THE IDENTIFICATION AND CHARACTERISATION OF EXCRETED PROTEINS DURING EARLY CHICK DEVELOPMENT

by CAROLINE CONNOLLY

Submitted in candidature for the degree of Ph.D. in the University of St. Andrews, August, 1992.

TABLE OF CONTENTS

DECLARATION

I, Caroline Connolly 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 Date 6/8/92

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on October 1st 1988 and as a candidate for the degree of Ph.D. in September 1989.

Signed Date 6/8/92

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

6/8/92

Signature of Supervisor Date

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made

and supplied to any bona fida library or research worker.

ABSTRACT

Proteins are transferred to medium conditioned by chick embryonic tissue. These secreted proteins are a diverse group of molecules including growth factors, proteases and glycoproteins. The majority of the secreted proteins present in conditioned medium are less than 70 kDa in molecular weight. Stage 17 to 22 embryos were used.

TGF-β is present in this medium as a 25 kDa active protein and is formed from the breakdown of an inactive molecular weight form. To obtain an adequate quantity of conditioned medium to carry out protein assays a proportion of 1 head : 6 wing buds : 6 leg buds : 3 tail buds were used. A spatial distribution of this growth factor was thought to exist in the conditioned medium of head, wing buds, leg buds and tail buds and was present at a ratio of 5 : 1.4 : 1.6 :1, respectively. This suggested that TGF-β is present in regions of increased developmental activity. When the amount of secreted protein was compared to the amount of total protein in secreting tissue the ratio of TGF-β in the head, wing, leg and tail buds was 1 : 1.3 : 1.9 : 1.8, respectively. This suggested that no significant distribution of the growth factor existed in these regions and that head conditioned medium contained more TGF-β

because there was more tissue present in the head than the limbs and tail.

During the investigation, loss of activity was observed for many of the proteins of conditioned medium. This led to the discovery of a single proteolytic activity in conditioned medium. From 2 to 8 days of development this activity was present in the head, wing buds, leg buds and tail of the embryo, at a constant level. Other stages were not tested. It is active at physiological temperature and a pH of 7.5. It does not fit into any of the four known classes of proteases and is not one of the known proteases associated with development. It is therefore suggested that it is of a novel class. Since the protease is present in the media conditioned by chick tissue at a constant level from days 2 to 8 of development and TGF-β is also present but in increasing amounts, a role the protease may have on TGF-β processing was investigated. The protease does not activate latent TGF-β or inactivate active TGF-β.
During an investigation into the effect TGF-B had on the proteolytic activity of the protease, to determine if TGF-β affected the proteolytic activity of the protease, it was discovered that TGF-B displayed proteolytic activity. The proteolytic properties of other growth factors were investigated to determine if proteolytic activity is a general property of growth factors.

An effect was observed when the growth factors EGF or FGF and the protease were incubated together but since the growth factors also displayed proteolytic activity, it was impossible to say which protein affected which.

Finally conditioned medium evokes a primary anchorage independent growth response in the presence of NRK 49F cells. This activity has been demonstrated to be due to a low molecular weight (less than 30 kDa), charged glycoprotein. Purification of a single molecule which produced this activity proved not to be possible and it is suggested that this activity requires the action of more than one glycoprotein. The glycoprotein mixture which displayed this primary response also possessed proteolytic activity.
It is suggested that many of the proteins of conditioned medium possess more than one type of activity as a means of increasing efficiency.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, John, for his help and enthusiasm. I also owe so much to my parents and sister for their encouragement and emotional support during the past few years. There are many people in the department who have made my stay in St. Andrews an interesting one, in particular, Dave, Fiona, Steve, Alison and Andy from the office; the birdy lot, especially Alex for listening to my moans and groans; Chris Cutler for his help with computers and overall support for the last 4 years, and the photographic unit for their quick and helpful service. Finally, for putting up with me during these last few stressful months, I would like to dedicate this thesis to Jaboury.

CHAPTER 1 INTRODUCTION

Cellular proliferation is potently stimulated by a family of hormonally active polypeptides, the growth factors. With the discovery of retroviral oncogenes (Bishop, 1983), and their cellular equivalents, knowledge of growth factors, their receptors and their mode of action is paving the way for understanding the functional nature of the molecular lesions which give rise to cancer cells (Der et al., 1983; Basrega et al., 1981; Burgess et al., 1983). Growth factors play important roles in control mechanisms *in vivo* that involve either autocrine or paracrine regulation of cell proliferation and differentiation (Goustin et al., 1986) but can also exert other effects unrelated to cell growth. The action of the growth factor and the range of cells which respond to it may both be diverse (Roberts et al., 1985).

Growth factors form part of a complex cellular signalling language in which the informational content resides not in individual peptides, but in the pattern of regulatory peptides to which the growth factor is exposed. Since growth factors can interact with each others activity they may be important to the control of complex morphogenetic events such as those that take place during embryonic development.

High specificity probes such as monoclonal antibodies or mRNA probes can be used to identify growth factors in tissues but this method may not

identify a protein in its active form since many important processing events of growth factors are known to occur post-transcriptionally, post-translationally or outside the cell. Additionally, the use of such probes can only identify known growth factors. An alternative method has therefore been employed to identify these exported, active growth factors. Bioassays are more sensitive than biochemical assays to identify novel growth factors. Such methods open up the possibility of mapping a single embryo for its distribution of growth factors, even before they are characterised. The growth factors detected by these means are necessarily those that are being secreted by the embryo, and therefore information is gained on secreted and processed growth factors rather than intracellular forms.

These exported proteins can be transferred to medium conditioned by chick embryonic tissue. It is the nature of some of these proteins which are explored in this thesis.

Since growth factors play important roles in embryonic development it is necessary to review them, in particular TGF-β which has many diverse roles in the embryo.

FGF is also discussed in this Chapter. The review of FGF emphasises the importance of growth factors on the activity of other growth factors.

Oncogene research is also looked at since by studying the effects of oncogenes in cancer, their

role in growth factor research and hence development has become of importance.
Finally the extracellular matrix is discussed since this is formed by secreted proteins and demonstrates the importance of such molecules in the architecture of the developing embryo. The matrix also provides a framework to study growth factors, proteolytic enzymes, glycoproteins and the interaction between these three classes of proteins in developing systems.
During the study a proteolytic enzyme proved to be of interest. Proteolytic enzymes are reviewed in Chapter 4.

1.0 THE TGF-β SUPERFAMILY

TGF-β is a multifunctional agent and represents a large family of factors with diverse activities. In 1987 the concept that TGF-β is prototypic of a superfamily of growth, differentiation and morphogenic factors became clear (Massague, 1987; Sporn et al., 1987) following the discoveries of the similarities between the inhibins, activins, Mullerian inhibiting substance, decapentaplegic product and TGF-β2. All these factors proved to be structurally related to TGF-β. The family now includes embryogenic morphogens and regulators of endocrine function, as well as specialized regulators of cell proliferation and differentiation. The distribution of TGF-β related

factors is widespread in organisms from fruit flies to humans, and their evolutionary conservation is unusually strict. These factors appear to be involved in many processes of tissue development and repair.

1.01 PROTOTYPE STRUCTURE

The structural prototype for the gene superfamily was a protein first isolated from human platelets as TGF-β (Assoian et al., 1983), cloned from a cDNA library (Derynck et al., 1985), and later named TGF-β1 (Cheifetz et al., 1987). Derynck et al., 1985 described TGF-β as a 25 kDa protein dimer that could be reduced to yield two 12 kDa chains of 112 amino acids, but which was only active in the dimer form. From cDNA clones of the human gene, TGF-β has been shown to be derived from a 390 amino acid precursor protein and contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum. In addition to this it is glycosylated (Derynck et al.,1985 and Purchio et al., 1988). The precursor cleavage site is a sequence of four basic amino acids immediately preceding the bioactive domain.

The precursor structure is shared by all known members of the family except the TGF-β4 precursor which lacks the signal sequence (Jakowlew et al., 1988)

Due to the presence of 18 half cysteine residues capable of forming 9 disulphide links the dimer is

TABLE 1 The TGF-β superfamily. The four known families of TGF-β are represented with the names and compositions of their bioactive dimers.

GENE	BIOACTIVE DIMERS name	composition
TGF-B family		
TGF-*B* 1	TGF-*B* 1	homodimer
TGF-*B* 2	TGF-*B* 2	homodimer
TGF-*B* 3	TGF-*B* 3	homodimer
TGF-*B* 4	cDNA only	
TGF-*B* 5	TGF-*B* 5	homodimer
	TGF-*B* 1, 2	heterodimer
Inhibin family		
alpha	Inhibin A	*a B* A dimer
	Inhibin B	*a, B* B dimer
B A	Activin A	*B* A homodimer
B B	Activin A B	*B* A.*B* B dimer
DPP/VG1 family		
Vg 1	cDNA only	
vgr-1	cDNA only	
DPP-C	c DNA only	
BMP-2	BMP homodimer	
BMP-3	homo or heterodimers	
BMP-4	cDNA only	
BMP-5	cDNA only	
BMP-6	homo or heterodimers	
BMP-7	homo or heterodimers	
MIS family		
MIS	homodimer	

both heat and acid stable. It is found to be unstable in the presence of the reducing agents dithiothreitol (Cheifetz et al., 1987) and β-mercaptoethanol (Roberts et al., 1983) which confirm the presence of disulphide bridges.
So far, the known TGF-β related factors can be grouped into four categories depending on their structural or functional relationship (Table 1). The region of the precursor which has shown the most sequence similarity between family members is in the C-terminal domain. The degree of amino acid sequence identity in this domain ranges from 25 to 90% between different family members. The N-terminal pro-region of a given factor may have been conserved between animal species but it is usually divergent between different factors (Derynck et al.,1986 and Cate et al., 1986). This region may be involved in the folding of the bioactive domain

1.02 TGF-β FAMILY

There have been five TGFs-β identified to date: TGF-β1; TGF-β2; TGF-β3; TGF-β5, and TGF-β1.2. They all show degrees of structural conservation (Kondaiah et al., 1990). TGF-β1 sequences show greater than 97% identity between various mammalian and avian species (Derynck et al., 1987). Conservation has also been shown at the genomic level. For instance the TGF-β1 gene from various mammalian species has a seven-exon structure which

is largely conserved in other TGF-β genes (Derynck et al, 1987, Van Obberghen-Schilling et al., 1987 and Derynck et al., 1988). This type of conservation suggests that TGFs-β arose by gene duplication. The multiplicity of TGF-β forms and the sequence conservation within each form through evolution suggest specific roles for each of the TGFs-β.

This phenomenon has also been seen for the proteases trypsin, chymotrypsin, elastase and thrombin where they show a high degree of sequence homology but differ in specificity,. TGFs-β may therefore have evolved differently to carry out similar work but acting on different substrates, or acting on similar substrates but in different ways. Graycar et al., 1989 demonstrated the similarities in activity between TGF-β1, 2 and 3 and Cheifetz et al., 1990 demonstrated that the activity varied according to the environment. Characterisation and analysis of their three dimensional structures should lead to understanding the nature of these similarities and differences

1.03 EXPRESSION

TGFs-B are expressed differently in different cells and this expression does not appear to be uniform among cells of the same lineage. Heine et al. (1987) and Miller et al. (1989) carried out experiments which showed that TGF-β expression is

active from embryonic development through to adulthood. An example of the above was seen in the mouse embryo where TGF-β1 mRNA was detected in lung, intestine, kidney mesenchymes, epithelial structures, osteocytes and centres of haematopoiesis (Lehnert and Akhurst, 1988; Wilcox and Derynck 1988). TGF-β2 mRNA was detectable in gastrointestinal and tracheal submucosae, blood vessels, skin, cartilage, and bone (Pelten et al., 1989).

1.04 CONTROL OF ACTIVITY

TGFs-β expression and activity is controlled at three levels:

1 Regulation of TGF-β at the transcriptional level.

2 Production of TGF-β as a latent factor.

3 Sequestration of activated TGFs-β by extracellular matrix and circulating proteins.

1.041 Transcriptional control

At the level of transcription, the TGF-β1 gene is stimulated by phorbol esters, probably via a protein kinase C-dependent pathway, and by TGF-β itself (Akhurst et al., 1988; Van Oberghen-Schilling et al., 1988). The TGF-β1 gene contains two transcriptional sites at the 5^1 end, and two

promoter sites. One of the promoters is located upstream of the first transcriptional start site and the other between the two start sites. Several transcriptional inhibitory regions are also found (Kim et al., 1989,1990).

The promoters contain transcriptional enhancer elements that responded to induction by phorbol esters and TGF-β1 or transactivation by AP-1. Activation via these elements is mediated by binding of the (Jun-Fos) Ap-1 complex.

Scotto et al., 1990 demonstrated the presence of other elements in the 3^{1}·flanking region which are responsive to phorbol esters. Expression of jun and fos are modulated by numerous factors including its own products and TGF-β1. These mechanisms, therefore, have the capacity to finely tune TGF-β1 expression in response to diverse stimuli.

1.042 Latent Factor Production.

The presence of latent forms of TGF-β were first reported in embryonic, human, mouse and chicken fibroblasts. They all released a biologically inactive or poorly active form of TGF-β when assayed for ability to induce anchorage independent growth (AIG) in NRK 49F cells (Pircher et al.,1984; Lawrence et al.,1984; Lawrence et al., 1985 Feige et al., 1991).

Pre-treatment which involved acidification followed by re-neutralisation allowed TGF-β to maintain AIG.

Three explanations were put forward to explain these observations:

1 A TGF-β inhibitor could have been inactivated due to the acidification. This was dismissed when serial dilutions of acid-neutralised conditioned medium into non-treated conditioned medium did not demonstrate any decrease in TGF-β as compared to similar dilutions in to fresh unconditioned medium. There was no reassociation of inhibitor to TGF-β in the untreated conditioned medium.

2 The acid-neutralisation could have altered the protein conformation of TGF-β. The cellular nature of the assay used was incapable of distinguishing such a possibility.

3 Finally the active form of TGF-β could be processed from a high molecular weight form (Pircher et al., 1984; Kryceve et al., 1985).

Exposure to extreme pH (<4 or >9), chaotropic agents (urea) or plasmin in vitro released active TGF-β from the latent complex (Lyons et al., 1988). The latent complex isolated from human platelets and fibroblasts consists of the mature TGF-β1 dimer plus two TGF-β1 pro-region polypeptides, disulphide linked to a glycoprotein of 125-160 kDa in platelets or 170-190 kDa in fibroblasts (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et

al., 1990). The pro-region polypeptides are disulphide linked to each other. The amino acid sequence was deduced for the 125-160 kDa glycoprotein by studying the cDNA sequence, and found to contain multiple EGF-like repeats in tandem. The function of this protein is not yet known. It does not prevent binding of activated TGF-β to cells, is not proteolytic nor is it found to be related to the TGF-β binding proteoglycan. The pro- region has many properties. In 1988 it was found by Gentry et al. to keep mature TGF-β1 in the latent state. It has been identified by Gray and Masson,1990 as being required for the correct folding of TGF-β1 during synthesis, and by Purchio et al.,1988 as possessing mannose-6-phosphate as well as the arg-gly-asp (RGD) sequence, which in fibronectin, vitronectin, laminin, and other other cell adhesion molecules recognises certain adhesion receptors of the Integrin class (Ruoslahti and Pierschbacher, 1987). The pro-region binds to mannose-6-phosphate receptors but it could not be established whether the RGD sequence could mediate binding of pro-TGF-β to integrins or whether binding mediated by RGD or mannose-6-phosphate could lead to activation of latent TGF-β1. The precise mechanisms which activate latent TGF-β are still unknown.

TGF-β is also found as an inactive complex in serum where it is attached to alpha-2-macroglobulin. It is thought that the binding to this molecule

provides a means of scavenging active free TGF-β that is released by platelets at sites of injury (O'Connor-McCourt and Wakefield, 1987).
Feige et al., 1991 demonstrated the importance of latent TGF-β. They observed that adrenocortical cells secrete TGF-β1 in a latent form, together with large amounts of alpha- 2-macroglobulin and they found that the physiological activation of latent TGF-β was the limiting step in controlling its action in the adrenal cortex.
The presence of an inactive complex is of importance since it ensures that active TGF-β is only generated in the appropriate environment.

It has been suggested that proteolytic action of plasmin or cathepsin D on TGF-β1 pro-region (Lyons et al., 1989; Sato and Rifkin, 1989), the removal of carbohydrate residues in this region (Miyazono and Heldin, 1989), and the action of acidic microenvironments in sites of wound healing and bone resorption, might contribute to activate latent TGF-β in vivo.

1.043 Sequestration of activated TGF-β by extracellular matrix components and serum proteins.

After release from the latent complex the active TGF-β can be bound by various proteins. It has been suggested that the cell rapidly (less than 3 min)

clears away any circulating activated TGF-β by binding to alpha-2- macroglobulin (Coffey et al., 1988; Wakefield, 1987).

High affinity binding of TGF-β to the core protein of the proteoglycan, or lower affinity interactions with abundant matrix components, might protect TGF-β from degradation, or function as a long term reservoir of TGF-β, or even act as a TGF-β clearance system.

1.05 OTHER MEMBERS OF THE TGF-β SUPERFAMILY.

1.051 Inhibins and Activins.

Inhibins and Activins were originally isolated from ovarian follicular fluid and both are able to modulate the production of follicle stimulating hormone (FSH) from pituitary cells. Inhibins are identified as heterodimers which inhibit production of pituitary FSH, gonadal sex steroids and placental hormones. The subunit structure is found to be αβA or αββ with the two β subunits showing 60% aminoacid sequence identity. The α subunit sequence is as divergent from the β subunits as it is from other members of the TGF-β family. The inhibins appear to repress FSH production by decreasing FSH-α and FSH-β mRNA levels (Attardi et al., 1989). The βA and ββ chains can pair with each other as well as forming dimers with the α subunit. The dimers of βA-βA and βA-ββ have opposite biological activity to that of the inhibins. These

dimers, called Activins, stimulate FSH production (Petraglia et al.,1989).
The antagonistic activity of inhibins and activins raises questions concerning their mode of action. Complex mechanisms appear to ensure tight control of activity for these factors as in the case of TGF-β.

1.052 Decapentaplegic, Vg1 and BMPs.

The biology of the TGF-β related factors underscores the role of certain members of the family as morphogens in arthropod and vertebrate developmental processes. The decapentaplegic (DPP) gene complex encodes important functions in embryonic as well as Drosophila pattern formation. Mutations in various regions of the DPP gene complex result in failed dorsal-ventral patterning during early embryogenesis, and defective patterning of larval imaginal disks (Spencer et al., 1982). The mutations affect cis-regulatory elements that control the expression of a set of overlapping transcripts. The product encoded by the transcripts has the predicted structure of a TGF-β related molecule with a C-terminal sequence that is 36% identical to the mature TGF-β1 sequence (Padgett et al.,1987).
Another member of the family, Vg1, was identified in the developing embryo of Xenopus laevis. The Vg1 product displays 38% homology to TGF-β1 and 50% homology to DPP.

involved in mesoderm induction

Another member of the family, extracted from bone and named Bone Morphogenetic Protein (BMP), induces proliferative and differentiative responses that culminate with the transient formation of cartilage followed by accumulation of bone with Haemapoietic marrow (Wozney, 1989). N-terminal sequencing and molecular cloning of these components identified them as members of the TGF-β superfamily with a similarity to DPP and Vg1.

1.053 Mullerian Inhibiting Substance

Mullerian Inhibiting Substance (MIS) was identified and purified based on its ability to induce regression of the primordium of female genitalia, the Mullerian duct, in mammalian male embryos. (Blanchard and Josso, 1974). The deduced sequence of MIS C-terminal domain is 25% identical to that of other TGF-β related factors. Purified from testes MIS is a disulphide-linked homodimer of glycosylated chains that in contrast to other TGF-β related factors contains the glycosylated N-terminal extension uncleaved from the C-terminal domain (Cate et al.,1986). Despite this recombinant MIS expressed in cultured cells undergoes TGF-β - like processing (Pepinski et al.,1988). It could be that the bioactive MIS is generated by cleavage of the precursor at the sites of action in vivo. It has also been proposed that MIS may promote testicular morphogenesis in addition to Mullerian duct regression. As with other TGF-β related

factors, progress in MIS research is revealing a broader range of actions than was originally anticipated.

1.06 BIOLOGICAL ACTIONS OF TGF-β

The majority of information on the activity of TGF-β is derived from studies on TGF-β1, 2, and 3. TGF-β has been described as a multifunctional peptide that controls proliferation, differentiation, and other functions, in many cell types (Roberts et al., 1990). Its multifunctionality was first observed in studies of its role in the control of cell proliferation. Roberts et al., 1981 measured TGFs-B ability to stimulate proliferation of NRK fibroblasts in soft agar. However it was later shown by Tucker et al.,1984, Roberts et al.,1985, and Moses et al., 1985 that it had inhibitory effects on proliferation in many primary or secondary cultures, including hepatocytes (Hayashi et al, 1985) and embryo fibroblasts (Anzano et al.,1986). In a cell of mesenchymal origin, whether TGF-β stimulates or inhibites proliferation is a function of all the growth factors in operation (Roberts, 1985). What is known of the molecular bases of the effects of TGF-β can be summarized as involving the regulation of two sets of genes : (1) genes encoding secreted proteins that contribute to the rate of accumulation or to the composition of

the extracellular matrix, and (2) genes encoding growth factors and perhaps differentiation factors. TGFs-β are involved in the control of cell proliferation, differentiation, cell adhesion and cell phenotype.

TGF-B stimulates or inhibits cell proliferation depending on the cell type and the environment. For example TGF-β1 slows the cell cycle of AKR-2B mouse fibroblasts (Shipley et al., 1985), but induces expression of PDGF-B in these cells (Leof et al., 1986). Thus when AKR-2B cells are placed in a mitogen rich medium, there is a net growth inhibitory effect of TGF-β1, but when AKR-2B cells are plated in a mitogen-free medium, there is a net growth stimulatory effect caused by TGF-β1-induced autocrine PDGF. The importance of the cellular environment in determining the response to TGF-β is underscored by the observation that the same cell type can be inhibited from proliferating when grown in monolayer cultures, or stimulated to proliferate in suspension cultures (Assoian and Sporn, 1986 ; Madri et al., 1988).

Since this growth factor stimulates cells to grow in soft agar it was named a transforming growth factor. The early preparations of TGFs (called sarcoma growth factor), however, contained TGF-β and TGF-α (Roberts et al., 1982). TGF-α was found to be the proliferative growth factor which acted through the EGF receptor to stimulate the

initiation of DNA synthesis. TGF-β and TGF-α act synergistically to stimulate anchorage independent proliferation of NRK cells (Anzano et al., 1982). One explanation put forward to explain this ability to stimulate AIG was that TGF-β induces the c-sis gene and therefore PDGF, as it did when the cells were grown attached to plastic (Leof et al., 1986). This is not sufficient to explain the action of TGF-β on cells in suspension culture, because PDGF acts synergistically with TGF-β to stimulate anchorage independent growth (van Zoelen et al., 1986). If TGF-β were to act by stimulating the production of PDGF, then the addition of PDGF to the assay would have had no effect on colony growth or would have had an additive, rather than synergistic, effect at subsaturating levels of TGF-β.

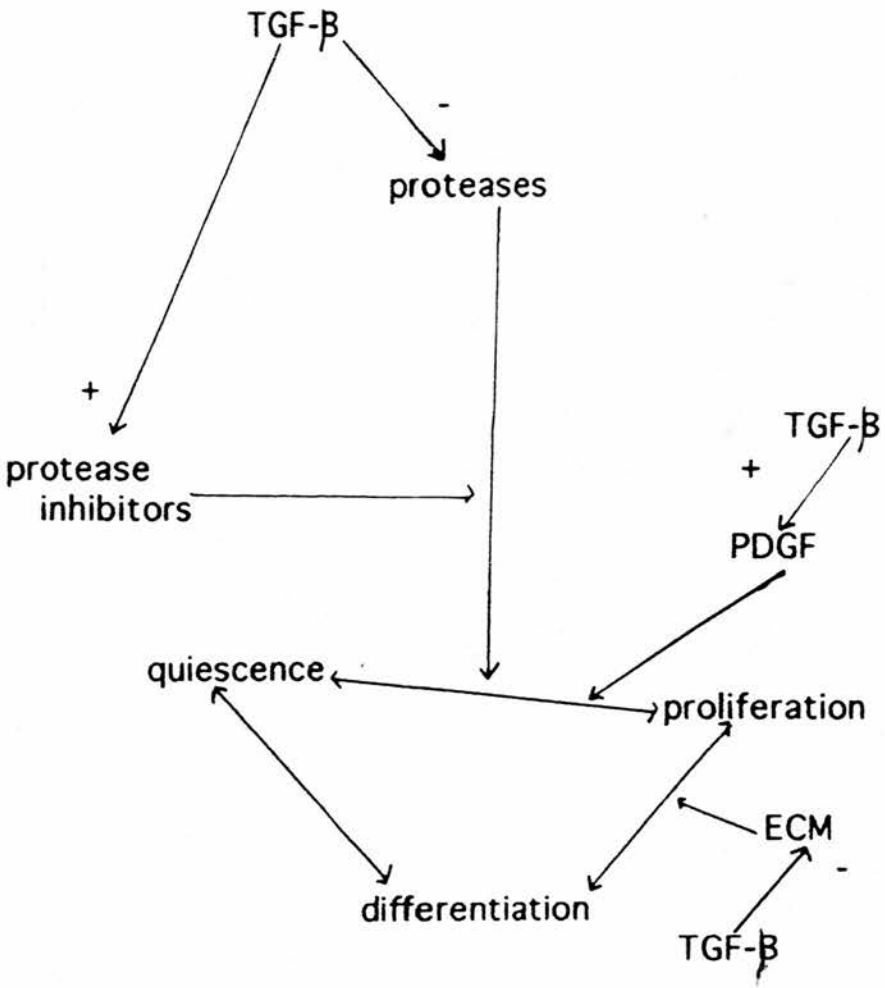
The regulation of gene expression by TGF-β, which results in an increase in the amount of extracellular matrix, is the most likely explanation put forward to explain the proliferative response TGF-β produces in the presence of PDGF and other growth factors. This activity of TGF-β is important for cells that are normally anchorage-dependent but which were grown in suspension. Anchorage-dependent cells, when they are unable to find an attachment site in suspension culture, may require an extensive network of extracellular matrix proteins in order to form a compact colony. By inducing genes which encode

protease inhibitors and repressing the expression of genes encoding proteases, TGF-β may decrease the extracellular expression of proteolytic activity and thereby acted as an extracellular matrix-sparing agent. The decreased proteolytic and increased expression of genes encoding extracellular matrix proteins that are directed by TGF-β result in the accumulation of extracellular matrix. This would allow the cells to proliferate in colonies by providing them with the necessary substratum. It has been suggested that the extracellular matrix-sparing activity may be the crucial activity by which TGF-β acts synergistically with proliferative growth factors such as EGF, TGF-α and PDGF to stimulate colony formation in soft agar (Roberts et al., 1985).

It is thought that since most cells possess TGF-β receptors, and different cell types produce different extracellular matrix proteins, a local increase in the concentration of TGF-β in the developing embryo could produce a profound change in both the composition and the amount of the extracellular matrix. The resulting changes would alter proliferation, differentiation and directed cell migration.

TGF-β has also been shown to inhibit anchorage - dependent proliferation of many cells in monolayer culture. Cell types which are inhibited in their proliferation by TGF-β include endothelial cells (Baird and Durkin, 1986), T lymphocytes (Kehrl et

Figure 1 The varied actions of TGF-β on cellular proliferation and differentiation. By altering the levels of several secreted proteins that affect proliferation and differentiation in different ways, TGF-β might act as a stimulator or an inhibitor of cellular growth or differentiation, depending on the strengths of the cellular responses to each extracellular signal.



al., 1986), keratinocytes (Coffey et al., 1988), intestinal epithelial cells (Lee et al., 1988), mammary epithelial cells (Silberstein and Daniel, 1987) and some fibroblasts (Kamijo et al., 1989). This inhibition of proliferation and morphogenesis by TGF-β is also observed in vivo. In the developing mouse mammary gland TGF-β inhibits proliferation (Silberstein and Daniel, 1987). Again, the ability of TGF-β to inhibit proliferation could be a consequence of its ability to induce c-sis and to alter the expression of genes encoding proteases and protease inhibitors. Scher, (1987) demonstrated that proliferation of many cells was stimulated by proteases and that the ability of proteases to stimulate proliferation was a function of their proteolytic activity. Proteases can stimulate cell proliferation by activating latent growth factors and cells can dampen the mitogenic effect of proteases by secreting protease inhibitors (Low et al., 1982). TGF-β can therefore, inhibit proliferation of certain cells in monolayer culture through its ability to decrease the overall proteolytic activity in the medium or the activity of particular proteases (Figure 1).

Whether cells respond to TGF-β with increased or decreased proliferation depends on their relative sensitivities to the action of extracellular proteases the PDGF produced in response to TGF-β, and the other growth factors in the environment. The cell's response to TGF-β and other growth

factors is, determined by the cells intracellular biochemical state, the receptors expressed on its surface and the regulatory molecules in its environment. It has been proposed that TGF-β regulates the expression of two important sets of genes : one set of TGF-β regulated genes encodes growth factors and differentiation factors, and another set encodes proteins that in one way or another impact on the extracellular matrix. Depending on the cell's biochemical condition and its environment, it would respond to TGF-β with increased or decreased proliferation.

TGF-β is also involved in the growth and development of bone and cartilage by stimulating the proliferation and differentiation of osteoblasts and chondrocytes (Pepper et al., 1990). Heine et al., 1987, demonstrated that one of the major sites of TGF-β synthesis and localisation in the foetus was in developing bone and cartilage. Madri et al., 1988 demonstrated that TGF-β is involved in angiogenesis. This growth factor is also shown to have wide reaching effects on cells of the immmune system. It inhibits proliferation of B lymphocytes (Kehrl et al., 1986), and induced IL-1-thymocytes (Ristow, 1986). Other biological actions include regulation of the differentiation of several steroidogenic cell types, such as the ovarian granulosa cell and the adrenocortical cell.

Directed movements of selected cells within the embryo are essential in early development. TGF-β is considered responsible for producing at least two types of secreted stimuli to direct the movement of cells through the embryo. The first is by the secretion of particular composition of extracellular matrix by cells in the specified path and the second type is by chemotactic action.

1.07 TGF-β RECEPTORS

Three receptors have been identified as TGF-β binding domains. Two are glycoproteins (receptors I and II) with molecular weights of 53 and 70-100 KDa. The other is a membrane proteoglycan called betaglycan. Receptors I and II bind TGF-β with higher affinity than betaglycan and are present at low levels in mammalian and avian cells. Betaglycan is broadly distributed but is not detectable in various cell types that respond to TGF-β including skeletal muscle myoblasts, haematopoietic progenitor cells, and vascular endothelial cells (Massague et al., 1990).

The formal identification of the receptor based on expressing its cDNA in receptor defective cells has not been accomplished. The distribution of the receptors and the isolation of resistant cell mutants have implicated receptors I and II as components of a signal-transducing TGF-β receptor complex.

A model in which a single receptor complex with two TGF-β binding subunits mediates multiple actions of TGF-β prevails at present over the hypothesis that the different responses to TGF-β were mediated by multiple unrelated receptor types (Massague et al., 1990).

Kimchi et al., 1988 suggested that the expression and dynamics of TGF-β receptors are, in general, not highly regulated. This is despite the fact that the receptor bound TGF-B was rapidly internalized and degraded (Frolik et al.,1984 ; Massague & kelly, 1986).

The most abundant cell-surface TGF-β binding component is found to be betaglycan (Fanger et al., 1986). Cheifetz et al., 1988 identified betaglycan as a 280-330 kDa integral membrane proteoglycan, consisting of a 200 kDa glycosaminoglycan chain and a 10 kDa fraction of N - linked glycans attached to a heterogeneous core polypeptide of 100-120 kDa. Betaglycan shows similar affinity for TGF-β1, 2, and 3 in many cell lines, unlike receptors I and II (Cheifetz & Massaague, 1989). Other studies by Segarini et al., 1987 have suggested that betaglycan has different affinities for TGF-β1 and 2.

The present favoured theory suggests that betaglycan is involved in ligand presentation to receptors I and II. It could function as a reservoir or clearance system for bioactive TGF-β.

1.08 MECHANISMS OF ACTION

There are four mechanisms which have been proposed for TGF-B action. These are cytoplasmic, nuclear, growth suppression, and differentiation control mechanisms.

1.081 Cytoplasmic Response

The primary signal transduction mechanism of TGF-β receptors is still not known. No-one has identified whether enzymatic activities or second messengers are involved in the mechanism of action.
Toxins have been reported to inhibit TGF-β1 induced c-sis expression. Cholera toxin inhibits the mitogenic response of AKR - 2B fibroblasts to TGF-β1, but does not alter other TGF-β responses or TGF-B1 binding to receptors (Howe et al., 1990). Muldoon et al., 1988 demonstrated that cytoplasmic responses such as activation of glycolysis, amino acid uptake and extracellular calcium levels were not directly coupled to TGF-β receptors, even although these activities have been observed in rat fibroblasts in response to TGF-β.

1.082 Nuclear Response

The nature of the nuclear response to TGF-β is very complex. TGF-B is found to regulate different transcriptional control elements. Transcriptional activation by TGF-β1 is mediated by a NF - 1 binding site in the collagen a2 (1) gene promoter

(Rossi, et al., 1988), by multiple AP - 1 binding sites in the TGF-β1 gene promoter (Kim et al., 1990) and by three distinct elements in the fibronectin gene promoter.

Some of the genes whose expression is regulated by TGF-β encode transcriptional factors or growth factors which then generate a secondary nuclear response. The type of response of a particular gene to TGF-β is dictated by cell specific determinants.

1.083 Growth Suppression

Many mechanisms have been proposed to explain the growth inhibitory action of TGF-β. It has been suggested that the growth suppression action is due to the ability of TGF-β to decrease expression of mitogenic inhibitors e.g. EGF receptors in NRK rat fibroblasts (Assoian, 1985). It was later found that this effect occurred in some cells but did not represent a general mechanism.

Other approaches have involved the identification of cell cycle events that are directly controlled by TGF-β action. TGF-β inhibits the cell cycle by lengthening the G1 phase (Lin et al., 1987). The most important event discovered from these experiments was the phosphorylation of the retinoblastoma gene product, RB. This product displays growth suppressor activity (Lee et al., 1987). It was suggested that TGF-β1 and RB function in a common growth inhibitory pathway in which TGF-

β1 attempts to keep RB in the growth suppressive state.

1.083 Differentiation mechanisms

It has only been in recent years that the events leading to terminal differentiation have begun to be defined. Heino and Massague, 1990, have demonstrated that TGF-β1 inhibited myoblast differentiation and that this is achieved by two mechanisms acting together. One mechanism leads to the blockage of gene expression, such as myogenin in L_6E_9 cells. The other mechanism is thought to involve TGF-β induced changes in cell adhesion.

1.1 FIBROBLAST DERIVED GROWTH FACTOR (FGF)

Fibroblast growth factor (FGF), is important in the development of various mesoderm derived tissues. FGF exists in two forms, basic and acidic FGF, and both interact with the same cell surface receptor (Neufeld and Gospodarowicz, 1986). This ability to share a receptor enables aFGF and bFGF to exert similar biological effects on a wide range of mesodermal and neuroectodermal cells to control both their proliferation and differentiation. The bioactivity of both aFGF and bFGF can be positively or negatively regulated by TGF-β, depending on the cell type (Globus et al., 1988). The ability of a tissue to synthesize FGFs and TGF-β and the amounts of these growth factors present in the microenvironment, can determine whether proliferation and differentiation are selectively enhanced or repressed by FGF.

1.11 FGF GENES

There is 55% structural homology between aFGF and bFGF and this suggests that they have evolved from a common ancestor. The bFGF gene is located on human chromosome 4 and the aFGF is located on chromosome 5 (Jaye et et al., 1986, Abraham et al., 1987). Southern blot

analysis of human genomic DNA has shown that there is only one bFGF and one aFGF gene. This implies that all the characterized or uncharacterized heparin binding endothelial cell mitogens related to bFGF or aFGF are probably the products of the bFGF or aFGF gene (Abraham et.al., 1987).

1.12 FORMS OF FGF

The primary translational product of the FGF gene contains 155 amino acids and has an apparent molecular weight of 18,000 Da (Jaye et al., 1986). Proteolytic cleavage results in the removal of the first nine amino acids from bFGF and fifteen from aFGF. Proteolytic cleavage continues to produce NH_2 truncated forms. Both forms of FGF lack classical consensus signal peptide sequences, a feature also characteristic of the precursors of interleukin-1a and interleukin-1β. The release of growth factors can occur following cell lysis but alternative mechanisms of secretion may involve the formation of complexes between the growth factor and carrier/binding proteins.

Larger molecular weight forms of FGF have also been reported. These include a 25,000 Da form (Moscatelli et al., 1987), and 22,000, 23,000 and 24,000 Da forms.

1.13 CONSERVED SEQUENCES

Since aFGF and bFGF are nearly identical in their biological activities and may act through the same receptors it is possible that they act through similar structures. bFGF appears to have been well conserved through evolution. Bovine and human bFGF differ in only two of their amino acids and have a sequence homology of 98.7% (Abraham et al., 1986). aFGF is less well conserved with fifteen amino acid differences between the human and bovine forms.

1.14 ONCOGENES

A number of oncogenes have been found that are structurally related to FGF (Delli-Bovi et al., 1987; De Lapeyriere et al., 1991; Dickson et al., 1984; Dickson et. al., 1989). These oncogenes have been identified in Kaposi's sarcoma (KS3 oncogene), human stomach cancer (hst oncogene), mouse mammary cancer (int-2 oncogene) and bladder cancer (FGF-5 oncogene).

From the study of these oncogenes it has been found that FGFs not only stimulate the division of mesoderm or neuroectoderm derived cells, but are also potent angiogenic agents

involved in the growth of new blood vessels (Gospodarowicz et al., 1987). New blood vessel formation is essential if solid tumours are to develop to a significant size. Production of FGFs or related proteins might contribute to the development of solid cancers because of the proteins' effects on cell division and angiogenesis.

The int-2 RNA has been identified in complex patterns in embryonic and extraembryonic tissues. This suggests multiple roles for int-2 in development, including regulation of the migration of early mesoderm and induction of the otocyst (Wikinson et al., 1988).

1.15 BIOLOGICAL ROLES

Accumulation of information has identified roles for FGFs in mesoderm induction and muscle formation in the early embryo (Slack, 1983), nervous system development (Togari et al., 1985), cartilage and bone formation (Globus et al., 1988), limb regeneration (Ratner et al., 1988), ovarian follicular morphogenesis (Gougeon, 1982), vascular development (Gospodarowicz et al., 1886) and in the extracellular matrix.

bFGF can act as a differentiation factor in early embryos and instruct tissues destined

to form ectodermal structures to differentiate into mesoderm instead.

The level of FGF in the body of the chick embryo is fairly constant from days 2-6 (Seed et al., 1988). The level increases so that by day 13, the embryo contains sevenfold more FGF than on days 2-6. In contrast, the level of FGF in the limb bud is higher than in the rest of the body until day 5, when it undergoes a decrease between days 5 and 7 (Seed et al., 1988). FGF delays the onset of differentiation of days 4-12 embryonic chick wing myoblasts (Seed et al., 1988). The limited period during which FGF-dependent myoblasts are found in the limb bud suggests they may play a role in early muscle morphogenesis. It also suggests that FGF may affect muscle development differently during various phases of embryogenesis.

Effects of FGFs on nerve cells has also been reported. bFGF can act as a differentiation factor for a rat pheochromocytoma (PC-12) cell line by inducing both neurite outgrowth and ornithine decarboxylase activity. The addition of bFGF to cultured rat cerebral cortical neurons enhances their survival (Morrison et al., 1986). From these results it was suggested that bFGF may function as a neurotropic agent in the CNS. FGF also has similar effects on dopaminergic and gabaergic

neurons of the CNS. Other types of neurons responsive to FGF include embryonic chick ciliary ganglia neurons and chick spinal cord neurons. Nerve cells from the peripheral or central nervous system (Janet et al., 1988), as well as astrocytes (Hatten et al., 1988). In addition to its effects on nerve and glial cells, bFGF could influence PNS and CNS development through its angiogenic properties.

Both bFGF and aFGF are mitogenic for bone cells (Globus et al., 1988). The effect of FGF is potentiated by TGF-β, which has a minimal effect by itself. bFGF, by increasing the osteocalcin content of the cell and decreasing alkaline phosphatase activity and collagen production, can influence the bone cell phenotype (Canalis et al., 1988). The bone cell derived bFGF is stored in an active form in the ECM and this suggests that bFGF may function as an autocrine or paracrine mitogen via its deposition into the bone ECM (Globus et al., 1989).

In addition to these properties FGFs affect the proliferation and differentiation of granulosa cells. bFGF can influence their hormonal response by inhibiting FSH. bFGF, through its angiogenic properties, is an important regulator in the early phase of

matured + enlarged ovarian follicle

corpus luteum development (Gospodarowicz et al., 1986).

bFGF has been detected in macrophages (Baird et al., 1985) and, following its release from damaged cells, it could play a crucial role in wound healing processes. In contrast to the growth factors PDGF and TGF-β , bFGF stimulates the proliferation of all the cell types involved in the wound healing process (Baird et al., 1985).

The high degree of structural conservation of bFGF between classes as different as mammals, birds and amphibians as well as its presence in all vertebrates studied to date, indicates that FGF could have a fundamental role *in vivo*.

1.2 GROWTH FACTORS, ONCOGENES AND CANCER

Knowledge that the transforming oncogenes of the acutely oncogenic retroviruses are homologous to cellular genes and are probably derived from them, brought several areas of research together with prospects for advances in virology, carcinogenesis, evolution and development.

Oncogenes were first discovered from the study of tumour viruses. They could cause cancer in either their natural hosts or experimental animals. In many cases, the cancer can be attributed to one or more genes within the DNA or RNA of the viral genome (Gallwitz et al., 1983). The study of retroviruses has helped in the understanding of oncogenic function.

Retroviruses are versatile carcinogens and cause every form of neoplasia known to plague animals. They can do this in at least two ways:

1 Some retroviruses carry oncogenes whose protein products can convert cells to cancerous growth by attacking crucial cellular events (Coffin et al., 1981), and

2 Some retroviruses do not carry oncogenes but can still cause cancer. When these retroviruses infect cells, the RNA of their genome is copied into DNA by reverse transcriptase. The viral DNA is then integrated into the chromosomal DNA of the host. Integration is potentially mutagenic and thus potentially carcinogenic.

Uncontrolled proliferation of cells occurs and this is the hallmark of tumour cells. Almost all oncogenes are altered versions of normal cellular genes (Proto-oncogenes) and their products are presumed to work, at least in part, by mimicking the products of the cellular genes from which they arose.

There are three sites in a growth control pathway at which oncogenic proteins can intervene to deliver a growth stimulus. Firstly, the protein itself might mimic a growth factor. The interaction of such a protein with a suitable receptor could stimulate growth in an autocrine fashion. Secondly the oncogenic protein might imitate an occupied growth factor receptor and thus provide a mitogenic signal in the absence of exogenous growth factors. Thirdly, the oncogenic protein might act on an intracellular growth control pathway uncoupling it fromthe need for an exogenous stimulus. There are oncogenic proteins acting at all three sites. The identification of oncogenes from the first and second categories came from comparisons of amino acid sequences. A strong homology was found between the product of the v-sis oncogene of simian sarcoma virus and PDGF (Olashaw et al., 1983). The *erb*-B protein can occupy the EGF receptor and therefore mimick its function. The identification of the third category came from the src family of oncogenes which encode proteins with tyrosine kinase activity. These enzymes phosphorylate

proteins that are normally the targets for the growth factor activated receptor protein tyrosine kinases.

Normal cellular genes (Proto-oncogenes) are thought of as crucial elements in growth regulation and differentiation because of their conservation throughout evolution and because of the well known growth deregulation effects produced by the transforming oncogenes. It is plausible that the normal counterparts of transforming oncogenes should be active during embryonic development and identification of a specific tissue or stage where proto-oncogenes are expressed should help to identify their roles.

Several proto-oncogenes have been found associated with cellular proteins and this has confirmed their importance to growth regulation. These specific proteins include growth factor or hormone receptors. (Muller, 1986; Hunter,1984).

The most well known of the tyrosine kinase family of proto-oncogenes is the EGF receptor which is the larger homologue of the *erb-β* protein. The oncogenic counterpart of the erb-β protein causes erythroblastosis (Downward et al., 1984).

The src proteins were the first tyrosine kinases to be discovered and form the archetype for this group of oncogene products. The transforming oncogene, *v-src*, encodes the transforming protein of the avian *Rous sarcoma* virus and the proto-oncogene, *c-sis*, encodes a similar 60kDa protein. Studies with *Rous*

sarcoma virus infections or *v-src* introduced into cultured cells have suggested that both differentiation and proliferation are induced by the gene.

Various oncogenes are structurally related to FGF (Delli-Bovi et al., 1987; De Lapeyriere et al., 1991). These oncogenes are a diverse group and have been identified in *Kaposi's sarcoma* (KS3 oncogene), human stomach cancer (*hst* oncogene), mouse mammary cancer (*int-2* oncogene) and bladder cancer (FGF-5 oncogene).

The protein product of *int-2*, an oncogene that was originally located in mouse mammary cancers caused by the mouse mammary tumour virus (Dickson et al., 1984), also belongs to the FGF family (Dickson et al., 1990). It resembles bFGF more closely than aFGF. In addition to *int-2* there are seven known members of the FGF family (Dickson et al., 1989).

Int-2 is a member of a group of genes which lie at common integration sites for the mouse mammary tumour virus in murine breast tumours. Insertion of a viral DNA on either side of the *int-2* gene results in its transcriptional activiation. The integration of the viral DNA appears to occur at random. It is thought that the transcriptionally active *int-2* gene, which is normally silent in the normal mammary gland, allows the cell to proliferate. The result is neoplasia.

Northern blotting and RNAase protection analysis have been used to detect *int-2* in the embryo. Adult

mouse tissues only contain small amounts of *int-2* RNA in the brain and testes. In the developing embryo this gene is found extensively from around day 7 of development until parturition. The number and diversity of these sites suggest multiple roles, consistent with functions such as mitogenesis, chemotaxis or induction of differentiation.

Studies looking at proto-oncogenes in developing systems will therefore be of importance not only in the regulation of developmental events but in the study of tumourogenesis.

1.3 THE ROLE OF THE EXTRACELLULAR MATRIX DURING DEVELOPMENT

The extracellular matrix (ECM) is a very important feature of development. It is a complex network formed outside cells from glycoproteins and proteoglycans secreted by the cells. The ECM provides a structural framework in the form of bone, cartilage and tendon. In addition to this it influences many biological processes including cell adhesion, migration, tissue morphogenesis, cell proliferation and cell differentiation. The ECM is composed of a basement membrane and interstitial connective tissue.

Basement membranes are extracellular structures which are found between such cells as endothelial and epithelial and around muscle, adipose and Schwann cells. They are mainly composed of types IV and V collagens, laminin, entactin, and proteoglycans, which interact to form a dense network. In the adult the basement membrane forms a barrier and thus compartmentalizes tissues and organs. It can also be selectively penetrated e.g. the glomerular basement membrane of the kidney is involved in filtering plasma and selectively retaining plasma proteins. The selectivity may depend on pore size and electrostatic forces within the matrix, due to the presence of charged molecules such as proteoglycans.

The basement membrane is important during embryonic development since it is involved in tissue architecture and epithelial cell polarity. This

membrane also plays a role in separating maternal and fetal environments in extraembryonic tissues such as the placenta, yolk sac and amnion.

The interstitial connective tissues contain several cell types in addition to those responsible for producing the bulk of the ECM (fibroblasts, osteoblasts and chondroblasts). These include macrophages, lymphocytes, granulocytes, melanocytes and nerve cells.

The major protein of connective tissue matrix is collagen. In addition to the various types of collagen, fibronectin , elastin, heparan sulphate proteoglycans and tenascin are found.

Both basement membranes and interstitial connective tissues are important structural features of embryonic and adult tissues. Defects in their synthesis and assembly can have profound effects on normal development. Indirect evidence for specific roles of the ECM in development comes from *in vitro* culture systems in which cells are grown in matrices of different composition. Li et al., 1987 demonstrated that primary mammary epithelial cells cultured on a complex matrix of basement membrane components show a more normal morphology and a much higher level of casein gene expression than cells grown on type I collagen or plastic. By altering with the ECM in vivo development was affected. In the white mutant of the Mexican axolotl pigment cell precursors fail to migrate from the neural crest through the subepidermal ECM. The

subepidermal matrix was adsorbed onto nucleopore carrier membranes, which were then grafted under the skin. Migration of pigment cell precursors occurred if they were reimplanted at an early stage of development. This suggested that the subepidermal matrix was defective in the mutant, rather than the neural crest cells (Perris et al., 1988). Some of the most direct evidence for the importance of the ECM comes from studies on mammalian development. These studies involved mutants in which ECM components were altered as a result of changes at the DNA level. An altered gene which codes for an ECM component and which affects development is the Mov-13 mouse line, in which a murine retrovirus has been inserted into the first intron of the a1 type I collagen gene (Harbers et al., 1984). This results in reduced synthesis and secretion of type I collagen and death of the embryos as a result of disruption of weakened blood vessel walls.

The quality and quantity of the ECM depends not only on the structural components such as collagen but also on the regulated expression of matrix-degrading proteases and their inhibitors. It is likely that the expression of proteases is closely regulated during embryonic development. Several secreted proteases including plasmin, plasminogen activators, collagenases and cathepsins participate in the degradation of the ECM (Tryggvason et al., 1987). Many of the matrix-degrading enzymes are

expressed in a latent form and require activation, possibly by proteolytic cleavage. Some of the enzymes require specific environmental conditions (e.g. acid pH). In addition there are specific inhibitors for these enzymes.

The condition of the ECM is influenced by the levels of structural proteins, matrix-degrading proteases, and inhibitors and activators of the proteases. These levels may be controlled by growth and differentiation factors acting indirectly through target cells. Therefore, growth factor modulation of the ECM could affect matrix remodelling and tissue morphogenesis during embryonic development.

1.4 AIMS

There are four aims to the project:

1 Identification and quantification of TGF-B in medium conditioned by chick embryonic tissue (Chapter 3).

2 Exploration and characterisation of a novel proteolytic activity discovered early in the experimental work . The nature of this activity is explored and characterised (Chapter 4).

3 Investigation into a possible role for this proteolytic enzyme in TGF-β processing along with mechanisms of activation of latent TGF-β (Chapter 5).
The proteolytic properties of other growth factors are investigated (Chapter 6). During an investigation into the effect TGF-β had on the proteolytic activity of the protease, TGF-β displayed proteolytic activity.

4 Exploration of the Anchorage Independent Growth (AIG) promoting properties of Conditioned Medium. The chick embryo produces this AIG response in a manner not consistent with known growth factors. The nature of this growth factor is explored (Chapter 7).

1.5 IMMEDIATE BACKGROUND

In 1988 McLachlan et al. identified the presence of a heat-stable protein that stimulates colony formation in NRK 49F cells in the presence of EGF, but not in its absence. This was suspected to be TGF-β.

TGF-β has been described as a 25kDa dimer which can be reduced to yield two 12.5kDa polypeptide chains (Derynck et al., 1985). Polyacrylamide gel electrophoresis was employed to assess if the TGF-β dimer existed within conditioned medium.

Conditioned medium was also shown to produce Anchorage Independent Growth (AIG) in the absence of any additional growth factors (Smith and McLachlan, 1990). This effect appeared to involve glycoprotein activity since it was suppressed by conconavalin A beads.

A principal method involved in exploring these aims was polyacrylamide gel electrophoresis and variations to this technique. The general methods are detailed in Chapter 2 and those associated with a particular Chapter are described in the Chapter concerned.

CHAPTER 2 METHODS

2.0 METHODS.

General methods are described in this Chapter. Methods which are explicit to protease purification and characterization are described in Chapter 4. Those methods concerned with glycoprotein purification are described in Chapter 7.

2.1 CONDITIONED MEDIUM PREPARATION

Fertilised chicken eggs (Muirfield Hatcheries, Kinross, Fife) were incubated blunt end up for four days at 37^{o}C in a humidified incubator to provide eggs between stages 17 and 22 according to the criteria of Hamilton and Hamburger (1951).
All the dissecting instruments and the eggs were cleaned with 70% alcohol. Entry into the eggs was made via the air space. Extra-embryonic membranes were removed to reveal the embryo, and two longitudinal incisions were made lateral to the midline. Anterior and transverse sections were made so that the embryo could be removed.
Microdissection of the embryo was performed under a Wild M8 dissecting microscope and involved removal of the head, wing buds, leg buds and tail buds by single transverse cuts using electrolytically sharpened tungsten needles.
To the central wells of a bacteriological grade 96 well plate (sterilin), 100ul of serum free Alpha Eagles Medium (Flow Laboratories) supplemented with 29.2ug/ml glutamine, 500ug/ml penicillin and 50IU/ml streptomycin were added. The dissected

embryonic regions were then placed beneath the surface of the medium such that each well contained either 1 head, 6 wings, 6 leg buds, or 3 tail buds. The plates were then incubated at 37°C, 5% CO_2 for 24h. Following this the contents of the microwell plates were centrifuged at 3000 rpm for 5 min. The pellets were discarded but the supernatants were retained in eppendorf tubes. The conditioned medium was stored at -20°C until required.

2.2 MICROWELL ASSAY FOR ANCHORAGE INDEPENDENT GROWTH

Various proteins isolated from conditioned medium were assayed for transforming activity using a microwell adaptation of the soft agar colony assay as described in Macintyre *et al*. 1988. Conditioned medium preparations, growth factors or control media were placed in the centre 60 wells of a 96 well plate to a volume of 24*ul* per well. Double strength alpha Eagles'medium containing 20% FCS with double strength supplements of glutamine (58.4 *ug*/ml), penicillin (100*ug*/ml) and streptomycin(100 i.u./ml), was mixed with an equal volume of 0.6% Bacto-Agar (Difco) at 40°C. Cells (NRK clone 49f) were centrifuged after trypsinisation and resuspended in fresh medium;they were then added to the agar-medium mixture such that 0.1ml added to each well gave a density of 200 cells. Plates were cooled at 4°C for 5min prior to incubation at 37°C for 9 days. Five replicates were set up at each

point and all experiments were coded and counted blind.

2.3 PROTEIN ASSAY

In order to assess the feasability of polyacrylamide gel electrophoresis of conditioned media, it was first necessary to determine the total protein content. Not only would this allow an estimation of the amount of sample to be applied to the gel, but also the most appropriate staining method required to visualise the proteins.

The protein assay chosen was essentially that of Bradford (1976) with modifications to volumes, which relies on the observation that the dye Coomassie Blue G-250, which exists as a brown colour when free in acid solution but becomes blue on binding to protein.

This method was chosen because it had a number of advantages over other methods. Firstly the procedure was both rapid and sensitive, providing results after 2 min and up to 1h without lengthy pretreatment of protein solutions. The sensitivity of the assay was such that 1 to 50 ug of protein could be detected (4 times more sensitive than the Lowry assay), although results within this range deviated slightly from linearity. This was due to an overlap in the spectrum of the two colour forms of the dye resulting in the background continually decreasing as more dye was bound to protein. Whilst the degree of curvature was only slight, its effect

was overcome by running the assay with a set of standards and calculating unknowns from the standard curve.

A further advantage of this method was the reduced interference of non-protein components, only large quantities of detergents causing abnormalities too great to overcome. This was an improvement over the interference associated with the Folin-Lowry assay in which a large number of materials caused significant abnormalities of results.

2.31 PROTEIN PREPARATION

Bovine Serum Albumin (1mg) was dissolved in phosphate buffered saline to give a final concentration of 25ug/ml. All chemicals were purchased from Sigma Chemicals, St Louis, MO. The solution was further diluted to provide protein standards ranging from 0 to 20 ug/ml in a final volume of 2400 ul.

100 ul samples of head, wing, leg and tail conditioned medium were diluted to a final volume of 2400 ul with PBS.

2.32 PROTEIN REAGENT PREPARATION.

The protein reagent, in which component concentrations were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid, was used as supplied by Bio-Rad Laboratories, Munich.

2.33 PROTEIN ASSAY.

To 2400 ul protein solution 600 ul of Bradford reagent was added. Following mixing the 20 ug/ml BSA standard was scanned between 400 and 700 nm on a Pye Unicam SP 1800 Ultraviolet Spectrophotometer to obtain the optimum absorbance wavelength. The remaining samples were measured at this wavelength (595 nm). Samples containing only antibiotic supplemented Alpha Eagles Medium were also measured to act as blanks and to determine any interference effects. The amount of protein in the standards were plotted against the corresponding absorbance resulting in a standard curve which was used to determine the amount of protein in conditioned media.

2.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

It is necessary to describe the types of electrophoresis employed since by altering the ingredients different characteristics of a protein can be examined.

SDS-PAGE is routinely used for the estimation of protein subunit molecular weights and for determining the subunit compositions of purified proteins.

The most popular electrophoretic method is the SDS-PAGE system developed by Laemmli. This is a

discontinuous system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strength. The buffer discontinuously acts to concentrate large volume samples in the stacking gel,resulting in better resolution than is possible using the same sample volumes in gels without stackers.

The Laemmli SDS-PAGE system is made up of four components. From the top of the cell downward, these are the electrode buffer, the sample, the stacking gel, and the resolving gel(Laemmli,1970). This system is an adaptation of an earlier method devised by Ornstein and Davis for fractionation of native serum proteins. The inclusion of SDS modifies the Ornstein-Davis technique since the properties of the detergent dominate the system. The complete denaturation and dissociation of proteins was not always desirable. In the experiments determining the molecular weight of TGF-B and in the purification of the proteolytic enzyme it was necessary to omit the reducing agent 2-mercaptoethanol and SDS from the sample preparation. This was found necessary so that the protein remained in an intact state. Any other alterations that were found necessary to the standard SDS system will be described in the sections where they apply.

2.41 GEL PREPARATION

Gels containing a 4% stacking gel and from 5-25% separating gel were prepared from the following stock solutions.

Separating gel buffer: 0.4% (w/v) SDS, 1.5M Tris-HCl
buffer pH 8.7

Stacking gel buffer: 0.4% (w/v) SDS, 0.5M Tris-HCl buffer pH 6.8

Reservoir buffer: 0.5% (w/v) SDS, 0.96M Glycine, 0.125M Tris-HCl. Before use this is diluted 1:5 with water to give the working pH of 8.3.

Acrylamide stock solution: 30% (w/v) Acrylamide (BDH), 0.8% (w/v) N,N' Methylene bis Acrylamide (Sigma Chemicals)

Polymerisation Catalysts: N,N,N',N'-Tetramethylethylenediamine (TEMED) was used directly as supplied (BDH).
Ammonium persulphate (BDH) was used as a 10% solution in water and was made up fresh for each run.

2.42 RECIPES FOR GELS

% Polyacrylamide gel	15	17.5	20
separating gel (ml)	10	10	10
water (ml)	10	6.7	3.3
Acrylamide Stock(ml)	20	23.3	26.7
TEMED (ul)	30	30	30
10% Ammonium persulphate (ul)	140	140	140

2.43 BSA STANDARD, TGF-B STANDARD AND MOLECULAR WEIGHT MARKER PREPARATION

BSA standards were prepared by serial dilutions of a 25ug/ml stock solution in sample buffer. Each of the samples were boiled and to 50ul of each sample 2.5ul of bromophenol blue was added.

TGF-B isolated from porcine platelets (Peninsula Labs Europe, Merseyside) was obtained as a 0.5ug lyophilised sample. For electrophoresis work it was dissolved in 100ul sample buffer without any mercaptoethanol, and 10ul (50ng) was loaded onto each lane of the gel, following the addition of 1ul of 1% bromophenol blue.

Molecular weight markers in the range 14,300 - 66,000 Da (Sigma Chemicals) and 12,300 - 78,000 Da (BDH Electran) and the 24,000 Da trypsinogen (Sigma Chemicals) were dissolved in sample buffer containing B - mercaptoethanol and heated at 100^{0}C for 3 min, allowed to cool, followed by the addition of 1% bromophenol blue.

2.44 GEL STAINING

2.441 Coomassie Brilliant Blue R-250

A 0.1% Coomassie Brilliant Blue R-250 (Sigma Chemicals) staining solution was made by dissolving 2.4g of the dye in glacial acetic acid (BDH Chemicals), methanol (BDH Chemicals), and double distilled water in the ratio 2 : 5 : 5. The solution was filtered through Whatman No. 1 filter paper and stored in a dark bottle. The gel was stained in this solution for 1h with gentle shaking and then destained in 10% acetic acid solution overnight with a change of solution after 30 min. Protein bands appeared blue.

2.442 Amido Black Stain

The gels were placed in 5 - 10 volumes of 7% (V/V) glacial acetic acid containing 0.1% (w/v) Amido Black. The gels were left in the stain for 1h and destained in 15% acetic acid overnight. Protein bands appeared blue/black.

2.443 Silver stain

Silver staining was carried out using a Sigma Kit for polyacrylamide gels (product no. Ag-25). Firstly, gels were fixed in a 30% ethanol / 10% glacial acetic acid solution for 50 min. This was followed by 3 x 10 min washes in distilled water and 30 min in a 0.5% silver nitrate solution. This was followed by a 30 sec water rinse before being placed in a 300 ml developer solution for 8 min

containing 30 ml sodium carbonate and 0.17 ml formaldehyde. When the darkest protein bands with minimum background staining had been produced, development was stopped by placing the gel in 3% glacial acetic acid for 5 min. After this time, the gels were washed for 3 x 10 min in distilled water. A 300 ml solution containing 2.0 ml potassium ferricyanide, 4.0 ml sodium thiosulphate and 0.17 ml sodium carbonate was poured onto the gel for 30 sec to remove any background colour. The gels were then washed in distilled water. If increased sensitivity was required the gels could be subject to the same process again.

2.5 DENSITOMETER SCANS

Stained gels were scanned using a Shimadzu Dual Wavelength Flying-Spot Scanner CS-9000 Densitometer connected to a Shimadzu DR-13 control/processor unit.

In order to obtaim the optimum wavelength value for scanning a gel, a protein band was placed in the beam of the densitometer and the transmission measured between 200 and 700 nm. The optimum wavelength was seen as the peak in this spectrum measurement, being 565 nm for Commassie Blue stained, 620 nm for amido black stained and 475 nm for silver stained gels.

Due to variations in the background staining of the gel, the densitometer was zeroed at the starting point of each scan. The lanes of the gel were then scanned and the protein peaks and their areas printed. The scanning parameters were set to: beam size 0.05 x 2.0 mm; drift line 0; PKF filter 2; minimum width 0, and minimum area 100.
From scans of molecular weight markers and determination of Rf values, the position of TGF-B was determined in each sample and the amount present quantified by comparison with peak area of the BSA or the TGF-B standards.

2.6 PURIFICATION OF FRACTIONS FROM POLYACRYLAMIDE GEL

A 17.5% gel was run containing conditioned medium, under non reducing conditions. A section of the gel was stained with silver stain to determine the position of protein bands. The other section of gel was incubated with Triton-X-100, to remove SDS, and then washed five times with distilled water. The gel was sectioned up 3 fractions; a 25 kDa fraction; > 25 kDa fraction, and < 25 kDa fraction. These pieces of gel were added to 4ml of DMEM (1%) and vigorously shaken at $4^{0}C$, overnight. The gel was then removed from the DMEM solution and the solution was freeze-dried.

CHAPTER 3 THE IDENTIFICATION, QUANTIFICATION AND LOCATION OF TRANSFORMING GROWTH FACTOR-BETA IN CHICK CONDITIONED MEDIUM

3.1 INTRODUCTION

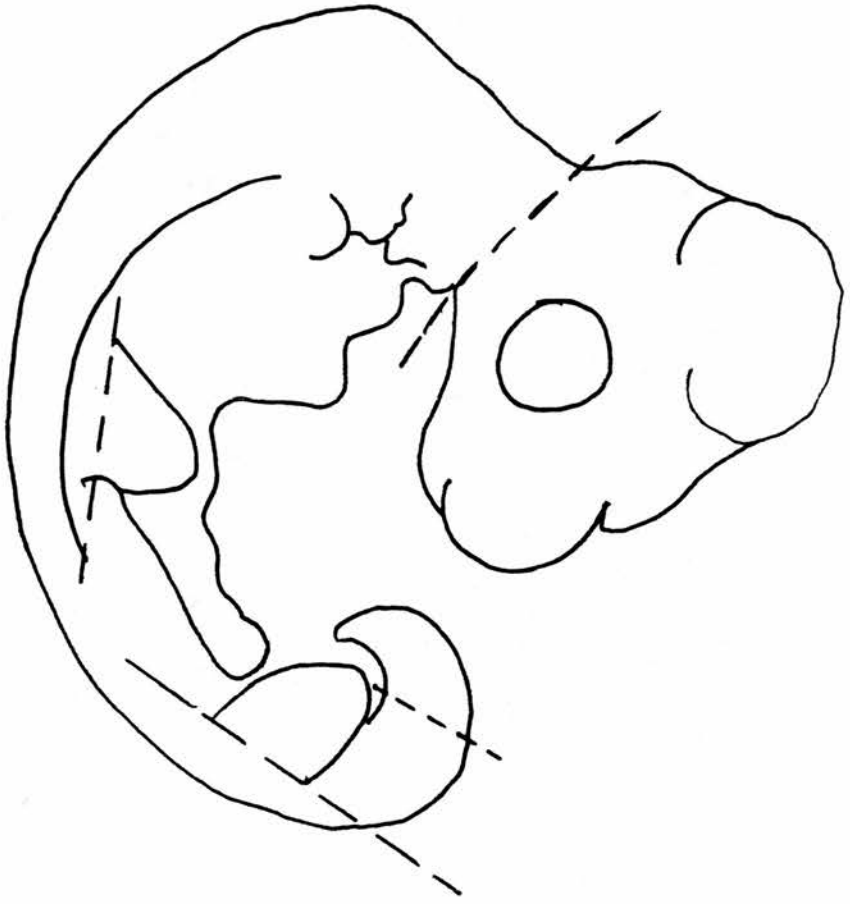
Smith et al., 1990 demonstrated that conditioned medium contains at least two different growth factors with transforming activity. One of these is heat stable and stimulates colony formation in NRK 49F cells in the presence of EGF, but not in its absence. This activity behaves like TGF-β, although this was not confirmed biochemically in the original paper.

The possible presence of TGF-B in Conditioned Medium was therefore investigated. Since TGF-β exists as a 25 kDa dimer which can be reduced to yield two 12.5 kDa proteins this property can be used to verify its presence. The technique of polyacrylamide gel electrophoresis is used to visualise this reduction.

Firstly, the number of proteins present in conditioned medium is investigated. It is not known how many proteins are secreted from chick tissue. Polyacrylamide gel electrophoresis is again used to determine the molecular weight range of the secreted proteins. Standard protein assays are used to quantify the amount of protein present in each of the regions of the chick.

Figure 3 demonstrates the regions of the chick embryo from where the secreted proteins are studied. The head, wing buds, leg buds and tail buds are used to make conditioned medium. The method of how to make conditioned medium is described in Chapter 2.

FIGURE 3 Diagramatic representation of chick embryo. Dashed lines represent regions which were used to make conditioned medium.



The amount of TGF-β present in conditioned medium can be calculated from densitometer scans of electrophoresed samples.

3.2 RESULTS

3.21 PROTEIN IDENTIFICATION

3.211 Proteins Within The 25kDa Range

The various proteins present in the medium conditioned by chick embryonic tissue were visualised using polyacrylamide gel electrophoresis. Figure 3.1 represents a sample of head conditioned medium which has been electrophoresed and demonstrates the range of proteins present. Electrophoresis was carried out in the absence of β-mercaptoethanol on 17.5% gels. Figure 3.2 contains electrophoresed samples of head, leg, wing and conditioned medium. All the samples were visualised with silver staining. A 17.5% polyacrylamide gel would separate proteins in the molecular weight range 10kDa to about 70kDa as seen in Figures 3.1 and 3.2. Head conditioned medium can clearly be seen to contain more proteins than wing and leg bud conditioned medium. Tail conditioned medium contained such a small amount of protein that they could not be seen.

3.212 Overall Protein Identification

By varying the amount of acrylamide crosslinker all the proteins present in conditioned medium could be visualised. 5, 7.5, 10, 15, 17.5, 20 and 25% polacrylamide gels were constructed. Proteins could not be visualised on the 5% gel which would separate proteins from about 150kDa to 350kDa. The proteins separated on the 7.5% gel are seen in

Figure 3.1 A 17.5% polyacrylamide gel of head conditioned medium, demonstrating the various proteins present from molecular weight 14 kDa to 70 kDa. CM represents head conditioned medium and M.W. represents the molecular weight markers. The 25 kDa protein band of conditioned medium is clearly seen.

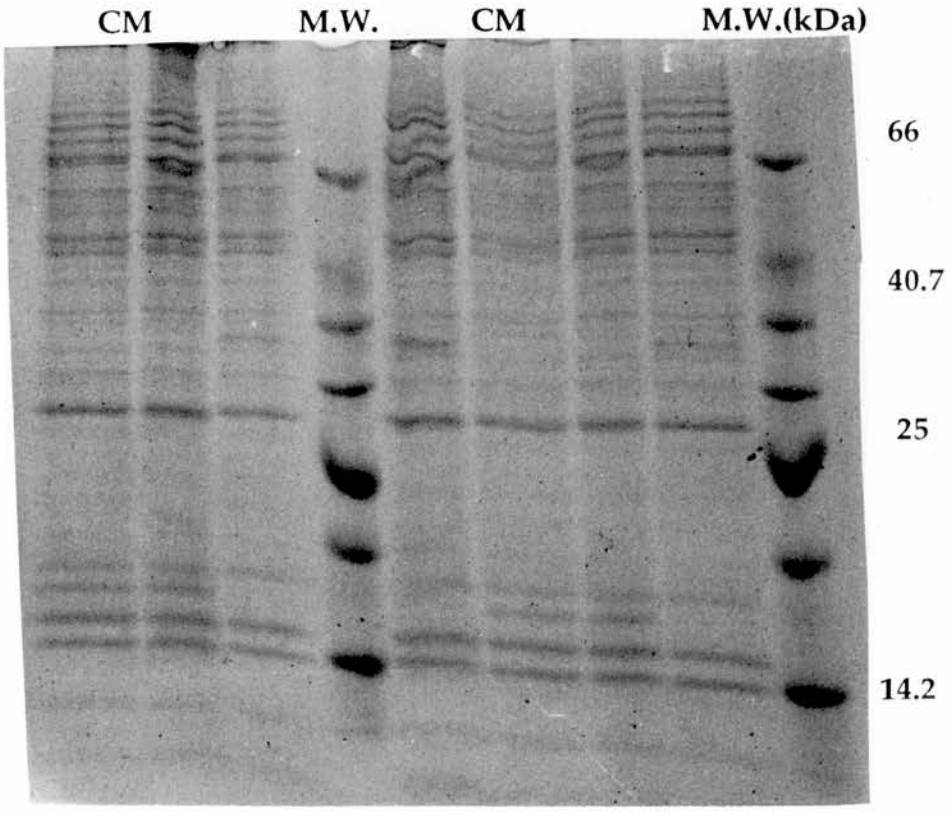

CM
M.W.
CM
M.W.(kDa)
66
40.7
25
14.2

Figure 3.2 A 17.5% polyacrylamide gel of head (HCM) , wing (WCM), and leg (LCM) conditioned medium. All samples were added at a concentration of 50 ng/50 ul. Commercially purified TGF-β (50 ng/10 ul) is also represented and shown not to be entirely pure. All the conditioned media are shown to possess a 25 kDa band which was later shown to be TGF-β.

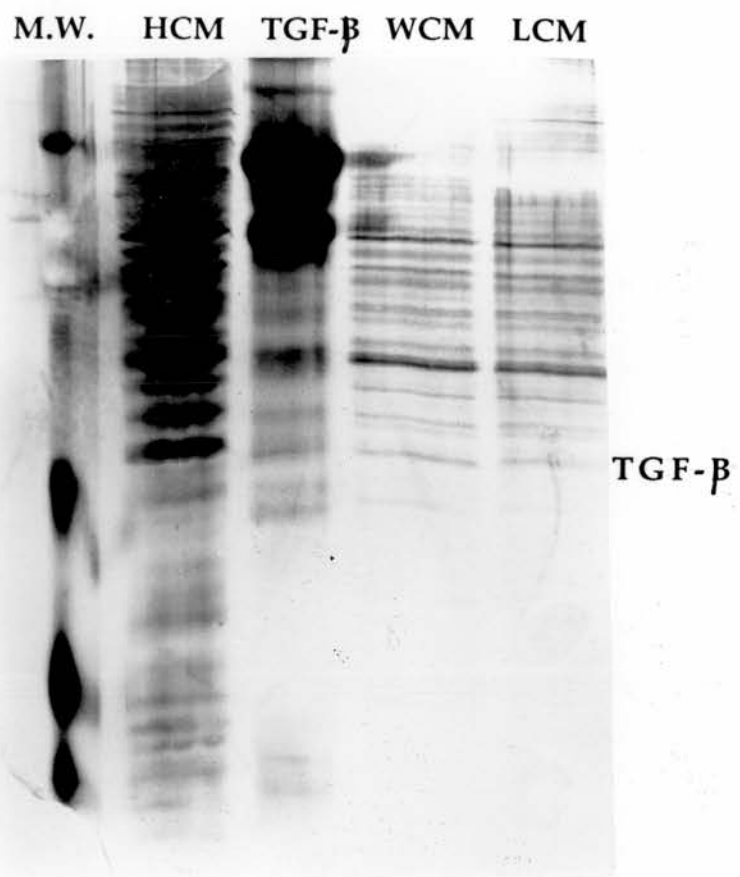

M.W.
HCM
TGF-β
WCM
LCM
TGF-β

Figure 3.3. The intensity of the bands are very faint. The 10, 15 and 17.5% gels all displayed fairly intense protein bands. These three types of gel separated proteins from 10 to 70kDa. The 20 and 25% gels which would separate proteins of molecular weight less than 10kDa contained very few faint bands.

Protein present in whole embryos (Figure 3.4) were also visualised. Whole embryos were homogenised in 100 ul of medium/buffer. However, when this was electrophoresed smearing of bands occurred indicating overloading. Dilutions had to be carried out before Figure 3.4 could be obtained

3.22 TOTAL PROTEIN CONTENT OF HEAD CONDITIONED MEDIUM.

To determine the amount of protein in various types of conditioned media a number of standards were assayed for protein amount by the method of Bradford, 1976. This assay relies on the observation that Coomassie Brilliant Blue G-250 is a brown/red colour when free in acid solution and becomes blue on binding to protein.

From absorbance measurements at 595nm of BSA standards a curve was constructed (Figure 3.5). From this curve the amount of protein present in conditioned media could be calculated.

The total amount of protein present in the head, wing, leg, and tail conditioned medium was determined. From Table 3 it was seen that the head conditioned medium contained the most protein. The

Figure 3.3 A 7.5% polyacrylamide gel of head conditioned medium (CM). Molecular weight markers (M.W.) range from 26 kDa to 116 kda. Conditioned medium is present at 20 ng/20 ul. Above molecular weight 116 kDa very few protein bands are seen. A higher concentration of conditioned medium did not reveal many more high molecular weight proteins.

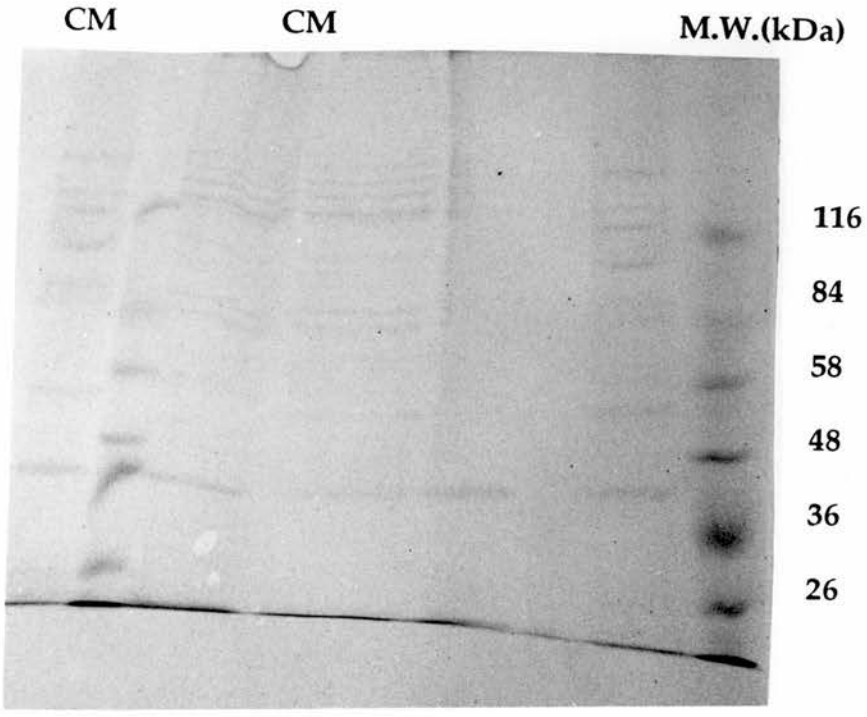

CM
CM
M.W.(kDa)
116
84
58
48
36
26

Figure 3.4 A 20% polyacrylamide gel of a whole embryo. A single embryo was homogenised and 100 ul of sample buffer was added. This sample was diluted to a concentration of 30 ng/ 100ul. There are so many proteins present that the bands are seen very close together.

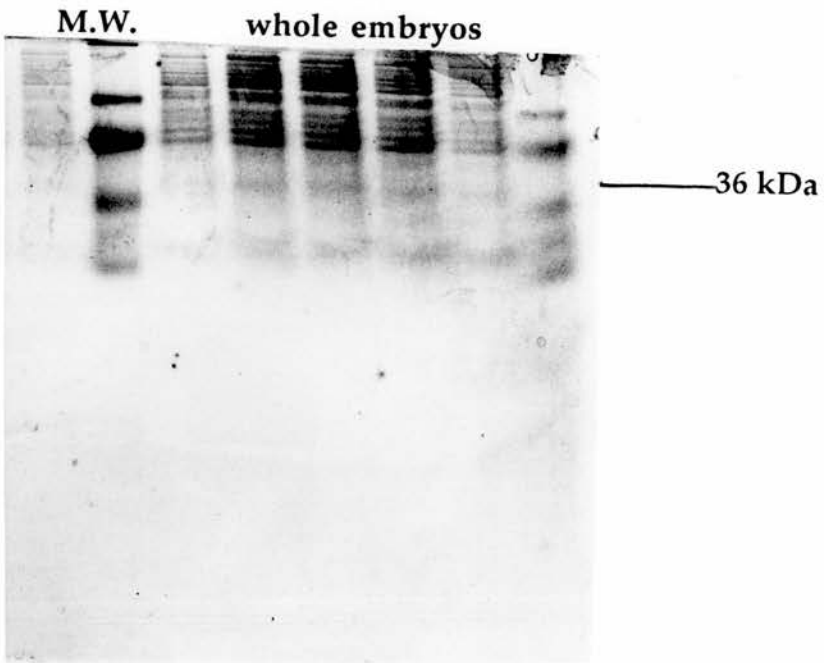

M.W.
whole embryos
36 kDa

Figure 3.5 A protein assay standard curve. By comparing the optical densities (O.D.) of BSA standards with the various conditioned media, the total amount of protein present in head, wing, leg and tail conditioned media can be calculated.

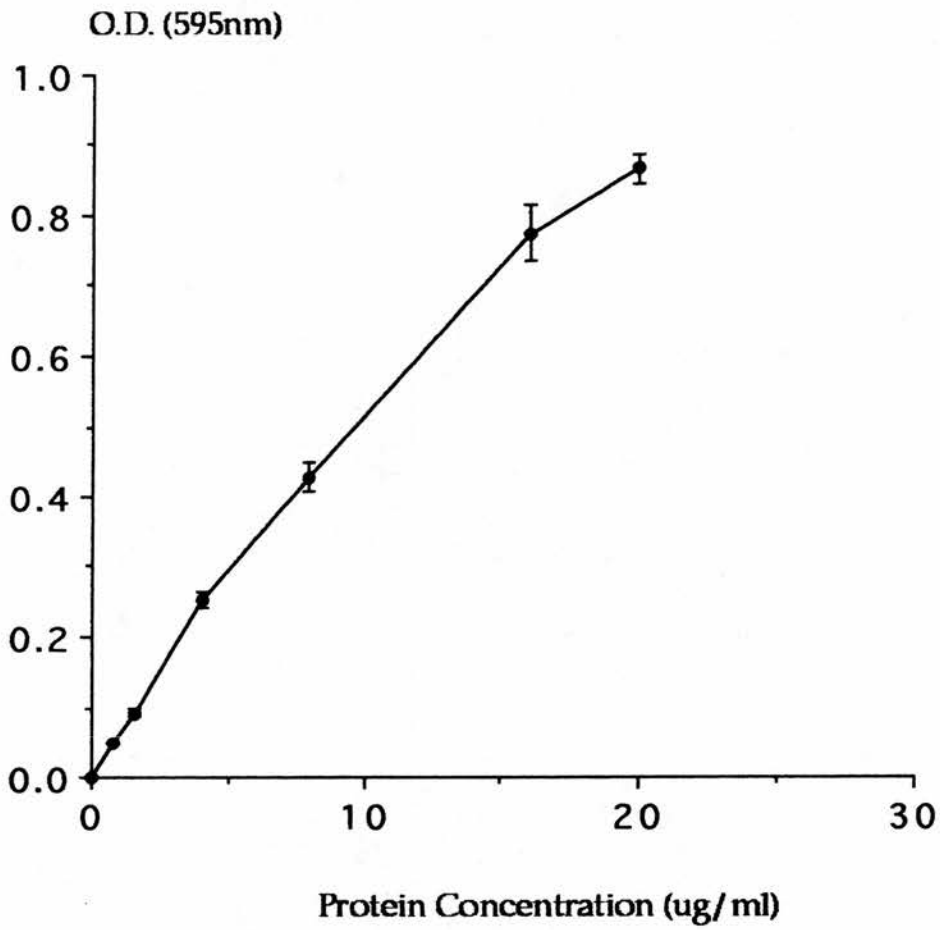

O.D. (595nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Protein Concentration (ug/ml)

Table 3 The protein amount present in head, wing, leg and tail conditioned media. Head conditioned medium contains more protein than the others. Leg conditioned medium contains more protein than wing conditioned medium probably because it develops at a faster rate.

CONDITIONED MEDIUM	PROTEIN AMOUNT (ug/ml)	RATIO
HEAD	335.0	2.8
WING	240.0	2.0
LEG	282.5	2.4
TAIL	120.0	1.0

ratio of protein from the head, wing, leg and tail was 2.8 : 2 : 2.4 : 1, respectively.

The next stage involved determining how much of the total protein present in each of the media was TGF-β. Before this could be achieved the position that TGF-β ran to on an SDS-polyacrylamide gel needed to be established.

3.23 AMOUNT OF PROTEIN PRESENT IN CHICK TISSUE

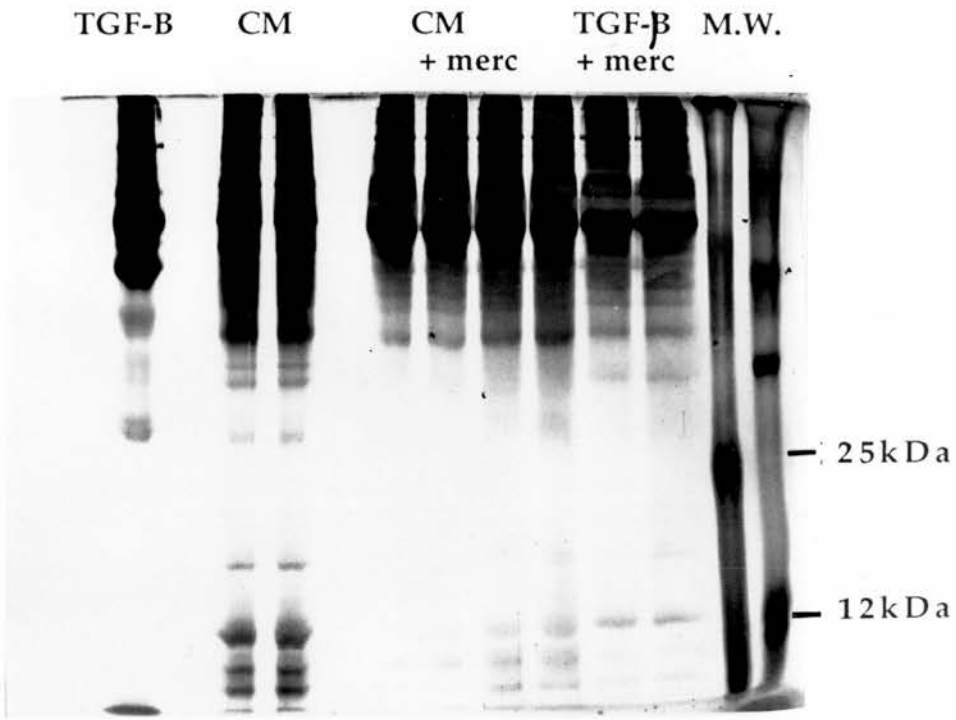
To determine the amount of protein present in the head, wing, leg and tail buds these regions were dissected from the trunk of the body of a stage 22 embryo and sonicated in 100ul of serum free medium. The amount of protein was then determined by the Bradford protein assay. The amount of protein present in head, wing, leg and tail tissue is 13190 ± 113 ug/ml, 2750 ± 32 ug/ml, 2130 ± 47 ug/ml, and 1382 ± 29 ug/ml, respectively.

3.24 TGF-β IDENTIFICATION

3.241 Determination Of The Position To Which TGF-B Runs On A Gel

Previous investigations by Derynck (1985), demonstrated that TGF-β was a 25Kd protein which under reducing conditions separated into two 12.5k Da fractions. Using commercially purified TGF-β the molecular weight was established by running on a 17.5% polyacrylamide gel molecular weight standards in a lane next to the TGF-β (Figure 3.6). The commercially purified TGF-β was shown to be 25Kd and when it was treated with the reducing agent 2-mercaptoethanol it split into 12.5Kd fractions

Figure 3.6 A 17.5% polyacrylamide gel representing the presence of TGF-β in conditioned medium. Commercially purified TGF-β was incubated with 2-mercaptoethanol (+ merc) and it is observed that the 25 kDa fraction splits and a 12.5 kDa band appears. The 25 kDa protein band present in head conditioned medium (CM) disappears in the presence of mercaptoethanol (+ merc). The presence of 12.5 kDa bands are not seen for CM + merc but these are later demonstrated to be present by densitometer analysis.


TGF-B
CM
CM
+ merc
TGF-β
+ merc
M.W.
25kDa
12kDa

(Figure 3.6). Figures 3.7 and 3.8 demonstrates the densitometer evidence of how mercaptoethanol breaks the commercially purified TGF-β into 12.5 kDa fractions. One way of detecting the presence of TGF-β in the conditioned medium was to subject the conditioned medium to reducing and non-reducing conditions and compare this with the commercially purified TGF-β subjected to the same conditions. From Figure 3.6 it can be seen that a 25Kd fraction is present in the conditioned medium and when this media was subjected to reducing conditions this fraction disappeared and a 12.5Kd fraction appeared. The densitometer scan of head conditioned medium shows this clearly (Figure 3.9).

3.242 Amount OF TGF-β Present In Conditioned Medium

The amount of TGF-β in conditioned media was determined by running the conditioned media under non-reducing conditions using a 17.5% polyacrylamide gel. Also subject to the electrophoresis were known amounts of Bovine Serum Albumin (BSA) and commercial TGF-β (Figure 3.10). Following electrophoresis the protein bands were visualized by the silver staining method rather than coomassie blue due to the lack of sensitivity of coomassie blue in detecting the proteins of conditioned media. The amount of TGF-β in conditioned media could be calculated by comparing the peak areas produced from the densitometer scans

Figure 3.7 Densitometer scan of commercially purified TGF-β. In the absence of mercaptoethanol a 25 kDa protein band is seen and in the presence of mercaptoethanol this band breaks down.

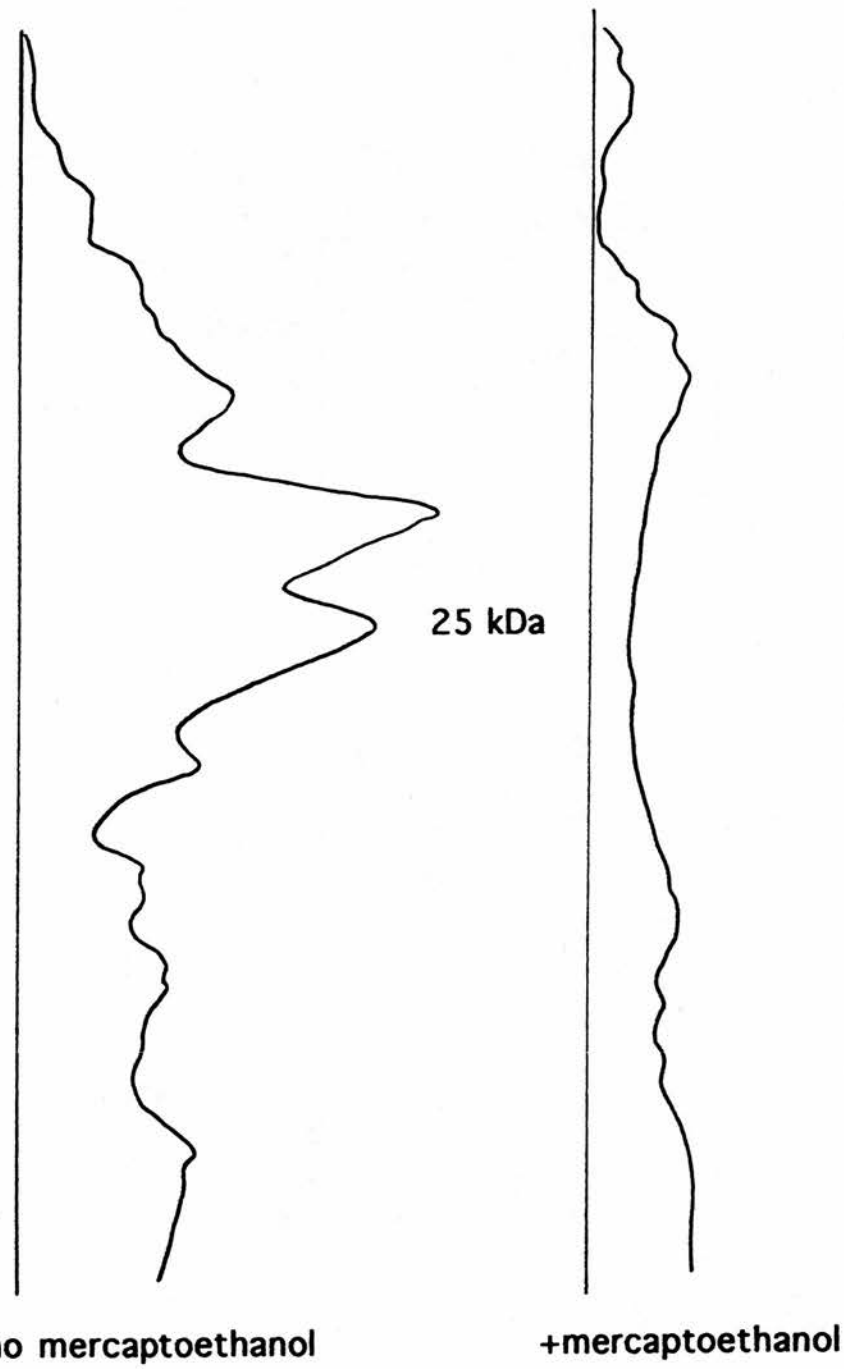

25 kDa
no mercaptoethanol
+mercaptoethanol

Figure 3.8 Densitometer scan of commercially purified TGF-β. In the absence of mercaptoethanol only a small amount of 12.5 kDa bands are seen. In the presence of mercaptoethanol the intensity of these proteins increase.

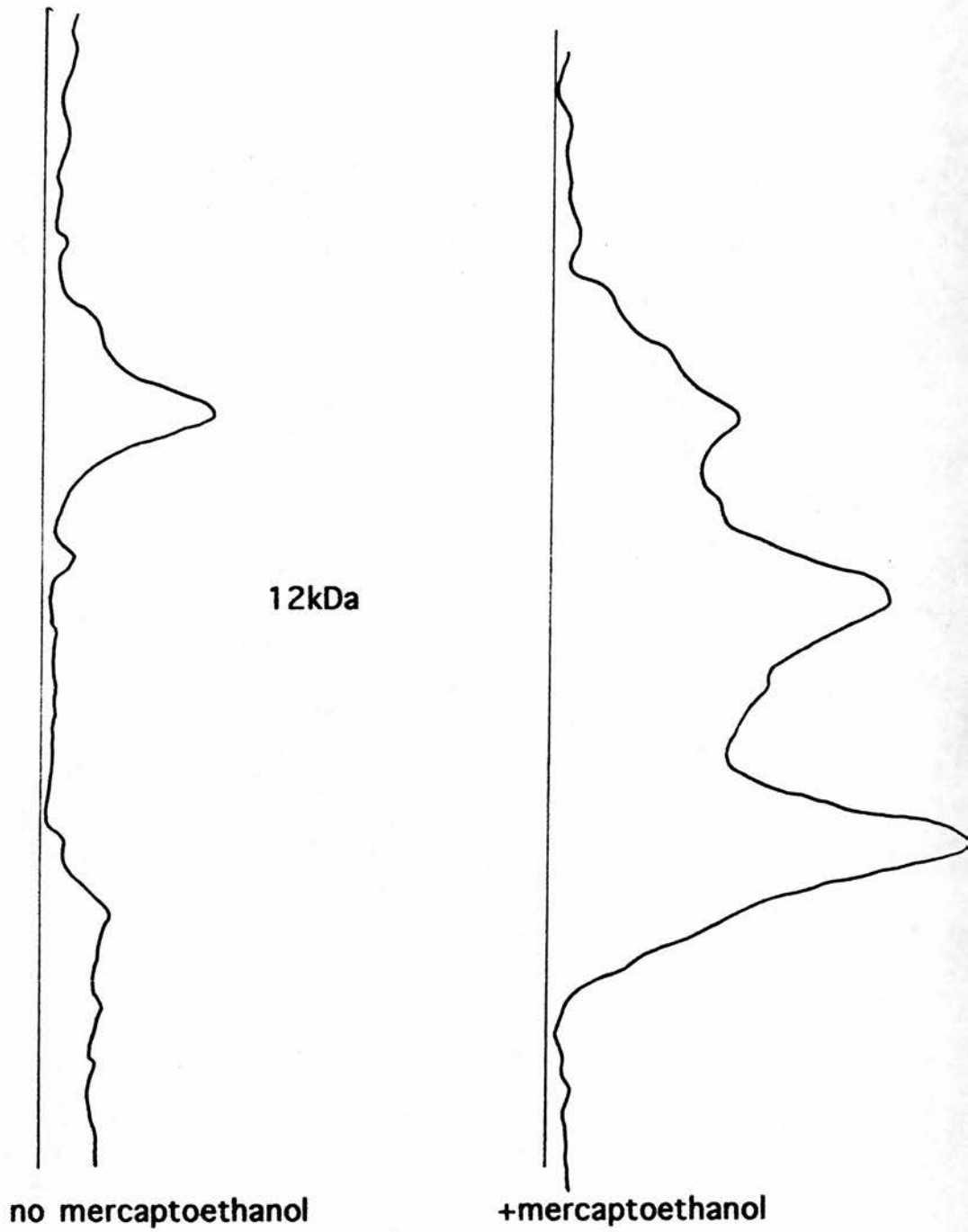

12kDa
no mercaptoethanol
+mercaptoethanol

Figure 3.9 Densitometer scan of head conditioned medium (HCM) in the absence and presence of mercaptoethanol. In the presence of mercaptoethanol the 25 kDa peak breaks down to form 12.5 kDa peaks, indicating the presence of TGF-β in conditioned medium.

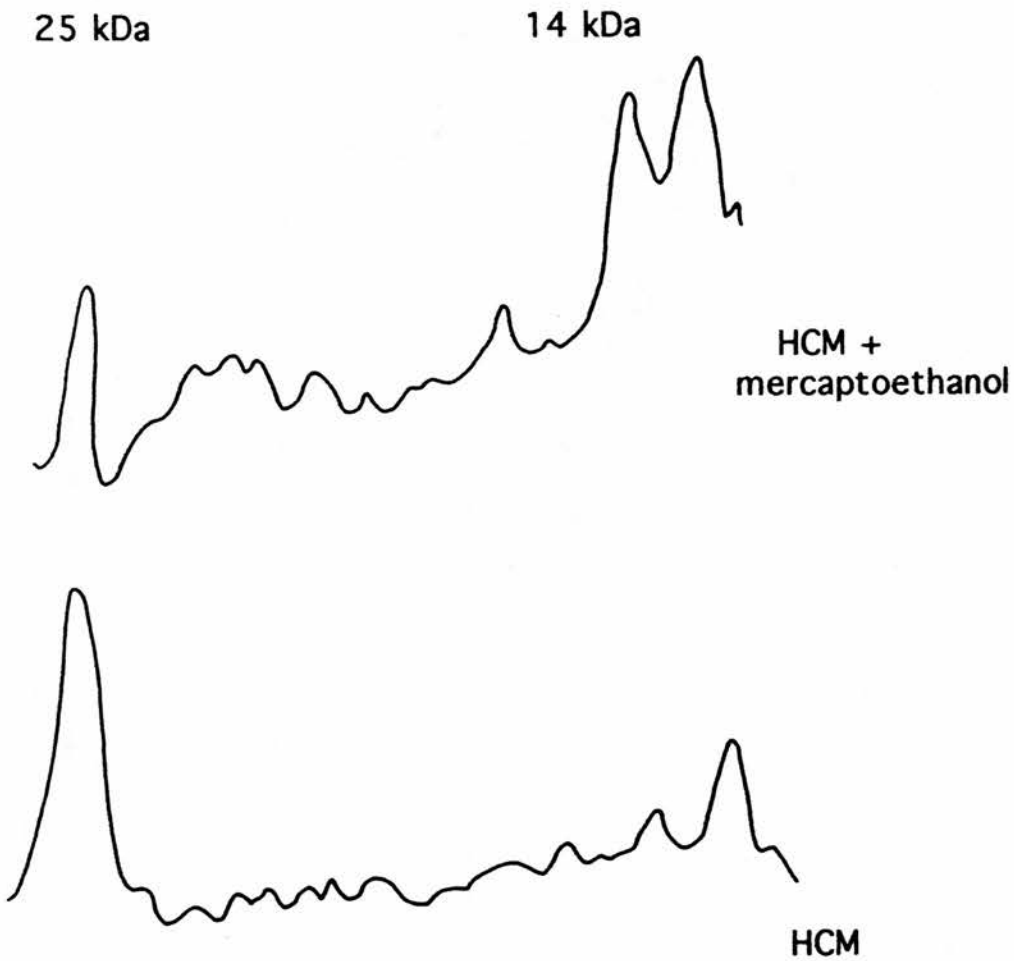
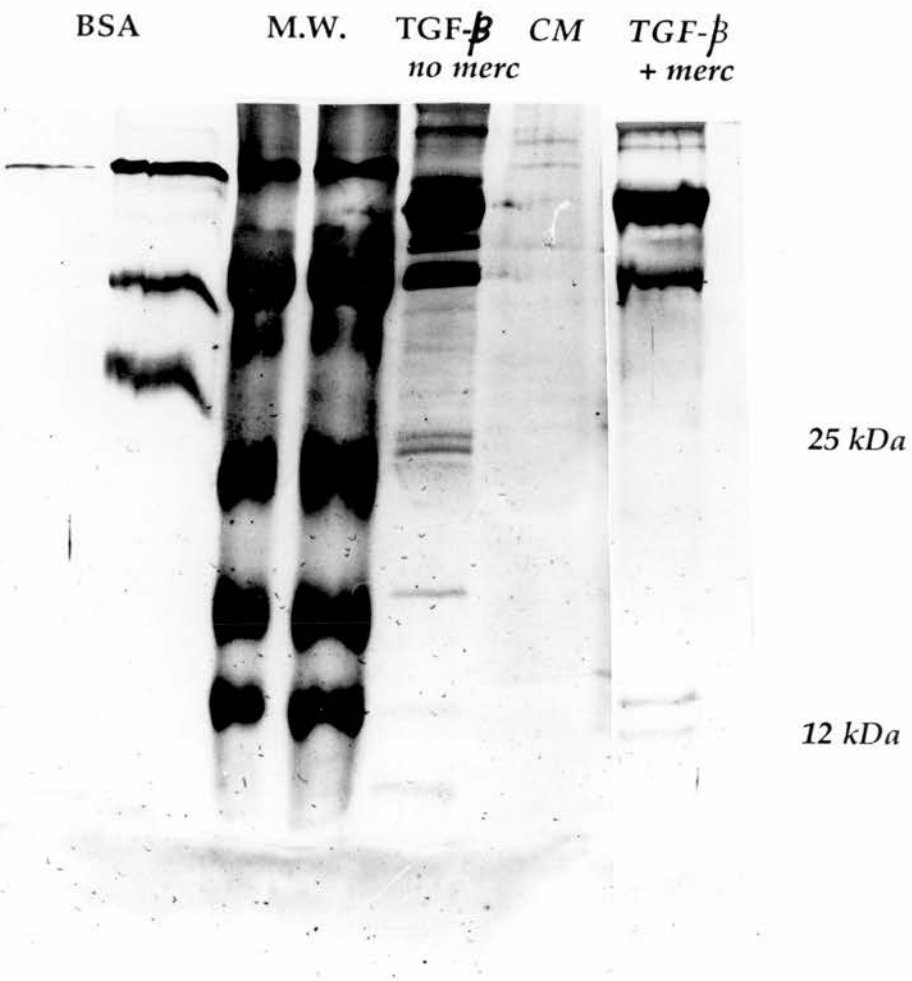

25 kDa
14 kDa
HCM +
mercaptoethanol
HCM

Figure 3.10 A 17.5% polyacrylamide gel of BSA and TGF-β. BSA present at 5 and 50 ng/50 ul were electrophoresed as was TGF-B (50 ng/10 ul). These bands were scanned by the densitometer to determine their peak areas. By comparing these scans to those of conditioned medium the amount of TGF-β could be calculated. Also shown is TGF-β in the absence and presence of mercaptoethanol. The 25 kDa band is shown to be broken down to 12.5 kDa bands in the presence of mercaptoethanol (+ merc).


BSA
M.W.
TGF-β
no merc
CM
TGF-β
+ merc
25 kDa
12 kDa

with the peak areas produced for the known amounts of BSA or commercial TGF-β (Figure 3.11). Figure 3.12 is a scan of the gel containing head, wing, leg, tail conditioned medium. The position of TGF-β in conditioned media is demonstrated. From the densitometer scans the amount of TGF-β in conditioned media was calculated (Table 3.1). The ratio of TGF-β in the head, wing, leg and tail was 5.0 : 1.4 : 1.6 : 1 respectively.

3.25 RATIO OF TGF-β IN CHICK TISSUE

When the amount of TGF-β present in conditioned medium is compared to the total protein present in the secreting tissue it is found that the ratio of TGF-β in the head, wing bud, leg bud and tail bud is 1 : 1.3 : 1.9 : 1.8, respectively.

Figure 3.11 The presence of TGF-β in conditioned media. The area of the 25 kDa peak from commercial TGF-β was calculated for a known amount of protein and from this the peak areas at 25 kDa produced by head (HCM), wing (WCM), leg (LCM) and tail (TCM) conditioned medium were converted into amounts of TGF-β present in conditioned media.

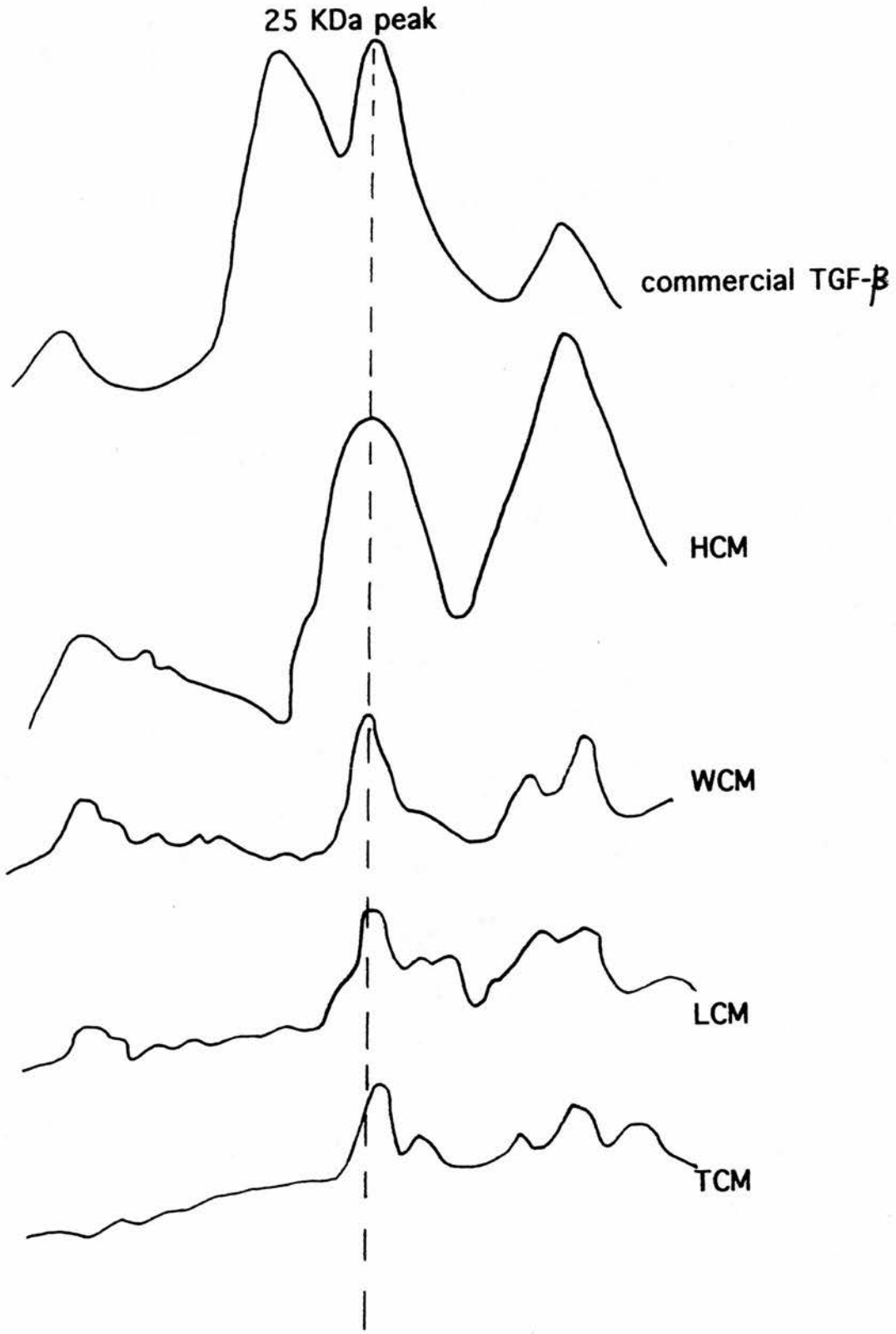

25 KDa peak
commercial TGF-β
HCM
WCM
LCM
TCM

Table 3.1 The amount of TGF-β present in conditioned media. Head conditioned medium is shown to contain more TGF-β than wing, leg and tail media.

CONDITIONED MEDIUM	TGF-β AMOUNT (ug/ml)	RATIO
HEAD	3643 ± 125	5.0
WING	996 ± 55	1.4
LEG	1167 ± 42	1.7
TAIL	702 ± 17	1.0

3.3 DISCUSSION

It was clear that conditioned medium was likely to contain a mixture of proteins, but the molecular weight range these covered was not known. Polyacrylamide gel electrophoresis identified proteins from about 10 kDa to about 200 kDa. The protein bands of molecular weight greater than 70 kDa stained very faintly, even when silver staining was used, which indicated that not many high molecular weight proteins were secreted from the embryo

Prior to the commencement of this investigation the presence of TGF-β in early chick embryo conditioned medium had not been positively identified, its activity only being inferred from the expression of TGF-β like activity on in vitro colony formation in soft agar (McLachlan et al., 1988).

Smith et al., 1990 demonstrated the presence of at least two different growth factors with transforming activity within conditioned medium. One of these was heat-stable and stimulated colony formation in NRK cells in the presence of EGF but not in its absence. This activity responded to a TGF-β like molecule. The other component was a heat-labile glycoprotein which had TGF-α like properties. Both were present from the intermediate primitive streak stage of development.

By the use of SDS-PAGE and densitometer scan analysis, a peak corresponding to TGF-β was positively identified in conditioned medium. It was identified as a 25 kDa dimer, made up of two 12.5 kDa polypeptide chains when electrophoresed on a polyacrylamide gel in reducing conditions. Initial studies by Macintyre et al., 1988 on transverse strips of stage 14 embryos did not reveal any distal-caudal distribution of TGF-β. The ratio of TGF-β in the the head, wing bud, leg and tail buds conditioned media is 5.0 : 1.4 : 1.6 : 1, respectively. However, when the amount of secreted TGF-β is compared to the total protein present in the secreting tissue, it is found that the ratio of TGF-β in the head, wing buds, leg buds and tail buds is 1 : 1.3 : 1.9 : 1.8, respectively. This indicates that more TGF-β is secreted from the head because it is a larger tissue but on a mass to volume ratio the wing, leg and tail buds contain more TGF-β. The differences in the amounts of TGF-β in the various regions is likely to be insignificant. This evidence therefore implies that areas of the chick embryo which are undergoing intense development do not contain any more TGF-β than areas developing at a slower rate. Since only a limited number of regions of the chick embryo were examined it is impossible to establish whether a spatial distribution of TGF-β occurs in the chick embryo.

It was demonstrated by Heine et al.,1987 that in the mouse embryo TGF-β was expressed in a unique pattern, both spatially and temporally. The specific pattern of histochemical staining of TGF-β in the mouse embryo appeared to correlate with specific morphogenetic and histogenetic events, particularly those involving cells and tissues of mesenchymal and mesodermal origin. Since the bulk of the vertebrate organism is composed of mesodermal cells and tissues it was apparent that TGF-β participated in some fundamental way in the basic architecture and organisation of almost the entire developing embryo.

Although this study demonstrated an important role for TGF-β in embryogenesis the study did not look at very early stages of development. Heine et al. studied embryos at 11d of gestation. Although TGF-β was found in areas such as connective tissue, cartilage and bone and it was very likely involved in the various developmental events, it was not established whether TGF-β was produced in these regions. In analyzing the developmental significance of TGF-β expression, it will not be sufficient to have determined the location and time period of TGF-β synthesis. Rather, it would also be necessary to determine the ratios of active and inactive TGF-β. The inclusion of TGF-β in an inactive complex is likely to be a very important determinant of the distribution of TGF-β activity, because it ensures that TGF-β activity is only

generated in the appropriate environment, which is in the presence of the appropriate proteases and/or low pH.

CHAPTER 4 THE IDENTIFICATION OF A NOVEL PROTEASE ACTIVITY IN CHICK CONDITIONED MEDIUM

4.1 INTRODUCTION

Proteases, the enzyme systems responsible for protein breakdown, are generally viewed as being vitally important for the development of the living organism.

Before the protease results presented here can be seen in context, it is first necessary to review the general nature of proteases and the types of identified to date, and which are associated with early development.

4.11 GENERAL NATURE OF PROTEASES

Protease is a term that originated from the 19th Century German Literature of Physiological Chemistry. The molecules themselves are presumed to have arisen in one of the earliest phases of biological evolution since even the most primitive of organisms must have required them for digestion and metabolism. Proteases are not only a physiological necessity but also a potential hazard since, if uncontrolled they can destroy the protein components of cells and tissues. Nature has therefore designed two mechanisms by which proteolytic enzymes are regulated. These are the activation of inactive precursors by limited proteolysis and the inactivation of protease

precursors by forming complexes with protein inhibitors.

The physiological importance of both these mechanisms has been demonstrated in certain disease states that are related to deficiences of functional precursors or protease inhibitors. For instance several types of familial haemophilias are due to deficiences in one or other of the plasma proteases that normally activate precursors in the blood coagulation cascade.

The basic function of proteases is the cleavage of peptide bonds. There are only a few ways in which this can be achieved. The most common mechanism is the polarization of the peptide bond by nucleophilic attack on the carbon-oxygen bond (either directly or mediated by a water molecule) assisted by the donation of a proton to the peptide with nitrogen. In proteolytic enzymes, certain amino acid residues fill the function of the nucleophiles and others act as proton donars.

Proteolytic enzymes play critical roles in the activation of many biological processes including blood coagulation and clot formation, platelet aggregation and release, fibrinolysis, complement activation, hormone production and cell division. In these activation processes the key initiation event involves limited proteolysis i.e. the conversion of inactive species to active forms by selective proteolytic cleavages involving proteases

which are normally very specific. The activation step is essentially irreversible since proteolysis is an exergonic reaction and under normal physiological conditions there are no simple biological mechanisms to repair a broken peptide bond. Such activation by limited proteolysis can occur in one step or a series of steps in which the product of each activation is itself a protease that can activate the next zymogen in the cascade. Where biological actions involve cascades, there are opportunities for large amplification effects in which the degree of amplification is largely determined by the number of steps in the cascade. Another important feature of biological events which are regulated by proteases is the possibility for modulation by certain protease inhibitors. By their ability to limit the extent, duration and site of protease action, they provide added specificity to the regulatory systems. The nomenclature used for identifying proteases is often misleading. The International Union of Biochemistry have recommended that the general term Peptidase should be used to describe any enzyme that hydrolyses peptide bonds in preference to the less acceptable but widely used term Protease. However many scientists still use the term protease Proteases may function either intracellularly or extracellularly. While all proteases are intracellular at some stage in their existence, some are synthesized for export to extracellular

spaces. Examples of extracellular proteases include the pancreatic proteases trypsin and chymotrypsin in addition to the more complex proteins of the blood coagulation and complement systems.

4.12 INTRACELLULAR PROTEASES

It is becoming clear that cellular proteolysis is a complex, highly controlled set of events that takes place in virtually all cell compartments. However little is known about the physiological substrates of cellular proteases.

Cellular proteases can range in size from about 20,000 to 800,000 daltons and therefore many of them are larger than most cellular enzymes. One reason for the synthesis of large proteinases that contain multiple subunits or domains is that these characteristics may be essential for the selectivity and regulatability of cellular proteolysis. Many large proteases have nonproteinaceous components some of which are glycoproteins or lipoproteins and some require divalent cations such as ATP. Some proteases have multicatalytic sites and cannot be easily categorized in one of the four major classes of endopeptidases.

The term protease is synonymous with peptide hydrolase and these terms include all enzymes that cleave peptide bonds (classified by the Enzyme

Commission of the International Union of Biochemistry as EC 3.4).

Proteases differ from almost all other enzymes in that their substrate specificities are extremely difficult to define and certainly do not represent an acceptable basis on which to classify the enzymes. Proteases can be subdivided into exopeptidases, whose action is directed by the amino or carboxy-terminus of the peptide, or endopeptidases, enzymes that cleave peptide bonds internally in peptides and usually cannot acc omodate the charged amino or carboxyl-termini amino acids at the active site.

Endopeptidase are classified according to their catalytic mechanisms. The Exopeptidases have been classified according to their substrate specificity.

4.121 Endopeptidases

Endopeptidases are classified according to essential catalytic residues at their active sites. There are four classes of proteinases: serine; cysteine; aspartic and metalloproteinases. The serine proteinases have a reactive serine at the active centre and it is the covalent binding of substrates to this active centre which demonstrates the catalytic mechanism of these proteinases. The cysteine proteinases contain an essential cysteine at the active centre and the aspartic proteinases

contain two aspartic residues at this centre. Metalloproteinases contain metal ions (usually zinc) at the active centre.

The class of proteinase is usually determined by the effect of proteinase inhibitors on enzyme activity. All serine proteases are inhibited by diisopropyl fluorophosphate, most by phenylmethanesulphonyl fluoride (PMSF), and some by chloromethyl ketones. The chloromethyl ketones and PMSF wil however also inhibit some of the cysteine proteinases. Low concentrations of *p*-hydroxymercuribenzoate (*p*CMB) and alkylating agents such as iodoacetate will inhibit the cysteine proteinases. Aspartic proteinases are inhibited by pepstatins (acylated pentapeptides isolated from actinomycetes). Chelating agents such as EDTA (ethylenediamine tetraacetic acid) inhibit metalloproteinases.

4.13 THE BIOCHEMICAL CHARACTERISTICS AND EVOLUTIONARY RELATIONSHIPS OF PROTEASES

In prokaryotes and eukaryotes serine proteases have been divided into two families: the chymotrypsin family and the subtilisin family. It is only the chymotrypsin family that has been found in eukaryotes however.

Much work on the evolutionary relationships of serine proteases has been done on the protease of the fibrinolytic and blood coagulation systems.

Studies carried out on the blood coagulation proteases have revealed that the large size of most of these proteases is probably as a result of gene fusion. The catalytic domains of the proteases are homologous to each other, as well as to other chymotrypsin family enzymes. The substrate specificities , however, are very different and the multiple noncatalytic domains confer different regulatory properties uppon these enzymes. Many of the plasma proteases contain pre-pro leader sequences, activation peptide domains, growth factor domains (homologous to Epidermal growth factor,EGF), Ca^{2+} binding domains, and kringle domains. Kringles are triple-looped, disulphide-crosslinked domains that may appear once or in multiple copies in one protein. They are thought to play a role in binding mediators such as membranes, other proteins, or phospholipids and in the regulation of proteolytic activity. There is a family of arginine-specific serine proteinases that are found in secretory or special granules in a number of cells and appear to have special processing functions. This group includes the gamma subunit of nerve growth factor and *B*-nerve growth factor endopeptidase.

The cysteine proteases that have been characterized fall into several evolutionary related families. The amino acid residues around the active-site cysteine (Cys 25 in papain) and an essential

histidine (His 159 in papain) have been highly conserved in the lysosomal cysteine proteases. Aspartic proteases have not been identified in prokaryotes but have been found in eukaryotes. Metallo-proteases are widely distributed in both eukaryotes and prokaryotes. Little is known about the evolutionary families of the metalloproteases because there is little amino acid data available.

4.14 PROTEASES OF THE EXTRACELLULAR MATRIX WHICH ARE ASSOCIATED WITH EARLY DEVELOPMENT

Unlike the identification and expression of growth factors the expression of proteases in development is much less studied. Evidence for proteolytic activity in early development comes from studies on the extracellular matrix.

Components of the matrix such as the basement membrane and interstitial connective tissue are involved in normal development. The way in which the extracellular matrix develops depends not only on these structural components but on the regulated expression of matrix degrading proteases and their inhibitors.

Possible roles for growth factor and protease involvement in matrix modelling during development comes from information obtained from cell and organ culture systems, or from normal or pathological processes in the adult such as tissue repair, angiogenesis and tumour invasion. In other words information is gathered from processes which mimic events during embryogenesis. The effects of oncogenes on protease production may also be relevant to understanding development since many oncogenes are modified components of normal growth factor signalling pathways.

Many proteolytic enzymes and their inhibitors are associated with the extracellular matrix. These include plasmin, plasminogen, plasminogen

activators (PA), PA inhibitors (PAIs), collagenases and cathepsins.

Plasminogen activator, a serine protease, is found intracellularly or attached to the cell surface of the matrix (Vassalli et al., 1985). Another serine protease, plasmin has been found to degrade most of the matrix components including laminin and fibronectin (Liotta et al., 1981) and also activates latent collagenase. Plasmin may therefore participate in the complete degradation of the ECM. Immunohistochemical studies have led to the discovery of an inhibitor of plasminogen activator, PAI-1. It was found to be homogeneously distributed in fibroblast and fibrosarcoma cells grown in culture dishes and closely associated with the pericellular space of endothelial cells (Levin and Santell, 1987). The interactions of plasmin, plasminogen, PA and PAIs with the ECM and with each other contribute to the site and extent of proteolysis which occurs.

Metalloproteases are also associated with the ECM. Three neutral metalloproteases have been identified : type 1 interstitial collagenase, type IV collagenase (gelatinase) and stromelysin (transin, proteoglycanase). Other metalloproteases include a 75 kDa collagenase produced by granulocytes (Hasty et al., 1986) and a 92 kDa gelatinase from macrophages and neutrophils.

Interstitial collagenase is the best characterized of the matrix-degrading metalloproteases. Human

skin collagenase is synthesized as a 54,092 Da prepro-enzyme and secreted as a 53 kDa pro-enzyme or as a glycosylated species of 57 kDa (Goldberg et al., 1986; Wilhelm et al., 1986). The final active form of collagenase is a stable 42 kDa protein.
In 1985, Chin et al., identified another metalloprotease which was capable of breaking down proteoglycans, laminin and fibronectin. This protease became known as stromelysin. Human stromelysin is synthesized as a 54 kDa molecule and undergoes various cleavages to produce a stable 45 kDa species (Wilhelm et al., 1987).
Human type IV collagenase was cloned in 1988 (Collier et al., 1988) and is a 72 kDa proenzyme. A 92 kDa type IV collagenase has also been cloned from transformed tissue culture cell (Wilhelm et al., 1989).

Other proteolytic enzymes which have been found associated with the ECM are the lysosomal proteases Cathepsin L, Cathepsin B and Cathepsin D.
Cathepsin L is a 39 kDa secreted glycoprotein with acid protease activity. It was originally identified in malignantly transformed mouse fibroblasts (Gottesman, 1978). It is also induced in fibroblasts by treatment with the tumour promoter TPA (Gottesman and Sobel, 1980) and growth factors such as PDGF and EGF (Scher et al., 1982) and FGF (Nilsen-Hamilton et al., 1980).

Cathepsin L is an acid-activatable protease with a broad substrate specificity. The role of Cathepsin L in ECM remodeling is not clear. The proenzyme and the active forms of the enzyme are actively secreted by macrophages and tumour cells but it is not yet clear whether the microenvironment is sufficiently acidic to allow proteolytic degradation by the enzyme, whose optimum pH is below 6. It is thought that an acid microenvironment exists close to the ECM or that inflammatory cells generate an environment of sufficiently low pH to allow proteolytic activity.
Cathepsin B is a lysosomal cysteine protease which is active at acidic pH. It is secreted by cancer cells as a 40 kDa proenzyme and requires a pepsin - like enzyme to produce the 25 kDa active form. Cathepsin B was first shown to degrade matrix proteins such as type 1 collagen, laminin, and proteoglycans in the early 1970s (Burleigh et al., 1974, Morrison et al., 1973, Roughley and Barrett, 1977). This enzyme is active at acidic pH. Since Cathepsin B activates latent type 1 collagenase its activity on the ECM may be amplified by its activation of metalloproteases.
Cathepsin D is an acidic protease found in the lysosomes of most cells especially phagocytic cells (Werb, 1988). Again, like the other lysosomal proteases it is secreted as a proenzyme and the 52 kDa molecule can be autoactivated at acidic pH to

give a 48 kDa species which is then broken down to 34 kDa and 14 kDa forms.

Since the medium conditioned by chick embryonic tissue displayed protein breakdown as identified by polyacrylamide gel electrophoresis and change in activity, a research programme was instituted to identify proteolytic activity within this medium. Proteolytic activity is identified using polyacrylamide gels containing known substrates of proteases as described in 4.2.
A single proteolytic activity was discovered. This enzyme was purified to investigate its nature and role.

4.2 METHODS

4.21 IDENTIFICATION AND ISOLATION OF PROTEOLYTIC ACTIVITY.

4.22 PURIFICATION OF PROTEASE

Preparative polyacrylamide gel electrophoresis, in a 15% resolving gel with a buffer system, according to Laemmli, was performed as in the case of an analytical gel , except that spacers of 3mm thickness were used.

Each gel was loaded with 3ml of head conditioned medium which had been dissolved in glycerol and water.

Electrophoresis was carried out at a constant current of 250 mA for 4.5h. After the run, a narrow vertical strip was cut from one end of the gel, the remaining part of the gel was stored at 4^0C. The strip was assayed to detect proteolytic activity according to the method of Foltmann et al. 1985. The gel strip was first treated with 2.5% Triton - X-100 for 30min.to remove the SDS and then equilibrated with 10mM Tris HCl pH 7.5 for 2h. The protease was detected by laying the strip on an agarose gel containing 1% skimmed milk powder as the substrate. After incubation for 6h at 37^0C, the proteolytic activity was detected as clear bands in a white agarose background. The section of the gel corresponding to the proteolytic activity was removed from the remaining part of the gel and finely chopped in 10mM TrisHCl pH7.5. The proteins

were eluted overnight at 4^{o}C with constant agitation.

4.23 DETERMINATION OF MOLECULAR WEIGHT

The molecular weight was determined by comparing the migration of the purified protease to that of protein standards on SDS-PAGE gels. The sample was mixed with an equal volume of sample buffer. Following electrophoresis the gel was stained with either amido black or silver nitrate.

4.24 DETERMINATION OF OPTIMUM pH

A total of 100 *ul* of the purified protease (8 *ug*/100 *ul*) was added to the assay mixtures containing 500 *ul* (0.1g/ml) of azocasein, solubilized in one of the following buffers : 0.5M acetate buffer (pH 4.0 - 5.5) ; 0.5M phosphate buffer (pH 5.5 - 8.0) ; 0.5M Tris HCl buffer (pH 7.0 - 9.0), or 0.5M bicarbonate buffer (pH 9.0 - 11.0). The reaction was terminated after an incubation of 5h at 37^{o}C by adding 500 *ul* of 20 % trichloroacetic acid and centrifuging at 12,000 x g for 5 min. The trichloroacetic acid soluble material was mixed with 150 *ul* of 6N NaOH and the absorbance was read at 375nm. Separate blanks were run for each pH step. The effect of pH on the denaturation of the protease was determined by by the azocasein assay. In this case the enzyme was incubated for 3h at room temperature in the buffers described above before incubation with the substrate at pH 7.0.

4.25 DETERMINATION OF OPTIMUM TEMPERATURE AND HEAT STABILITY

The optimum temperature for enzyme activity was determined by the azocasein assay at pH 7.0 in 50mM tris hydrochloride buffer carried out at 25, 30, 37,45 and 60^{0}C. The temperature stability was measured as described above, except that the protease was incubated at various temperatures for 30 min before enzyme activity was measured.

4.26 EFFECT OF INHIBITORS ON THE PROTEOLYTIC ACTIVITY

Protease activity was measured in the presence of various known protease inhibitors: EDTA; phenylmethylsulphonyl fluoride (PMSF); SDS; 2-mercaptoethanol; dithiothreitol (DTT); aprotinin; pepstatin; $MgCl_2$; CaCl; 1,10 Phenanthroline; L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane or E-64. The enzyme was preincubated for 30min at 37^{o}C in the presence of the inhibitor. The substrate azocasein (500*u*l, 0.1g/ml) was then added to the mixture and this was incubated for a further 5h at 37^{o}C. The activity was measured and compared to that of the control by the azocasein assay.

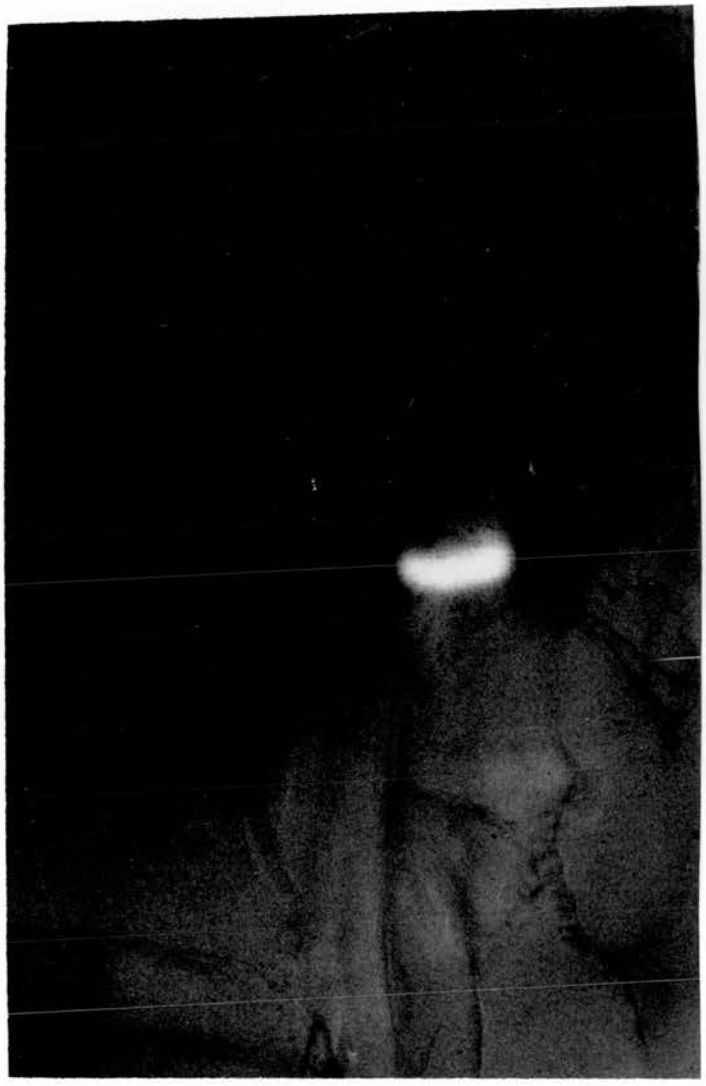
4.3 RESULTS

4.31 IDENTIFICATION OF PROTEASE

Medium conditioned by chick embryonic tissue has been demonstrated to possess a wide variety of proteins with a range of molecular weights (Figure 3). Bovine serum albumin can be seen around 66,000 daltons and TGF-B at 25,000.

The protease was initially detected by visualisation on a polyacrylamide gel with the substrate gelatin incorporated. Gels were incubated at $37^{0}C$ and over a range pH values. A single proteolytic activity was observed upon amido black staining only when the gel was incubated at pH7.5. The activity was detected as a clear band against a black background(Figure 4). This single activity was present in the embryo from first to the eighth day of incubation at $37^{0}C$. Table 4 shows the amount of protease present from day 4 to day 8. Earlier stages were impossible to use for purification due to the tiny amount of material available. For purification of the protease from the gel, gelatin was not incorporated; instead the protease was detected by laying a strip of the gel containing the electrophoresed sample on an agarose plate with 1%

Figure 4 A single proteolytic activity present in conditioned medium. A general proteolytic substrate, gelatin, was incorporated into a polyacrylamide gel prior to the addition of conditioned medium. Following eletrophoresis the gel was stained with a 1% amido black solution. The protease digested the gelatin and showed up as white band against a black background.



40.7 KDa
PROTEASE

skimmed milk incorporated as the substrate. The position of the protease was located since the protease digests the milk substrate and a clear band is observed.

To assess the temporal changes in protease expression the changes in the amount of protease produced needed to be monitored. This can be achieved by preparing head conditioned medium from several embryo stages, calculating the total amount of protein present at each stage, purifying the protease from each stage and calculating the percentage of protease present as a whole. Once the samples were purified they were weighed using an analytical balance and the amount of protease was determined using the Bradford protein determination assay.

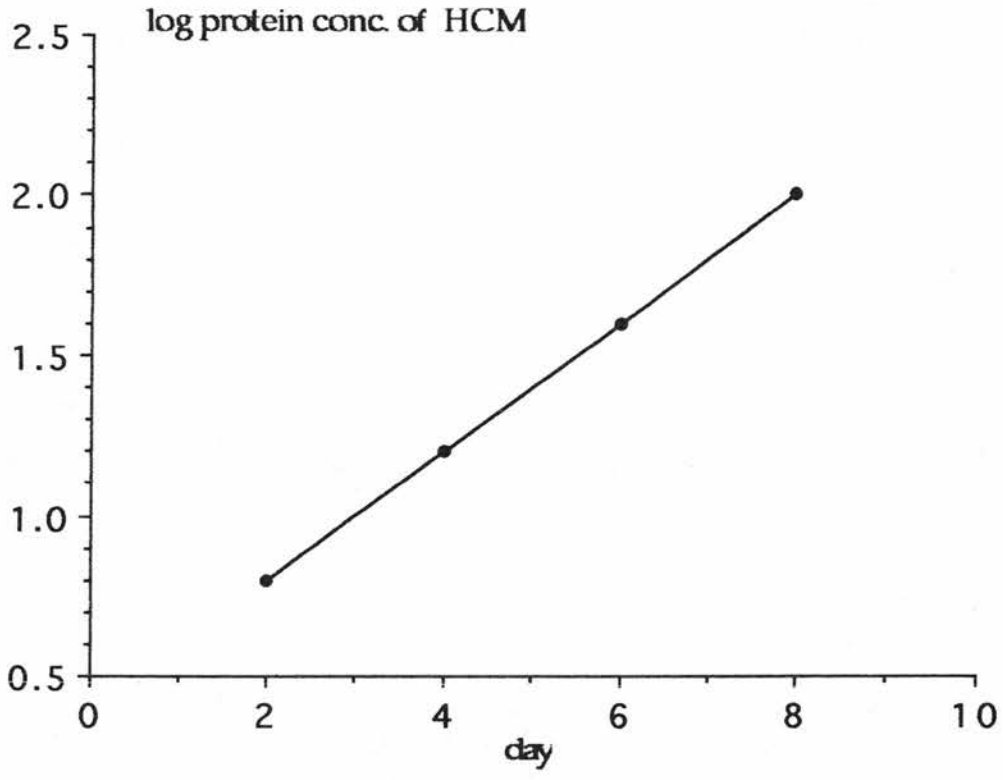
Protein loss was not calculated but since all samples were subjected to the same procedure it can be assumed that the protein loss is consistent in all samples. From Table 4 it can be seen that as the embryonic stage increases the amount of protease present also increases. From the results it can be seen that a semi-logarithmic plot of total protein concentration versus time produces a straight line (Figure 4.1)

This indicates that the total protein amount of head conditioned medium increases exponentially.

Table 4 The amount of protease in conditioned medium. The amount of protease increases from day 4 embryos to day 8. As a percentage of the total amount of protein present at each of these stages, the amount of protease remains relatively constant.

stage (day)	protein conc of purified protease (ug/ml)	protein conc. of HCM (ug/ml)	protease as % of total conc.
4	0.32	13.75	2.3 ± 0.59
6	1.15	37.2	3.1 ± 0.55
8	3.74	114.1	3.3 ± 0.18

Figure 4.1 Log total protein concentration of head conditioned medium from embryos which are at stages 2 to 8 days of embryonic development.


log protein conc. of HCM
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
10
day

This is probably due to the increase in the amount of material as the stage of the embryo increases. The expression of the protease does not follow a similar exponential path. The protease does not seem to show any marked increase in amount between 4 and 8 days of embryonic development.

4.32 CHARACTERISATION OF THE PROTEASE

4.321 MOLECULAR WEIGHT DETERMINATION

When the purified protease from each of the different stages of development were subject to electrophoresis on a 15% polyacrylamide gel a single protein band was found at 40.7KD as visualised by silver staining (Figure 4.2). The protease was not broken down by the reducing agents Dithiothreitol and β-mercaptoethanol, which would split any disulphide bridges, indicating that the protease exists as a single polypeptide chain.

4.322 OPTIMUM PH DETERMINATION

The optimum pH at which the enzyme worked as determined by the azocasein assay was found to be pH7.5 (Figure 4.3). The protease was active over

Figure 4.2 A 40.7 kDa purified protease from head conditoned medium. The molecular weight markers (M.W.) range from 14.2 to 66 kDa. Trypsinogen (TRYP) is also observed with a molecular weight of 24 kDa.

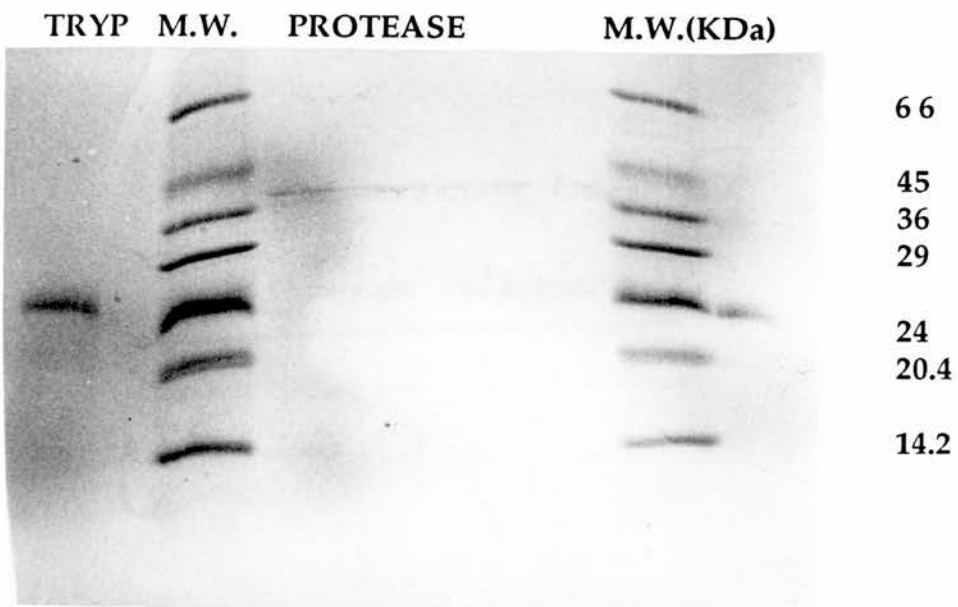
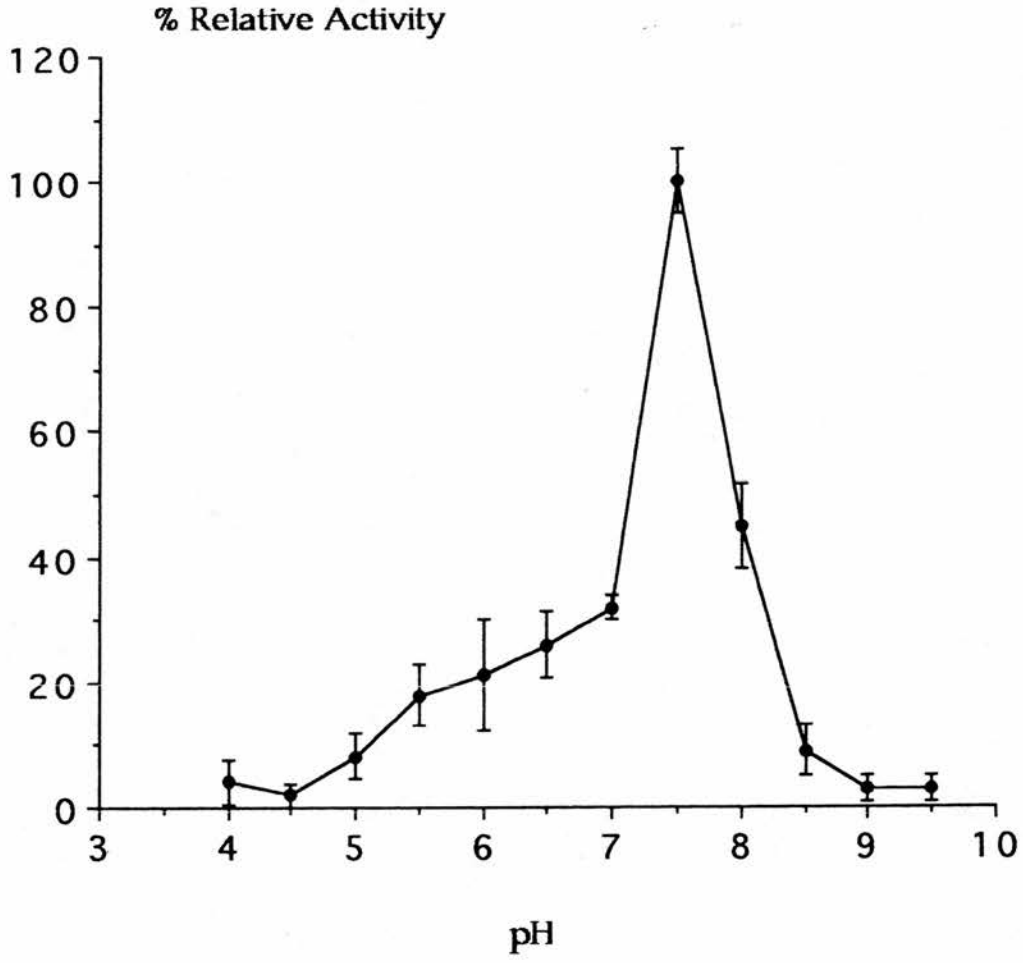

TRYP
M.W.
PROTEASE
M.W.(KDa)
66
45
36
29
24
20.4
14.2

Figure 4.3 Optimium pH of 7.5 for proteolytic activity of the enzyme isolated from conditioned medium. The activity was assayed for by using the substrate azocasein and detecting any change spectrophotometrically.


% Relative Activity
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH

the pH range 7.0 to 8.5. To assess the pH at which the enzyme was denatured the protease was preincubated at various pHs for 3h at room temperature and then mixed with the substrate azocasein. From Figure 4.4 the only pHs which led to denaturation of enzyme activity were pH 4, 4.5, 5 and 9.5 i.e. extremes of pH. The enzyme showed no activity towards the substrate at these pH values. The protease therefore functioned at physiological pH. The enzyme was still active when left at room temperature for several hours indicating that it is very stable.

4.323 OPTIMUM TEMPERATURE DETERMINATION

The optimum temperature at which the enzyme worked was again determined by the azocasein assay and the assay was carried out at pH7.5 (Figure 4.5). The protease was added to the substrate and they were incubated at 0, 15, 25, 37, 45 and 60^{o}C. The optimum temperature was found to be 37^{o}C. From 0 to 25^{o}C there was virtually no activity and from 45^{o}C the activity was inhibited.

4.33 EFFECT OF INHIBITORS ON PROTEASE ACTIVITY.

Table 4.2 shows the effect of various inhibitors on the activity of the protease. The blank is set

Figure 4.4 pH at which the proteolytic activity was inhibited. Extremes of pH (less than pH 5.0 and greater than pH 9) resulted in inhibition of the proteolytic activity.

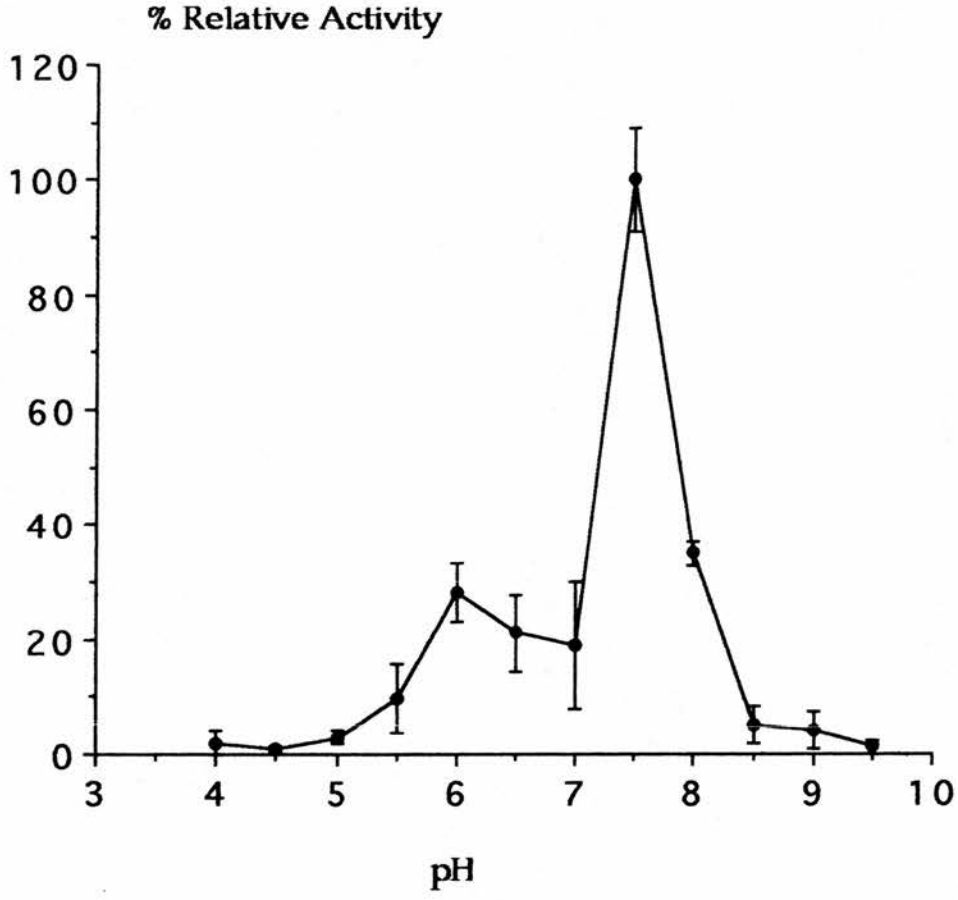
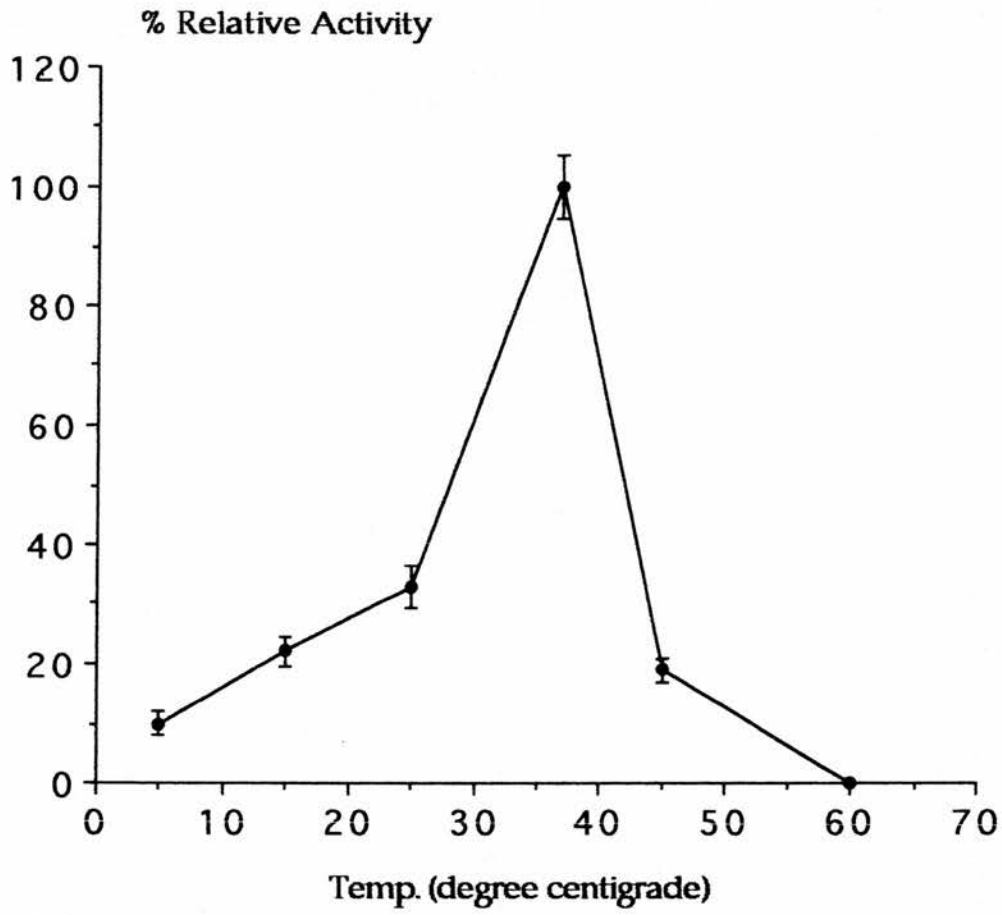

% Relative Activity
120
100
80
60
40
20
0
3
4
5
6
7
8
9
10
pH

Figure 4.5 Optimum temperature for proteolytic activity. The enzyme was active from less than 5^0C to about 50^0C. The optimum temperature for activity was 37^0C. The activity was measured by the ability of the enzyme to digest azocasein and the change was measured spectrophotometrically. The activity was inhibited from about 45^0C.


% Relative Activity
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Temp. (degree centigrade)

to include the protease operating without any inhibition and its activity is taken as 100%. From Table 4.1 and Table 4,2 it can be seen that EDTA, iodoacetate and $CaCl_2$ inhibited the activity of the enzyme.

EDTA is an inhibitor of metalloproteases. Another inhibitor of metalloproteases, 1, 10, phenanthroline did not markedly reduce the activity of the protease. When the protease was incubated with iodoacetate the amount of activity dropped by almost 40% which strongly indicated a cysteine protease. However, when L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, (E-64), which is an irreversible inhibitor of cysteine proteases, was incubated with the enzyme virtually no activity was lost.There was also almost no loss in activity when PMSF or Aprotinin were used as inhibitors indicating that the protease did not belong to the serine class, and since Pepstatin had no effect on activity, it could be concluded that the enzyme did not contain an aspartic residue at the active centre.

Table 4.2 represents the other factors which were used to determine protease activity. SDS did not appear to affect the activity even although it would bind to the protein and distort the shape. Mercaptoethanol and DTT resulted in an increase in

Table 4.1 Effect of inhibitors on proteolytic activity. Values are referred to as relative activity,compared to value in absence of inhibitors (considered as 100% activity). Iodoacetate and EDTA are the only inhibitors which produce any marked reduction in proteolytic activity.

Inhibitor	Concentration (mM)	Nature	% Relative Activity
EDTA	20	Inhibits metalloproteases	83 ± 4.2
1, 10 Phen anthroline	10	Inhibits metalloproteases	98 ± 2.2
Iodoacetate	10	Inhibits cysteine proteases	63 ± 3.9
E-64	10	Inhibits cysteine proteases	97 ± 2.8
PMSF	10	Inhibits serine proteases	95 ± 2.9
Aprotinin	2	Inhibits serine proteases	95 ± 2.8
Pepstatin	2	Inhibits aspartic proteases	98 ± 3.0

Table 4.2 Response of protease activity to various factors. Values are referred to as relative activity, compared to value in absence of inhibitors (considered as 100% activity). DTT, mercaptoethanol and $MgCl_2$ all lead to an increase in activity and $CaCl_2$ leads to a decrease.

Factor	Concentration (mM)	Nature	% Relative Activity
SDS	20	Distorts protein shape	99 ± 7.0
DTT	20	Breaks S-S bonds	112 ± 3.6
Mercapto ethanol	20	Breaks S-S bonds	116 ± 1.4
$MgCl_2$	2	Required by particular protease	105 ± 5.3
CaCl2	2	Required by particular protease	78 ± 6.9

activity. $MgCl_2$ did not appear to affect the activity.

4.4 DISCUSSION

During the course of work which involved identifying TGF-β, it was suspected, due to the visual breakdown of proteins as observed during polyacrylamide gel electrophoresis, that proteolytic action might be taking place within the conditioned medium.

Using gelatin, a general proteolytic substrate incorporated into a polyacrylamide gel, a single proteolytic activity was identified. This enzyme was active under physiological conditions and appeared to exist as a single polypeptide chain of molecular weight 40.7 kDa after one dimensional gel electrophoresis and isoelectric focussing.

It seems very likely that inhibitors are very important in the control of proteases. It is also possible that changes in protease activities in cells are due to changes in the inhibitor rather than the protease. Any attempt to understand the biological role for a proteolytic enzyme must take into account the possible existence of inhibitors. Inhibitors can therefore be used to determine what type of protease is present within the developing embryo and may give an insight into how the protease functions.

EDTA decreased the activity of the protease and this therefore suggested that the enzyme was of the metallo protease class. EDTA is also an excellent chelator of Ca^{2+}. This ion is required by several proteases for activity, including those of the

cysteine class. Another inhibitor of metalloproteases, 1, 10, phenanthroline did not reduce the activity of the protease markedly. This would indicate that EDTA chelated Ca^{2+} and that it was this ion which was required by the protease for activity. Since iodoacetate reduced the activity of the protease this indicated a cysteine protease. However, when E-64 was incubated with the enzyme virtually no activity was lost. E-64 is a non-competitive irreversible inhibitor of cysteine proteases and is insensitive to pH between 5 and 9. E-64 is specific for the active site of the cysteine protease whereas iodoacetate will bind to any structural S-H group. Another indication that the protease could be of the cysteine class was when the protease was incubated with $CaCl_2$. Some cysteine proteases require Ca^{2+} for activity but will undergo autolysis in the presence of a higher than normal calcium concentration. This was the effect observed when $CaCl_2$ was added to the protease.

There was almost no loss in activity when PMSF or Aprotinin were used as inhibitors indicating that the protease did not belong to the serine class, and since Pepstatin had no effect on activity, it could be concluded that the enzyme did not contain an aspartic residue at the active centre.

SDS did not appear to affect the activity even although it would bind to the protein and distort the shape. SDS cannot therefore affect the activity

of the enzyme even if it can alter the shape. This could be because the active centre is not on the surface of the molecule. Mercaptoethanol and DTT resulted in an increase in activity probably because they would break disulphide bridges and make the active centre more acessible to the substrate. $MgCl_2$ did not appear to affect the activity.

The protease therefore does not fit into any of the four main classes of proteases. Since the protease is produced by the early embryo it is thought that it may play a role in developmental events.

CHAPTER 5 THE EFFECT OF HEAT TREATMENT AND THE NOVEL PROTEASE ON TGF-β LATENCY

5.1 INTRODUCTION

This chapter investigates the activation of TGF-β from its latent form. The role the proteolytic enzyme isolated from conditioned medium may have on TGF-β processing was of particular interest.

Latency is observed in many growth factors and proteins. Proteolytic enzymes play important roles in the activation of many inactive species to active ones by selective cleavages that are normally very specific. For instance, FGF is initially synthesised as a 155 amino acid precursor molecule (Jaye et al., 1986) before proteolytic cleavage converts it into its active form. Latency is also observed for proteolytic factors. Cathepsin D, an aspartyl protease found in lysosomes, is synthesised as a 52 kDa promolecule, and is autoactivated at acidic pH. Latency in proteins seems essential so that the active, intact molecule can reach its site of action.
For a review on latent TGF-β refer to chapter 1. Below is an account of proteolytic involvement in the activation of latent TGF-β.

5.11 EVIDENCE OF PROTEOLYTIC INVOLVEMENT IN THE ACTIVATION OF TGF-β

A latent TGF-β complex is released by platelets when they are stimulated to degranulate by thrombin (Wakefield et al., 1988). Latent TGF-β is found in many tissues and released by many cells in culture

(Roberts et al.,1982 ; Lawrence et al., 1984). In its latent form TGF-β may be buried within the complex because it is not recognised by anti-TGF-β1 antibody.

The complex has been proposed to be a delivery system for TGF-β.

The latent TGF-β is activated by acid environments and by proteolytic action as occurs during blood clotting, during wound healing or foetal development.

TGF-β may be activated by proteases released into the wound area by cells that had been stimulated to proliferate by growth factors such as PDGF, FGF, and EGF.

Both serine and thiol proteases are released in response to stimulation by these growth factors. Plasmin, a neutral serine protease, which is most commonly associated with the proteolytic breakdown of fibrin clots (Astrup, 1975), and an other serine protease, plasminogen activator (PA), degrade extracellular matrix proteins and therefore play a role in tumour invasion, ovulation and matrix remodeling during development (Balian et al.,1979 ; O'Grady et al., 1981).

The Lysosomal protease Cathepsin D is proposed to be involved in the release of active growth factors from precursors (Lyons et al.,1988).

Lyons et al., 1988 suggested that cell-associated proteases were likely candidates in the activation of latent TGF-β. Plasmin and Cathepsin D were found

to generate 25kDa bands in fibroblast conditioned medium. These bands corresponded to the active form of TGF-B as demonstrated by immunoprecipitation analysis of radiolabeled cell-conditioned medium. Elastase and thrombin and other cell-associated proteases did not produce 25kDa bands in the medium. Plasmin was found by Lyons to activate the same pool of latent TGF-β as that activated at pH 4.5. Such mild acid conditions would not be expected to cleave the Arg-Arg site that must be cleaved for TGF-β processing (Derynck et al., 1985). Therefore, plasmin activation is probably mediated at another site. The possibility that plasmin functions as a physiological regulator of TGF-β was supported by the observations which showed TGF-β as a regulator of extracellular PA activity. TGF-β enhanced the production of endothelial-type PA inhibitor (Laiho et al., 1986) and decreased the mRNA levels and extracellular activity of urokinase- type PA in cultured lung fibroblasts (Keski-Oja et al., 1988). This was thought to provide a negative feedback control mechanism.

The pathway proposed for the mechanism is that Cell-derived plasminogen activators convert plasminogen to plasmin which in turn activate TGF-β, allowing binding to specific cell surface receptors. Among other subsequent biological effects, TGF-β increased the production the production of endothelial-type PA inhibitor and

decreased PA levels resulting in lower PA activity, less plasmin production, and diminished activation of TGF-β. Proof of this mechanism would require purification of the latent form of TGF-β as well as examination of cell membrane and extracellular matrix preparations for TGF-β activating capability followed by purification of molecules responsible for such activity.

Since the presence of TGF-β has been confirmed in conditioned medium (Chapter 3) and a latent form has been reported in many tissues (Lawrence et al.,1985), the presence of this inactive molecule was sought. The technique of Densitomet ry which is described in Chapter 2, is used to determine if the amount of TGF-β increases upon heat treatment and if higher molecular weight complexes are broken down.

Fractions of conditioned medium which did not contain the TGF-β band were purified from polyacrylamide gels. All protein bands above the 25kDa band were heat treated. These were then assayed for AIG activity using a microwell assay (described in Chapter 2) to determine if TGF-β had indeed been formed due to the heat treatment.

The proteolytic activity, characterised in Chapter 4 was incubated with the high molecular weight fractions to determine if this is the enzyme *invivo* which activates TGF-β. This proteolytic activity

occurs in the same regions of the embryo as TGF-β and at the same stages of development.

5.2 RESULTS

5.21 EFFECT OF HEAT TREATMENT ON CONDITIONED MEDIUM

5.211 DENSITOMETER ANALYSIS

Various polyacrylamide gels were run containing head conditioned medium subjected to heat treatment and reducing conditions. These gels were then scanned using the densitometer to determine if heat treatment increased the amount of TGF-β present and decreased the higher molecular weight bands.
In the absence of reducing agents head conditioned medium which was heat treated contained more TGF-β (Figure 5). When conditioned medium was reduced to yield the 12.5kDa forms and then heat treated the 25 kDa form reappeared indicating that it was formed from a higher molecular weight complex. Heat treatment decreased the amount of proteins in the molecular weight range 60-70 kDa (Figure 5.1) and increased lower molecular weight bands (Figure 5.2).

5.212 MICROWELL ANALYSIS

Medium conditioned by the presence of chick embryonic tissue showed the ability to initiate anchorage independent growth (AIG) in NRK cells. The addition of EGF enhanced this effect (Table 5).

Figure 5 Densitometer scans of heat-treated and non heat-treated conditioned medium. When conditioned medium was heat-treated the TGF-β band (25 kDa) increased in amount.

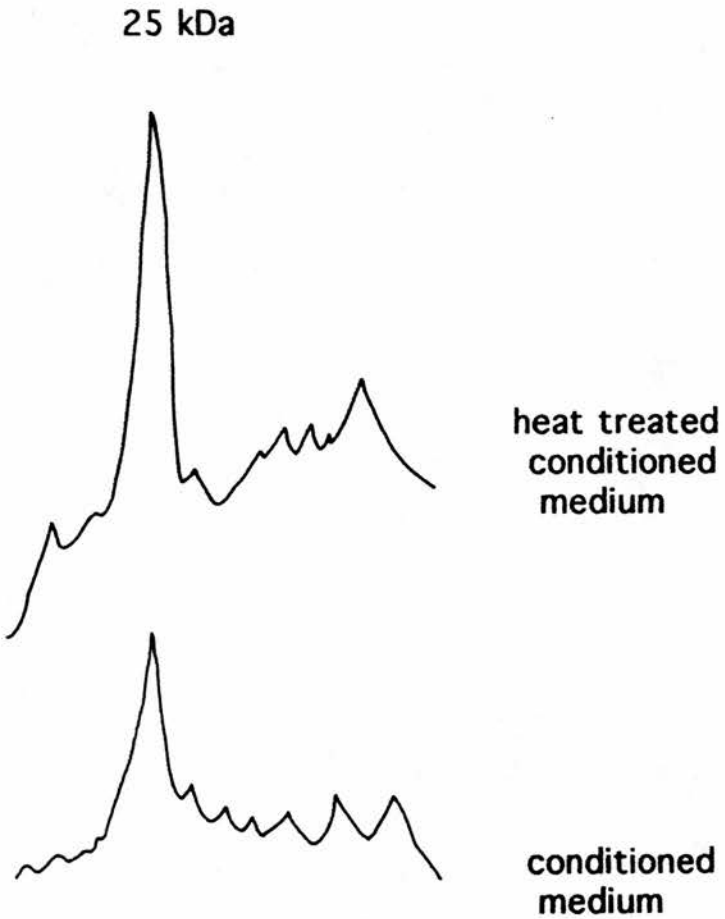

25 kDa
heat treated
conditioned
medium
conditioned
medium

Figure 5.1 Densitometer scans of high molecular proteins of conditioned medium. When conditioned medium was heat-treated proteins with molecular weights of about 66 kDa were broken down.

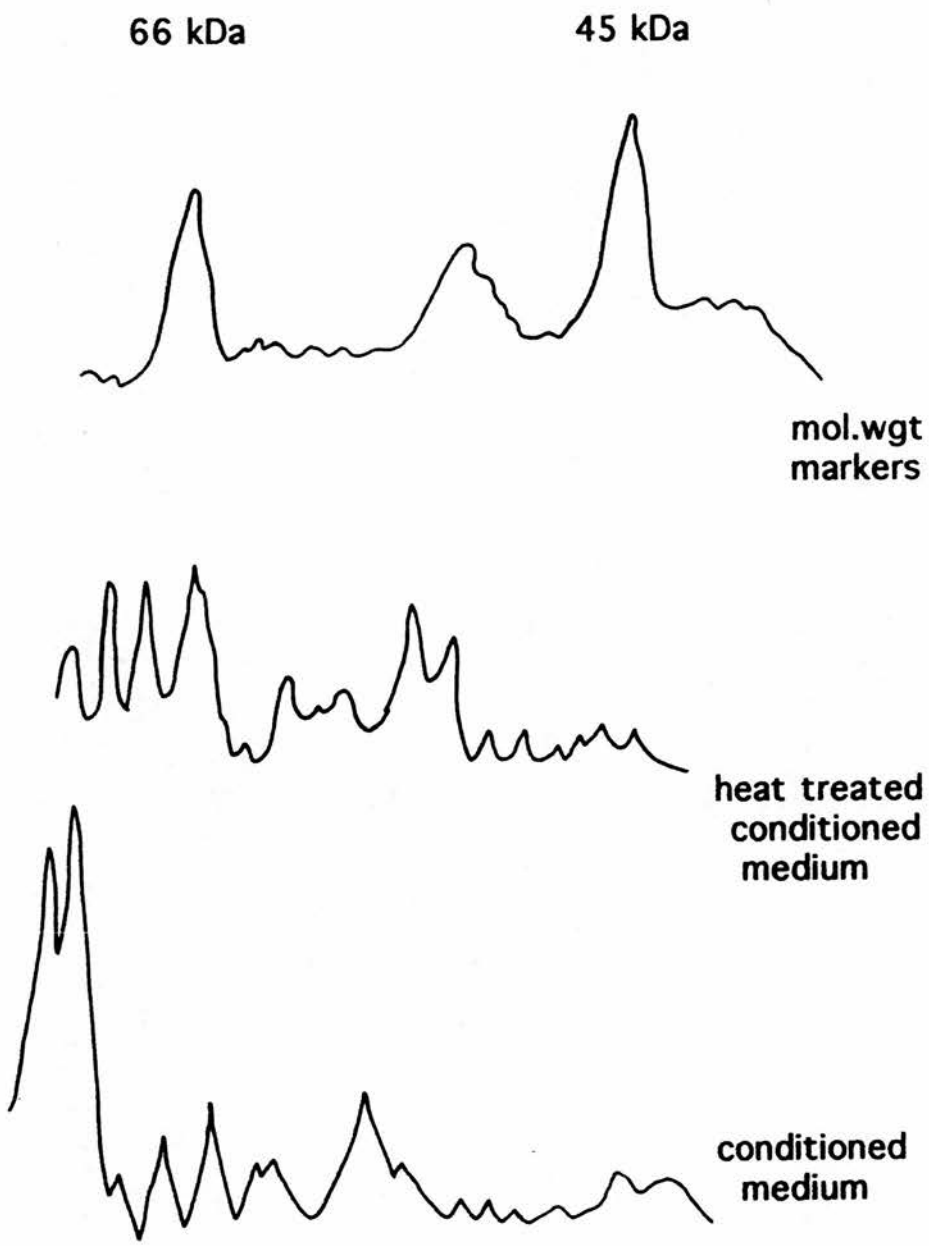

66 kDa
45 kDa
mol.wgt
markers
heat treated
conditioned
medium
conditioned
medium

Figure 5.2 Densitometer scans of low molecular weight proteins of conditioned medium. When conditioned medium was heat-treated proteins such as TGF-β increased in amount.

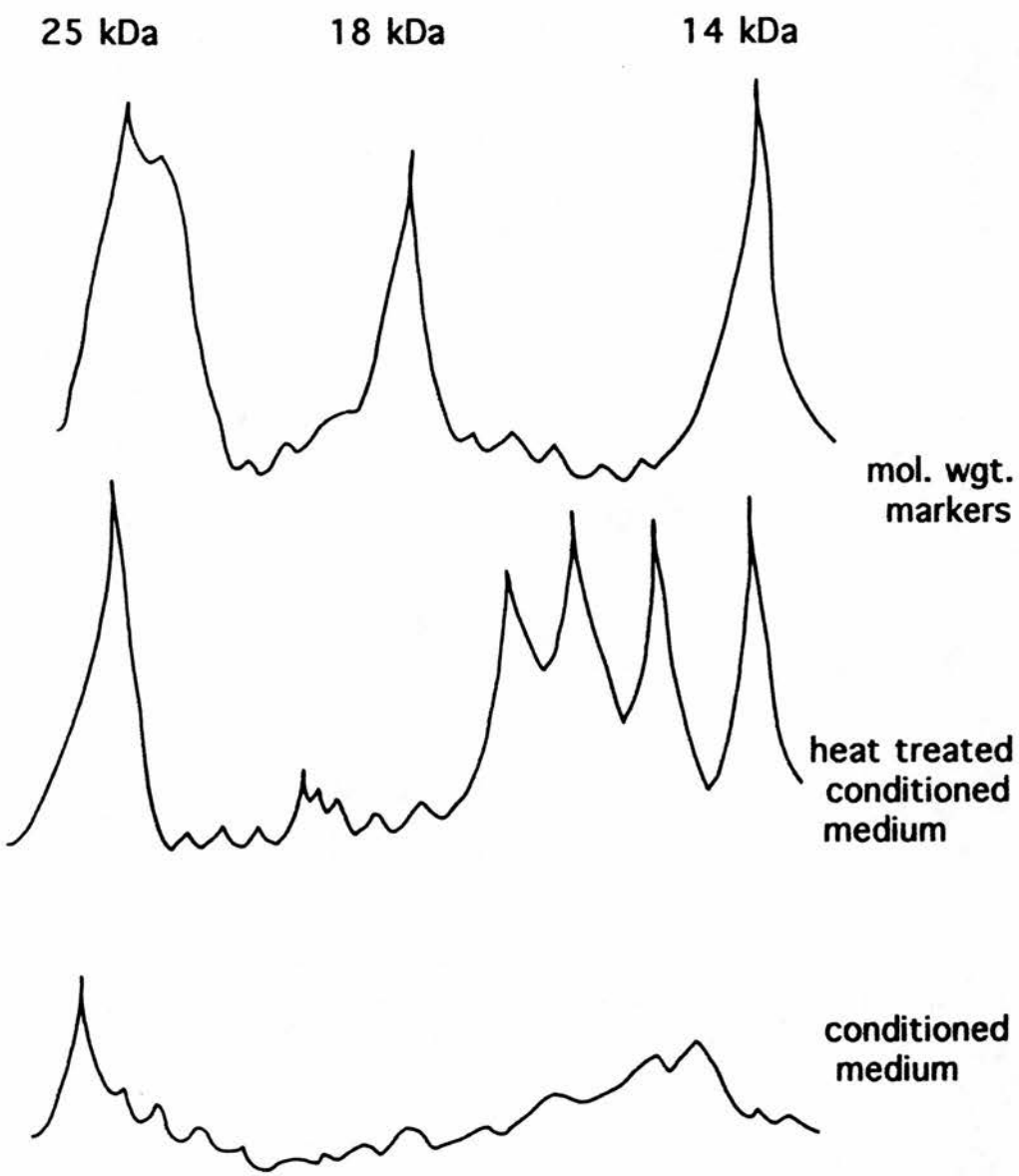

25 kDa
18 kDa
14 kDa
mol. wgt.
markers
heat treated
conditioned
medium
conditioned
medium

Table 5 Effect of heat treatment on conditioned medium. Colony numbers obtained with conditioned medium, heat-treated conditioned medium (CM) and control medium. The assay was conducted in the presence of NRK cells. The results are given as the mean of five replicates, with standard deviations in brackets. Conditioned medium produces colonies in the absence of any additional growth factors but this activity is removed upon heat-treatment. Heat-treated conditioned medium in the presence of EGF produces a greater number of colonies than non heat-treated conditioned medium with EGF. This effect is significant since heat-treated conditioned medium is in the presence of 0.2ng/ml of EGF rather than 1ng/ml. At 0.2ng/ml EGF the number of colonies produced by heat-treated conditioned medium is at the limits of the assay.

It was suggested that a TGF-β like molecule was present.

	Without supplements	With added TGF-β (1ng/ml)	With added EGF
Conditioned medium	40.0 (4.5)	6.6 (1.8)	93.2 (8.3) (1ng/ml)
Heat-treated CM	0.0 (0)	0.0 (0)	103.0 (21.9) (0.2ng/ml)
Control medium	0.0 (0)	0.0 (0)	42.7 (6.7) (1ng/ml)

The primary AIG promoting effect was removed by heating conditioned medium to 100^{0}C for 3 min. The heat treatment of conditioned medium resulted in a significant increase on the synergistic effect of conditioned medium on EGF. The EGF concentration for heat-treated conditioned medium was 0.2ng/ml and 1ng/ml for non-heat-treated medium. In the presence of 0.2ng/ml EGF the number of colonies produced by heat-treated conditioned medium was at the limits of the assay.

This suggested that conditioned medium contained a TGF-β like molecule which was heat stable and upon heat treatment its activity was enhanced.

To determine if heat treatment resulted in the breakdown of latent TGF-β, fractions of molecular weight > 25 kDa were purified from conditioned medium and assayed for AIG (Figure 5.3).

It was observed in the presence of EGF the fraction heat treated resulted in an increase in colony number and without EGF there were virtually no colonies. A similar fraction which was not heat treated did not produce a significant effect in the presence of EGF, unlike whole conditioned medium. This indicated that there was a significant increase in colony numbers between the heat and non-heat treated fractions in the presence of EGF and that the primary AIG effect of conditioned medium must be in the molecular weight range less than 25kDa.

Figure 5.3 The colony promoting activity of all the proteins above 25 kDa. Proteins above the position of TGF-β were eluted from a polyacrylamide gel and assayed for anchorage independent growth in the presence of NRK-49F cells. EGF was present at 1 ng/ml. In the absence of EGF both heat and non-heat treated fractions did not produce any colonies. In the presence of EGF the heat-treated fraction produced an increased number of colonies.

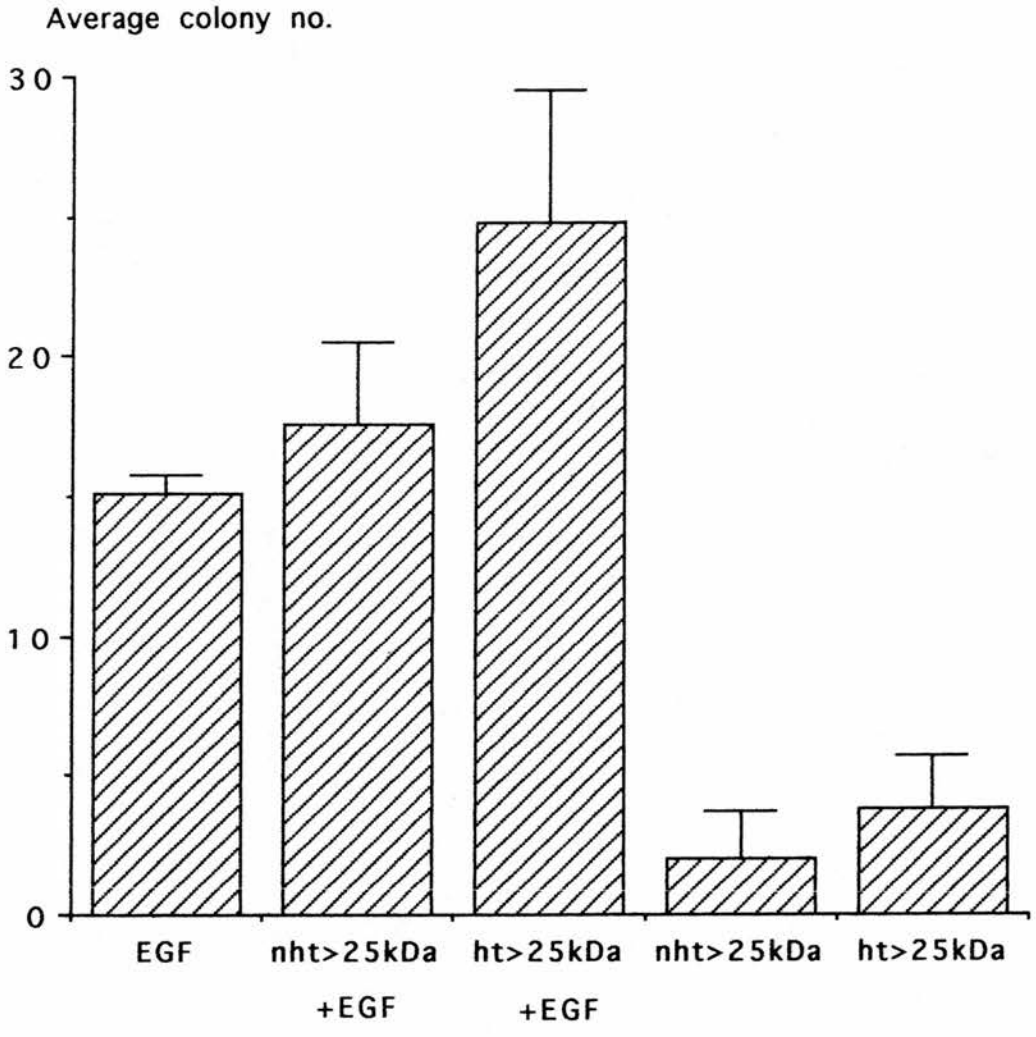
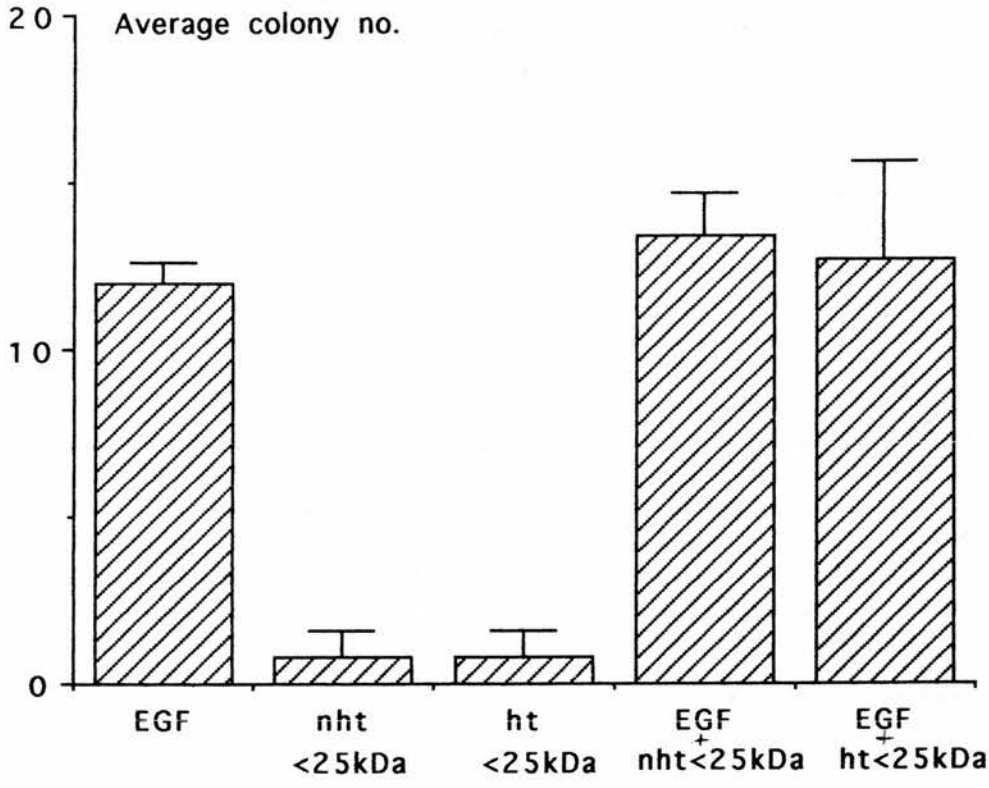

Average colony no.
30
20
10
0
EGF
nht>25kDa +EGF
ht>25kDa +EGF
nht>25kDa
ht>25kDa

Figure 5.4 The colony promoting properties of proteins less than 25 kDa. The protein bands less than 25 kDa were eluted from a polyacrylamide gel and the colony promoting activity was measured in the presence of NRK cells. In the presence and absence of EGF both heat-treated and non- heat-treated fractions did not produce colonies. EGF was present at 1 ng/ml.


20
Average colony no.
10
0
EGF
nht <25kDa
ht <25kDa
EGF + nht<25kDa
EGF + ht<25kDa

To determine if this primary effect was present in the molecular weight range less than 25KDa fractions purified from the gel were assayed in the microwell assay for AIG. No response was observed in the absence of EGF.

5.22 EFFECT OF THE 40.7kDa PROTEASE ISOLATED FROM CHICK HEAD CONDITIONED MEDIUM ON AIG

The most effective way to determine if the protease isolated from conditioned medium had any role in the activation of latent TGF-B, was to incubate the protease with conditioned medium and then assay in the microwell assay.

The proteolytic enzyme had no effect on AIG in the absence of any additional growth factors. The affect of the protease on activation of TGF-B was first studied in whole conditioned medium. From Figure 5.5 it was observed that the protease had no effect on the colony promoting activity of EGF. The protease was incubated with heat-treated and non-heat-treated conditioned medium with no change in their colony promoting properties. In the presence of EGF there were virtually no differences between the number of colonies when heat-treated and non-heat-treated conditioned medium were incubated with the protease (Figure 5.6). It was therefore

Figure 5.5 The effect of the protease from conditoned medium on the colony promoting ativity of EGF. The protease (P) had no effect on EGF when assayed in the presence of NRK cells.

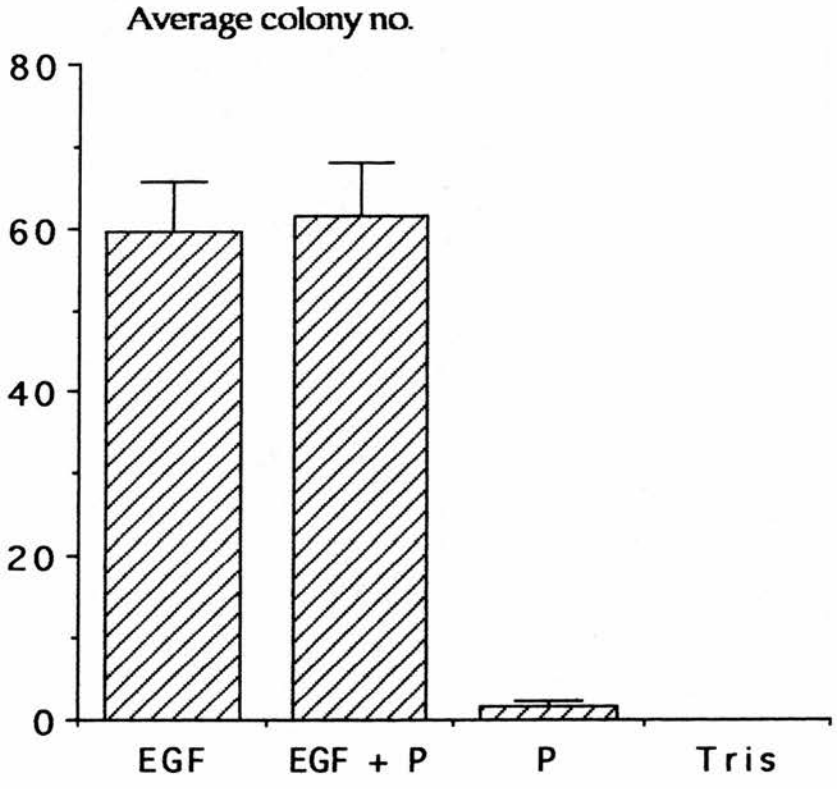
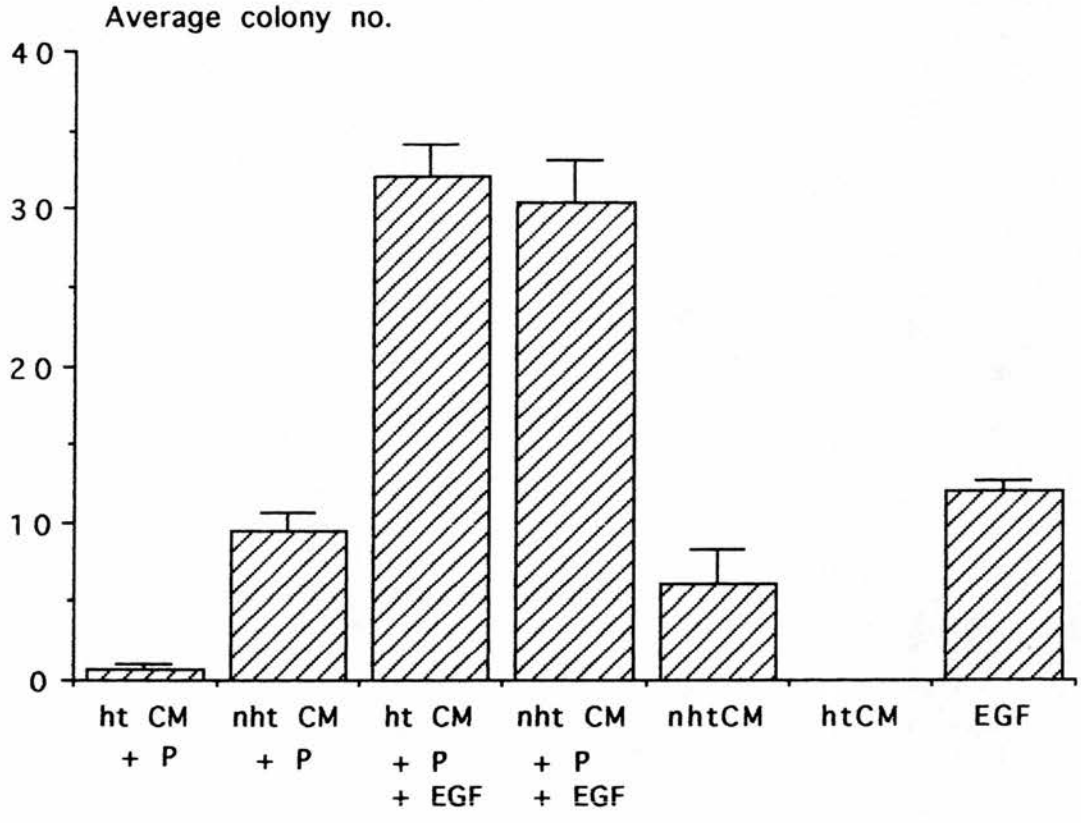

Average colony no.
80
60
40
20
0
EGF
EGF + P
P
Tris

Figure 5.6 The effect of the protease isolated from conditioned medium on the colony promoting activity of conditioned medium. The protease (P) had no effect on heat- treated conditioned medium (ht CM) or non heat-treated conditioned medium (nht CM) when they were assayed for AIG in the presence of NRK cells. There was no difference in the colonies for heat-treated and non-heat-treated conditioned medium, in the presence of EGF and protease, indicating that the protease did not activate latent TGF-β. The protease was present at 10 ug/ml and EGF at 1ng/ml.


Average colony no.
40
30
20
10
0
ht CM + P
nht CM + P
ht CM + P + EGF
nht CM + P + EGF
nhtCM
htCM
EGF

concluded that the protease had no effect on converting latent TGF-β into its active form. The sample purified from the gel which included all proteins of molecular weight > 25KDa were also tested for AIG in the presence of the protease (Figure 5.7). Colonies were again not affected when the protease was mixed with heat or non-heat treated fractions. The ability to promote AIG was not destroyed by the protease unlike the effects of other proteolytic enzymes such as trypsin which removed the EGF enhancing effect (data not shown).

5.23 EFFECT OF PROTEASE ON ACTIVE TGF-β

The proteolytic enzyme did not alter the response of TGF-β in the microwell assay (Figure 5.8). From Figure 5.8 it was also observed not to have any effect on the EGF enhancing effect on TGF-β. The enzyme therefore was not involved in converting latent TGF-β into its active form or in the breakdown of active TGF-β.

Figure 5.7 The effect of the proteolytic enzyme on the colony promoting activity of high molecular weight proteins purified from polyacrylamide gels. The protease (P) did not produce any difference on the EGF enhancing effect of both heat-treated (ht > 25 kDa) and non heat-treated (nht > 25 kDa) fractions, indicating it did not convert latent TGF-β into its active form.

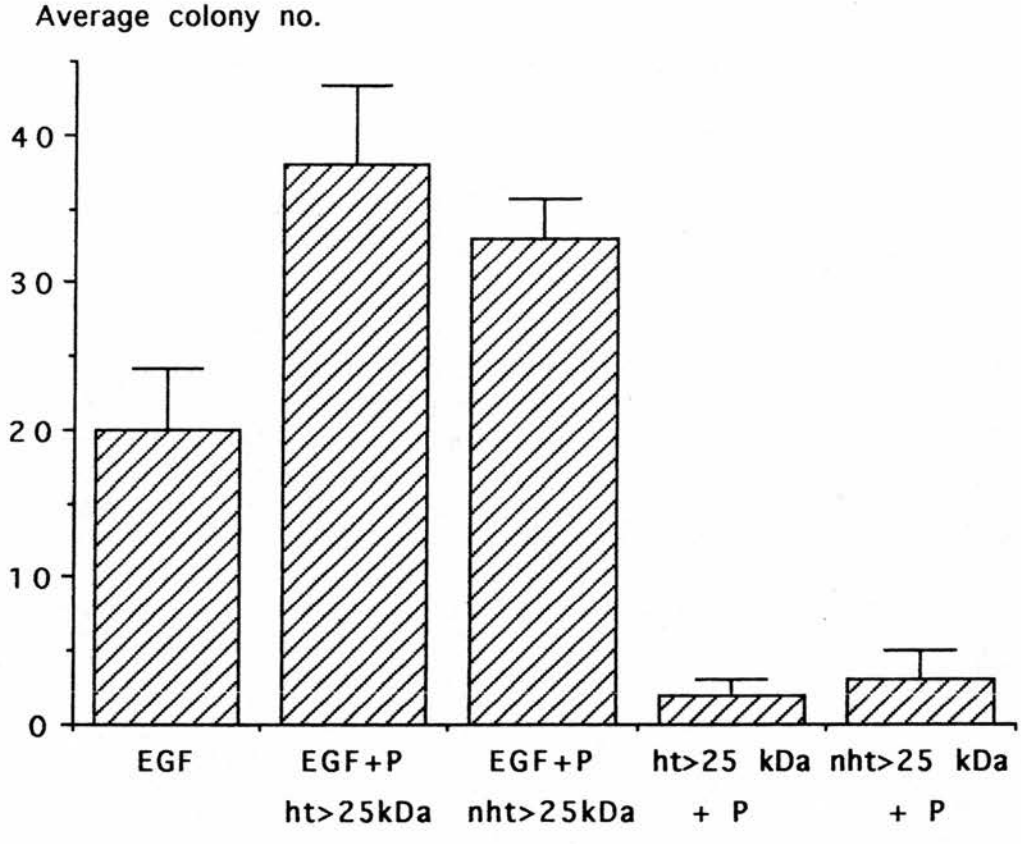
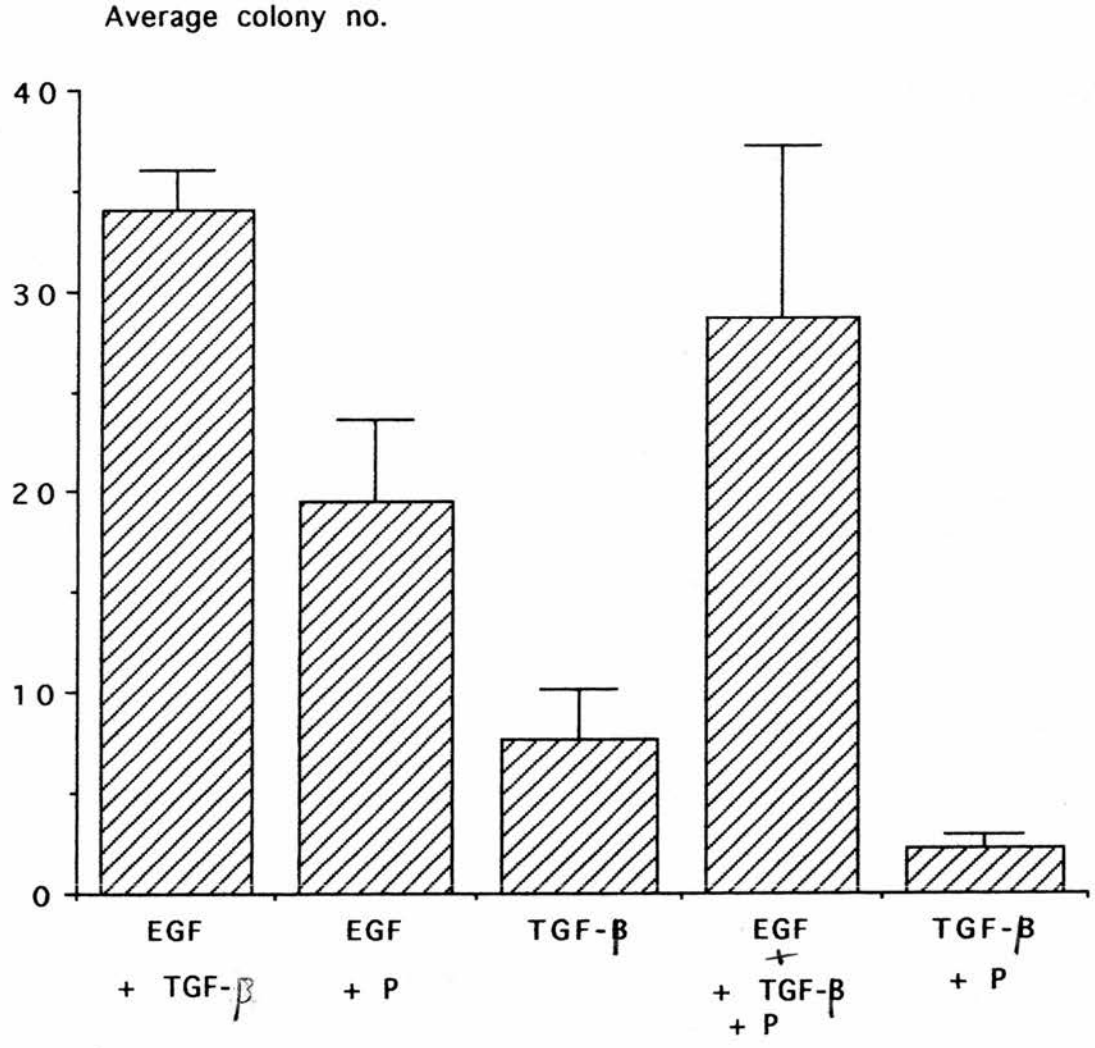

Average colony no.
40
30
20
10
0
EGF
EGF+P ht>25kDa
EGF+P nht>25kDa
ht>25 kDa + P
nht>25 kDa + P

Figure 5.8 The effect of the proteolytic enzyme on TGF-β. The protease (P) did not affect TGF-β when assayed in the presence of NRK cells. It did not affect the EGF enhancing properties on TGF-β.


Average colony no.
40
30
20
10
0
EGF + TGF-β
EGF + P
TGF-β
EGF + TGF-β + P
TGF-β + P

5.3 DISCUSSION

Densitometer scans show that the amount of TGF-β increased when conditioned medium was heat treated. Heat treatment also decreased the amount of high molecular weight proteins and increased lower molecular weight ones. This indicated that TGF-β was probably formed from the breakdown of a higher molecular weight protein, despite previous work by Miyazono et al., 1989 who demonstrated that heat treatment did not activate latent TGF-B.

Confirmation of heat activation of latent TGF-β was studied in microwell assays for AIG, where high molecular weight fractions isolated from conditioned medium, which were heat-treated, displayed TGF-β activity in the presence of EGF. These fractions did not display activity in the absence of heat-treatment. This suggests that heat treatment results in the production of TGF-B within conditioned medium. The identity of the high molecular weight latent form of TGF-β was investigated.

Very small amounts of the exported proteins with high molecular weights were present in the conditioned medium. It was not possible, therefore, to scan for these proteins as they could barely be seen on a polyacrylamide gel.

As mentioned earlier chick embryonic conditioned medium has been demonstrated to possess a wide variety of proteins, with a range of molecular

weights. One such protein was identified, being unique in its possession of proteolytic activity and is discussed in Chapter 4. The biological significance of this protease seemed of some interest. Within conditioned medium in addition to TGF-β there has been demonstrated the presence of a labile, inactive pool of TGF-β. If indeed TGF-β is playing an important role in developmental processes occuring in the early stages of the chick embryo, regulation of its activity would be of prime importance. The active TGF-β present in conditioned medium had already undergone this controlled activation. The discovery of the proteolytic activity within conditioned medium was therefore of importance in assessing any role it might play in the modulation of TGF-β activity invivo.

The mechanisms involved in the processing of growth factors from their inactive export forms to the active state in the extracellular environment remains obscure. Proteolytic involvement has repeatedly been postulated but *in vivo* evidence for their existence has so far been lacking. It was thought that this new protease may belong to this class of growth factor processing enzymes. However, the protease isolated from conditioned medium did not increase the amount of TGF-β present in conditioned medium and therefore did not activate the latent complex of TGF-β.

Finally since both the protease and TGF-β were active at the same temporal span of development, the effect of the protease on the active TGF-β molecule was investigated . Commercially purified TGF-B was incubated with the protease and the colony promoting activity of TGF-β was investigated. Latent TGF-β was assumed not to be present in the commercially purified TGF-β sample and therefore could not be processed to produce any additional TGF-β. The proteolytic enzyme did not alter the colony promoting activity of TGF-β in the presence of EGF. The protease was not therefore involved in the clearance of activated TGF-β from the cell.

In conclusion active TGF-β has been identified in chick embryonic conditioned medium and its amount increased upon heat treatment of this media indicating that it was formed from the breakdown of a higher molecular weight fraction.

The identification of a proteolytic activity within the same temporal span as TGF-β suggested a processing role for this enzyme. It was shown, however not to be involved in the activation of latent TGF-β or TGF-β breakdown.

CHAPTER 6 THE PROTEOLYTIC PROPERTIES OF GROWTH FACTORS

6.1 INTRODUCTION

In this Chapter the proteolytic properties of commercially purified growth factors are explored. Below is an account of various proteins with known growth factor activity and growth factors which contain proteolytic activity. This emphasises the complexity of assessing the effect of growth factor or proteolytic action on each other.

6.11 PROTEINS WITH GROWTH FACTOR PROPERTIES.

[illegible] discovered that proteases such as trypsin, pronase and ficin were able to initiate DNA synthesis and cell proliferation. The mitogenic action of trypsin was confirmed in a number of investigations in the 1970s. It was observed that when trypsin was added to cultures of nonproliferating secondary chick embryo fibroblasts there was an increase in DNA synthesis, mitosis and a twofold increase in cell number. In 1975 Chen and Buchanan reported that the addition of purified thrombin to non proliferating cultures of secondary chick embryo fibroblasts stimulated DNA synthesis and cell division. Thrombin also stimulates the proliferation of cultured nondividing normal human fibroblasts, secondary mouse embryo fibroblasts and Chinese hamster lung cells.

Other studies have indicated that proteins associated with the extracellular matrix have

growth factor activity. Laminin, which is a multidomain glycoprotein, has mitogenic activity for 3T3 cells and PAM212 cells ((Panayotou et al., 1989). This study was controlled for EGF contamination and demonstrated that macromolecules from the matrix can directly induce localized cell proliferation in developing tissues.

Cathepsin D which is an aspartyl protease associated with the extracellular matrix (Capony et al., 1987). This protein is secreted by oestrogen treated MCF7 human breast cancer cells and is produced by oestrogen receptor-negative breast carcinoma cell lines (Rochefort et al., 1987). The cathepsin D mRNA and protein are induced in oestrogen receptor-positive cells by treatment with oestrogens and high doses of androgens, but not by glucocorticoids or progestins. Anti-oestrogens inhibit DNA synthesis and block induction of the protein. It has therefore been reported that Cathepsin D may be a mitogen for human breast cancer cells. Mechanisms for the protease mediated mitogenesis have been proposed. These include the release of growth factors from precursors which are mediated by the protease or that Cathepsin D can act on cells directly via specific receptors, as in the case of thrombin.

Growth factor like activity was also found for fibronectin, another important matrix protein. Fibroblast replication is regulated by exogenous signals provided by growth factors, mediators that

interact with the target cell surface and signal the cell to proliferate. Growth factors can be grouped into two types, competence or progression factors. Competence factors such as FGF or PDGF act early in the G1 phase and render the cells responsive to progression factors. Progression factors such as insulin and insulin-like growth factors act in late G1 and signal the cell to continue through the cell cycle and replicate. Bitterman et al., 1983 has suggested that the extracellular matrix can replace the requirements for a competence factor in certain cell types. Fibronectin has been shown to stimulate fibroblast replication in a dose dependent manner in the presence of a fixed dose of a progression factor. Fibronectin also signalled growth arrested fibroblasts to traverse G1 and come closer to S phase. These observations suggest that fibronectin may provide at least one of the signals by which the matrix conveys the "competence" that allows fibroblasts to replicate in the presence of an appropriate progression signal.

Martin and Timpl, 1987 also demonstrated that laminin contained growth factor-like activity, by stimulating cell movement.The results indicated that laminin was chemotactic when in solution and haptotactic when on surfaces but in either form is able to guide cell movement.

Another protein of the matrix, Thrombospondin, which mediates cell to cell and cell to matrix

interactions, has been shown to contain an amino acid region with close homology to EGF (Majack et al., 1986).

6.12 GROWTH FACTORS WITH PROTEOLYTIC PROPERTIES

The biologically active NGF molecule consists of two identical 13 kDa polypeptide chains. This dimer is stored in an inactive form and has the subunit structure of (alpha,gamma, beta $)_2$. The gamma subunit is a proteolytic enzyme whereas the alpha subunit inhibits this protease. NGF is synthesized as a prohormone containing the alpha and beta subunits and is then cleaved to produce the active form by the gamma subunit.

EGF is also isolated as a high molecular weight complex and is associated with a specific arginine enteropeptidase known as the EGF binding protein. In the presence of this protease EGF has increased mitogenic activity, There have been no reports indicating that EGF has proteolytic activity. There have also been studies carried out on the proteolytic activites of FGF, IGF and PDGF but they have not been conclusive.

Therefore, although many proteins and proteolytic enzymes have been identified as promoting growth factor like activity, no growth factor has been shown to display proteolytic activity. NGF has been demonstrated to possess a subunit of its structure

which is proteolytic but its only function is in the processing of NGF.

This Chapter looks at the proteolytic properties of commercially purified growth factors by measuring their response to the substrates azocasein and azoalbumin spectrophotometrically (as described in the method section-6.2). From this the optimum concentration at which the growth factor displays proteolytic activity can be determined.
The effect these growth factors have on the proteolytic activity of the protease is also investigated by incubating the components with the protease, as described in section 6.2, and then assaying for activity in the azocasein assay.
Protease-like structures within growth factors have not been sought for growth factors and the activity of these structures has not been investigated before.

6.2 METHODS

6.21 EFFECT OF VARIOUS PROTEINS ON THE ACTIVITY OF THE PROTEASE

The protease 7.6mg/ml was incubated with a number of proteins : head conditioned medium; TGF-B; EGF; bFGF; glycoprotein mixture , for 1h . Since all of the samples tested were in solution in serum free medium, the protease was also incubated with this as a control. Following this hour the mixture was then incubated with the azocasein and incubated for a further 5h. The samples were then subject to the azocasein assay as before.

6.3 RESULTS

6.31 OPTIMUM ACTIVITY OF PROTEINS OF CONTITIONED MEDIUM

6.311 OPTIMUM CONCENTRATION FOR ACTIVITY FOR THE PROTEASE

Increasing amounts of protease were incubated with azocasein substrate (Figure 6). As the enzyme concentration increases the amount of azocasein broken down increases until the amount of enzyme is too great for the amount of substrate.
At 10ug/ml of protease the amount of breakdown of substrate is at its greatest.

6.312 OPTIMUM PROTEOLYTIC ACTIVITY OF TGF-B

TGF-B showed proteolytic activity when assayed in the presence of azocasein or azoalbumin . It did not show any of this activity when assayed with casein or gelatin directly on a polyacrylamide gel. From Figure 6.1 it was seen that the proteolytic activity of TGF-β increased until about 5ng/ml and then the activity levelled.

6.313 OPTIMUM PROTEOLYTIC ACTIVITY OF EGF

EGF also displayed proteolytic activity when assayed in the presence of the substrates azocasein

Figure 6 Optimum working concentration of protease. The concentration at which the protease was most active against azocasein was 11 ug/ml. The response was measured spectrophotometrically at optical density 375 nm.

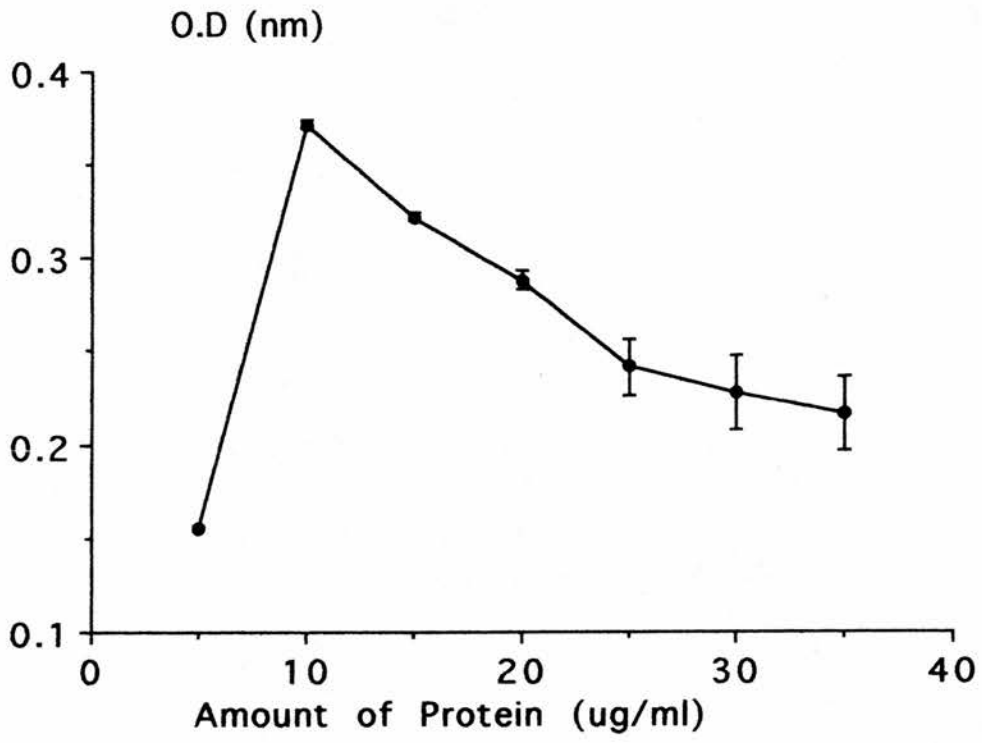
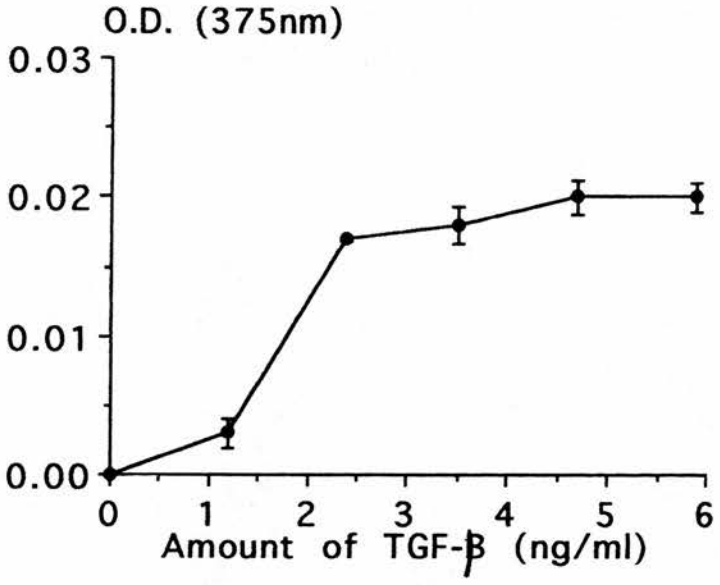

O.D (nm)
0.4
0.3
0.2
0.1
0
10
20
30
40
Amount of Protein (ug/ml)

Figure 6.1 Optimum proteolytic concentration of TGF-β. The concentration at which TGF-β was most active proteolytically was 5 ng/ml as determined by the azocasein assay.


O.D. (375nm)
0.03
0.02
0.01
0.00
0
1
2
3
4
5
6
Amount of TGF-β (ng/ml)

and azoalbumin. The activity was still shown to be increasing at about 11ng/ml of EGF (Figure 6.2). EGF could not be detected on a polyacrylamide gel in the presence of casein or gelatin. .

6.314 DOSE RESPONSE OF OTHER FACTORS

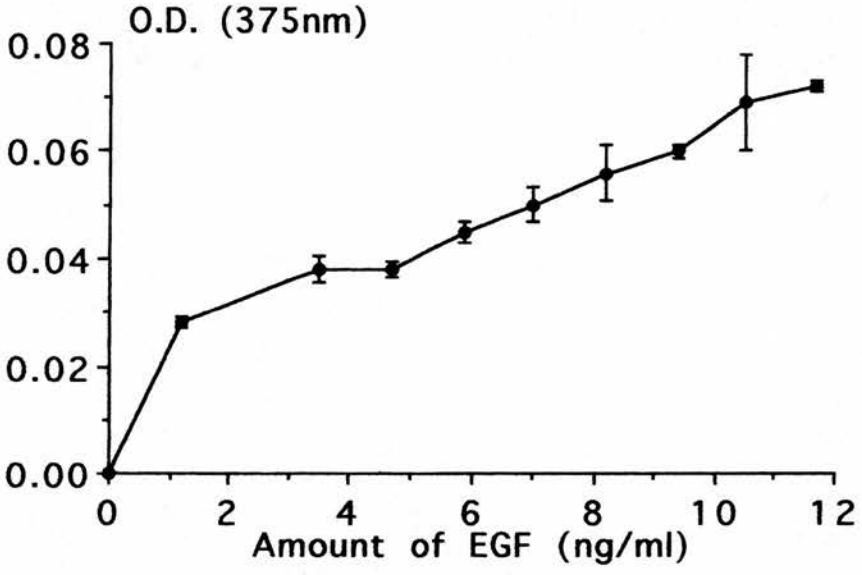
It was impossible to determine an optimum working concentration with head conditioned medium and the glycoprotein mixture because both contained many proteins, each of which would have their own optimum concentration for activity.

6.32 PROTEOLYTIC ACTIVITY OF CHICK TISSUE.

Whole embryos were mixed with 200ul of serum free medium and the cells were broken into by sonicating for 10min. The activity was detected by running the whole extract on a polyacrylamide gel and then laying the gel on an agarose gel with skimmed milk incorporated. The entire gel turned clear upon incubation with the gel containing the electrophoresed whole embryo indicating that it contained many proteases.
The same process was carried out on heads of the chick embryo. Again the entire gel containing the skimmed milk substrate turned clear.

6.33 PROTEOLYTIC ACTIVITY OF COMMERCIALLY PURIFIED GROWTH FACTORS

Figure 6.2 Optimum proteolytic concentration of EGF. The concentration at which EGF was most active proteolytically was not determined. It was still increasing at 11 ng/ml when assayed in the azocasein assay.


O.D. (375nm)
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
12
Amount of EGF (ng/ml)

TGF-β, EGF and FGF all displayed proteolytic activity when assayed in the presence of azocasein or azoalbumin (Figure 6.3). At a concentration of 5ng/ml TGF-β contained the most activity followed by EGF and FGF. Head conditioned medium also contained this activity (Figure 6.3).

6.34 EFFECT OF GROWTH FACTORS ON THE PROTEOLYTIC ACTIVITY OF THE PROTEASE.

The samples tested were incubated with the proteolytic enzyme in the presence of excess substrate. When TGF-β was mixed with the protease the activity was that of the protease alone. TGF-β displayed 30% more activity than the protease when incubated alone.

A similar picture was seen when EGF and FGF were mixed with the protease prior to assaying for proteolytic activity (Figures 6.4, 6.5 and 6.6). For EGF the activity dropped to below that of either EGF or the protease when these two factors were incubated prior to assaying for proteolytic activity. In the case of FGF the response when it was mixed with the protease was the same as that of the growth factor in the absence of protease. Conditioned medium (Figure 6.7) together with the protease produced a response slightly greater than either the protease or conditioned medium.

The values represented by EX are the expected results if the growth factors and protease had no effect on each other.

Figure 6.3 Proteolytic activity of commercially purified growth factors and conditioned medium. Conditioned medium (CM), TGF-β, EGF, FGF and conditioned medium all displayed activity. All samples were present at 5 ng/ml.

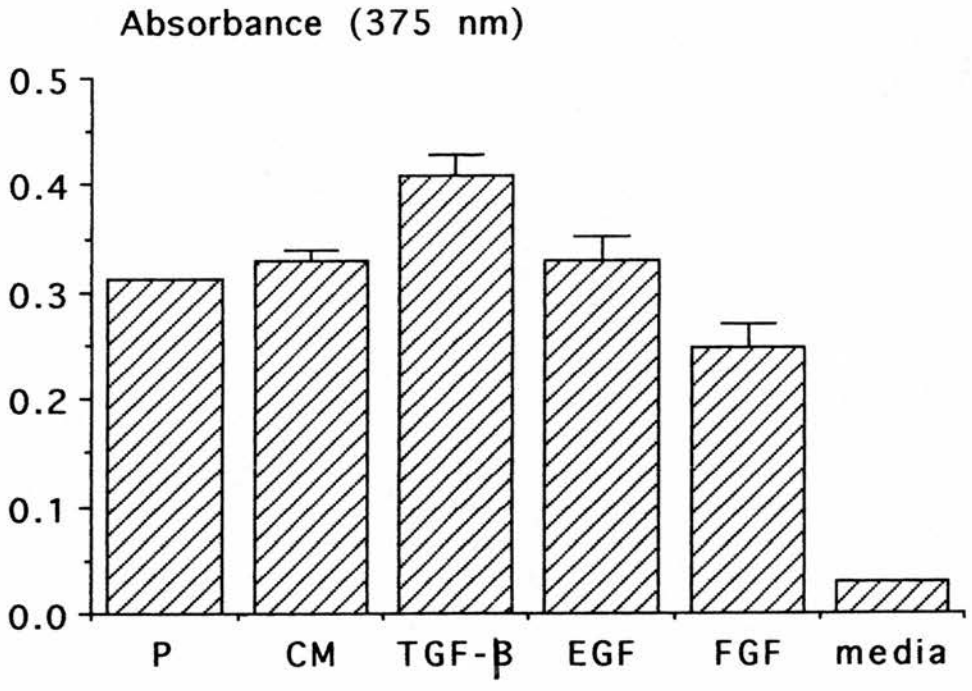

Absorbance (375 nm)
0.5
0.4
0.3
0.2
0.1
0.0
P
CM
TGF-β
EGF
FGF
media

Figure 6.4 Effect of TGF-β on proteolytic activity. TGF-β contained 30% more proteolytic activity than the protease (P). The expected response (EX) if the two proteins had no effect on each other, was that of the protease plus that of TGF-β. This was not observed.

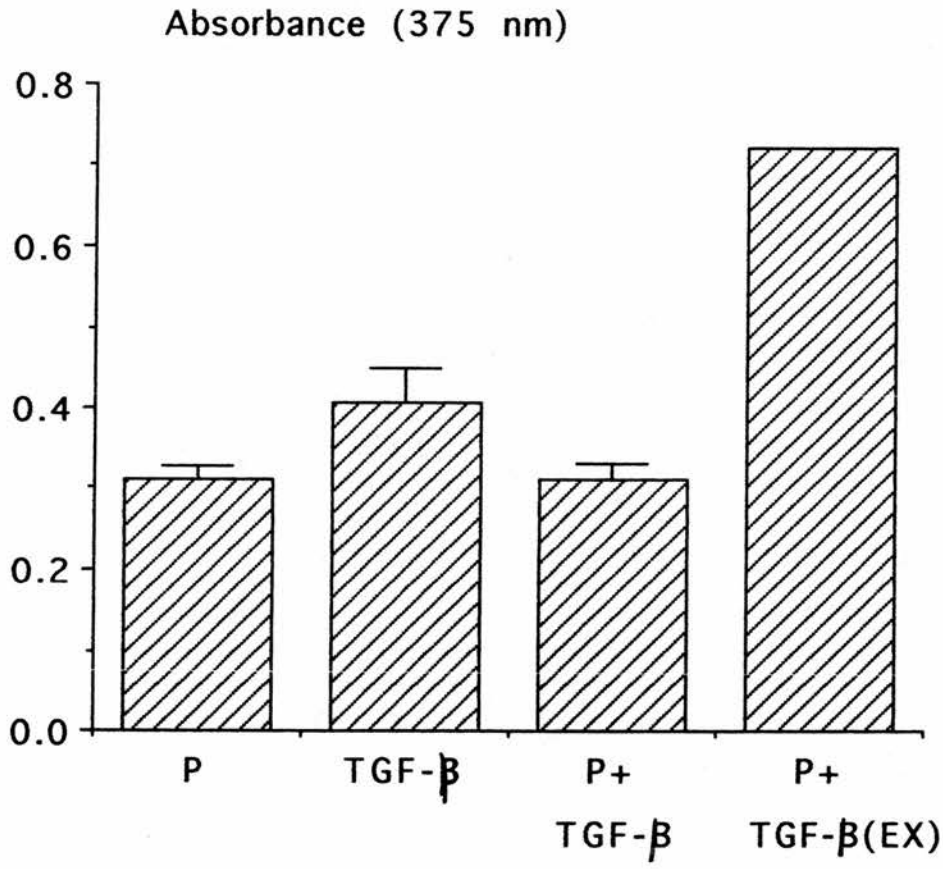

Absorbance (375 nm)
0.8
0.6
0.4
0.2
0.0
P
TGF-β
P+ TGF-β
P+ TGF-β(EX)

Figure 6.5 The effect of EGF on proteolytic activity. The protease (P) and EGF were incubated together prior to assaying for activity. Together they produced a response which was less than that of either EGF or the protease. The expected result (EX), in the presence of excess substrate, was that of protease plus that of EGF.

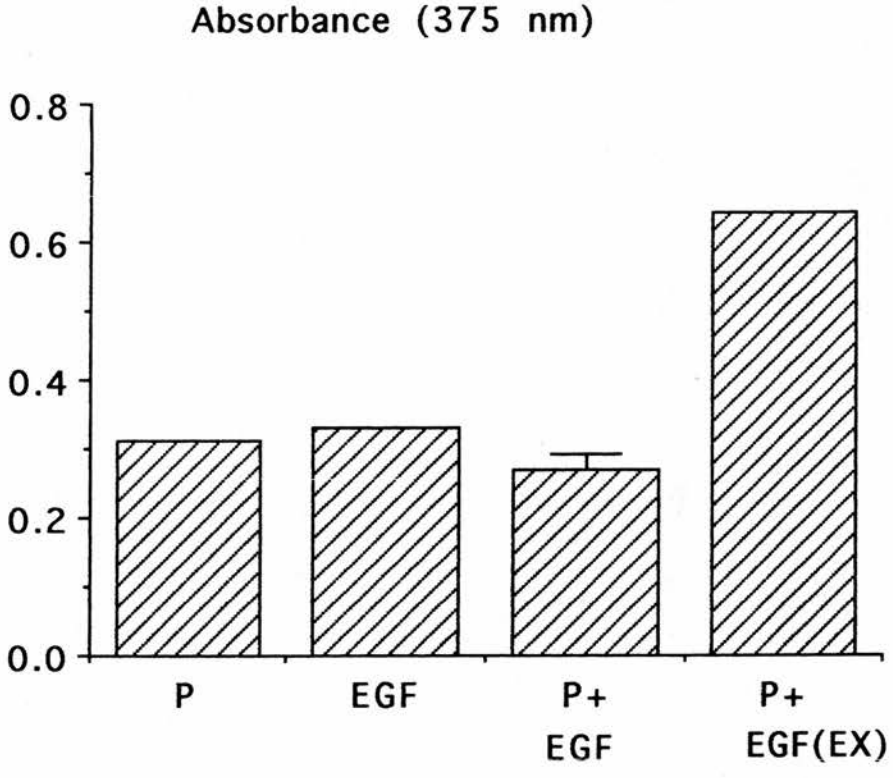

Absorbance (375 nm)
0.8
0.6
0.4
0.2
0.0
P
EGF
P+
EGF
P+
EGF(EX)

Figure 6.6 The effect of FGF on proteolytic activity. The protease (P) and FGF were incubated together prior to assaying for activity. Together they produced a response which was the same as that for FGF alone. The expected result (EX), in the presence of excess substrate, was that of protease plus that of FGF.

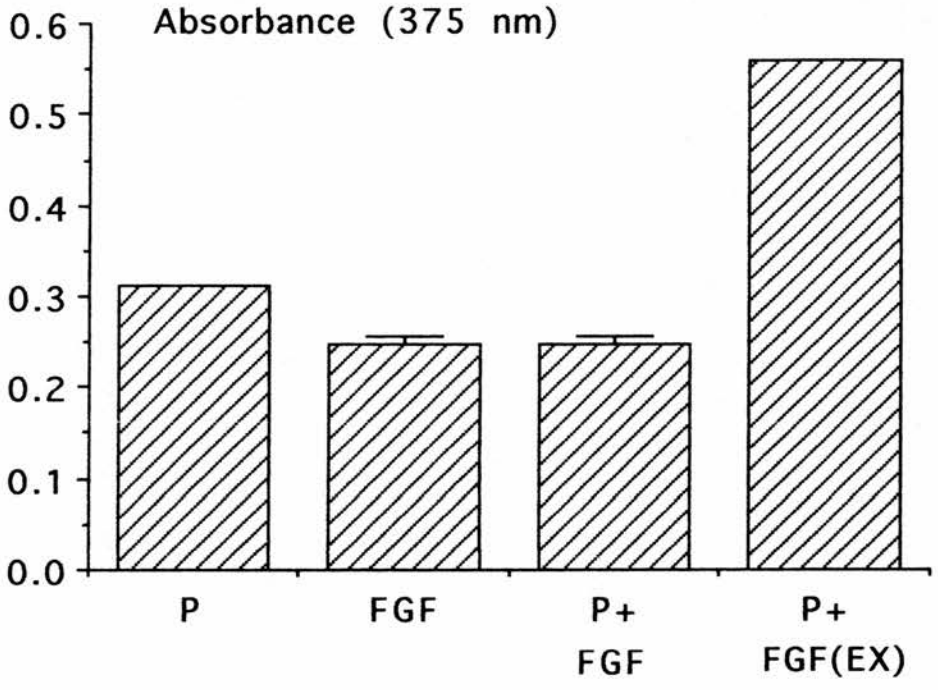
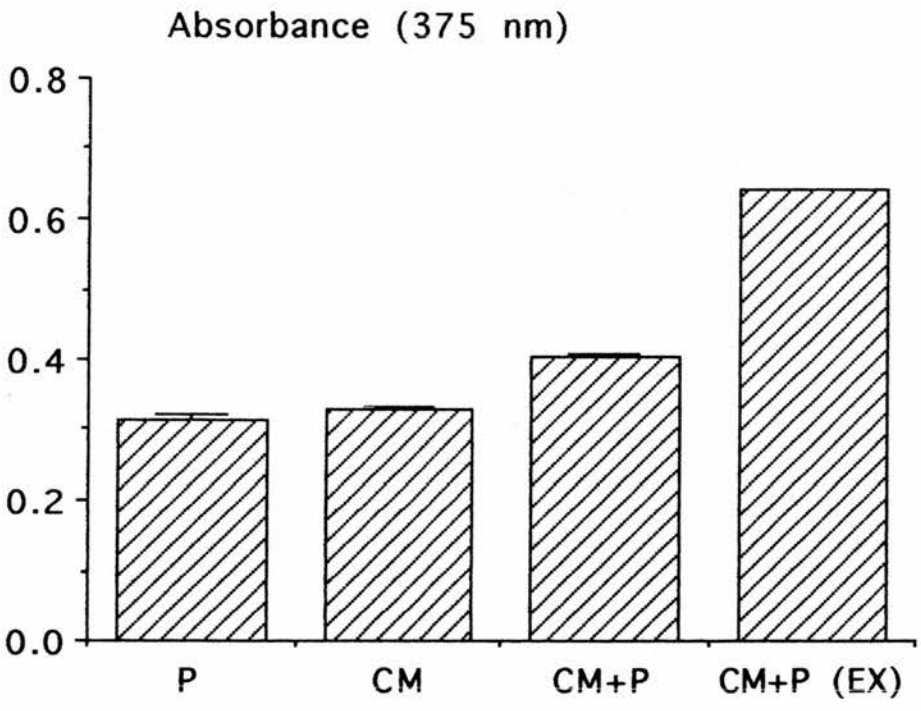

Absorbance (375 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P
FGF
P+ FGF
P+ FGF(EX)

Figure 6.7 The effect of conditioned medium on proteolytic activity. The protease (P) and conditioned medium (CM) were incubated together prior to assaying for activity. Together they produced a response which was slightly more than either protease or conditioned medium alone. The expected result (EX), in the presence of excess substrate, was that of protease plus that of CM.


Absorbance (375 nm)
0.8
0.6
0.4
0.2
0.0
P
CM
CM+P
CM+P (EX)

6.4 DISCUSSION

A role for the proteolytic enzyme was further investigated. The effect the protease had on growth factors which are known or expected to be present in conditioned medium was looked at. During the investigation TGF-β displayed proteolytic activity. The proteolytic activity of other growth factors was investigated.

TGF-β, EGF and FGF displayed proteolytic activity towards the substrates azocasein and azoalbumin. Conditioned medium also contained proteolytic activity. The proteolytic components of the latent forms of growth factors have been looked at (Stryer, 1981) but suprisingly the proteolytic properties of active growth factors have not been recorded.

TGF-β contained the greatest activity towards the substrates. All the growth factors tested were active at pH 7.5 and at 37^{0}C.

The proteolytic activity of conditioned medium could be an additive effect of the proteolytic activity of growth factors present in conditioned medium.

The addition of these growth factors to the protease therefore made analysis of the results more complicated.

If the protease and TGF-β had no effect upon each other the activity observed would be that of TGF-β plus that of the protease (Figure 6.4). The same

would be expected for all the other factors tested (Figures 6.5, 6.6).

The protease purified from the conditioned medium is solely a proteolytic enzyme since it was detected by traditional proteolytic detection methods. The commercial growth factors and probably the growth factors present in conditioned medium likely contain a proteolytic subunit. Under the correct conditions these growth factors display proteolytic activity rather than growth factor activity.

Since the growth factors and the protease displayed proteolytic activity it was unclear which protein affected the other. Excess substrate was used and time for reaction was not a limiting factor. It is proposed that the protease plays some part in growth factor processing but from this assay it is not clear whether it is the growth factor or the protease which is processing the other.

It may be that when growth factors such as TGF-β, EGF and FGF are present in the same environment as the protease, an inhibitory effect occurs where either the growth factors or the proteases activity is reduced. This may function as a control mechanism to slow down various biological events. The spatial and temporal distribution of TGF-β and the proteolytic enzyme isolated from chick conditioned medium, indicate that these two factors are probably involved in the regulation of some event in the developing chick. Both have been

demonstrated to possess proteolytic activity. The activity isolated from conditioned medium has been shown to be active against a wide range of proteolytic substrates and is easily visualised on polyacrylamide gel. TGF-β and other growth factors were not visualised on polyacrylamide gels but were shown to contain proteolytic activity toward azocasein and azoalbumin. Since it is only exported proteins which are present in the medium it is possible that more of the proteolytic enzyme was exported between 4 and 8 days of development than any other component.

When a whole embryo fraction was stained for proteolytic activity the entire gel was digested indicating that many of these proteins were not present in the conditioned medium or were present in such small amounts that they could not be detected (6.32).

Since all the growth factors tested contained proteolytic activity and since it has been described that some growth factors can inhibit and others stimulate proteases, it is impossible to say from the method adopted what the effect is. It is possible to say that protease and all the growth factors tested did result in a drop in the expected activity.

CHAPTER 7 AN INVESTIGATION INTO THE PRIMARY ANCHORAGE INDEPENDENT GROWTH PROMOTING RESPONSE OF CONDITIONED MEDIUM

7.1 INTRODUCTION

One of the initial aims of the project was to explore the nature of the proteins of conditioned medium involved in Anchorage Independent Growth particularly the protein which produced an Anchorage Independent Growth promoting response in the absence of any additional growth factors.

Medium conditioned by chick embryonic tissue contains at least two different growth factors with transforming activity. One of these has been identified as TGF-β and the other component has been described as a heat labile glycoprotein which has TGF-α-like properties.

Conditioned medium promotes anchorage independent growth in NRK-49F cells in the absence of added EGF and this primary growth promoting effect is removed by heating conditioned medium to $100^{0}C$ (Smith and McLachlan, 1990). This primary effect in conditioned medium can be detected as early as the intermediate primitive streak stage (Hamburger and Hamilton stage 3) and is present in all the stages tested up to Hamburger and Hamilton stage 27. Most of this primary anchorage independent growth promoting activity is found at the cranial end of the main body axis, in the region of the developing brain. This effect is removed partially but not entirely by 10KDa and 30KDa molecular weight exclusion filters. It was identified as possessing glycoprotein characteristics since conconavalin A

beads incubated with conditioned medium removed its activity.

This Chapter investigates the nature of the heat-labile glycoprotein component of conditioned medium which displays TGF-alpha like properties. Two different approaches are used to purify the glycoprotein component and are described in the methods section. The glycoprotein components obtained from the purification procedures are analysed for the AIG promoting effect in the microwell assay (described in Chapter 2). Glycoproteins are detected visually using an adaptation of polyacrylamide gel electrophoresis. The proteolytic content of the glycoproteins is then looked at since other components of conditioned medium which were not expected to have proteolytic activity displayed activity towards azocasin and azoalbumin.

7.2 METHODS

7.21 IDENTIFICATION OF THE PRIMARY EFFECT

7.211 PURIFICATION OF THE PRIMARY EFFECT WITHIN THE CONDITIONED MEDIUM

Nylon fibre was firstly sterilised by placing in oven at 130^{0}C for 2h. This was then fitted into the base of a sterile 5ml plastic syringe. The conconavalin A was then poured on top of this and the column was equilibrated using 0.01M Tris HCl buffer, containing 0.1M NaCl pH7.5. To the nozzle of the syringe a piece of plastic tubing, with an adjustable clip controlled the flow of liquid. At the top end of the syringe, a stopper with a piece of tubing inserted connnected the column to the resevoir buffer. The Head Conditioned Medium (2ml) was added to the top end of the column and allowed to run through by adjusting the clips at the top and bottom of the column. Since the head conditioned medium is pink in colour its passage through the conconavalin A can be easily visualised. Samples were collected immediately the conditioned medium was added. Once the medium had passed through the column, 5ml of methyl-*a*-mannoside (0.1M) was added to the column and allowed to pass through. Methyl-*a*- mannoside cleaves the glycoproteins attached to the column. A further 5ml of reservoir buffer was passed through. Only four samples were collected: buffer as soon as head conditioned medium was added; remaining head conditioned medium which did not

bind to the column; any proteins which were released when the methyl-*a*- mannoside was added, and the remaining proteins which detached from the column when more reservoir buffer was added. THe samples were then freeze-dried and redissolved in a smaller volume of serum free medium. The samples were then subject to the microwell assay.

7.22 DETECTION OF GLYCOPROTEINS FROM HEAD CONDITIONED MEDIUM AND SAMPLES FROM CONCONAVALIN A COLUMN

Head conditioned medium and the samples from the conconavalin column were electrophoresed on a 15% polyacrylamide gel for 3h. Following electrophoresis the gel was immersed in 12.5% TCA for 30 min. and then rinsed in distilled water for 30 sec. It was then immersed in 1% periodic/3% acetic acid for 50min and left in distilled water overnight. The following day the gel was immersed in Schiffs reagent for 50min in a dark cupboard, followed by 3x 10min washes in 0.5% metabisulphite. The excess stain in the gel can be removed by several washes in distilled water. It can then be stored in 5% acetic acid.

7.23 IDENTIFICATION OF THE PRIMARY EFFECT WITHIN THE GLYCOPROTEIN MIXTURE.

All of the glycoprotein mixtures including head conditioned medium were subject to the microwell assay for anchorage independant growth. The three glycoprotein samples collected from the

conconavalin A column were assayed to determine if they could promote anchorage independent growth without the addition of any growth factors.

7.3 RESULTS

7.31 PURIFICATION OF THE PRIMARY EFFECT

Two very different methods were employed to purify the primary anchorage independent growth promoting effect of conditioned medium. The first method involved running polyacrylamide gels containing conditioned medium and cutting strips of proteins out and eluting directly from the gel by vigorous shaking at 4^0C in serum free medium. Fractions with molecular weights greater than or equal to 25kDa did not show the primary anchorage independent effect but still produced the EGF enhancing effect. Since earlier results had suggested that the primary effect was in the molecular weight range of about 10kDa, the fractions eluted from the gel with molecular weights less than 25kDa were subject to the microwell assay in the presence of NRK-49F cells (Figure 5.4). There was no response observed in the absence of EGF. The amount of protein present in this fraction was found to be 253 ug/ml.

A second approach to purify this primary anchorage growth promoting effect was employed. Since the high temperatures of electrophoresis were thought to have inactivated the effect the second method was carried out entirely at 4^0C. Conconavalin A columns were constructed in sterile plastic syringes and equlibrated. Conditioned medium was freeze-dried and redissolved in small amounts of

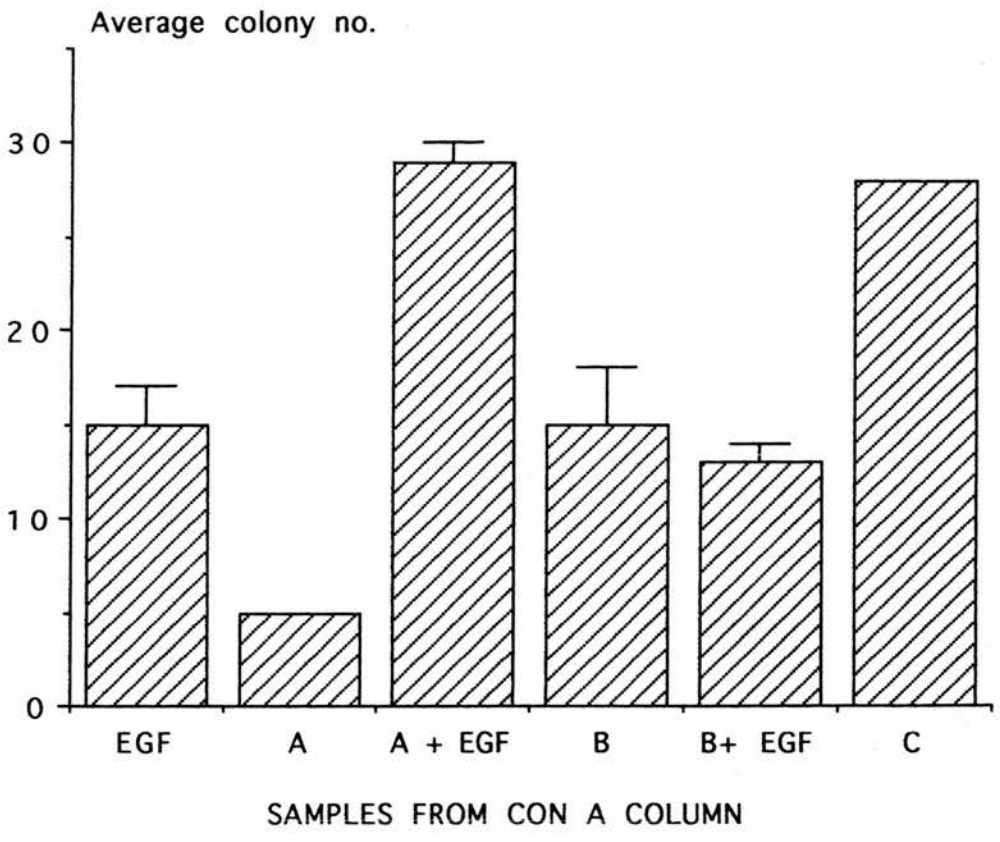
buffer before being applied to the column. The passage of the medium through the column was easy to follow since the medium was a pink colour. Elution of the glycoproteins involved the addition of a general enzyme to release the glycoprotein. These collections were then analyzed in the microwell assay. Three samples were collected. The first sample collected contained all the head conditioned medium which did not bind to the column. The second sample contained all the proteins which would have been released following the addition of enzyme to cleave glycoproteins and the third sample contained the proteins washed down after the initial release of glycoproteins.

7.32 DETECTION OF PRIMARY AIG EFFECT

The three samples collected from the conconavalin A column were assayed for anchorage independence in the microwell assay in the presence of NRK-49F cells (Figure 7).

When head conditioned medium was run down the column the sample collected did not possess the primary growth promoting effect indicating that it was bound to the column. When the cleaving enzyme was added, the glycoprotein with the primary effect was released and when assayed it produced colonies in the absence of EGF. When more buffer was added to the column more of this primary effect was released. The fraction containing the primary promoting growth effect was not enhanced to produce

Figure 7 The primary anchorage independent growth promoting properties of the glycoprotein components of conditioned medium. Samples collected from a conconavalin A column were assayed for colony promoting activity in the presence of NRK cells. Head conditioned medium was run through the column (A) to allow the glycoproteins to bind. The fraction collected (A) contained TGF-β and was enhanced to produce colonies in the presence of EGF. When the cleaving enzyme was applied to the column the primary response was released as represented by B and C.


Average colony no.
30
20
10
0
EGF
A
A + EGF
B
B+ EGF
C
SAMPLES FROM CON A COLUMN

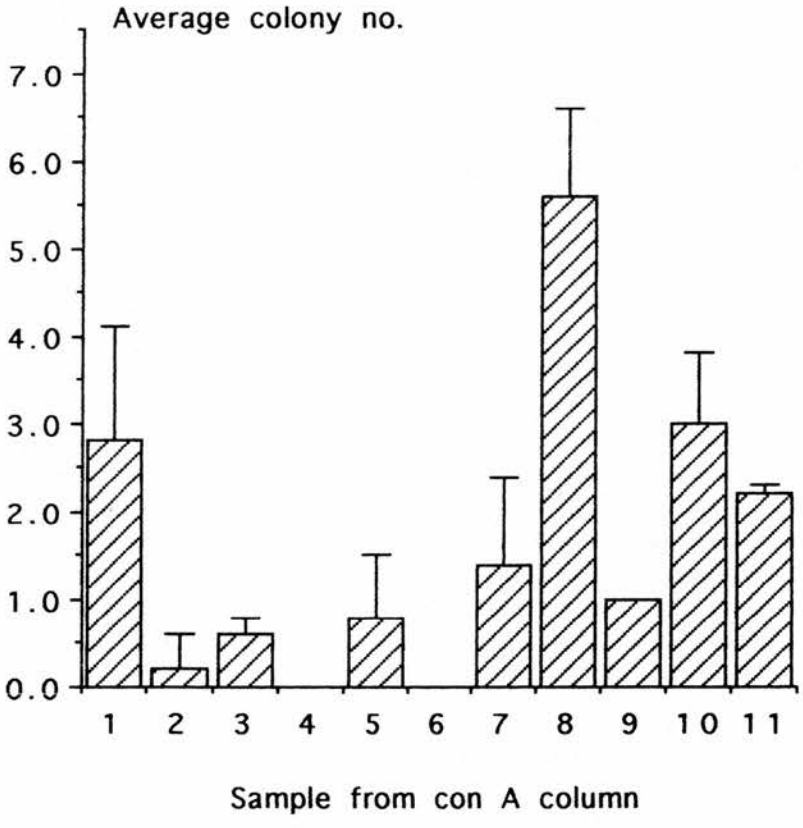
more colonies in the presence of EGF. The first sample released from the column, which contained head conditioned medium without the glycoprotein content, produced an increased number of colonies in the presence of EGF. This implied that TGF-B had not bound to the column. This sample did not produce the same amount of colonies as whole conditioned medium. This again indicated that the primary effect had been separated from the EGF enhancing effect.

To further purify the primary effect from all the other glycoproteins the sample containing the greatest amount of primary anchorage independent growth was applied to another conconavalin A column and the steps in the purification were repeated. This time eleven separate samples were collected and each was assayed for the primary effect (Figure 7.1). Some of the samples purified contained a very small amount of the effect but this effect was not as obvious when bulk samples were collected. This indicated that the primary anchorage independent growth promoting effect of conditioned medium is not the result of a single glycoprotein but rather a combination of two or more.

7.33 VISUAL IDENTIFICATION OF GLYCOPROTEINS

An adaptation of the standard SDS-polyacrylamide gel electrophoresis procedure was undertaken to

Figure 7.1 Further purification of the primary effect. The sample containing most of the the primary producing effect was added to a conconavalin A column and 11 fractions were collected (1 - 11) and assayed for colony promoting activity. They majority of the samples produced very small responses, which implied that more than one protein was required to produce the primary response.


Average colony no.
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1 2 3 4 5 6 7 8 9 10 11
Sample from con A column

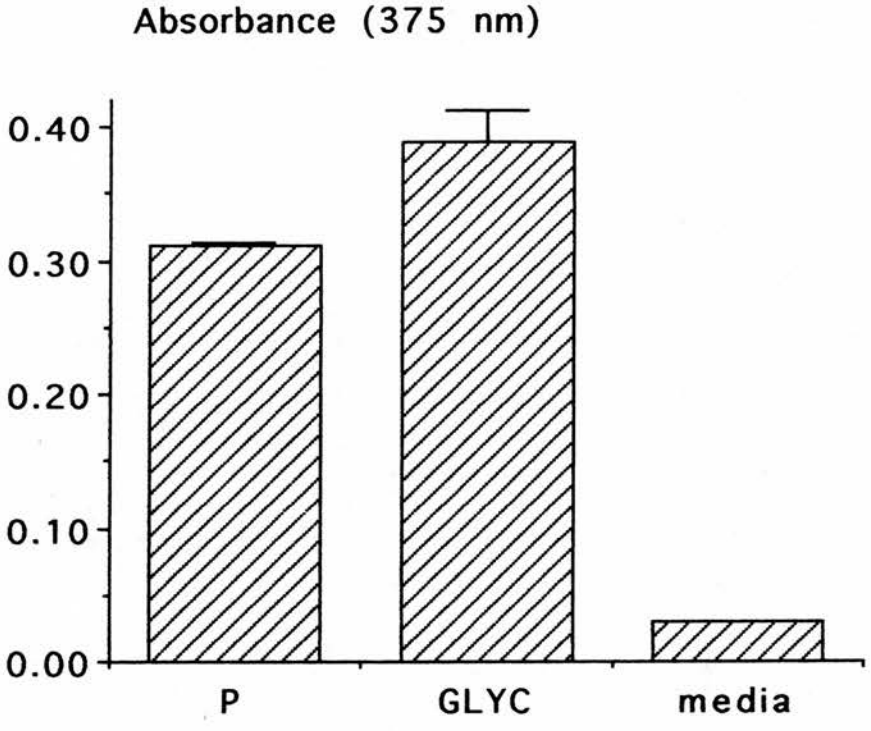
identify glycoproteins only. This method uses a dye which binds only to proteins with carbohydrate components. The fractions isolated from the conconavalin A column were applied to the gel electrophoresed and stained. No glycoproteins could be visualised. Whole conditioned medium was therefore subject to the same procedure. A number of very faint bands were observed. This again emphasises the difficulty in applying standard biochemical techniques to very small amounts of material. From the polyacrylamide gels it is obvious that glycoproteins are present within the conditioned medium from about 10kDa in molecular weight to about 70kDa.

These results therefore suggest that the primary anchorage independent growth effect is due to glycoprotein activity but not that of just one glycoprotein.

7.34 PROTEOLYTIC ACTIVITY OF GLYCOPROTEIN MIXTURE

Out of interest the glycoprotein mixture containing the primary growth promoting response was tested for proteolytic activity. A dose response curve could not be constructed since more than one protein was present. Proteolytic activity was identified (Figure 7.2) and this suggests that the glycoproteins involved in anchorage independent growth also contain proteolytic activity.

Figure 7.2 The proteolytic activity of the glycoprotein mixture isolated from conditioned medium. The glycoprotein mixture (GLYC) contained proteolytic activity.


Absorbance (375 nm)
0.40
0.30
0.20
0.10
0.00
P
GLYC
media

7.4 DISCUSSION

Earlier evidence identified the primary AIG promoting response of conditioned medium as a heat labile, trypsin sensitive charged glycoprotein (Smith and McLachlan, 1990). This protein did not appear to be EGF, since it stimulates AIG in NR6 cells which lack functional EGF receptors and is inhibited rather than promoted by additional TGF-β. It does not compete with radiolabelled EGF for EGF-receptor sites on 3T3 cells. This primary effect could not be due to TGF-α as the above arguments for EGF also apply to TGF-α. In addition TGF-α is heat stab le.

FGF-like growth factors have transforming activity (Rappolee et al., 1988). AIG is not stimulated in NRK cells either by acidic FGF or basic FGF or both together. FGF also reduces the AIG response of NRK cells to EGF. bFGF induces additional AIG in NR6 cells but this activity is removed by ant-bFGF, while the corresponding activity of conditioned medium is not. FGF does not appear to be the major component of the primary AIG effect.

PDGF has also been ruled out since it is heat stable (Assoian et al., 1984).

This primary response was not produced by fractions isolated from polyacrylamide gels. Initially it was thought that since during polyacrylamide gel electrophoresis there is a temperature increase this may have been responsible for inactivating the

effect. However the temperature never reaches 100^{0}C which is the temperature at which the activity is destroyed. The second reason put forward to explain why there was no effect produced from these purified low molecular weight fractions was that more than one factor was required and it had a molecular weight greater than 25 kDa. This reasoning appeared to hold true when conditioned medium was run down a conconavalin A column and bulk fractions were collected. The glycoproteins released from the column together produced the primary anchorage independent growth promoting effect. This response was not due to the presence of TGF-β since this came through the column first and gave EGF enhancing effect. the glycoproteins containing the primary effect were further separated only a few of the fractions contained a trace of the effect. This demonstrated that more than one glycoprotein was required to produce this growth promoting effect. One of these proteins may be the major contributor but requires the presence of one or more glycoproteins for activity.

The glycoprotein mixture isolated from conditioned medium which contained the primary anchorage independent growth promoting effect also displayed proteolytic activity.

The proteolytic activity that the glycoprotein mixture displays may be important in its promotion

of growth, by activating or deactivating events in the growth process.

CHAPTER 8 CONCLUSIONS

Medium conditioned by chick embryonic tissue contains many diverse secreted proteins such as growth factors, proteolytic enzymes and glycoproteins which display various biological activities.

The identification of the growth factor TGF-β in the medium was the first aim of the project. Through polyacrylamide gel electrophoresis and densitometer analysis TGF-β was identified as a 25 kDa protein and composed of 12.5 kDa subunits. Four regions of the embryo were examined for TGF-β activity. The head, wing buds, leg buds and tail buds were used to make conditioned medium and they were all tested for the amount of TGF-β they possessed. The ratio of TGF-β in the head, wing, leg and tail regions of the embryo was 1 : 1.3 : 1.9 : 1.8, respectively. These results suggest that the head secretes more TGF-β because it is a larger tissue. These results do not establish whether a spatial distribution of the growth factor exists in the chick embryo.

Initial studies by Macintyre et al., 1988 on transverse strips of stage 14 chick embryos did not reveal any distal-caudal distribution of TGF-β. However, it was demonstrated by Heine et al., 1987 that in the mouse embryo TGF-β was expressed in a spatial pattern. This study did not establish, however, whether TGF-β was produced in these regions in addition to being expressed. The presence of the inactive TGF-β complex needs to be

established since the presence of this complex implies that TGF-β is being generated.
The TGF-B present in conditioned medium has been shown to be formed from the breakdown of higher molecular weight proteins indicating that an inactive complex is present.

Early in the investigation a single proteolytic activity was discovered in conditioned medium. Gelatin, incorporated into a polyacrylamide gel, provided a means of assessing the presence of proteolytic activity. In the presence of the protease, gelatin was digested and when the gel was stained with amido black the digestion showed up as a clear band against a black background.
Since this was only a single activity it was suggested that it possessed an important developmental role. Following purification, characterization of this enzyme revealed it was most active at physiological temperature and a pH of 7.5. To determine the class of protease, the enzyme was incubated with a range of inhibitors. From these investigations the enzyme was found not to belong to the four main classes of protease which are cysteine proteases, metallo proteases, aspartic proteases and serine proteases. This therefore suggested a novel enzyme.
The only other known proteolytic enzymes associated with development are those concerned with the extracellular matrix. The identification of

proteases concerned with the matrix has led to the discovery of an even wider range of events involved in embryonic development. The enzyme isolated from conditioned medium was not one of the serine proteases associated with the matrix. These proteases (plasmin, plasminogen, and plasminogen activators) were ruled out since although they are active at neutral pH, their inhibitors, PMSF and Aprotinin did not result in any loss of activity for the protease from conditioned medium.

It was also thought that this enzyme could belong to the metalloproteases associated with the matrix. Collagenase with a molecular weight of 42 kDa and active at neutral pH seemeed the most likely possibilty. However, collagenase is inhibited by 1, 10 phenanthroline, a general inhibitor of metallo proteases and the enzyme isolated from conditioned medium is not.

It is also unlikely that the protease belongs to the lysosomal family of matrix enzymes since they all require an acidic environment for activity and the conditioned medium enzyme is not functioning at its maximium activity under acidic conditions.

It was thought that many of these proteolytic enzymes associated with matrix remodelling and architecture would be found in conditioned medium. This was not observed. Many proteolytic enzymes were present in the whole tissue since when the polyacrylamide gel containing the whole embryo extract was placed on an agarose gel with casein

incorporated, the entire agarose gel turned clear, indicating proteolytic digestion. The enzymes associated with the matrix may be present in conditioned medium in such small amounts that they cannot be detected by the methods used.

The amount of the protease present in head conditioned medium remained constant between stages 14 and 22 (Hamburger and Hamilton stages). These were the only stages tested for activity. Between these stages the head region of the embryo undergoes many developmental processes including the development of the brain and the eye. The presence of the proteolytic enzyme at these stages may suggest an important role for it in brain or eye development. However, other stages and regions need to be tested to determine if the amount of protease remains constant throughout embryonic development or there are times of increased or decreased activity. The amount of growth factors present in the embryo are known to be spatially and temporally regulated. The level of FGF remains constant from days 2-6 of development and then it increases. By day 13 the embryo contains an increased amount of FGF. By regulating the amount of FGF the embryo can delay the onset of some processes and stimulate others.

The proteolytic enzyme and TGF-β, for the stages tested, occurred over the same temporal span. This suggested a possible role for the enzyme in TGF-β processing.

The proteolytic enzyme did not activate latent TGF-B within conditioned medium and it did not degrade active TGF-β. The proteolytic enzyme was not therefore the *in vivo* molecule which activated TGF-B at its site of action and was not involved in the clearance of active TGF-β from the cell.

A further role for the proteolytic enzyme was pursued. Commercially purified growth factors were incubated with the protease and their effect on its proteolytic activity was investigated. The assay used was sensitive to slight changes in protein amount. The results proved difficult to interpret since the growth factors tested, namely TGF-β, FGF and EGF, displayed proteolytic activity. Previous work has looked for the presence of proteolytic activity in growth factors through amino acid analysis of their precursors. There appears to be no records of growth factors being subject to proteolytic methods of assessing activity. For these three growth factors it seems that proteolytic activity is a general feature of their make up.

When the growth factors were incubated with the protease and the activity was measured in the presence of excess substrate an effect was evident for all the factors tested. From the assay used it is unclear which component affected the activity of the other.

Since three very different growth factors produce a change in proteolytic response in the presence of the enzyme isolated from conditioned medium, it is possible that the protease has a more general role in the development of the chick embryo than expected.

It cannot be ruled out that it is the growth factors which are regulating the activity of the protease. In developmental systems there have been many reports on growth factor regulation of proteolytic activity but not of proteolytic regulation of growth factor action.

For example there have been many cases reporting the increase in plasminogen activator secretion by EGF (Lee and Weinstein, 1978; Eaton and Baker, 1983; Jetten and Goldfarb, 1983; Hamilton et al., 1984;). EGF has also been shown to increase the stimulation of plasminogen activator (Laiho et al., 1986). TGF-β induces plasminogen activator-1 (Laiho et al., 1987). Interstitial collagenase and stromelysin are induced by PDGF, EGF (Kerr et al., 1988) and interleukin-1 (Frisch and Ruley, 1987). TGF-β has an inhibitory effect on stromelysin (Matrisian et al., 1985).Madri et al., 1988 demonstrated that TGF-β is involved in protease production during angiogenesis.

In mammalian development regulation of proteolytic enzymes by growth factors is seen during embryo implantation. The migration of the blastocyst

through the uterine stroma depends on the action of proteolytic enzymes, especially metalloproteases. Brenner et al., 1989 have suggested that most embryonic proteolytic enzymes are metalloproteases. By altering the temperature and pH of the assay, the effect of the growth factors and the protease on each other may be elucidated. The effect of the growth factors on one particular protein can be different in different cellular environments. By incubating the protease and growth factors under acidic conditions, any effect observed would be due to the growth factor since the protease is inactive under acidic conditions.

From previous work on proteins and proteolytic enzymes, it is clear that growth factor research and proteolytic systems are both required for normal development.

One of the initial aims of the project was to explore the nature of the primary growth promoting effect of conditioned medium. This effect did not require any additional growth factors for activity and was destroyed by heat treatment. It was also shown not to be EGF, TGF-alpha, FGF or PDGF. Since this effect has been shown to be removed by conconavalin A beads it contains glycoprotein activity.

This activity was expected to be the result of a low molecular weight activity since the activity

was removed by 10 kDa and 30 kDa molecular weight exclusion filters.

Purification of low molecular weight proteins from conditioned medium did not produce the anchorage independent growth response when the samples were assayed in a microwell assay in the presence of NRK 49F cells. Conconavalin A columns separated the activity down to a group of proteins. Further separation of these proteins resulted in reduced amounts of activity. This indicated that probably more than one glycoprotein was required to produce the primary growth response.

Of the glycoproteins known to be present in the developing embryo many are produced as a result of growth factor action indicating the network of activities occuring in the developing embryo.

Out of interest the glycoprotein mixture which produced the anchorage independent growth promoting response was tested for proteolytic activity and it was discovered that it possessed proteolytic activity.

This is also seen for Cathepsin L which is a 39 kDa secreted glycoprotein and contains acid protease activity. It has a broad substrate specificity (Gal and Gottesman, 1986). In human tissues Cathepsin L is the cysteine protease with the greatest activity against collagen and proteoglycans and can degrade collagen. EGF and FGF can raise the levels of this glycoprotein and TGF-β can decrease the levels.

Cathepsin L emphasises the complexity of a single glycoprotein in the developing embryo.

The developing embryo therefore secretes many proteins which possess more than one type of activity. This multi activity characteristic of many of the proteins of the chick embryo may be necessary in the interests of economy. The numerous processes occuring in the developing chick involve many energy consuming processes. By producing proteins with more than one function, more than one activity and different activities in different environments, the embryo conserves energy.

The four main aims of the project were :
(1) Identification and quantification of TGF-B in medium conditioned by chick embryonic tissue; (2) Exploration and characterisation of a novel proteolytic activity discovered early in the experimental work; (3) An investigation into the role of this proteolytic enzyme in TGF-β processing. and mechanisms of activation of latent TGF-β. The proteolytic properties of other growth factors was also investigated, and (4) The exploration of the Anchorage Independent Growth (AIG) promoting properties of Conditioned Medium. Of these aims TGF-β has been identified and quantified. The protease has been characterised and it has been shown not to belong to any of the Known classes of protease. A role for it has not yet been

identified. From this investigation it looks as if more than one glycoprotein is required to produce the primary response. The nature of the primary effect has not yet been identified.

FURTHER RESEARCH

The proteolytic enzyme isolated from conditioned medium is currently being sequenced. Once the sequence is known, sequence homologies will be sought through the use of a data base, in the expectation that this will shed light on its functions and evolutionary relationships. Knowledge of the sequence will also allow the identification of functional elements within the structure which control its activity. This may provide insights into the natural substrate and action of the molecule.

The spatial and temporal distribution of the enzyme can be explored using polyclonal antibodies. The substrate specificity of the protease will also be investigated using chromogenic peptides to determine the cleavage site of the enzyme.

REFERENCES

Abraham, J.A.,Whang, L., Tumolo, A., Mergia, A., and Fiddes, J.C. 1986 Human basic fibroblast growth factor: nucleotide sequence and genomic organization. **EMBO J. 5: 2523-2528.**

Abraham, J.A.,Whang, L., Tumolo, A., Mergia, A., and Fiddes, J.C. 1987 Molecular Biology of Homo Sapiens, **51: 657-668. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.**

Akhurst, R.J., Fee, F., Balmain, A. 1988 Localized production of TGF-B mRNA in tumour promoter-stimulated mouse epidermis. **Nature 331 : 363-365.**

Anzano, M.A.,Roberts, A.B., Meyers, C.A., Komoriya, A., Lamb,L.C.,Smith, J.M. and Sporn, M.B. 1982. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. **Cancer res. 42, 4776-4778.**

Anzano, M.A., Roberts, A.B., Smith, J.M., Sporn, M.B. and Delarco, J.E. 1983. Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type alpha and type beta transforming growth factors. **Proc. Natl. Acad. Sci. U.S.A.. 80, 6264-6268.**

Anzano, M.A., Roberts, A.B. and Sporn, M.B. 1986. Anchorage-independent growth of primary rat embryo cells is induced by platelet-derived growth factor and inhibited by type-beta transforming growth factor. **J. Cell Physiol. 126, 312-318.**

Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T., Leder, P., Cochran, B.H. and Stiles, C.D. 1984 Functional role for c-myc in mitogenic response to platelet-derived growth factor. **Nature (London) 310, 655-660.**

Assoian, R.K. 1985 Biphasic effects of type B transforming growth factor on epidermal growth factor receptors in NRK fibroblasts. **J. Biol. Chem. 260 : 9613-9617.**

Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M., Sporn, M.B. 1983 Transforming growth factor-beta in human platelets. **J. Biol. Chem. 258 : 7155-7160.**

Assoian, R.K., Grotendorst, G.R., Miller, D.M. and Sporn, M.B. 1984 Cellular transformation by coordinated action of three peptide growth factors from human platelets. **Nature 309 : 804-806.**

Assoian, R.K., Sporn, M.B. 1986 Type-beta transforming growth factor in human platelets : release during platelet degranulation and action on vascular smooth muscle cells **J. Cell Biol. 102 :1712-1733.**

Astrup, T. 1975 In "Proteases and Biological Control" (E. Reich, D.B. Rifkin, and E. Shaw, eds.), **343-355 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.**

Attardi, B., Keeping, H.S., Winters, S.J., Kotsuji, F., Maurer, R. A. Tren, P. 1989 Rapid and profound suppression of messenger ribonucleic acid encoding follicle stimulating hormone B by inhibin from primate Sertoli cells. **Mol. Endocrinol. 3 : 280-287.**

Baird, A., and Durkin, T. 1986. Inhibition of endothelial cell proliferation by type beta-transforming growth factor: interactions with acidic and basic fibroblast growth factors. **Biochem. Biophys. Res. Commun. 138, 476-482.**

Baird, A., Mormede, P. and Bohlen, P. 1985 Immunoreactive fibroblast growth factorin cells of peritoneal exulate suggests its identity with macrophage derived growth factor. **Biochem. Biophys. Res. Commun. 126 : 358-364.**

Balian, G., Click, E.M., Crouch, E., Davidson, J.M. and Bornstein, P. 1979 Isolatation of a Collagen-binding fragment from fibronectin and cold-insoluble globulin. **J. Biol. Chem. 254 : 1429-1432.**

Baserga, R., ed. 1981 Tissue Growth Factors, Springer-Verlag, Berlin.

Bell, K.M. 1986 The preliminary characterization of mitogens secreted by embryonic chick wing bud tissues *in vitro* **J. Embryol. exp. Morph. 93 : 257-265.**

Bishop, J.M. 1983. Cancer genes come of age. **Cell, 32, 1081-1020.**

Bitterman P.B., Rennard, S.I., Aldberg, S., and Crystal, B.G., 1983 Role of Fibronectin as a growth factor for fibroblasts. **J. Cell Biol. 97 : 1925-1932.**

Blanchard, M., Josso, N. 1974 Source of anti-Mullerian hormone synthesized by the fetal testis : Mullerian

inhibiting activity of fetal bovine Sertoli cells in culture. **Pediatr. Res. 8 : 968-971.**

Blatti, S.P., Foster, D.N., Ranganathan, G., Moses, H.L., and Getz, M.J. 1988 **Proc. Natl. Acad. Sci. U.S.A. 85 : 1119**

Bowen-Pope, D.F., Di Corletto, Ross, R.J. 1983 Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. **Cell biol. 96, 679-683.**

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. **Anal. Biochem., 72, 248-254**

Brenner, C. A., Adler, R.R., Rappolee, D.A., Pedersen, R.A., and Werb, Z. 1989 Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. **Genes Devl. 3 : 848-859.**

Bullough, W.S. 1965. Mitotic and functional homeostasis : a speculative review. **Cancer Res. 25, 1683-1727.**

Burgess, A.W. and Nicola, N.A. 1983 Growth Factors and Stem Cells, Academic Press, Sydney.

Burleigh, M.C., Barrett, A.J., and Lazarus, G.S. 1974 A lysosomal enzyme that degrades native collagen. **Biochem. J. 137 : 387-398.**

Canalis, E., Centrella, M. and McCarthy, T. 1988 Effects of basic fibroblast growth factor on bone formation in vitro. **J. Clin. Invest. 81 : 1572-1577.**

Capony, F., Morisset, M., Barrett, A.J., Capony, J.P., Broquet, P., Vignon, F., Chambon, M., Louisot, P., and Rochefort, H. 1987 Phosphorylation, glycosylation, and proteolytic activity of the 52-kDa estrogen-induced protein secreted by MCF7 cells. **J. Cell Biol. 104 : 253-262.**

Carpenter, G. & Cohen, S.A. 1979 Epidermal growth factor. **Ann. Rev. Biochem. 48, 193-216.**

Cate, R.L., Mattaliano, R.J., Hession, C., Tizad, R., Farber, N.M., et al.,1986 Isolation of the bovine and human genes for Mullerian inhibiting substance and

expression of the human gene in animal cells. **Cell 45: 685-698.**

Chandrasekhar, S. and Harvey, A.K. 1988. Transforming growth factor-*B* is a potent inhibitor of IL-1 induced protease activity and cartilage proteoglcan degradation. **Biochem. and Biophys. Res. Commun., 157, 1352-1359.**

Cheifetz, S., Andres, J.L., Massague, J. 1988 The transforming growth factor-B receptor type III is a membrane proteoglycan. Domain sructure of the receptor. **J. Biol. Chem. 263 : 16984-16991.**

Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., et al 1990. Determinants of cellular responsiveness to three transforming growth factor-B isoforms. Role of serum factors and distinct TGF-B receptor subsets. **J. Biol. Chem. 265 : 20533-20538.**

Cheifetz, S., Like, B. and Massague, J. 1986. Cellular distribution of type I and type II receptors for transforming growth factor-*B*. **J. Biol. Chem., 261. 409-415.**

Cheifetz, S., Massague, J. 1989 The TGF-B receptor proteoglycan. Cell surface expression and ligand binding in the absence of glycoasminoglycan chains. **J. Biol. Chem. 264 : 12025-12028.**

Cheifetz, S., Weatherbee, J.A., Tsang, M.L.-S., Anderson, J.K., Mole, J.E., Lucas, R. and Massague, J. 1987. The transforming growth factor-*B* system, a complex pattern of cross reactive receptors and ligands. **Cell, 48. 409-415.**

Chiang, C.-P., and Nilsen-Hamilton, M. 1986 Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-beta on the production of secreted proteins by murine 3T3 cells and human fibroblasts. **J. Biol. Chem. 261 : 10478-10481.**

Chin, J.R., Murphy, G., and Werb, Z. 1985 Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization, and substrates. **J. Biol. Chem. 260 : 12367-12376.**

Chou, I.-N., Cox, R., and Black, P.H., 1979 Studies on the mechanism of plasminogen activator synthesis/release by Swiss 3T3 cells. **J. Cell. Physiol. 100 : 457-466.**

Coffey, R. J. Jr., Bascom, C. C., Sipes, N.J., Graves-Deal, R., Weissman, B.E., Moses, H.L. 1988. Selective

inhibition of growth relatde gene expression in murine keratinocytes by transforming growth factor B. **Mol. Cell Biol. 8 : 3088-3093.**

Coffin,J.M., Varmus, H.E., Bishop, J.M., Essex, M., Hardy, W.D., Martin, G.S., Rosenberg, N.E., Scolnick, E.M., Weinberg, R.A. and Vogt, P.K. 1981 **J. Virol 40, 953-957.**

Cohen, S. 1982 in Accomplishments in Cancer Research (Fortner, J.G. and Rhoads, J.E., eds), p. 76, Lippincott, Philadelphia.

Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant, G.A., Seltzer, J.L., Kronberger, A., He, C., Bauer, E.A., and Goldberg, G.I. 1988 H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. **J. Biol. Chem. 263 : 6579-6587.**

Corvera, S. and Czech, M.P. 1985. Mechanism of insulin action on membrane protein recycling: a selective decrease in the phosphorylation state of insulin-like growth factor II receptors in the cell surface membrane. **Proc. Natl. Acad. Sci. U.S.A. 82 : 7314-7318.**

Delarco, J.E. and Todaro, G.J. 1978. Growth factors from murine sarcoma virus transformed cells. **Proc. Natl. Sci. USA, 75. 4001-4005.**

Delli-Bovi, P., Curatola, A.M., Kern, F.G., Greco, A., Ittman, M., Basilico, C. 1987 An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. **Cell 50: 729-737.**

De Lapeyriere, D., Marics. I., Adelaidem, J., raybaud, F., Courlier, F., Rosnet, O., Benharrach, D., Mattei, M.G., and Birnbaum, D. 1991 Fibroblast growth factor gene expression in AIDS-Kaposi's sarcoma detected by in situ hybridization. **Am-J-Pathol 138(1) : 9-15**

Denhardt, D.T., Hamilton, R.T., Parfett, C.L.J., Edwards, D.R., St. Pierre, R., Waterhouse, P., and Nilsen-Hamilton, M 1986 Close relationship of the major excreted protein of transformed murine fibroblasts to thiol-dependent cathepsins. **Cancer Res. 46 : 4590-4593.**

Der, C.J., Krontiris, T.G. and Cooper, G.M. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and

Kirsten sarcoma viruses 1982. **Proc. Natl. Acad. Sci. usa 79, 3637-3640.**

Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. 1985. Human transforming growth factor-*B* cDNA sequence and expression in human tumour cell lines. **Nature, Lond., 316. 701-705.**

Derynck, R., Jarrett, J.A., Chen, E.Y., and Goedal, D.V. 1986. The murine transforming growth factor-B precursor. **J. Biol. Chem. 261:4377-4379.**

Derynck, R., Lindquist, P.B., Lee, A., Wen, D., Tamm, J., et al. 1988 Anew type of transforming growth factor-B, TGF-B3. **EMBO J. 7 : 3737-3743.**

Derynck, R., Rhee, L., Chen, E.Y., Van Tilburg, A. 1987 Intron-exon structure of human transforming growth factor-B precursor gene. **Nucleic Acid Res. 15: 3188-3189.**

Dickson, C., Acland, P., Smith, R., Dixon, M., Deed, R., MacAllan, D., Walther, W., Fuller-Pace, F., Kiefer, P. and Peters, G. 1990 Characterization of int-2 : a member of the fibroblast growth factor family. **J. Cell Sci. Suppl. 13 : 87-96.**

Dickson, C., Dixon, M., Deed, R., Acland, P., Moore, R., Whyte, A., Peters, G. 1989 Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. **Molec. Cell Biol. 9 : 4896-4902.**

Dickson, C., Smith, R., Brookes, S., and Peters, G. 1984 Tumorogenesis by mouse mammary tumour virus : proviral activation of cellular gene in the common integration region int-2 **Cell 37 : 529-536.**

Doolittle, R. F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.A. 1983 Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor **Science 221, 275-277.**

Downward, J., Yarden, Y., Mays, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. 1984 Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. **Nature (London) 307, 521-527.**

Eaton, D.L., and Baker, J.B. 1983 Phorbol ester and

mitogens stimulate human fibroblast secretions of plasmin-activatable plasminogen activator and protease nexin, an antiactivator/antiplasmin. **J. Cell Biol. 97 : 323-328.**

Edwards, D.R., Murphy, G., Reynolds, J.J., Whitham, S.E., Docherty, A.J.P., Angel, P., and Heath, J.K. 1987 Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. **EMBO J. 6 : 1899-1904.**

Ellingsworth, L.R., Braman, J.E., Fox, K., Rosen, D.M., Bentz, H., Piez, K.A. and Seyedin, S.M. 1986 Antibodies To The N-Terminal Portion Of Cartilage Inducing Factor A and TGF-B. **J. Biol. Chem. , 261: 12362-12367.**

Every, D. 1981 Quantitative Measurement Of Protease Activities In Slab Polyacrylanide Gel Electrophoretograms. **Anal. Biochem. 116: 519-523.**

Fanger, B.O., Wakefield, L.M., Sporn, M.B. 1986 Structure and properties of cellular receptor for transforming growth factor type-beta. **Biochemistry 25 : 3083-3091.**

Feige, J.J., Cochet, C., Savona, C., Shi, D.L., Keramidas, M., Defaye, G., Chambaz, E.M. 1991 Transforming growth factor beta-1: an autocrine regulator of adrenocortica; steriodogenesis. **Endoc. Res 17(1-2): 267-279.**

Fine, L.G., Holley, R.W., Nasri, H. and Badie-Dezfooly, B. 1985 BSC-1 Growth Inhibitor Transforms A Mitogenic Stimulus Into A Hypertrophic Stimulus For Renal Proximal tubule Cells: Relationship to Na+/H+ antiport. **Proc. Natl. Acad. Sci. USA. 82: 6163-6166.**

Foltmann et al., 1985. Detection of proteases by clotting of casein after gel electrophoresis. **Analytical Biochemistry, 146 : 353-360.**

Frisch, S.M., and Ruley, H.E. 1987 Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone **J. Biol. Chem. 262 : 16300-16304.**

Frolik, C.A., Wakefield, L.M., Smith, D.M., Sporn, M.B. 1984 Characterization of a membrane receptor for transforming growth factor-B in normal rat kidney fibroblasts. **J. Biol. Chem. 259 : 10995-10,000.**

Gal, S., and Gottesman, M.M. 1986 The major excreted protein (MEP) of transformed mouse cells and cathepsin L

have similar protease specificity. **Biochem. Biophys. Res. Commun. 139 : 156-162.**

Gal, S., and Gottesman, M.M. 1986 The major excreted protein of transformed fibroblasts is an activable acid-protease. **J. Biol. Chem. 261 : 1760-1765.**

Gallwitz, D., Donath, C and Sander, C 1983 A yeast gene encoding a protein homologous to the human c-has/bas proto-oncogene product. **Nature 306, 704-706**

Gentry, L.E., Lioubin, M.N., Purchio, A.F., Marquardt, H. 1988 Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide **Mol. Cell Biol. 8 : 4162-4168.**

Globus, R., Patterson-Buckendahl, O., and Gospodarowicz, D. 1988 Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor beta. **Endocrinology (Baltimore) 123: 98-105.**

Globus, R., Plouet, J. and Gospodarowicz, D. 1989 Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular matrix. **Endocrinology (Baltimore) 124 : 1539-1547.**

Goldberg, G.I., Wilhelm, S.M., Kronberger, A.M., Bauer, E.A., Grant, G. A., and Eisen, A.Z. 1986 Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. **J. Biol. Chem. 261 : 6600-6605.**

Goetinck, P.F. 1983 In " Cartilage" (B.K. Hall, ed.), Vol.111. pp. 165-189. Academic Press, New York.

Goetinck, P.F. 1988 Studies on the avian chondrodysplasia mutant, nanomelia. **Pathol. Immunopathol. Res. 7, 73-75.**

Goetinck, P.F., and Winterbottom, N. 1991 In " Biochemistry and Physiology of the Skin" (L. Goldstein, ed), 2nd ed. Oxford Univ. Press, New York.

Gospodarowicz, D. 1990 Fibroblast growth factor and its involvement in developmental processes. **Curr. Top. Dev. Biol. 24 : 57-93.**

Gospodarowicz, D. 1987 Isolation and characterization of acidic and basic fibroblast growth factor. **Methods**

Enzymol. 147: 106-119.

Gospodarowicz, D., Massoglia, S., Cheng, J., Fujii, D.K., 1986, Effect of Fibroblast Growth Factor and Lipoproteins on the Proliferation of Endothelial Cells Derived From Bovine Adrenal Cortex, Brain Cortex and Corpus Luteum Capillaries, **J. Cell. Physiol., 127** (1) : **121-136.**

Gottesman, M.M. 1978 Transformation dependent secretion of a low molecular weight protein by murine fibroblasts. **Proc. Natl. Acad. Sci. U.S.A. 75 : 2767-2771.**

Gottesman, M.M., and Sobel, M.E. 1980 Tumour proteins and Kirsten sarcoma virus increase synthesis of a secreted glycoprotein by regulating levels of translatable mRNA. **Cell 19 : 449-455.**

Gougeon, A. and Lefevre, B. 1982 Evolution of the Diameters of the Largest Healthy and Atretic follicles during the Human Menstrual Cycle. **J. Reprod. Fertil. 69 (2) : 497-502.**

Goustin, A.S.,Leof, E.B., Shipley, G.D., and Moses, H.l. 1986 Growth factors and cancer. **Cancer Res. 46:1015-1029.**

Grant, G.A., Eisen, A.Z., Marmer, B.L., Roswit, W.T., and Goldberg, G.I. 1987 The activation of human skin fibroblast procollagenase. Sequence identification of the major conversion products. **J. Biol. Chem. 262 : 5886-5889.**

Gray, A.M., Mason, A.V. 1990 Requirement for activin A and transforming growth factor-B1 pro-regions in homodimer assembly. **Science 247 : 1328-1330.**

Graycar, J.L., Miller, D.A., Arrick, B.A., Lyons, R.M., Moses, H.L., Derynck, R. 1989 Human transforming growth factor-B3 : recombinant expression, purification and biological activities in comparison with transforming growth factors B1 and B2. **Mol. Endocrinol. 3 : 1977-1986.**

Gregory, H. 1975 Isolation and structure of urogastrone and its relationship to epidermal growth factor. **Nature 257, 325-327.**

Hamburger, V. and Hamilton, H.L. 1951 A Series Of Normal Stages In The Development Of The Chick **Embryo. J. Morph., 88:49-92.**

Hamilton, R.T., Delgado, M.A., Kyoung-Shim, J., Denhardt, D.T., and Nilsen-Hamilton, M. 1990

Hamilton, J.A., Lingelbach, S., Patridge, N.C., and Martin, T.J. 1984 Stimulation of plasminogen activator in osteoblast-like cells by bone-resorbing hormones. **Biochem. Biophys. Res. Commun. 122 : 230-236**

Hamilton, R.T., Nilsen-Hamilton, R.T., and Adams, G. 1985 Superinduction by cycloheximide of mitogen-induced secreted proteins produced by Balb/c 3T3 cells. **J. Cell. Physiol. 123 : 201-208.**

Harbers, K., Kuehn, M., Delius, H., and Jaenisch, R. 1984 Insertion of retrovirus into the first intron of alpha 1(I) collagen gene to embryonic lethal mutation in mice. **Proc. Natl. Acad. Sci. U.S.A. 81 : 1504-1508.**

Hasty, K.A., Hibbs, M.S., Kang, A.H., and Mainardi, C.L. 1986 . Secreted forms of human neutrophil collagenase. **J. Biol. Chem. 261 : 5645-5650.**

Hatten, M. E., Lynch, M., Rydel, R.E., Sanchez, J., Joseph-Siverstein, J., Moscatelli, D. and Rifkin, D.B. 1988 In vitro neurite extension by granule neurons is dependent upon
astroglial-derived fibroblast growth factor. **Dev. Biol. 15 : 280-289.**

Hayashi, I., Carr, B.I. 1985. DNA synthesis in rat hepatocytes: inhibition by a platelet factor and stimulation by an endogenous factor. **J. Cell. Physiol. 125 : 82-90.**

Heine, U.I., Munoz E.P., Flanders, K.C., Ellingsworth, L.R., Lam, H.-Y.P., Thompson, N.L., Roberts, A.B. and Sporn, M.B. 1987. Role Of Transforming Growth Factor-B In The Development Of The Mouse Embryo. **J. Cell. Biol., 105: 2861-2876.**

Heinegard, D., Franzen, A., Hedbom, E., and Sommarin, Y. 1986 Common structures of the core proteins of interstitial proteoglycans. **Ciba Found. Symp. 124 : 69-88.**

Heinmark, R.L., Twardzik, D.R., and Schwartz, S.M. 1986 Inhibition of endothelial regeneration by type-beta transforming growth factor from platelets. **Science 233 : 1078-1080.**

Heino, J., Massague, J. 1990 Cell adhesion to collagen and decreased myogenic gene expression implicated in the control of myogenesis by TGF-B. **J. Biol. Chem. 265 : 10181-10184.**

Howe, P.H., Cunningham, M.R., Leof, E.B. 1990 Distinct pathways regulate transforming growth factor B1-stimulated proto-oncogene and extracellular matrix gene expression. **J. Cell. Physiol. 142 : 39-45.**

Hunter, T. 1984 Oncogenes and proto-oncogenes: How do they differ ? **J. natn. Cancer Inst. 73: 773-786.**

Ibbotson, K.J., Harrod, J., Gowen, M., D'Souza, S., Smith, D.D., Winkler, M.E., Derynck, R., and Mundy, G.R. 1986 Human recombinant transforming growth factor alpha stimulates bone
resorption and inhibits formation in vitro. **Proc. Natl. Acad. Sci. U.S.A. 83 : 2228-2232.**

Ignotz, R.A. and Massague, J. 1985. Type-B Transforming Growth Factor Controls The Adipogenic Differentiation Of 3T3 Fibroblasts. **Proc. Natl. Acad. Sci. USA, 82; 8530-8534.**

Ikawa, H., Trelstadt, R.L., Hutson, J.M., Mangonara, T.F., Donahue, P.H., 1984 Changing Patterns Of Fibronectin, Laminin, Type 4 Collagen And A Basement Membrane Proteoglycan During Rat Mullerian Duct Regression. **Dev. Biol. 102: 260-263.**

Jakowlew, S.B., Dillard , P.J., Sporn, M.B., Roberts, A.B. 1988. Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor beta 4 from chicken embryo chondrocytes. **Mol. Endocrinol. 2: 1186-1195.**

Janet, T., Grothe, C., Pettmann, B., Unsicker, K. and Sensenbrenner, M. 1988 Immunocytochemical demonstration of fibroblast growth factor in cultured chick and rat neurons. **J. Neurosci. Res. 19 :195-201.**

Jaye, M., Howk, R., Burgess, W., Ricca, G.A., Chiu, I.M., Ravera, M.W., O'Brien, S.J., Modi, W.S., Maciag, T., and Drohan, W.N. 1986 Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. **Science 233: 541-545.**

Jetten, A.M., and Goldfarb, R.H. 1983 Action of epidermal growth factor and retinoids on anchorage-dependent and independent growth of nontransformed rat kidney cells.
Cancer Res. 43 : 2094-2099.

Jullien, P., Berg, T.M., Lannoy, C., Lawrence, D., 1988 Bifunctional Activity Of TGF-B On The Growth Of NRK-49F Cells, Normal And Transformed By Kirstein Murine Sarcoma Virus. **J.Cell Physiol. 136: 175-181.**

Jullien, P., Berg, T.M., Lawrence, D.M., 1989 Acidic Cellular Environments: Activation Of Latent TGF-B And Sensitisation Of Cellular Responses To TGF-B And EGF. **Int. J. Cancer. 43: 886-891.**

Kamata, N. et al. 1986 Growth-inhibitory effects of epidermal growth factor and overexpression of its receptors on human squamous cell carcinomas in culture. **Cancer Res. 1648-1653.**

Kamijo, R., Takeda, K., Nagumo, M., and Konno, K. 1989 Suppression of TNF-stimulated proliferation of diploid fibroblasts and TNF-induced cytotoxicity against transformed fibroblasts by TGF-beta. **Biochem. Biophys. Res. Commun. 158 : 155-162.**

Kanzaki, T., Olofsson, A., Moren, A., Wernstedt, C., Hellman, U., et al. 1990 TGF-B1 binding protein : A component of the large latent complex of TGF-B1 with multiple repeat sequences. **Cell 61 : 1051-1061.**

Kehrl, J.H., Robrts,A.B., Wakefield, L.M., Jakowlew,S.B., Sporn,M.B., Fauci, A.S. 1986 TGF-B Is An Important Immunomodulatory Protein For Human B Lymphocytes. **J. Immunol. 137: 3855-3860.**

Kerr, L.D., Holt, J.T., and Matrisian, L.M. 1988 Growth factors regulate transin gene expression by c-fos-dependent and c-fos-independent pathways. **Science 242 : 1424-1427.**

Keski-Oja, J., Lyons, R.M., and Moses, H.l. 1987..Immunodetection and modulation of cellular growth with antibodies against native transforming growth factor-beta 1. **J.cell. Biochem. Suppl. 11A:60**

Keski-Oja, J., F. Blasi, E.B. Leof, and H.L. Moses. 1988. Regulation of the synthesisand activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor-B. **J. Cell Biol. 106 : 451-459.**

Kelleher, P.J., Juliand, R.L., 1984 Detection Of Proteases In Polyacrylamide Gels Containing Covalently Bound Substrates. **Anal. Biochem. 136: 470-475.**

Kim, S.-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K.Y., et al. 1990 Autoinduction of TGF-B1 is mediated by the AP-1 complex. **Mol. Cell. Biol. 10 : 1492-1497.**

Kim, S.-J., Jeang, K.T., Glick, A., Sporn, M.B., Roberts, A.B. 1989 Promoter sequences of the human transforming growth factor-B1 gene responsive to transforming growth factor-B1 autoinduction **J. Biol. Chem. 264 : 7041-7045.**

Kimchi, A., Wang, X.-F., Weinberg, R.A., Cheifetz, S., Massague, J. 1988 Absence of TGF-B receptors and growth inhibitory responses in retinoblastoma cells. **Science 240 : 196-198.**

Kimmelman, D. and Kirschner, M. 1987. Synergistic Induction Of Mesoderm FGF And TGF-B And The Identification Of An mRNA Encoding For FGF In The Early Xenopus Embryo. **Cell, 51: 869-877.**

Knabbe, C., Lippman, M.E., Wakefield, L.M., Flanders, K.C., Kasid, A., et al. 1987 Evidence that transforming growth factor-B is a hormonally regulated negative growth factor in human breast cancer cells **Cell 48 : 417-428.**

Kondaiah, P., Sands, M.J., Smith, J.M., Fields, A., Roberts, A.B., et al., 1990. Identification of a novel transforming growth-B (TGF-B5) mRNA in Xenopus embryo. **Cell 48: 417-428.**

kryceve-Martinerie, C., Lawrence, D., Crochet, P., Jullien, P., Vigier, P., 1985 Further Study Of BTGFs Released By Virally Transformed And Non-Transformed Cells. **Int.J. Cancer. 35: 553-558.**

Laemmli, U.K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227,680
Laiho, M., De Caprio, J.A., Ludlow, J.W., Livingston, D.M., Massague, J. 1990 Growth inhibition by TGF-B linked to suppression of retinoblastoma protein phosphorylation. **Cell 62 : 175-185.**

Laiho, M., O. Saksela, P.A. Andreasen, and J. Keski-Oja. 1986. Enhanced production and extracellular deposition of the endothelial type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-B. **J. Cell Biol. 103 : 2403-2410.**

Lawrence,D., Pircherr, R., Jullien, P., 1985 Conversiion

Of A High Molecular Weight Latent TGF-B From Chick Embryo Fibroblasts Into A Low Molecular Weight Active BTGF Under Acidic Conditions. **Biochem. Biophys. Res. Comm. 133: 1026-1034.**

Lawrence, D., Pircher, R., Kryceve, C., Jullien, P., 1984 Normal Embryo Fibroblasts Release TGFs In A Latent Form. **J.Cell Physiol. 121: 184-188.**

LeBaron, R.G., Esko, J.D., Woods, A., Johansson, S., and Hook, M. 1988 Adhesion of glycosaminoglycan-deficient chinese hamster ovary cell mutants to fibronectin substrata. **J. Cell Biol. 106 :945-952.**

Lee, G., Ellingsworth, L.R., Gillis, S., Wall., R., and Kincade, P.W 1987 Beta transforming growth factors are potential regulators of B lymphopoiesis. **J. Exp. Med. 166 :1290-1299.**

Lee, L.S., and Weinstein, I.B. 1978 Epidermal growth factor, like phorbol esters, induces plasminogen activator in Hela cells. **Nature (London) 274 : 696-697.**

Lehnert, S.A. and Akhurst, R.J. 1988 Embryonic Pattern Expression Of TGF beta type-1 RNA Suggests Both Paracrine And Autocrine Mechanisms Of Action. **Development, 204: 263-273.**

Leof, E.B., Proper, J.A., Goustin, A.S., Shiplley, G.D., Dicorletto, P.E., Moses, H.L., 1986 Induction Of cSis mRNA And Activity Similar To PDGF By TGF-B. A Proposed Model For Indirect Mitogenesis Involving Autocrine Activity. **Proc. Natl. Acad. Sci. 83: 2453-2457.**

Levin, E.G., and Santell, L., 1987 Association of a plasminogen activator inhibitor (PAI-1) with the growth substratum and membrane of human endothelial cells. **J. Cell Biol. 105 : 2543-2549.**

Li, M., Aggeler, J., Farson, D.A., Hatier, C., Hassel, J., and Bissell, M.J. 1987 Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. **Proc. Natl. Acad. Sci. U.S.A. 84 : 136-140.**

Lin, T., Blaisdell, J., Haskell, J.F. 1987 Transforming growth factor-B inhibits Leydig-cell steroidogenesis in primary culture. **Biochem. Biophys. Res. Commun. 146 : 387-394.**

Linzer, D.I.H., and Nathans, D. 1985 A new member of

the prolactin-growth hormone gene family expressed in mouse placenta. **EMBO J. 4 : 1419-1423.**

Liotta, L.A., Goldfarb, R.H., Brundage, R., Siegal, G.P., Terranova, V., and Garbisa, S. 1981 Effect of plasminogen activator (Urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. **Cancer Res. 41 : 4629-4636.**

Low, D.A., Scott, R.W., Baker, J.B., and Cunningham, D.D. 1982 Cells regulate their mitogenic response to thrombin through release of protease nexin. **Nature (London) 298 : 476-478.**

Lyons, K., Graycar, J.L., lee, A., Hashmi, S., Lindquist, P.B., et al., 1989 Bgr-1, a mammalian gene related to Xenopus Vg-1, is a member of the transforming growth factor gene superfamily. **Proc. Natl. Acad. Sci. U.S.A. 86 : 4554-4558.**

Lyons, R.M., Keski-Oja, J., Moses, H.L, 1988 Proteolytic Activation Of Latent TGF-B From Fibroblast Conditioned Medium. **J. Cell. Biol. 106: 1659-1665.**

Macintyre, J., Hume, D.D., Smith, J., McLachlan, J.C., 1988 A Microwell Assay For Anchorage Independent Growth. **Tissue AndCell 20: 331-338.**

Madri, J.A., Williams. S.K., Wyatt, T. and Mezzio, C. 1983. Capillary Endothelial Cell Cultures: Phenotypic Modulation By Matrix Components. **J. Cell Biol. 97: 153-165.**

Madri, J.A., Pratt, B.M., Joseph, L.B. and Tucker, A.M. 1988 Phenotypic Modulation Of Endothelial Cells By Transforming Growth Factor-B Depends Upon Composition And Organisation Of The Extracellular Matrix. **J. Cell Biol. 106: 1375-1379.**

Majack, R.A., Cook, S.C., and Bornstein, P. 1986 Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. **Proc. Natl. Acad. Sci. U.S.A. 83 : 9050-9054.**

Makela, T.P., Alitalo, R., Paulsson, Y., Westermark, B., Heldin, C.-H., and Alita;o, K. 1987 Regulation of platelet-derived growth factor gene expression by transforming growth factor beta and phorbol ester in human leukemia cell lines. **Mol. Cell. Biol. 7 : 3656-3662.**

Martin, G.R., Timpl, R. Laminin and other basement membrane components. **Ann. Rev. Cell. Biol. 3 : 57-85.**

Massague, J., 1987 The TGFB Family Of Growth And Differentiation Factors. **Cell 49: 437-438.**

Massague, J., Boyd, F.T., Andres, J.L., Cheifetz, S. 1990 Mediators of TGF-B action : TGF-B receptors and TGF-B binding proteoglycans. **Ann. NY Acad. Sci. 593 : 59-72.**

Massague, J., Kelly, B. 1986 Internalization of transforming growth factor-B andits receptor in Balb/c 3T3 fibroblasts. **J. Cell. Physiol. 128 : 216-222.**

Massague, J. and Like, B. 1985 Cellular receptors for type beta transforming growth factor **J. Biol. Chem., 260 : 2636-2645.**

Masui, T., Wakefield, L.M., Lechner, J.F., LaVeak, M.A., Sporn, M.B., Harris, C.C., 1985 Type B TGF Is The Principal Differentiation Inducing Serum Factor For Normal Human Bronchial Cells.
Proc. Natl. Acad. Sci. 83: 2438-2442.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M.-C., and Breatnach, R. 1985 Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. **EMBO J. 4 : 1435-1440.**

Matrisian, L.M., Leroy, P., Ruhimann, C., Gesnel, M.C., Breatnach, R., 1986 Isolation Of The Ocogene And EGF Induced Transin Gene: Complex Control In Rat Fibroblasts. **Mol. Cell.Biol. 6: 1679-1689.**

McLachlan, J.C., Macintyre, J., Hume, D.D., Smith, J., 1988 Direct Demonstration Of Production Of TGF Activity By Embryonic Chick Tissue. **Experentia 44:351-352.**

Mercola, M., Stiles, C., 1988 Growth Factor Superfamilies And Mammalian Embryogenesis. **Development 102: 451-460.**

Miller, D.A., Lee, A., Matsui, Y., Chen, E.Y., Moses, H.L., Derynck, R. 1989 Complementary DNA cloning of the murine transforming growth factor-B3 (TGF-B3) precursor and the comparative expression of TGF-B3 and TGF-B1 messenger RNA in murine embryos and adult tissues. **Mol. Endocrinol. 3 : 1926-1934.**

Milwidsky, A., Kaneti, H., Finci, Z., Laufer, N., Tsafriri, A., Mayer, M. 1989 Human follicular fluid protease and antiprotease activities: a suggested correlation with ability of oocytes to undergo invitro fertilization. **Fertil. Steril. 52(2); 274-280.**

Miyazono, K., Heldin, U., 1989 Role Of Carbohydrate Structures In TGF-B1 Latency. **Nature 338: 158-160..**

Miyazono, K., Hellmann, U., Wernstadt, C., Heldin, C.H., 1988 Latent High Molecular Complex Of TGFB1 **J.Biol. Chem. 263 : 6407-6415.**

Morgan, D.A., Ruscetti, F.W. and Gallo, R.C. 1976 Selective *in vitro* growth of T lymphocytes from normal human bone marrows. **Science 193, 1007-1008.**

Morrison, R.I.G., Barrett, A.J., Dingle, J.T., and Prior, D. 1973 Cathepsins B1 and D : Action of human cartilage proteoglycans. **Biochim. Biophys. Acta 302 : 411-419.**

Morrison, R.S., Sharma, A., DeVeillis, J. and Bradshaw, R.A. 1986 Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. **Proc. Natl. Acad. Sci. U.S.A. 83 (19) : 7537-41**

Moscatelli, D., Joseph-Silverstein, J., Manejias, R., and Rifkin, D.B. 1987 Mr 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. **Proc. Natl. Acad. Sci. U.S.A. 84:5778-5782.**

Moscatelli, D., and Rifkin, D.B. 1988 Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. **Biochim. Biophys. Acta 948 : 67-85**

Moses, H.L., Tucker, R.F., Leof, E.B., Coffey, R.J.Jr., Halper, J., Shipley, G.D. 1985. Type B transforming growth factor is a growth stimulator and growth inhibitor. In Cancer cells, ed. J.Feramisco, B. Ozanne, C.Stiles, 3: 65-75. New York. Cold Spring Harbour Press.

Mulder, K., Levine, A.E., Hinshaw, N.H., 1989 Up Regulation Of c-myc Gene In A Transformed Cell Line Appraching Stationary Phase Growth In Culture. **Cancer Res. 49: 2320-2326.**

Muldoon, L.L., Rodland, K.D., Magun, B.E. 1988 Transforming growth factor B and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in Rat-1 fibroblasts. **J. Biol. Chem. 263 : 18834-18841.**

Muller, R., Muller, D., Verrier,B., Bravo, R. and Herbst, H 1986 Evidence that exprssion of c-fos protein in amnion cells is regulated by external signals. **EMBO J. 5: 311-316.**

Neufeld, G., and Gospodarowicz, D. 1986 Basic and acidic fibroblast growth factors interact with the same cell
surface receptors. **J. Biol. Chem. 261: 5631-5637.**

Nicola, N.A., Metcalf, D., Matsumoto, M. and Johnson, J.R. 1983 Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. **J. Biol. Chem. 258, 9017-9023.**

Nilsen-Hamilton, M., Shapiro, J.M., Massoglia, S.L., Hamilton, R.T. 1980 Selective stimulation by mitogens of incorporation of ^{35}S methionine into a family of proteins released into the medium by 3T3 cells. **Cell 20 : 19-28.**

O'Connor-McCourt, M.D., Wakefield, L.M., 1987 Latent TGFB In Serum. **J. Biol. Chem. 262: 14090-14099.**

O'Grady, R.L., Upfold, L.I., and Stephens, R.W. 1981 Rat mammary carcinoma cells secrete active collagenase and active latent enzyme in the stroma via plasminogen activator **Int. J. Cancer 28 : 509-515.**

Okada, F., Yamaguivhi, K., Ichihara, A., Nakamura, T., 1989a One Of Two Subunits Of Masking Protein In Latent TGF-B Is Part Of A Pro TGF-B. **FEBS Letters 242: 240-244.**

Okada, F., Yamaguivhi, K., Ichihara, A., Nakamura, T., 1989b Purification And Structural Analysis Of A Latent Form Of TGF-B From Rat Platelets. **J. Biochem. 106: 304-310.**

Olashaw, N.E., Pledger, W.J. 1983 Association of platelet derived growth factor induced protein with nuclear material. **Nature 306: 272-274.**

Olashaw, N.E., Pledger, W.J., 1983 Association of platelet-derived growth factor-induced protein with nuclear

material. **Nature 306 : 272-274.**

Oreffo, R.O.C., Mundy, G.R., Sevedin, S.M., Bonewald, L.F., 1989 Activation Of The Bone Derived Latent Form Of TGF-B Complex By Isolated Osteoclasts. **Biochem. Biophys. Res. Comm. 158: 817-823.**

Padgett, R.W., St Johnston, R.D., and Gelbart, W.M. 1987 A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-B family. **Nature (Lond) 325, 81-84.**

Panaretto, B.A., Leish, Z., Moore, G.P. & Robertson, D.M. 1984 Inhibition of DNA synthesis in dermal tissue of merino sheep treated with depilatory doses of mouse epidermal growth factor. **J. Endocr. 100, 25-31.**

Panayotou, G., End, P., Aumailley, M., Timpl, R., and Engel, J. 1989 Domains of laminin with growth-factor activity. Cell 56 : 93-101.

Parish, D.C., Tuteja, R., Gainer, M., Penglah, Y., 1986 Purification And Characterisation Of A Paired Basic Residue Specific Prohormone Converting Enzyme From Bovine Pituitary Neural Lobe Secretory Vesicles. **J. Biol. Chem. 261: 14392-14397.**

Park, A.J., Matrisian, L.M., Kells, A.F., Pearson, R., Yuan, Z.Y., Navre, M. 1991 Mutational analysis of the transin (rat stromelysin) autoinhibitor region demonstrates a role for residues surrounding the cysteine switch. **J. Biol. Chem. 266 (3) : 1584-1590.**

Pelton, R.W., Nomura, S., Moses, H.L., Hogan, B.L.M. 1989 Expression of transforming growth factor-B-2 RNA during murine embryogenesis. **Development 106 : 759-768.**

Penttinen, R.P., Koyayashi, S., Bornstein, P. 1988 Transforming growth factor-B increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. **Proc. Natl. Acad. Sci. U.S.A. 85 : 1105-1108.**

Pepinsky, R.B., Sinclair, L.K., Chow, E.P., Mattialano, R.J., Mangonara, T.F., Donahue, P.K., Cate, R.L., 1988 Proteolytic Processing Of Mullerian Inhibiting Substance Produces A TGFB Like Fragment. **J. Biol. Chem. 263: 18961-18964.**

Pepper, M.S., Belin, D., Montesano, R., Orchi, L., Vassali, J.D. 1990 TGF-B1 modulates bFGF-induced

proteolytic and angiogenic properties of endothelial cells *invitro* **Cell Biol. 111 (2) : 743-758.**

Perris, R., von Boxberg, Y, Lofberg, J. Local Embryonic Matrices Determine Region Specific Phenotypes in Neural Crest Cells. 1988 Science 241 : 86-89.

Petraglia, F., Vaughan, J., Vale, W. 1989 Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placenta cells. **Proc. Natl. Acad. Sci. U.S.A. 86 : 5114-5117.**

Pilatte, Y., Bigman, J., Lambre, C.R., 1987 Lysosomal And Cytosolic Sialidases In Rabbit Alveolar Macrophages: Demonstration Of Increased Lysosomal Activity After Invivo Activation With *Bacillus Calmette- Guerin.* **Biochem. Biophys. Acta. 923: 150-155.**

Pircher, R., Jullien, P., Lawrence, D., 1986 BTGF Is Stored In Human Blood Platelets As A Latent High Molecular Weight Complex. **Biochem. Biophys. Res. Comm. 136: 30-37.**

Pircher, R., Lawrence, D., Jullien, P., 1984 Latent bTGF In Non Transformed And Kirsten Sarcoma Virus Transformed And Normal Rat Kidney Cells Clone 49F. **Cancer Res. 44: 5538-5543.**

Pollanen, J., Saksela, O., Salonen, E.-M,m Andreasen, P., Nielsen, L., Dano, K., Vaheri, A. 1987 Distinct localizations of urokinase-type plasminogen activator and its type 1 inhibitor under cultured human fibroblasts and sarcoma cells. **J. Cell Biol. 104 : 1085-1096.**

Potter, V.R. 1983. Cancer as a problem in intercellular communication: regulation by growth-inhibiting factors (Chalones). **Prog. Nucleic Acid Res. Mol. Biol. 29, 161-173.**

Prats, H., Kaghad, M., Prats, A.C., Klagsburn, M.,m Lelias, J.M., Liauzum, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A. and Caput, D. 1989 **Proc. Natl. Acad. Sci. U.S.A. 86 : 1836-1840.**

Proper, J.A., Bjornson, C.L. and Moses, H.L. 1982 Mouse embryos contain polypeptide growth factor(s) capable of inducing a reversible neoplastic phenotype in non-transformed cells in culture **J. Cell Biol., 110 : 169-174.**

Purchio, A.F., Cooper, J.A., Brunner, A.M., Lioubin, M.N., Gentry, L.E., et al., 1988 Identification of mannose -6-phosphate in two two asparagine linked sugar chains on recombinant transforming growth factor-B1 precursor. **J. Biol. Chem. 263: 14211-14215.**

Rabin, M.S., Doherty, P.J., and Gottesman, M.M. 1986 **Proc. Natl. Acad. Sci. U.S.A. 83 : 357-360.**

Rappolee, D.A., Brenner, C.A., Schultz, R., Mark, D. and Werb, Z. 1988 Developmental expression of PDGF, TGF-a and TGF-B genes in preimplantation mouse embryos. **Science 241 : 1823-1825.**

Ratner, N., Hong, D.M., Lieberman, M.A., Bunge, R.P., Glaser, R. 1988 The neuronal cell surface molecule mitogenic for Swchann cells is a heparin binding protein **Proc. Natl. Acad. Sci. USA. 85 (18) : 6992-6996.**

Rechler, M.M. and Nissley, S.P.A. 1985. The nature and regulation of the receptors for insulin-like growth factors. **Ann. Rev. Physiol. 47, 425-452.**

Ristow, H.J. 1986 BSC-1 growth inhibitor type B transforming growth factor is a strong inhibitor of thymocyte proliferation. **Proc. Natl. Acad. Sci. USA 83 : 5531-5534.**

Rizzino, A. 1988 Transforming growth factor-beta: multiple effects on cell differentiation and extracellular matrices. **Dev. Biol. 130 : 411-422.**

Robb, R.J. 1984 Immunol. Today 5, 203-209.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., Sporn, M.B., 1981 A New Class Of TGF Potentiated By EGF: Isolation From Non-Neoplastic Tissue. **Proc. Natl. Acad. Sci. 78: 5339-5343.**

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. 1985 Type B TGF A Bifunctional Regulator Of Cellular Growth. **Proc. Natl. Acad. Sci. 82: 119-123.**

Roberts, A.B., Frolik, C.A., Anzano, M.A. and Sporn, M.B. 1983. Transforming growth factors from neoplastic and nonneoplastic tissues. **Fedn. Proc., 42, 119-123.**

Roberts, A.B.,Lamb, L.C., Newton, D.L., Sporn, M.B.,

DeLarco, J.E. and Todaro, G.J. 1980 Transformig growth factors : isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extration. **Proc. Natl. Acad. Sci. USA, 77, 3494-3498.**

Roberts, A.B., Sporn, M.B. 1990 The transforming growth factor-betas. In "Peptide Growth Factors and their Receptors", ed. M. Sporn, A.B. Roberts. Heidelberg: Springer-Verlag.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Rehrl, J.H. and Fanci, A.S. 1986 Transforming growth factor type beta: rapid induction of fibrosis and angiogenes is in vivo and stimulation of collagen formation in vitro. **Proc. Natl. Acad. Sci. USA, 83, 4167-4171.**

Robey, P.G., Young, M.F., Flanders, K.C., Roche, N.S., Kondaihiah, P., Reddi, AQ.H., Termine, P., Sporn, M.B.,ROberts, A.B. 1987 Osteoblasts Synthesise And Respond To TGF-B *In Vitro*. **J. Biol. Chem. 105: 457-463.**

Rochefort, H., Capony, F., Garcia, M., Cavailles, V., Freiss, G., Chambon, M., Morisset, M., and Vignon. F. 1987 Estrogen-induced lysosomal proteases secreted by breast cancer cells: a role in carcinogenesis? **J. Cell. Biochem. 35 : 17-29.**

Rosa, F., Roberts, A.B., Danielpour, D., Dart, L.l., Sporn, M.B. and David, I.B., 1988 Mesoderm induction in amphibians : the role of TGF-B2 like fators. **Science 239 : 783-785.**

Rossi, P., Karsenty, G., Roberts, A.B., Roche, N.S., Sporn, M.B., de Crombrugghe, B. 1988 A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-B. **Cell 52 : 405-414.**

Roughley, P.J., and Barrett, A.J. 1977 The degradation of cartilage proteoglycans by tissue proteinases : proteoglycan structure and its susceptibility to proteolysis. **Biochem. J. 167 : 629-637.**

Ruoslahti, E., Pierschbacher, M.D. 1987 New perspectives in cell adhesion : RGS and integrins. **Science 238 : 491-495.**

Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D.B. 1988 Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. **J. Cell. Biol. 107 :**

743-751.

Sandberg, M., Vuorio, T., Hirvonen, H., Alitalo, K. and Vuorio, E. 1988 Enhanced expression of TGf-B and c-fos mRNAs in growth plates of developing human long bones. **Development 102 : 461-470.**

Sato, Y., Rifkin, D.B. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells : Activation of a latent transforming growth factor-B1 like molecule by plasmin during co-culture. **J. Cell Biol. 109 : 309-315.**

Scher, W. 1987 The role of extracellular proteases in cell proliferation and differentiation. **Lab. Invest. 57 : 607-623.**

Scher, C.D., Hendrickson, S.L., Whipple, A.P., Gottesman, M.M., and Pledger, W.J. 1982 Cold Spring Harbour Conf. **Cell Proliferation 9 : 289-303.**

Schlesinger, M.J. 1986. The dynamic state of heat shock proteins in chicken embryo fibroblasts. **J.Cell. Biol. 103, 321-325.**

Scotto, L., Vaduva, P.I., Wager, R.E., Assoian, R.K. 1990 Type B1 transforming growth factor gene expression. A corrected mRNA structure reveals a downstream phorbol ester responsive element in human cells. J. **Biol. Chem. 265 : 2203-2208.**

Seed, J., and Hauschka, S.D. 1988. Clonal analysis of vertebrate myogenesis. VIII. Fibroblasts growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development. **Dev. Biol. 128: 40-49.**

Seed, J., Olwin, B.B., and Hauschka, S.D. 1988 Fibroblast growth factor levels in the whole embryo and limb bud during chick development. **Dev. Biol. 128; 50-57.**

Segarini, P.R., Roberts, A.B., Rosen, M.D., Seyedin, S.M. 1987 Membrane binding characteristics of two forms of transforming growth factor-B. **J. Biol. Chem. 262 : 16455-14662.**

Shipley, G.D., Tucker, R.F., Moses, H.L. 1985 Type B-transforming growt factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S-phase after prolonged prereplicative interval. **Proc. Natl. Acad. Sci. U.S.A. 82 : 4147-4151.**

Shipley, G.D., Pittlekow, M.R., Wille, J.J., Scott, E.E., Moses, H.L., 1986 Reversible Inhibition Of Human Prokeratinocytes Proliferation By Type Beta TGF Growth Inhibitor In Serum Free Medium. **Cancer Res. 46: 2068-2071.**

Silberstein, G.B., Daniel, C.W. 1987 Reversible inhibtion of mammary gland growth by transforming growth factor-B. **Science 237 : 291-293.**

Slack, J.M. and Smith, J.C., Dosalisation and Neural Induction : Properties of the Organiser in Xenopus Laevis. 1983 J. **Embryol. Exp. Morphol. 78 : 299-317.**

Smith, J., McLachlan, J.C., 1989 Developmental Pattern Of Growth Factor Production In Chick Embryo Limb Buds. **J. Anat. 165: 159-168.**

Smith, J., McLachlan, J.C., 1990 Identification Of A Novel Growth Factor With Transforming Activity Secreted By Individual Chick Embryos. **Development 109, 905-910.**

Spencer, F.A., Hoffmann, F.M., Gelbart, W.M. 1982 Decapentaplegic : A gene complex affecting morphogenesis in *Drosophila melanogaster*. **Cell 28 : 451-461.**

Spiro, R.C., Casteel, H.E., Laufer, D.M., Reisfeld, R.A., and Harper, J.R. 1989 Post-translational addition of chondroitin sulfate glycosaminoglycans. Role of N-linked oligosaccharide addition, trimming, and processing. **J. Biol. Chem. 264 : 1779-1786.**

Sporn, M.B., Roberts, A.B., 1985 Autocrine Growth Factors And Cancer **Nature 313: 745-747.**

Sporn, M.B., Roberts, A.B., Wakefield, L.M., 1987 Some Recent Advances In The Chemistry And Biology Of TGF-B. **J. Cell Biol. 105; 1039-1045.**

Sporn, M.B., Roberts, A.B., Wakefield, L.M., Assoain, R.K., 1986 TGF-B: Biological Function And Chemical Structure. **Science 233:532-534.**

Sporn, M.B., Roberts, A.B., Wakefield, L.M., and de Crombrugghe, B. 1987 Some recent advances in the chemistry and biology of transforming growth factor-beta. **J. Cell Biol. 105 : 1039-1045.**

Sporn, M.B. and Todaro, G.J. 1980. New Engl. J. Med.

303, 878-880.

Stiles, C.D. 1983 The molecular biology of platelet-derived growth factor. **Cell 33, 653-655.**

Strassmann, G., Cone , J.L., Arthur, P.M., Guertin, M., Herrfeldt, J., 1989 Effect Of Platelet Derived Growth factor (TGF) Type B On Murine Inflammatory Mononuclear Phagocytes: Increased Fibronectin Production. **Cell.Immun. 121: 306-316.**

Taylor, J.M., Cohen, S., Mitchell, W.M., 1970 EGF: High And Low Molecular Weight Forms. **Proc. Natl. Acad. Sci. 67:164-171.**

Togari, A., Dickens, G., Kuzuya, H., Guroff, G. 1985 The effect of fibroblast growth factor on PC12 cells. **J. Neurosci. 5 (2) : 307-316.**

Tryggvason, K., Hoyhtya, M., and Salo, T. 1987 Proteolytic degradation of extracellular matrix in tumor invasion. **Biochim. Biophys. Acta 907 : 191-217.**

Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W. 1984 Growth inhibitor from BSC-1 cells is closely related to the platelet type B transforming growth factor. **Science 226, 705-707.**

Van Obberghen-Schilling, E., Roche, N.S., Flanders, K.C., Sporn, M.B., Baker, C.C.1988. Transforming growth factor-B1 positively regulates its oqn expression in normal and transformed cells. **J. Biol. Chem.263: 7741-7746.**

van Zoelen, E.J.J., van Oostwaard, T.M.J., and de Laat, S.W. 1986 PDGF-like growth factor Induces EGF Potentiated Phenotypic Transformation of Normal Rat Kidney Cells in the Absence of TGF-beta. **J. Biol. Chem. 261 : 5003-5009.**

Vassalli, J.-D., Baccino, D., and Belin, D. 1985 A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. **J. Cell Biol. 100 : 86-92.**

Wakefield, L.M., Smith, D.M., Flanders, K.C., SpornM.B., 1988 Latent TGF-B From Human Platelets. **J. Biol. Chem. 263; 7646-7654.**

Wakefield, L.M., Smith, D.M., Masui, T., Harris, C.C.

and Sporn, M.B. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-B **J. Cell Biol. 105, 965-975.**

Waterfield, M.D., Scrace, G.T., Whittle, N., Sroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J.S. and Deuel, T.F. 1983 nature (London) 304, 35-39.

Werb, Z. 1988 In " Textbook of Rheumatology " (W.N. Kelly, E.D. Harris, Jr., S. Ruddy, and C.B. Sledge, eds.), Chap. 18 Saunders, Philadelphia, Pennsylvania.

Wilcox, J.N., Derynck, R. 1988 Developmental expression of transforming growth factors alpha and beta in mouse fetus. **Mol. Cell. Biol. 8 : 3415-3422.**

Wilhelm, S.M., Collier, I.E., Kronenberger, A., Eisen, A.Z., Marmer, B.L., Grant, G. A., Bauer, E.A., and Goldberg, G.I. 1987 Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. **Proc. Natl. Acad. Sci. U.S.A. 84 : 6725-6729.**

Wilhelm, S.M., Eisen, A.Z., Teter, M., Clark, S.D., Kronberger, A., and Goldberg, G. 1986 Human fibroblast collagenase: glycosylation and tissue-specific levels of enzyme synthesis. **Proc. Natl. Acad. Sci. U.S.A. 84 : 6725-6729.**

Wilkinson, D.G., Peters, G., Dickson, C., and McMahon, A.P. 1988 Expression of the FGf-related proto-oncogene int-2 during gastrulation and neuralation in the mouse. **EMBO J. 7 : 691-695.**

Wozney, J.M. 1989 Bone morphogenetic proteins. **Prog. Growth Factor Res. 1 : 267-280.**

Togari, A., Dickens, G., Kuzuya, H., Guroff, G. 1985 The effect of fibroblast growth factor on PC12 cells. **J. Neurosci. 5 (2) : 307-316.**

Tryggvason, K., Hoyhtya, M., and Salo, T. 1987 Proteolytic degradation of extracellular matrix in tumor invasion. **Biochim. Biophys. Acta 907 : 191-217.**

Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W. 1984 Growth inhibitor from BSC-1 cells is closely related to the platelet type B transforming growth factor. **Science 226, 705-707.**

Van Obberghen-Schilling, E., Roche, N.S., Flanders, K.C., Sporn, M.B., Baker, C.C.1988. Transforming growth factor-B1 positively regulates its oqn expression in normal and transformed cells. **J. Biol. Chem.263: 7741-7746.**

van Zoelen, E.J.J., van Oostwaard, T.M.J., and de Laat, S.W. 1986 **J. Biol. Chem. 261 : 5003-5009.**

Vassalli, J.-D., Baccino, D., and Belin, D. 1985 A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. **J. Cell**

beta in mouse fetus. **Mol. Cell. Biol. 8 : 3415-3422.**

Wilhelm, S.M., Collier, I.E., Kronenberger, A., Eisen, A.Z., Marmer, B.L., Grant, G. A., Bauer, E.A., and Goldberg, G.I. 1987 Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differential expression in normal and tumorigenic cells. **Proc. Natl. Acad. Sci. U.S.A. 84 : 6725-6729.**

Wilhelm, S.M., Eisen, A.Z., Teter, M., Clark, S.D., Kronberger, A., and Goldberg, G. 1986 Human fibroblast collagenase: glycosylation and tissue-specific levels of
enzyme synthesis. **Proc. Natl. Acad. Sci. U.S.A. 84 : 6725-6729.**

Wilkinson, D.G., Peters, G., Dickson, C., and McMahon, A.P. 1988 Expression of the FGf-related proto-oncogene int-2 during gastrulation and neuralation in the mouse. **EMBO J. 7 : 691-695.**

Wozney, J.M. 1989 Bone morphogenetic proteins. **Prog. Growth Factor Res. 1 : 267-280.**