$) (Fig. 2B). In the presence of captopril, 45.18% ± 6.17% of cells were in S phase at time 0, and the proportion did not significantly change after 24 h of incubation at 47.74% ± 5.41% ($p > 0.05$) (Fig. 3A). In the presence of AcSDKP, 40.19% ± 2.56% of HPP-CFC-1 were in S phase and 31.82% ± 2.95% ($p > 0.05$) were still cycling after 24 h of in vitro incubation (Fig. 3B). However, after the addition of AcSDKP (10^{-9} M) in the presence of captopril, there was a significantly reduced proportion of HPP-CFC-1 in S phase at the end of the 24-h incubation period. While at time 0, 42.12% ± 3.70% of cells were in S phase, only 10.27% ± 1.86% ($p < 0.001$) (Fig. 3B) were in S phase after 24 h.

AcSDKP Directly Blocks Cell Entry Into S Phase

Treating cells for one h with Ara-C kills cell in S phase [25]. Therefore, addition of captopril and AcSDKP soon after Ara-C treatment gives an insight to the mode of action of the peptide. After an initial one-h incubation with Ara-C, there are no cells in S phase at the beginning of the subsequent incubation period. Consequently, the percentage of cells in S phase following this second incubation in the presence or absence of captopril and AcSDKP is the result of cell entry during the second incubation period. Cells were incubated for four h after the initial S phase suicide with Ara-C to allow a large proportion to enter S phase. As shown in Figure 4, the proportion of cells in S phase in the absence of captopril and AcSDKP was 28.44% ± 2.50%. However, there was a significant reduction in cell entry into S phase in the presence of captopril and AcSDKP; only 5.44% ± 1.38% ($p < 0.001$) had entered S phase after four h of incubation.

Discussion

In vitro incubation with or without ACE inhibitors had no effect on the cycling status of HPP-CFC-1 from both normal

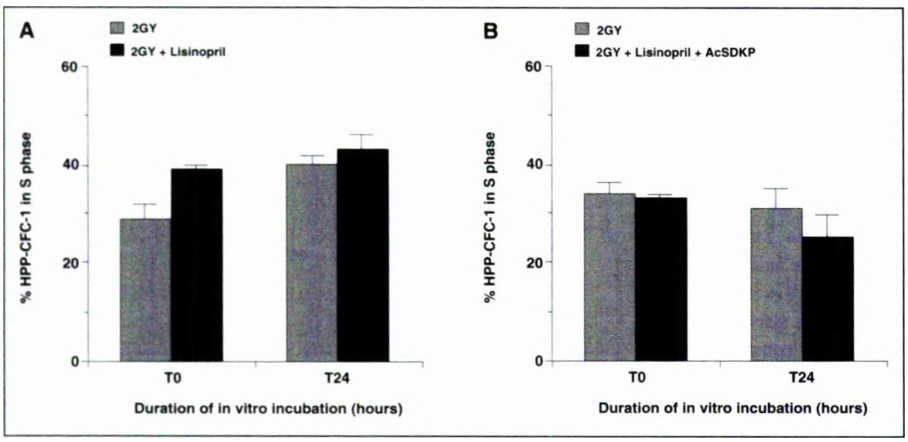

Figure 2. Bone marrow cells removed one day after 2 Gy irradiation were incubated in the presence and absence of lisinopril. *The proportion of HPP-CFC-1 in S phase in irradiated bone marrow cells remained high after in vitro incubation in the presence or absence of lisinopril, where* n *is the number of individual experiments. A)* ± *lisinopril* (n = *4*) , *(error bars =* ± *SE). Following addition of AcSDKP the proportion of HPP-CFC-1 in S phase in the presence of lisinopril was not significantly reduced* (p > *0.05). B)* ±*AcSDKP + lisinopril* (n = *4), (error bars =* ±*SE).*

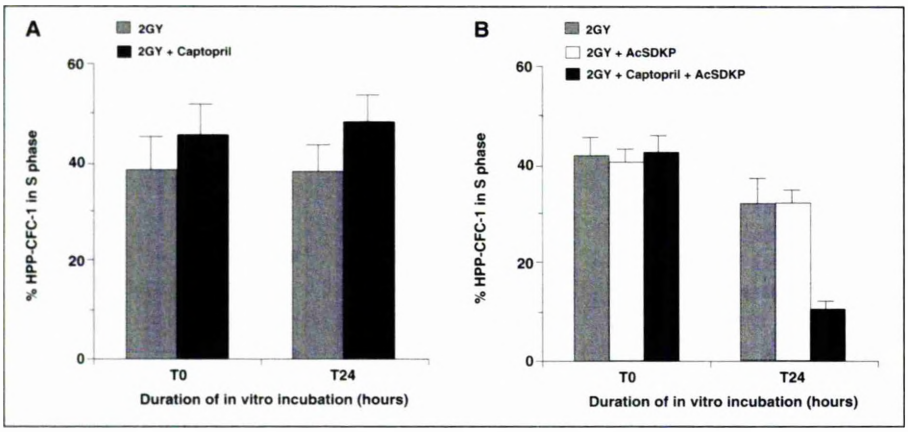

Figure 3. Bone marrow cells removed one day after 2 Gy irradiation were incubated in the presence and absence of captopril. *The proportion of HPP-CFC-1 in S phase in irradiated bone marrow cells remained high after in vitro incubation in the presence or absence of captopril, where* n *is the number of individual experiments. A)* ± *captopril* (n = *4*) , *(error bars =* ± *SE). Following addition of AcSDKP the proportion of HPP-CFC-1 in S phase in the presence of captopril was significantly reduced* (p > *0.001). B)* ± *AcSDKP + captopril* (n = *8) AcSDKP had no effect on stem cell cycling both with short and long term incubation.* (n = *4), (error bars =* ± *SE).*

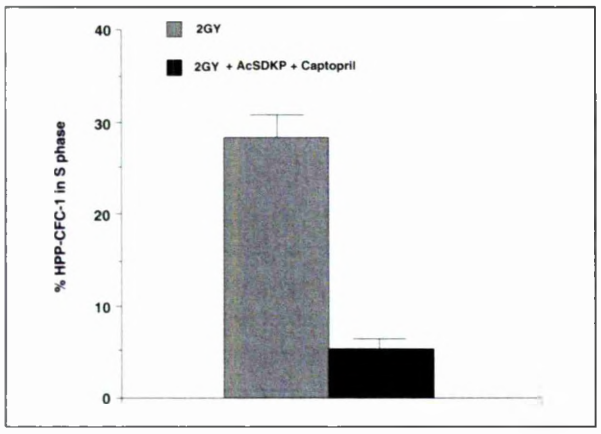

Figure 4. Bone marrow cells removed one day after 2 Gy irradiation were incubated with Ara-C for one h, washed then incubated for a further four h with AcSDKP plus captopril or with medium. *The proportion of HPP-CFC-1 that had entered S phase in the absence of AcSDKP plus captopril was high. However, a combination of AcSDKP and captopril blocked cell entry into S phase and the proportion in S phase was low* (p < *0.001)* (n = *4, error bars =* ± *SE).*

or irradiated bone marrow cell suspensions. Incubating cells in the presence of AcSDKP alone did not reduce the proportion of cells in S phase, either after a short or a long incubation period. It has been shown previously that AcSDKP on its own has no inhibitory effect on the proportion of HPP-CFC in S phase in regenerating bone marrow following short incubation periods in serum-free supplemented Dulbecco's medium [9]. AcSDKP has a half life of approximately 3.5 h in FCS [24]. This presumably explains the lack of a noticeable inhibitory effect with the peptide alone. These results indicate that stem cells once induced into cycle remain in cycle until a high enough concentration of inhibitors can switch them out of cycle. The fact that stem cells incubated in the presence of AcSDKP and captopril are switched out of cycle while more remain in cycle when incubated in the presence of AcSDKP and lisinopril suggests the importance of AcSDKP in the regulation of cell cycle if its degradation is prevented. The inhibitory effect of captopril and AcSDKP on regenerating bone marrow also suggests that the peptide may have a direct

inhibitory effect on cycling hematopoietic stem cells. However, an indirect effect cannot be excluded as whole bone marrow preparation was used. It is possible that this action is mediated by opposing the effect of stimulators of stem cells as previously shown by *Robinson et al.* [9]. AcSDKP and captopril blocks the cycling of CFU-S-12 in vitro from regenerating bone marrow [13], and now we have shown that cycling HPP-CFC-1 are also inhibited.

The difference in the ability of the two ACE inhibitors to prevent AcSDKP degradation can be attributed to their varying influence on the two catalytic domains of ACE. Captopril binds preferentially to the NH_2-terminal domain of ACE in vitro [20], and it also has the lowest IC_{50} (10^{-9} M) for inhibition of AcSDKP hydrolysis by ACE. At 10^{-8} M captopril inhibits 88% of ACE activity as measured by the hydrolysis of AcSDKP, while lisinopril inhibits only 80%. Enalaprilat, another ACE inhibitor, inhibits 85% of ACE activity on AcSDKP hydrolysis at the same concentration [19]. Thus, it is initially surprising that a differential effect of the two ACE inhibitors was observed in vitro. However, some in vitro work has shown lisinopril to have greater inhibitory potency than captopril on the activity of N-active site when AcSDKP is a substrate [27], but this is highly dependent on Cl^- concentration and pH [20]. Thus, lisinopril will not be active in culture medium at the N-active site and AcSDKP action will be compromised.

Captopril is the ACE inhibitor which is most frequently associated with development of neutropenia [28, 29]. Moreover, people at risk of neutropenia are those with renal, collagen, vascular, or autoimmune diseases suggesting high levels of ACE. Since captopril inhibits the N-active catalytic site strongly, it is possible that there is an increase in AcSDKP levels during captopril therapy. *Azizi et al.* [22] have demonstrated an increase in AcSDKP concentration following in vitro administration of captopril. This could lead to the switching of cycling stem cells out of cycle and/or blocking of stem cell recruitment into cycle and thus contributing to the neutropenia. Captopril, but not lisinopril in vitro, suppresses IL-1β–induced synthesis of tumor necrosis factor, and IL-1β-induced synthesis of IL-1α by human peripheral blood mononuclear cells [30]. Since IL-1α is involved in the recruitment of primitive stem cells [31], it implies that captopril on its own may influence cytokine-mediated stem cell proliferation. Captopril and the ability of AcSDKP to inhibit stem cell proliferation in vitro, if realized, could have tremendous implications for purging neoplastic cells from autologous bone marrow transplants.

Administration of the partially purified fractions of fetal calf bone marrow probably containing AcSDKP has been shown to protect mice from the cytotoxic effects of Ara-C [32]. AcSDKP given at an appropriate time after Ara-C administration in vivo prevents colony-forming units-spleen entry into S phase [10]. Therefore, it has rightly been suggested that AcSDKP may be acting at G_0/G_1 S boundary from the fact that the peptide protects murine hematopoietic cells from the cytotoxic effects of Ara-C. It has now been shown for the first time that AcSDKP in vitro blocks cycling HPP-CFC-1 entry into S phase. In addition, this investigation shows that AcSDKP not only blocks recruitment of stem cells into S phase but also switches them out of cycle. Thus, AcSDKP is active at the G_1/S boundary. The possibility that AcSDKP may be active in other phases of the cell cycle cannot be excluded in these studies, which are only investigating the proportion of cells in S phase. It has been suggested that inhibition cell entry into S phase may be mediated by counteracting one or a few key steps of the mitogen signal transduction pathways [33]. AcSDKP has been shown to inhibit the G_0/G_1 to S phase transition using continuous cell lines.

This study has shown that ACE inhibitors (captopril), presumably by prevention of AcSDKP degradation, act in concert with the peptide to switch cycling hematopoietic stem cells out of cycle. This could be one of the reasons for bone marrow suppression observed after captopril therapy. As captopril is active on the N-domain of ACE, and ACE activity on angiotensin-I is mainly restricted to the C-domain, it should be possible to minimize hematotoxicity by using C-domain-specific ACE inhibitors for treatment of hypertension.

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