

CYTOGENETIC DAMAGE, ONCOGENIC
TRANSFORMATION AND P53 INDUCTION IN HUMAN
EPITHELIAL CELLS IN RESPONSE TO IRRADIATION

Mark Armitage

A Thesis Submitted for the Degree of PhD
at the
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Mark Armitage

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

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Declaration

I, Mark Armitage, hereby certify that this thesis has been composed by myself, and 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.

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

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

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ABSTRACT

Ionizing radiation can have several different effects on cells, some are almost instantaneous such as the generation of DNA damage, other cellular responses take a matter of minutes or hours - DNA repair protein induction/activation, and others may take months or even years to be manifested - carcinogenesis.

Human epithelial cell lines derived from both normal, non-neoplastic tissues and from a malignant source were cultured in order to examine several effects of ionizing radiation on such cell types. Cells not from a malignant source were previously immortalized by viral infection or by transfection with viral sequences.

Simian virus 40 immortalised uroepithelial cells (SV-HUC) were found to be approximately a factor of two fold more radioresistant than cells of malignant origin (T24) in terms of unrepaired clastogenic damage i.e. assessment of micronuclei levels following irradiation.

SV-HUC lines unlike T24 cells are non-tumourigenic when inoculated into nude athymic mice. SV-HUC lines proved very resistant to full oncogenic transformation using radiation and chemical carcinogens. However, morphological alterations and decreased anchorage dependant growth was observed in post carcinogen treated cells after appropriate cell culture conditions were utilized. The progression from this phenotype to a fully tumourigenic one was not recorded in this study.

The ability of ionizing radiation to induce increased levels of the nuclear phosphoprotein p53 was also assessed using several different cell lines. SV-HUC and T24 cell lines failed to exhibit any increased p53 stabilization following irradiation. One cell line, a human papilloma virus transformed line (HPV) did show an approximate two fold increase of the wild type p53

protein after treatment with radiation. Only the cell line HPV showed any cell cycle delay, resulting in accumulation of cells in the G_2/M compartment in post irradiation cell cycle analysis. The status of p53 was also assessed i.e. wild type or mutant conformation in all the above cells lines and two other control lines HOS (a human osteosarcoma cell line) and H Tori-3 (SV40 immortalised thyroid epithelial cells).

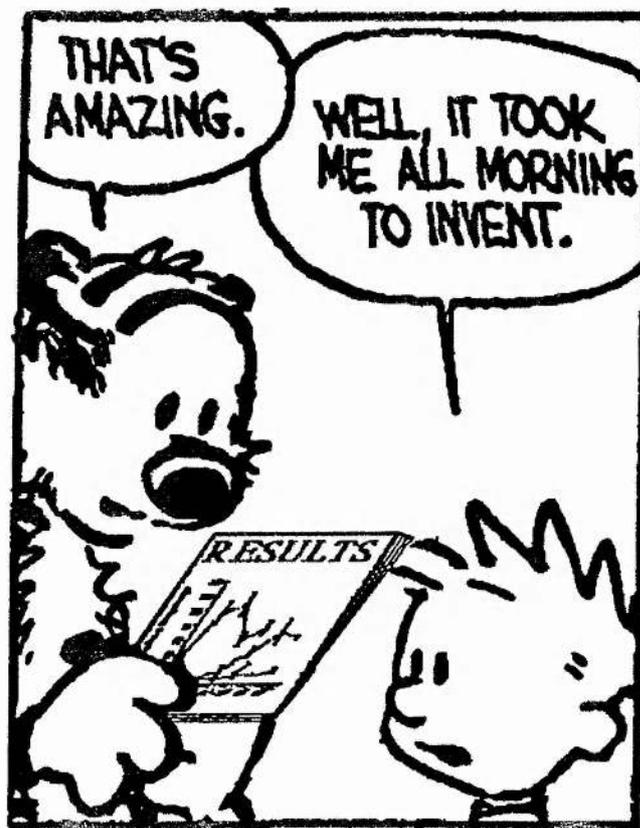


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CHAPTER 1

INTRODUCTION

- 1.1 Human Carcinogenesis - Historical and Epidemiological Studies.
- 1.2 The use of rodent cell lines to study transformation *in vitro*.
- 1.3 The use of human cell lines to study transformation *in vitro*.
- 1.4 The multistep nature of carcinogenesis.
 - 1.4a. Carcinogen interaction with DNA.
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 - Tumour suppressor gene inactivation.
 - 1.4d. Initiation, clonal expansion and promotion.
- 1.5 Aims of thesis.

INTRODUCTION

1.1 Human Carcinogenesis - Historical and Epidemiological Studies

Cancer has been described as a genetic disease at the somatic cell level (Bodmer 1988), occurring chiefly in cells of epithelial origin. Estimates place the proportion of adult human cancers arising in epithelial tissues at >90% (Cairns 1975). This is not entirely surprising when the nature of such self-renewing epithelia is considered, a continual but usually controlled multiplication of dividing cells in basal layers replacing cells lost at the epithelial surface. It is this escape from normal cellular constraints and controlled growth initiated by somatic mutations, spontaneous or induced, that may be termed cancer.

The generation of human cancer due to physical and chemical environmental factors has been known for over a century; Marie Curie's death thought to be from leukaemia, due to her work with radium and Percival Pott's identification of scrotal cancer in children chimney sweeps due to their exposure to soot (1775). More recently there has been an expanding body of evidence implicating ionizing radiation exposure with increased rates of human cancer. Radium dial painters who ingested significant quantities of this α emitter were often found to suffer from osteosarcomas in later life due to the incorporation of radium into bones since it lies in the same group of the periodic table as calcium. Atomic bomb attacks on Hiroshima and Nagasaki generated an excess of cancers in the surviving populations due to γ and neutron exposure (Reviewed by Hall 1988). Within the last few years much controversy has been generated by the apparent increased rates of leukaemia and non-Hodgkin's lymphoma around nuclear reprocessing

plants such as Sellafield in West Cumbria (Gardner *et al.*, 1990). More accurately, there appear to be raised levels of leukaemia amongst the children of male workers exposed to occupational radiation prior to conception of these children.

However, many other varying factors such as diet, age, sex and genetic predisposition to cancer only serve to complicate the already limited data from such human *in vivo* studies. To gain a better understanding of the effects of radiation on mammalian and human cells it was necessary to turn to the use of cell culture and in particular experiments using immortal cell lines.

1.2 The use of rodent cell lines to study neoplastic transformation *in vitro*.

The development of cell lines with an indefinite life span in culture which were not malignant in origin, enabled a great deal of investigation to be performed using defined conditions and closely controlled carcinogen exposures. Much of our present knowledge concerning a whole range of radiation induced cellular changes is largely based on observations made in such cell lines.

The cell lines initially generated were derived from rodent fibroblast material which existed as primary cultures. However, after a careful regime of sub-culturing an immortal population of cells having survived "crisis" was generated (Reznikoff *et al.*, 1973a). Such cells required no further treatment to generate a population with an indefinite life span in culture. Cell lines commonly used include; C3H 10T¹/2, Balb/c - 3T3 and Chinese Hamster Ovary or CHO cells.

Soon after such cell lines were established it became possible, through treatments with chemical carcinogens and later radiation, to facilitate

oncogenic or neoplastic transformation of such cells. That is to say, that cells maintained in culture generated dense type colonies (type II and III foci) on a confluent monolayer which when reintroduced into a mouse of the same strain as the initial parent cell type, generated tumours (Reznikoff *et al.*, 1973b, Terzaghi and Little 1976). Also it became apparent that such cell lines were relatively easy to transform into an oncogenic phenotype from the non-tumourigenic parental phenotype. It is also worth noting the relatively simple treatment protocol used to generate such transformed foci when compared to the procedure used for human transformation work (see section 1.3).

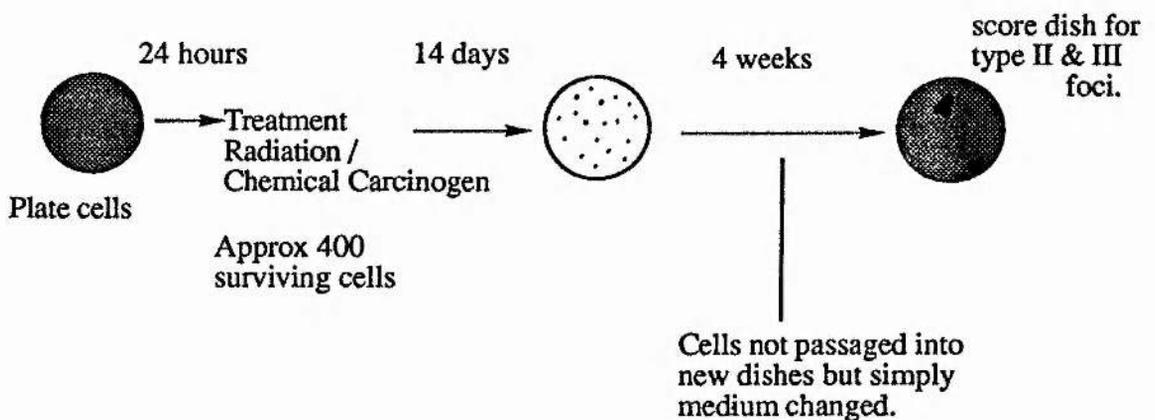


FIG 1.1. Protocol for a cell transformation assay using C3H 10T¹/2 cells. A duplicate series of plates were also treated in an identical manner but harvested after 14 days and the cell number determined. This enables transformation frequencies per surviving cell to be determined.

Taken from Hall and Hei 1986.

Such cell lines are still extensively used to study the effects of a variety of different parameters on oncogenic transformation due to the highly reproducible results when standardised procedures are followed (Meeting Report 1985). Some of the more recent applications of such cell lines have

included the effects of particles of defined LET (linear energy transfer; Hei et al., 1988), restriction endonucleases (Bryant and Riches 1989) and cell cycle dependence (Cao *et al.*, 1992, Miller *et al.*, 1992) on oncogenic transformation.

1.3. The use of human cell lines to study oncogenic transformation *in vitro*.

Despite the large amount of data generated concerning the responses of rodent fibroblast cell lines in transformation studies it remains unclear how accurately this relates to the human situation .

Firstly interspecies differences must be considered. Rodent cells exhibit a far higher degree of chromosomal instability than human cells do. Rodent cells acquire immortality and generate permanent cell lines spontaneously, human cells do not but instead need a foreign agent such as a virus to become immortal, i.e. cell crisis does not lead to the establishment of a cell line in human cell culture. Finally even when human cells have been immortalised they are often much more resistant to oncogenic transformation with either radiation or chemical carcinogens when compared to rodent cell lines (reviewed by Reznikoff *et al.*, 1986).

Cell type may also be another important factor when deciding on the suitability of model systems and their relevance to neoplastic development in humans. As mentioned earlier >90% of all human cancers originate in epithelial tissues, however all of the rodent cell lines discussed above are fibroblast in origin. Therefore a seemingly more suitable cell type to study would be one of human epithelial origin.

Initially only human cells that were from malignant sources could be cultured indefinitely since they were already immortal, but such cell lines are of no real significance when *in vitro* oncogenic transformation is being

studied. With advances in cell culture methodology, more complex and defined media and the development of immortalisation techniques human cells could now be maintained indefinitely in culture. Immortalisation of human material usually relies on the infection of a primary tissue explant with a DNA tumour virus or at least a plasmid containing sequences from such a virus (Christian *et al.*, 1987, Dipaolo *et al.*, 1989, Thraves *et al.*, 1990). Human cells are nonpermissive for many such tumour viruses such as Simian Virus 40 (SV40) i.e. they are not capable of permitting a lytic infection which generates more virus particles. However the early genes of this virus coding for the T-antigen protein, do integrate into the human cell's DNA. The production of this viral oncoprotein in the human cell leads to the generation of an immortal phenotype. This T-antigen of SV40 was reported to bind to a 53,000 Dalton protein (p53) in immortalised cells by Lane and Crawford (1979). The modification of function of p53 was found to be a vital switch able to transform a cell with limited life span in culture into an immortal one (the functions of p53 are considered more fully in Chapter 5).

Human papilloma virus although acting in a slightly different way since it can produce a lytic infection in human cells, also is able to immortalise cells since its E6 protein can abrogate p53 function (Lechner *et al.*, 1992, Mietz *et al.*, 1992). Cells immortalised by such methods are non-tumourigenic (Christian *et al.*, 1987, Dipaolo *et al.*, 1989, Thraves *et al.*, 1990 plus see Chapter 4).

As with rodent cell lines full oncogenic transformation using chemical carcinogens was reported soon after such cell lines had been established (Reznikoff *et al.*, 1988, Rhim *et al.*, 1990, Bookand *et al.*, 1992).

Tumourigenicity in such cases was checked for by tumour formation in athymic nude mice which have a deficient immune system and so allow

growth of cells which are non-self. The cell culture procedure following carcinogen exposure differed considerably from that used to study rodent oncogenic transformation.

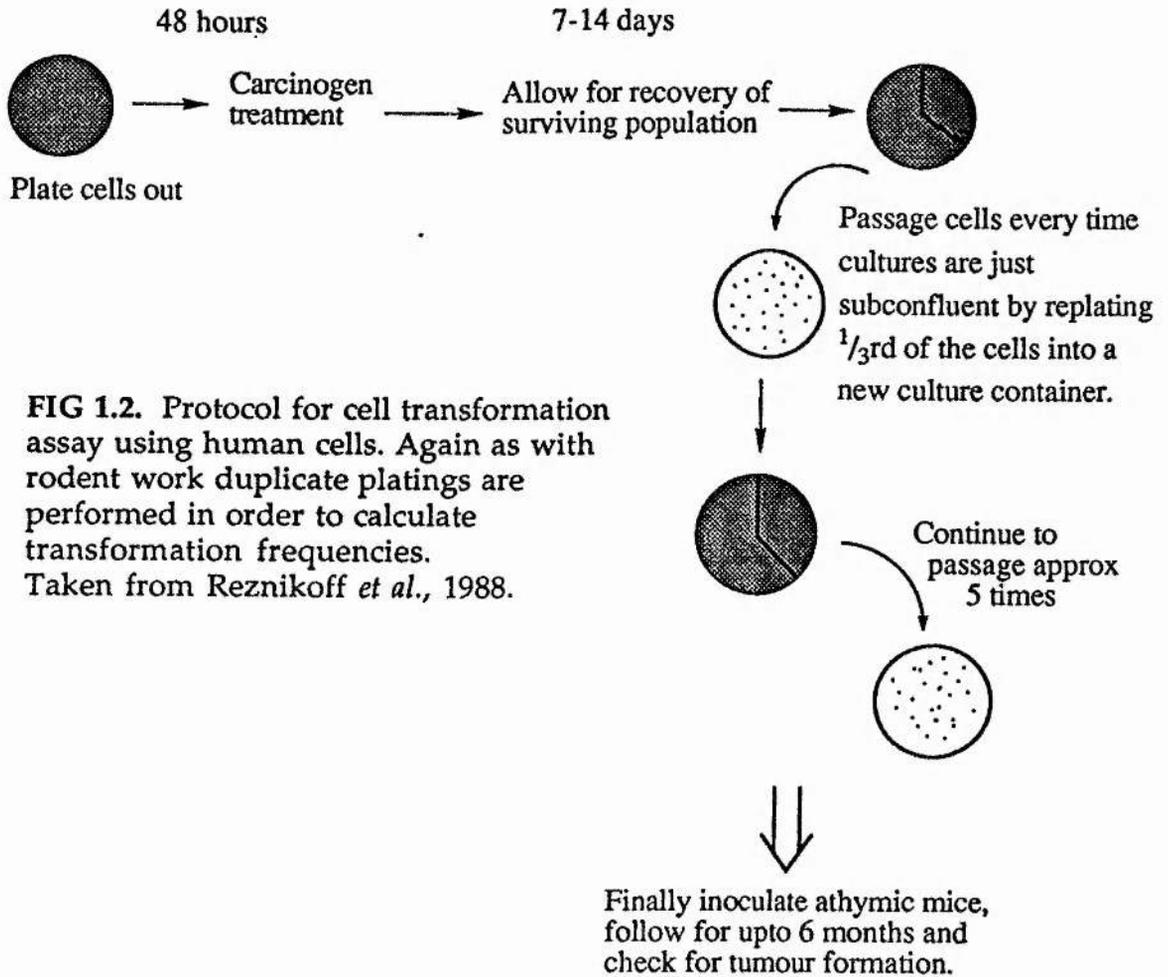


FIG 1.2. Protocol for cell transformation assay using human cells. Again as with rodent work duplicate platings are performed in order to calculate transformation frequencies. Taken from Reznikoff *et al.*, 1988.

Despite chemical carcinogen induced neoplastic transformation being achieved in such human epithelial cell lines, reports of radiation inducing a similar phenotype were not observed. The few exceptions to this were one isolated report of a human fibroblast being transformed by X-rays (Borek 1980) and the work carried out on human hybrid cells by Sun and Redpath. This

hybrid cell line is the result of fusing a purported cervical carcinoma cell line (HeLa cells) and a normal skin fibroblast. Such cells are initially nontumourigenic but do readily undergo radiation induced oncogenic transformation after a treatment protocol similar to that used for the rodent transformation systems (Redpath *et al.*, 1987, Sun *et al.*, 1988, Redpath and Sun 1990). However, these cells must be considered "preneoplastic" and hence far less refractory to radiation induced transformation than their "normal" human counter parts. What this transformation system may in fact be studying is the loss of a single tumour suppressor gene supplied by the fibroblast cell, which is masking the previously expressed tumourigenic phenotype of the HeLa cell.

1.4. The multistep nature of carcinogenesis.

Cancer development is in most cases a prolonged, slow process by biological standards often lasting from one to two-thirds of the life span of the organism before it's manifestation (Farber and Rubin 1991). It is now widely acknowledged that cancer arises not from a single event within a cell, but via a multistep mechanism requiring the co-operation of a number of different cellular processes (Faber 1984). The only exceptions to this are the rapidly generated tumours formed as a result of oncogene containing retroviral infections (Benchimol 1992). Environmental exposure to biological, chemical & physical agents and genetic predisposition all serve to determine an individuals susceptibility to cancer. However, a series of common if not entirely universal cellular alterations in rodent and human, *in vivo* and *in vitro* systems has so far been elucidated:-

1.4a. Carcinogen interaction with DNA.

Any potential carcinogen must ultimately interact with DNA and cause mutations that are then fixed in the surviving cell population. With radiation acting as the carcinogen it is absorbed by the nucleus within 10^{-15} seconds and the damaging chemical processes involving free radical reactions are over within 10^{-5} seconds, i.e. its effect on DNA is very rapid (Hall 1988). Chemical carcinogens on the other hand are often metabolised by cells before their carcinogenic effects are exercised by such enzyme complexes as cytochrome P-450 and N-acetyltransferase (Harris 1991, Swaminathan and Reznikoff 1992). Such processes often have a genetic component associated with them and polymorphism of detoxifying enzymes is not uncommon between different individuals. Mechanisms such as these serve to regulate the rate and quantity with which DNA adducts are formed. (A more detailed description of the types of DNA adducts generated by the different carcinogens used in this study is given in section 4.8).

Several other species which are not enzyme complexes can also help to modify the extent of DNA adduct formation. Glutathione and other sulfhydryl containing compounds have a known mechanism of action acting as efficient scavengers of free radicals so exhibiting radioprotection properties. Recently a range of other, less likely candidates has been identified such as vitamin C, vitamin E, β -carotene and selenium which are dietary constituents that appear to act by limiting the extent of free radical reactions with DNA and so reduce the extent of possible oncogenic damage (von Sonntag 1987, Henderson *et al.*, 1991).

1.4b. DNA repair.

Once DNA damage has occurred whether it be deletion, base substitution/alteration, chain rupture etc... spontaneous or induced, the fate

of such a cell is now dependant on the repair processes that can be brought to bear in an attempt to rectify such damage. Cells containing damaged DNA in a vital coding region, i.e. one which codes for an essential protein or a control element such as a promoter or enhancer region for such a gene, now have three possible alternatives.

1. Successful repair of the damaged sequence - no phenotypic effect.
2. Non-repair or misrepair of the damaged sequence - cell death.
3. Non-repair or misrepair of the damaged sequence - cell death does not result but the damage is now fixed as a mutation, this may then contribute towards carcinogenesis.

A full overview of DNA repair mechanisms is outside the scope of this thesis but it should be noted that mammalian cells like simpler prokaryotic organisms, appear to have a number of different types of repair processes. These often complex processes requiring recognition, incision, excision and re-synthesis procedures can be engaged to deal with differing extents of damage as well as DNA damage of different types. These include; short-patch repair capable of operating on the major damage products of ionizing radiation and alkylating agents, long-patch repair which operates in response to UV like damage and "inducible or adaptive" repair in response to pretreatments of a carcinogen prior to a larger "challenge" dose (Hanawalt *et al.*, 1979, Morimoto *et al.*, 1986, Teoule 1987).

1.4c. Unrepaired DNA damage.

Unrepaired DNA lesions may be expressed in a number of different ways following genetic injury.

1. **Cytogenetic damage.** The generation of acentric fragments which no longer associate with other intact DNA at mitosis and nuclear division is one

manifestation of such damage, these include chromosome & chromatid aberrations and micronuclei. Although DNA damage which is expressed in this form is often not as carcinogenic as say base substitutions, since the cell usually cannot cope with a large loss in genetic material and so such losses are lethal to the cell. Cytogenetic aberrations of this nature do serve to provide an indication of the extent of certain types of unrepaired genetic damage, screening for such aberrations often forms the basis of risk assessment following radiation exposure. Recently Kadhim *et al* (1992) have demonstrated that exposure of human cells to α irradiation can result in the transmission of chromosomal instability to daughter cell progeny several cell divisions later. This finding may have important implications for radiation induced leukaemia (see section 1.1).

2. Alteration of proto-oncogene function. There are now more than 30 oncogenes that are known to participate in human cancer formation (Busch 1990). Proto-oncogenes are cellular genes which code for proteins that control cell proliferation and differentiation within the cell (Wynford-Thomas 1991). Mutation within such a gene or misexpression (usually acting in dominant fashion) can lead to the generation of a neoplastic phenotype. They may be activated by a single point mutation such as the *Ha-ras* oncogene where a single base pair alteration at codon 12 leads to the substitution of a different amino acid in place of the usually present glycine (Tabin *et al.*, 1982, Balmain *et al.*, 1984). Alternatively they may be activated not by a structural alteration in the protein which is coded for by the oncogene but modified expression. A now well known example of this is the over expression of the *c-myc* oncogene usually located on chromosome 8q. However, when this oncogene is juxtaposed next to the Ig H region of chromosome 14q by a reciprocal

translocation Burkitt's lymphoma results. This is thought to result from the Ig H enhancer region increasing expression of *c-myc* (Klein and Klein 1987).

Oncogenes may be broadly split into three different groups depending on their site and mode of action.

- a. An altered growth factor receptor molecule located on the plasma membrane. Example; *c-erb B2* codes for a truncated epidermal growth factor receptor molecule that leads to constitutive tyrosine kinase activity (Wright *et al.*, 1989, Arronson 1991).
- b. Modification of signal transduction proteins which link growth factor/growth factor receptor molecule binding to the generation of an intracellular signal such as increases in inositol triphosphate, diacylglycerol and Ca^{2+} by regulation of adenylate cyclase. Example; *Ha-ras* codes for a protein which shares a large degree of homology with G proteins. Mutation of the *Ha-ras* oncogene results in the subsequent 21 KDa or p21 protein being irreversible bound to GTP (guanosine triphosphate) and hence in a constantly active state (Levinson 1987).
- c. Nuclear oncogenes. Modification of proteins which in turn alter the nuclear transcription rates of other genes. Example; *fos* oncogene which codes for a protein that controls the induction of immediate early genes regulating $G_0 \rightarrow G_1$ transition and subsequent progression through the cell cycle (Verma 1987).

Hence a cell containing activated oncogenes may acquire growth autonomy and no longer be dependent on extracellular mitogenic stimuli to induce cell division and growth.

3. Tumour suppressor gene inactivation. If oncogenes act by gain of function mutations behaving in a dominant fashion, then tumour suppressor genes can be said to act by a loss of function behaving in a recessive manner.

Initially tumour suppressor genes were often observed to be absent or in a homozygous mutant conformation in tumour cells. Tumour suppressor genes may be characterised by the following definition: "a genetic element whose loss or inactivation allows a cell to display one or another phenotype of neoplastic growth deregulation" (Weinberg 1991). At the biochemical level they appear to act as transducers of negative cellular proliferation signals i.e. down regulation of cell growth (Levine 1990, Lane 1992, Hartwell 1992).

Oncogenes do not normally show a familial inheritance since their gain of function acting in a dominant way is enough to cause sufficient abnormal embryonic development that lethality results, hence they are not found as germ line mutations. Alternatively tumour suppressor genes may be present as germ line mutations that are capable of transmitting to subsequent generations an increased susceptibility of neoplasia since they act in a recessive manner. This allows heterozygous individuals to develop normally and to sometimes reach reproductive maturity. Most known tumour suppressor genes have now also been found in association with genetically inheritable traits that increase an individuals risk of cancer.

Examples: The retinoblastoma gene (RB) located on chromosome 13q14 is found in a disease sharing the same name which generates neoplasia of the retina. In the familial form of the disease a mutant RB allele is passed on from a genetically affected parent or else is generated at gametogenesis. In the sporadic form of the disease the mutant allele is generated after conception (Levine 1990). Wilms tumour, typified by familial kidney neoplasia, shares a striking similarity to the retinoblastoma story except the gene in this case is located on chromosome 11p13. The p53 gene located on chromosome 17p13.1 has also been found to be involved in a familial disease called Li-Fraumeni syndrome. The behaviour of this commonly found

tumour suppressor gene is not strictly recessive nor dominant, but acts in a "dominant negative" fashion (Oren 1992, see section 5.2).

This recently discovered class of genes which appears to play a very central role in neoplasia, should in the near future, provide many further insights concerning the pathways and mechanisms used by cells in the development of cancer.

1.4d. Initiation, clonal expansion and promotion.

A single mutation on its own may not be capable of generating a neoplastic phenotype, but if the mutant cell has gained a selective advantage over other non-mutant cells eg. an increased growth rate, then this cell forms an increasing sub-population which can serve as a pool for secondary oncogenic alterations, i.e. clonal expansion.

There still remains considerable uncertainty as to the nature of the initiating step(s) in carcinogenesis. Experiments performed with C3H 10T1/2 cells by Reznikoff *et al.* (1973b) and Kennedy *et al.* (1980) indicated that for both chemical and radiation induced neoplastic transformation, the transformation frequency for type II and III foci was inversely proportional to the number of cells plated if the number of cells exceeded approximately 400 cells per 100mm dish. Also, if a culture of 10T1/2 cells was allowed to recover following irradiation, split and replated at different cell densities, then the number of transformed colonies observed per dish remained constant. From this it was concluded that most if not all cells originally exposed to the carcinogen had become "initiated/ altered" in some way and capable of transmitting to subsequent daughter cells an increased risk of transformation. This also led to speculation that this initial heritable change was perhaps not genetic after all, or that mutation in any one of a large number of genes could

produce an initiated state. Further support of this was indicated by the observation that protease inhibitors, which should have no effect on existing mutations, reverted initiated cells to their original uninitiated state (Kennedy 1985). Although evidence such as this concerning the nature of initiation events may tend to question the validity of genetic involvement in oncogenic transformation, it is also worth noting that such data has been generated using just rodent systems which are easily transformed. The situation in human cells is even less clear but it has been suggested that rodent cell lines may need as few as two events to generate neoplastic transformation, whereas human cell lines perhaps require as many as seven independent events to generate a similar phenotype (Farber and Rubin 1991).

Clonal expansion of already initiated cells also appears to be a vital consideration in *in vitro* transformation assays since the subsequent growth of carcinogen treated cells appears to greatly influence the transformation frequency (Yao *et al.*, 1990).

Promotion of initiated cells, i.e. the stage of cellular alteration which leads to either oncogenic transformation *in vitro* or tumour formation *in vivo*, may occur spontaneously, but can also be enhanced by agents termed "tumour promoters". Probably the best studied class of tumour promoters are the phorbol esters and in particular TPA (12-O-tetradecanoyl-phorbol-13-acetate; the mode of action of this compound is considered in section 4.7). Promotion permanently alters initiated cells into a transformed tumourigenic state, this has been observed both *in vitro* and *in vivo*. Treatment of irradiated C3H 10T1/2 cells with TPA increased transformation frequencies (Kennedy 1978) and application of TPA to mouse epidermal tissue after treatment with the carcinogen DMBA (dimethylbenzanthracene) caused benign papillomas to undergo progression into malignant carcinomas. Often

Ha-ras and subsequently *c-myc* oncogenes co-operate to bring about this phenotype (Balmain 1985).

Hence, carcinogenesis is the accumulation of sometimes several alterations all acting in unison to enable a cell to escape from normal cellular constraints, such as contact inhibition or reduced cell cycling times and ultimately leads to cancer formation. The study of carcinogenesis has many facets requiring the amalgamation of several lines of research involving hundreds of different techniques, only a very limited number of which it has been possible to comment upon and use in this study.

1.5 Aims of the thesis.

Ionizing radiation generates many wide ranging alterations within a cell, some permanent others just temporary. The objective of this study was to examine in particular, three aspects of human cellular responses to radiation.

1. The assessment of cytogenetic damage following irradiation in different human epithelial cell lines, using both immortal but non-tumourigenic cells and cells derived from similar human tissue but which were from a malignant source. This was carried out in order to gain an indication of differential radiosensitivities between normal and malignant human cell types.
2. The ability of radiation to induce neoplastic transformation in immortal but non-tumourigenic human epithelial cells and how this might differ from chemical induced transformation.
3. The induction of wild type p53 protein in response to irradiation of human epithelial cells of different origins and how the induction of this protein might influence post irradiation cell cycle delay so as to allow for increased DNA repair.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture.

2.2 Protocol for Carcinogen Treatments.

2.3 Cytogenetic Assessment of Radiation Induced Damage.

2.4 Transformation Experiments.

2.5 Immunochemistry and ELISA Tests.

2.6 Immunoprecipitation and Western Blot Analysis of Mutant p53 protein.

2.7 Flow Cytometric Analysis of Cell Cycling Following Irradiation.

MATERIALS AND METHODS

2.1 CELL CULTURE.

2.1a. SV-HUC lines.

Three SV-HUC lines were used, designated SV-HUC-1 or PC, NT11 and BC16. These cell lines were derived from an eleven year old male road accident victim's urethral tissue which was judged to be free of any malignancy. The Human Uroepithelial Cells were immortalised by infection with Simian Virus 40 generating non-senescent cell lines that do not shed the live virus, i.e. non-permissive. The immortalisation of these cell lines was performed by Dr.C.A. Reznikoff's group, University of Wisconsin, Dept of Human Oncology (Christian *et al.*, 1988).

The culture media used was Hams F12 medium (Gibco) supplemented with 1% fetal calf serum (FCS; Globepharm), human insulin (250 units/ml; Wellcome), hydrocortisone (1µg/ml; Sigma), iron free human transferrin (5µg/ml; Sigma), nonessential amino acids (0.1mM; Gibco), dextrose (15mM; Sigma), penicillin (100 units/ml; Glaxo) and streptomycin (100µg/ml; Evans). This medium is referred to as F12+ 1% FCS. Cells were grown in plastic 75 cm² or 25 cm² tissue culture flasks, gassed with 5% CO₂ in air and incubated at 37°C. Alternatively cells could be grown in 100mm petri dishes in a humidified incubator at 37°C in a 5% CO₂ in air atmosphere. However, over prolonged periods of incubation fungal contamination became a problem using this second technique. Cells were covered with 10 mls of medium in the case of 100mm dishes & 75 cm² flasks and 5 mls in 25 cm² flasks. (For all 25 cm² flasks the amount of each solution added was always half that given to the 75 cm² flasks). The medium was changed three times per week.

Cells were routinely dispersed and passaged before they reached confluence using 0.1% EDTA (Sigma) in Hanks' balanced salts solution pH 7.2. Cultures

had F12 media removed and rinsed in 2mls 0.1% EDTA, this removed and 5mls of fresh 0.1% EDTA added before incubating at 37°C for 20 mins. The flasks tapped to dislodge cells and the cell suspension added to an equal volume of F12+ 1% FCS before centrifugation at 1000rpm for 10 mins. A later alteration of this method was to add 1ml of 0.05% trypsin (Gibco) with 0.2% EDTA in PBS to the 0.1% EDTA after the 20 min incubation for approximately 60 seconds. This removed far more of the attached cells and increased the cell viability. However, long incubation times in trypsin (10mins+) resulted in cell death and greatly reduced viability (see Appedix Table A.1).

2.1b. Malignant bladder carcinoma cell line.

The cell line used MGH U1, has in fact been shown to be the bladder carcinoma cell line T24 by DNA fingerprinting tests (Masters *et al.*,1988). These cells were cultured in a similar way except the medium used was RPMI (Northumbria Biologicals Ltd) supplemented with 5% FCS, antibiotics and L-glutamine (2.0 mM; Flow Laboratories). Cells were removed from flasks using 0.01% recrystallized trypsin (Cooper Biomedicals) with 0.2% EDTA in PBS at 37°C for 10 mins.

2.1c. HPV cell line.

This cell line was immortalised by transfecting human foreskin explant tissue with a Human Papilloma Virus 16 recombinant plasmid construct (pMHPV16d). The immortalization was carried out by Dr. J A. DiPaolo, Laboratory of Biology, National Cancer Institute, Bethesda, MD. (DiPaolo *et al.* 1989). Cells were again cultured in a similar way to the SV-HUC and T24 lines except the medium used was 50% Hams F12/ 50% Dulbeccos (Gibco) supplemented with 7% FCS, hydrocortisone 1µg/ml and antibiotics. The cells were passaged with 0.05% trypsin (Gibco) with 0.2% EDTA in PBS at 37°C for 10 mins. This line was used

as an example of a non SV40 immortalised but non tumourigenic epithelial cell line with which to compare p53 data from the SV-HUC lines.

2.1d HOS cell line. This cell line was derived from a Human Osteosarcoma and has been shown to be mutant for p53 Romano *et al.*, 1989. Cells were provided by Dr. B. Vojtesek, Dept of Biochemistry, University of Dundee. The culture medium used was Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% FCS and 2 mM L-glutamine maintained at 37°C in 5% CO₂ in air atmosphere. Cell dispersal was carried out using the EDTA/trypsin technique described in section 2.1a. HOS cells were used as a positive control when checking for the presence of mutant p53 protein.

2.1e H Tori-3 cell line. Cells were derived from human female thyroid epithelial tissue (Lemoine *et al.*, 1989) and was provided by Dr. D. Wynford-Thomas, Dept of Pathology, University of Wales College of Medicine, Cardiff. The cell culture procedure used was identical to that used for HPV cells. H Tori-3 cells were used as an SV40 immortalised control cell line with which to compare SV-HUC data.

2.2 PROTOCOL FOR CARCINOGEN TREATMENTS

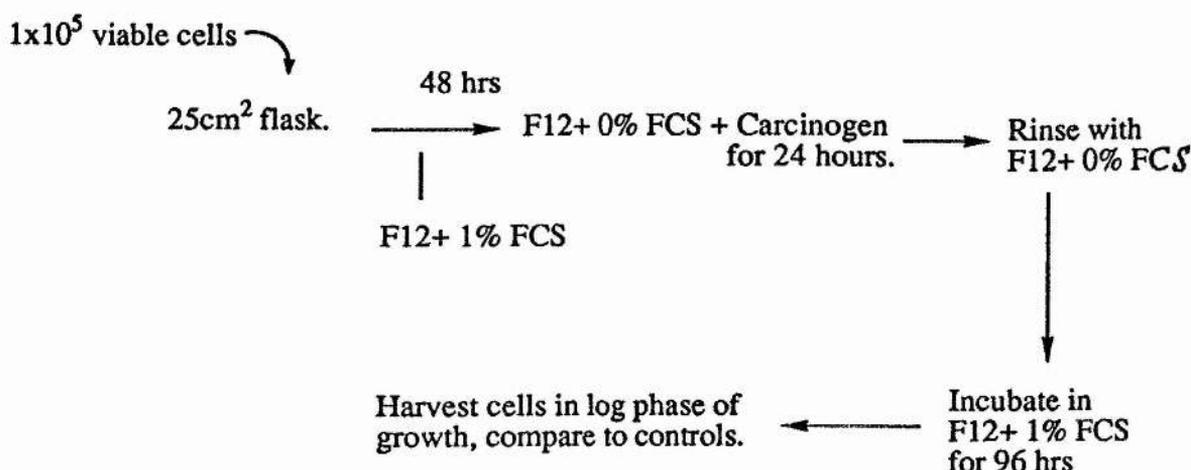
2.2a. Cytotoxicity assesment of carcinogen treatments. (Figure 2.1).

Since the SV-HUC lines do not readily form a viable single cell suspension the use of standard colony type assays could not be relied upon to give an accurate indication of cytotoxicity in these cell lines. Therefore a "regrowth assay" was utilized which did not depend on the generation of a single cell suspension.

1×10^5 viable cells were plated in 25cm^2 flasks in F12+ 1%FCS medium for 48 hours, after which this medium was removed and replaced with F12+ 0%FCS. The carcinogen treatment (radiation or chemical) was now carried out and cultures left for a period of 24 hours, before removing old media rinsing with F12+ 0%FCS and finally F12+ 1%FCS added back. The cultures were then incubated for 96 hours before cells were removed, counted and compared to control groups. Cell viability was checked by incubating 1ml of cell suspension with $50\mu\text{l}$ of 2% fast green (BDH) in 0.15M NaCl for 20 mins at 37°C . Viable cells remained unstained whereas dead cells turned green in colour. Cell counts were carried out using haemocytometers. Triplicate samples were used for each cytotoxicity determination and calculated relative to cell survival from control cultures.

A similar protocol was used when determining cell viability with different cell culture techniques i.e. different media and dispersal methods. The cells were always incubated in F12+ 1%FCS for the first 24 hours to allow cell attachment before being replaced with trial media. Cultures were again incubated for 96 hours before harvesting and comparison to control samples (see Appendix Figure A.1)

For the cell line T24 where a viable single cell suspension could be obtained a colony assay was used to calculate a survival curve following X-irradiation. This was carried out in 100mm plastic petri dishes. The number of cells plated was adjusted so that 100-200 colonies per dish were obtained after a six day incubation period. Dishes were then fixed in methanol for 10 mins and stained in 10% Giemsa for 10 mins. Colonies of greater than 50 cells were scored.

Fig 2.1 Cytotoxicity Assay.**2.2b. Radiation treatments.**

Cells were irradiated whilst attached to flasks. Initially cells were given various doses of 250 kv X-rays (0.5 mm Cu filtration). The dose rate was 0.74 Gy/min. Later, due to failure of the X-ray facility, a gamma irradiator (Caesium 137 isotope) was used to deliver the same doses of radiation. The dose rate was 4.6 Gy/min. Doses were checked using a ferrous sulphate technique (Frankenburg, 1969).

2.2c. Chemical carcinogen treatments.

All chemical carcinogens were dissolved in dimethylsulphoxide (DMSO; BDH). The final concentration of DMSO in cell culture was 0.5% with control groups being given the same amount of solvent without the carcinogen. All carcinogen treatments lasted for a period of 24 hours in serum free medium except that for 3-methylcholanthrene (MCA). MCA treatment lasted for 48 hours, 24 hours in F12+ 0%FCS medium after which serum was added back to the cultures upto 1% FCS again for a further 24 hours before rinsing and fresh F12+ 1%FCS medium added.

2.3 CYTOGENETIC ASSESMENT OF RADIATION INDUCED DAMAGE.

2.3a. Micronucleus assay.

The cytogenetic response to radiation of SV-HUC lines and the T24 cell line was studied using the micronucleus assay. Micronuclei (MN) were assayed using the cytokinesis block technique (Fenech & Morley 1985). Briefly, immediately after irradiation cytochalasin B (Cyto B; Sigma; stock solution 3mg/ml in DMSO) was added to give a final concentration of 3µg/ml. Cultures were then incubated for 48 hours before harvesting with EDTA or trypsin.

2.3b. Preparation of slides.

Slides were prepared by centrifugation using a Shandon Cytospin 2 (800 r.p.m. for 10 min) and fixed in methanol (10 min). Slides were then stained in 56% Jenners in water for 5 min, rinsed in water, 25% Giemsa in water for 10 min and again rinsed in water. Acridine Orange staining was performed on selected slides to check the accuracy of the Jenner/Giemsa staining technique using a method described by Tinwell and Ashby (1989). Briefly, Jenner/Giemsa stained slides were destained in methanol: acetic acid (3:1) overnight before staining with Acridine Orange 0.125 mg/ml dissolved in phosphate buffer (0.66% potassium phosphate monobasic + 0.33% potassium phosphate dibasic). Slides were stained for 60 s before two washes in phosphate buffer the first for 10 min and the second for 15 min before mounting in buffer. Fluorescence microscopy was performed using 450-490 nm wavelength light. A total of 200 Jenner/Giemsa stained binucleate cells were scored per sample per experiment.

2.3c. Karyotyping of cells. SV-HUC lines were grown to mid-confluence and treated with 0.1µg/ml demicolcemid (Sigma) for 6 hours. Cells were removed

from flasks, spun, incubated with 10 ml of 75mM KCl for 10 min at 37°C before fixing with cold acetic acid / methanol (1:3). Preparations were washed three times in acetic acid / methanol, left overnight at 4°C and resuspended in a small volume of fixative. Chromosome spreads were prepared by dropping aliquots of cell suspension onto clean, wet, cold slides followed by the same size aliquot of fixative. Slides were air dried and stained directly with 3% Giemsa (BDH) in water or used for G-banding. For G-banding slides were treated with a 0.125 % trypsin solution (Gibco) in 0.85% NaCl buffer for 10 seconds, rinsed three times in buffer before staining with 3% Giemsa. 50 metaphase spreads were scored to gain modal chromosome numbers.

2.4 TRANSFORMATION EXPERIMENTS.

2.4a. Protocol for SV-HUC transformation experiments. (Figure 2.2).

The carcinogen treatment was carried out as described previously. Some carcinogen treatments were carried out twice with a recovery period and split of the cells between each treatment. When split the cells were replated at a concentration of 1×10^6 cells per flask, left for 48 hours and again treated with the carcinogen as above. The cultures were then allowed to continue growing until almost confluent whereupon they were split and used to reseed three new flasks at approx 1.5×10^6 cells per flask. These cultures were again allowed to almost reach confluence, split $1/3^{\text{rd}}$ and new flasks seeded except that each flask was now used to reseed just one flask each i.e. no further "bulking up" of cells. This continued passaging was carried out six times following final carcinogen treatment over a period of eight weeks with medium changed three times per week. At the sixth split the three flasks of the same treatment were pooled, resuspended in 0.2ml of F12+ 0% FCS medium ready for inoculation into nude

mice. Cells of similar treatments were not pooled during the experiment except if they were derived from the same initial flask following carcinogen treatment.

The effect of postconfluent growth inhibition (i.e. cells maintained at G_0) following carcinogen exposure (as with rodent cells) was also investigated. 1×10^6 cells were plated and treated using an identical carcinogen exposure protocol as described above. Cultures were then grown to confluence before passaging and a second carcinogen exposure, or held at a confluence depending on the treatment protocol used (Figure 2.3). Once such cultures had attained confluent inhibited growth they were maintained in this state for a period of eight weeks and the medium changed three times per week. Cellular transformation in such cultures was assayed by the formation of dense "transformed colonies" growing on top of a growth inhibited or plateau phase monolayer of cells. Using the EDTA/trypsin method cells could be removed, inoculated into nude mice or checked for colony formation in 0.3% agarose. These dense type colonies could be seen without staining but a sample were fixed with methanol for 10 min and stained with 10% Giemsa in water for 10 min. The larger of these colonies could be picked off and transplanted into a new culture flask where upon they continued to grow as a dense/tight colony of cells. After a further 6-8 week incubation period such colonies could be fixed, sectioned and stained in a similar way to that for the tumour biopsies from the nude mice. These colonies were also inoculated into nude mice and checked for tumour formation.

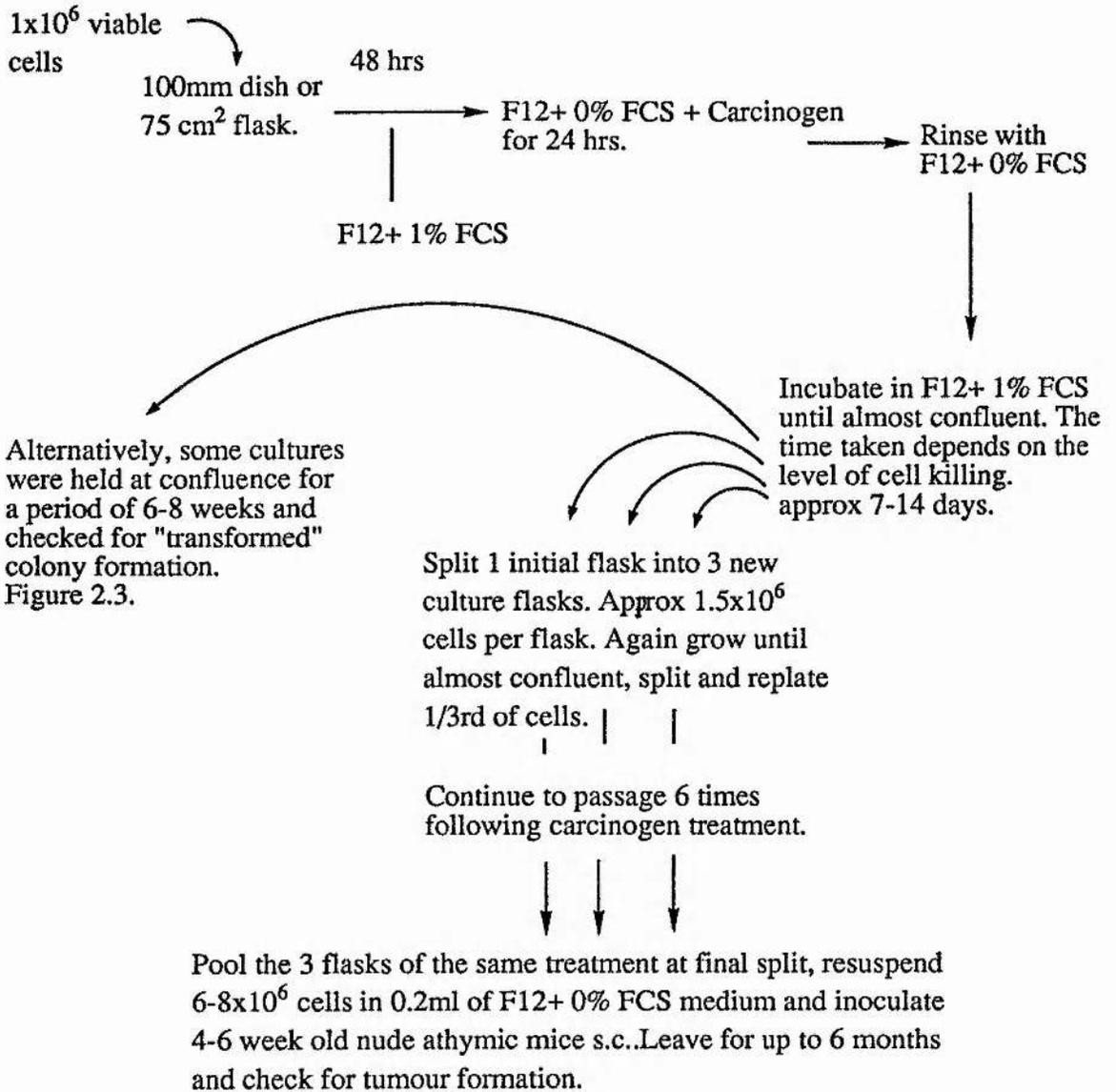
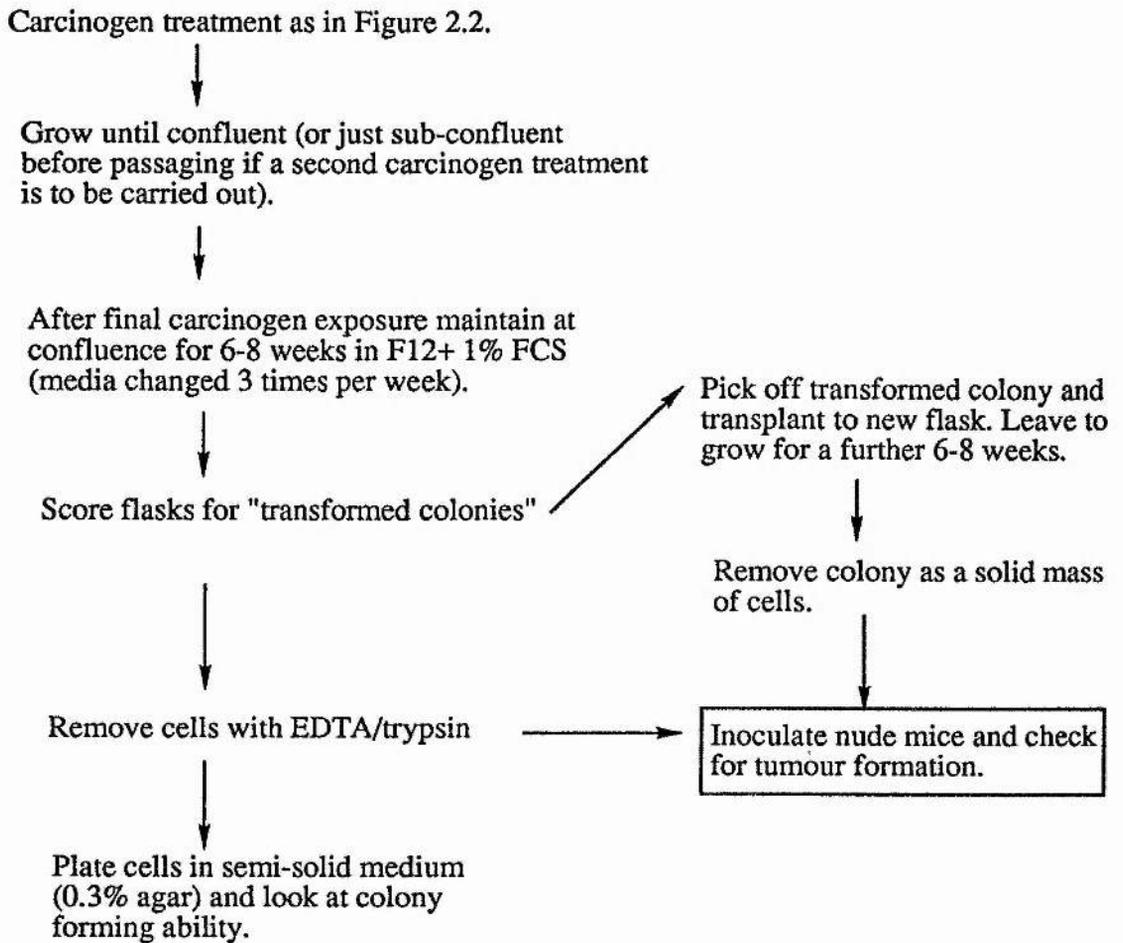
Fig 2.2 Transformation experiment protocol.

Fig 2.3. Experimental protocol for postconfluent growth inhibition transformation.



2.4 b. Inoculation of cells into nude mice.

The ability of control and carcinogen treated cells to form tumours was assayed by inoculation of $6-8 \times 10^6$ cells into 4-6 week old athymic nude mice. Mice were originally from OLAC Ltd; strain MF1-nu/nu (athymic), but once a breeding stock was established experimental animals were taken from this stock. The cells were injected suspended in a volume of 0.2mls of F12+ 0% FCS subcutaneously mid way between the fore and hind limbs in lateral position. Animals were examined weekly for tumour formation and sacrificed if any

tumour reached >1 cm in diameter, if necrosis occurred or after six months i.e. full term experiment. Tumour biopsies were split into two halves. One half was fixed, sectioned and stained with haematoxylin and eosin or monoclonal antibodies to human cytokeratin and the SV40 T-antigen to confirm the origin of any tumours obtained. The other tumour section was dissected into 1mm cubes and placed with a small amount of F12+ 1% FCS medium on the base of a culture flask to try and generate a cell line. The cell line T24 was used as a positive control for tumour formation in the nude mice but the medium used was RPMI.

2.4 c. Growth of cells in 0.3% agar.

The ability of cells to grow in semi-solid medium was assayed for both control cells and those showing a dense type colony appearance from the transformation experiments where cultures were left at confluence. 1×10^5 viable cells per 60mm dish were plated in 2ml of F12+ 1%FCS medium containing 0.3% agarose over 2ml of F12+ 1% FCS, 0.5% agarose underlayer. Dishes were incubated in 95% air 5% CO₂ atmosphere at 37°C for 21 days. Five 1cm³ fields were scored per plate per treatment for colonies of greater than 50 cells. Triplicate plates were prepared per treatment.

2.5 IMMUNOCHEMISTRY AND ELISA TESTS.

2.5 a. Immunocytochemical staining with substrate specific monoclonal antibodies.

Cells from three main sources were stained with antibodies; Fixed and sectioned biopsys from nude mice, cells grown on glass cover slips and cells grown on plastic petri dishes. The antibodies used were against three main protein complexes.

1. The SV40 T-antigen to indicate that the cells concerned were immortalised by SV40. The antibody used was a mouse monoclonal designated PAb 405 which reacts with a region of the T-antigen close to the C terminus of the protein.
2. Human cytokeratin to indicate that cells were of human origin. The antibody used was a mouse monoclonal which reacts with several forms of human cytokeratin (DAKO M821)
3. The p53 protein in both wild type & mutant conformations and following radiation treatment. All antibodies were mouse monoclonals.

ANTIBODY	SPECIFICITY
PAb 240	Reacts with human, mouse, rat, hamster, bovine and chicken. Epitope lies between amino acids 156-214. Will not bind to p53 when it is complexed to SV40 T-antigen. PAb 240 recognises mutant but not wild type p53, also bind to HSP70. All known activating mutations in p53 result in a common conformational change which are detected by PAb 240.
PAb 246	Reacts with mouse only. Epitope is between amino acids 88-109 but will not react with p53 having mutations which lie between 120-240, i.e. mutations here have a conformational effect elsewhere. Will bind to p53 when complexed with T-antigen but not with HSP70.
PAb 421	Not specific to one species, mouse, human etc. Binds to C terminus of mutant and wild type p53.
DO-1	Reacts with human, monkey bovine but not mouse. Mutant and wild type p53 detected but greater affinity for wild type than PAb 421. Epitope in N-terminus region aa 1-45.

Table 2.1. Taken from Gannon *et al.*, 1990, Levine 1990 and Vojtesek *et al.*, 1992.

However, all antibodies will react both with wild type and mutant forms of p53 if the protein is denatured i.e. after fixation. Hence the use of ELISA tests was required to determine between mutant and wild type protein and also in quantitative assays. All antibodies unless otherwise stated were obtained from

quantitative assays. All antibodies unless otherwise stated were obtained from Dr. D. Lane, Dept of Biochemistry, University of Dundee. See table 2.1 for details of p53 antibody epitope specificity.

The protocol in all cases remained essentially the same except no fixing was required in biopsy samples. The method described initially is for all primary antibodies except M821, where differences in methodology apply they will be described later.

2.5 a.1. SV40 T-antigen and wild type p53 protein staining

Cells were fixed in cold methanol/acetone (1:1) for 2 min in the case of all PAb and DO-1 monoclonals before being allowed to air dry. The primary antibody was then used neat in the form of hybridoma culture supernatant or ascites fluid diluted 1:500 in PBS, incubated at room temperature for 1 hour in a humidified atmosphere. Specimens rinsed three times in PBS before the secondary antibody with 5% FCS to prevent non specific binding added for a further 1 hour at room temperature. The secondary antibody was a rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugated polyclonal antibody (P161 DAKO) diluted 1:100 in PBS if the section was to be viewed with an ordinary light microscope. If the section was to be examined under fluorescence with a confocal microscope then the secondary antibody was a sheep anti-mouse IgG FITC (Fluoresein Isothiocyanate) conjugated polyclonal antibody (S121-201 Scottish Antibody Production Unit-SAPU) diluted 1:20 in PBS. The secondary antibody was then washed off as above before developing with 3', 3-diaminobenzidine (DAB; Sigma) in the case of the HRP IgG antibody. DAB was used at a concentration of 1mg/ml in water with 0.03% nickel sulphate (to enhance a black colouration) and activated with 0.06% H₂O₂ and left for 5-10 min in the dark before removing the DAB solution. Specimens for use with the confocal microscope (krypton/argon mixed gas laser, wavelength 488 nm) could be

viewed directly but were mounted in 90% glycerol/10% PBS with 25mg/ml N-propyl gallate (Sigma) as an anti-fading agent.

2.5 a.2. Human cytokeratin staining.

Immunocytochemical staining using anti-cytokeratin antibodies was carried out on samples fixed in cold acetone for 20 min and then rehydrated in 1% sheep serum (SAPU) in PBS for 30 min. The primary antibody M821 was incubated at 37°C for 1 hour diluted 1:25 in 1% SSPBS. Rinsed three times in PBS and the secondary antibody a sheep anti-mouse HRP conjugated polyclonal antibody (S081-201; SAPU) diluted 1:20 in 1% SSPBS added for 1 hour at 37°C. The remainder of the protocol is now the same as for the PAb antibodies.

2.5 b. Enzyme-linked immunoabsorbant assay (ELISA) for p53 protein.

(Figure 2.4).

ELISA tests were performed to quantify the level of wild type p53 protein present after radiation treatment. The technique can be split into three separate procedures.

1. Generation of a protein extract by cell lysis.
2. Measurement of the total amount of cellular protein in the extract.
3. The ELISA itself.

2.5 b.1. Cell lysis; Cell lines SV-HUC-1 (PC) and HKc were irradiated in 75cm² flasks as described previously. Three sub-confluent flasks per data point were used. Cells were then harvested, washed and spun (1000xg for 10 mins) three times in PBS before finally pelleting the cells and freezing at -70°C. Cell lysis was carried out in NET lysis buffer (50 mM Tris; BRL, 150mM NaCl, 5mM EDTA; Sigma, 1% NP-40; BDH, 350µg/ml phenylmethylsulfonyl fluoride; BRL. Harlow and Lane,1988). 1ml of NET buffer added to the cell pellet, vortexed and left on ice for 30 mins before centrifugation at 10,000xg for 30

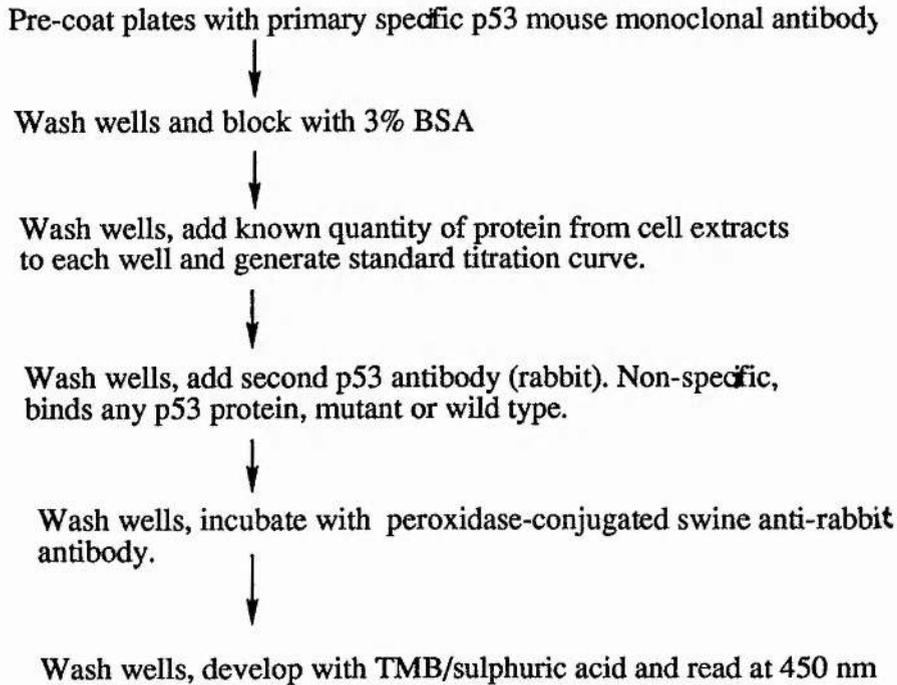
mins at 4⁰C. The supernatant containing the protein extract was then diluted in NET buffer through a series of nine 50% dilutions to generate samples for a titration curve.

2.5 b.2. Measurement of the total amount of cellular protein. This was assayed using Bradford dye (BioRad). Briefly, a 1:20 fold dilution of the protein extract in water was carried out. Then 1µl of this diluted cell extract added to 100µl of water and 1ml of Bradford dye added. The absorbance was then measured at 595 nm compared to a control of 100µl water plus 1ml of Bradford dye. A reading of 0.095 corresponding to 1mg/ml of protein. The amount of cell extract added to each well was then be adjusted so that all wells of the same dilution received the same quantity of protein.

2.5 b.3. ELISA. The assay itself was performed in flexible PVC 96-well microtitre plates (Falcon). Plates were coated with the primary antibody (D0-1 or PAb) over night in a humidified atmosphere with 50µl per well of 30µg/ml antibody (neat) if hybridoma culture media or ascities diluted 1:500 in coating buffer (0.1M Na₂CO₃/NaHCO₃, pH 9). Plates were then rinsed in PBS and blocked for 2 hours with 3% Bovine serum albumin (BSA-ELISA grade; Sigma) in PBS at room temperature. The BSA was then removed and rinsed with PBS (x1), PBS+0.1% NP-40 (x3), PBS (x1) before the cell extract added for 2 hours at 4⁰C. Two wells in each row remained free of protein extract and received just lysis buffer which served as controls. The same washing procedure was carried out followed by addition of rabbit antiserum to p53, CM-1 (David Lane) diluted 1:500 in 1% BSA for 2 hours at 4⁰C. This antiserum contained the second p53 antibody which is non-specific and binds all the different forms of p53 protein. After washing using the same protocol, peroxidase-conjugated swine antiserum to rabbit immunoglobulins (DAKO; P217) diluted 1:500 in 5% FCS in PBS was added for 2 hours at 4⁰C. Again washed using the same method and visualisation carried out using tetramethylbezidine (TMB; Sigma). TMB dissolved in DMSO at 10mg/ml was diluted 1:100 in substrate buffer (0.1M

$\text{Na}_2\text{HPO}_4/0.1\text{M}$ citric acid, pH 6.0) add activated with 0.06% H_2O_2 . 50 μl of this solution was added to each well, left in the dark for 20 mins and the reaction stopped with 1M H_2SO_4 (50 μl per well). The plate was then analysed on an automatic plate reader at 450 nm.

Fig 2.4. Summary of procedure for ELISA.



2.6 IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS OF MUTANT p53 PROTEIN. (Figure 2.5).

Cell lines were checked for the presence of mutant p53 protein using immunoprecipitation and western blot analysis followed by subsequent antibody staining of the nitrocellulose membrane. Again the technique can essentially be split into three procedures.

1. Generation of a protein extract and immunoprecipitation with specific monoclonal antibodies.
2. Polyacrylamide Gel Electrophoresis (PAGE) and electrophoretic transfer onto a nitrocellulose membrane.
3. Antibody staining of the nitrocellulose membrane coupled with visualisation of bound antibodies.

2.6 a. Generation of protein extract and immunoprecipitation.

The protein extract was produced using exactly same protocol as described for ELISA experiments i.e. NET lysis buffer at 4⁰C (see section 2.5b.1.). Following cell lysis, extracts (600µl aliquots) were pre-adsorbed with 40µl protein G-Sepharose (Sigma) washed in NET lysis buffer to remove the ethanol. This was carried out on a rotating wheel at 4⁰C for 40 min and the protein G-Sepharose pelleted by centrifugation at 14K for 2 min. 200µl of cell extract was then incubated with 1µl of ascities containing the primary specific monoclonal, DO-1, PAb 240 or anti-retinoblastoma (Rb) mutant protein antibodies (IF8; D. Lane, Dept of Biochemistry, University of Dundee). The IF8 antibody was used as an "irrelevant antibody" to check for non-specific binding of the second anti-p53 antibody CM1. This incubation was performed at 4⁰C over night. To this was added 20µl of protein G-Sepharose for 40 mins at 4⁰C on a rotating wheel to precipitate out the antibody-p53 protein complex. Following immunoprecipitation the samples were spun at 14K for 5 min and the sediment washed 4 times in lysis buffer. An equal volume (50µl) of sample buffer was then added to the protein G-Sepharose - antibody - p53 protein complex, boiled for 5 min and spun at 14K for 8 min. (Sample buffer 2x concentrate pH 6.8; 4% sodium dodecyl sulphate (SDS; BDH), 120 mM Tris (BDH), 20% glycerol (Serva) and 0.002% bromophenol blue (Sigma)). 40ml of this sample could then be loaded onto the 10% acrylamide gel.

2.6 b. Electrophoresis and electrophoretic transfer.

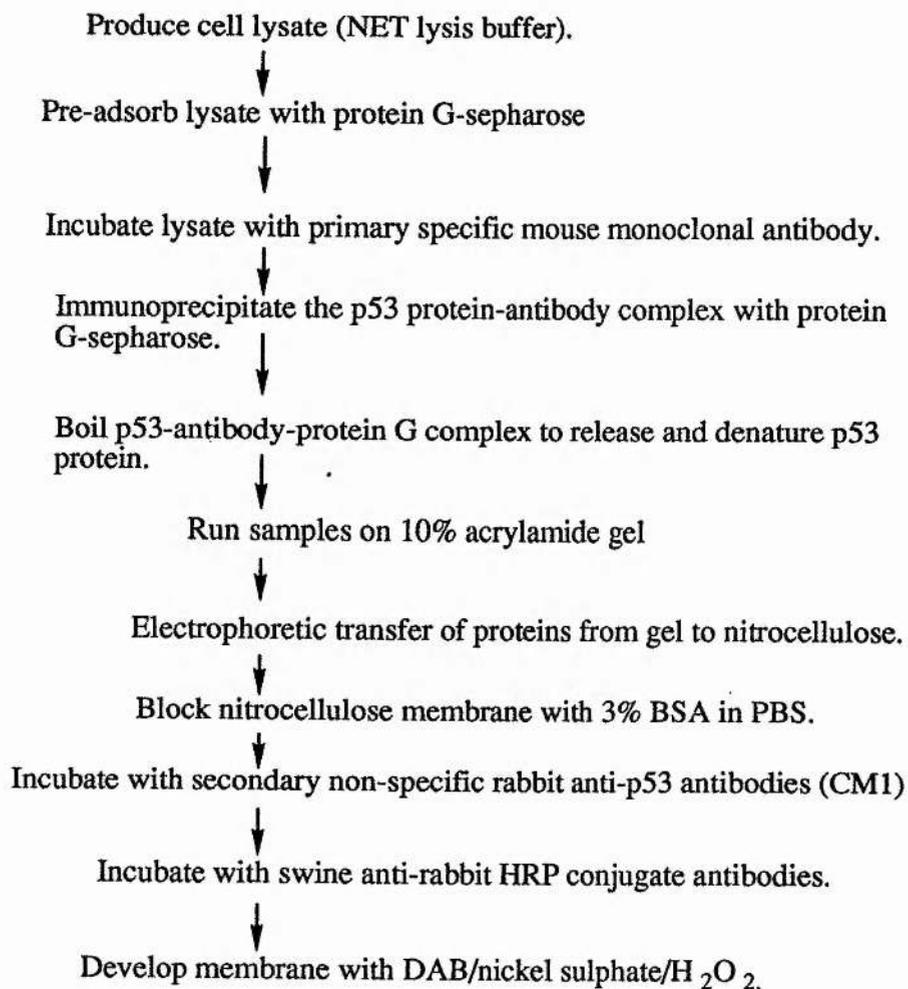
Samples were run on a 10% SDS-PAGE gel with a stacking gel of 4%, 1.5mm thickness. 10% gel constituents; 12.5ml of 1.5M Tris pH 8.8, 0.5ml of 10% SDS, 16.67ml of 30% acrylamide (Sigma), 20.33 ml of distilled water and polymerized with 400 μ l of APS (ammonium persulphate; Fluka) and 40 μ l of TEMED (N,N'-methylene-bis-acrylamide; Sigma), approx 1 hour to set. 4% gel constituents; 2.5ml of 0.5M Tris pH 6.8, 1.3ml of 30% acrylamide, 0.1ml of 10% SDS, 6.1 ml of distilled water, polymerized with 50 μ l of APS and 10 μ l of TEMED. The gel was run at 36mA for approximately 3 hours on a Gibco/BRL apparatus with a running buffer of 0.025 M Tris, 0.24 M glycine and 0.1% SDS.

Electrophoretic transfer was carried out using a semi-dry technique with a transfer buffer of 0.025 M Tris, 0.192 M glycine, 20% methanol and 0.0375% SDS. The apparatus used was an LKB 2117 Multiphor II electrophoresis unit at 0.8 mA per cm² for 2 hours to transfer protein onto nylon backed nitrocellulose (Hybond-C extra; Amersham).

2.6 c. Immunoblotting of nitrocellulose with antibodies and visualisation.

The nitrocellulose membrane was first incubated with 3% BSA in PBS for 2 hours at room temperature to block nonspecific binding sites. The second nonspecific, anti-p53 antibody (CM1) diluted 1:500 in 3% BSA in PBS was added for 1 hour at 37⁰C. Following four washes in PBS, HRP conjugated swine anti-rabbit immunoglobulins diluted 1:500 in 3% BSA in PBS was incubated for a further 1 hour at 37⁰C. After washing in PBS visualization was carried out using DAB/H₂O₂/nickel sulphate as described for immunocytochemical staining (10ml of 1mg/ml DAB, 150 μ l 3% NiSO₄, 15 μ l of 30% H₂O₂; see section 2.5a.).

Fig. 2.5. Summary of procedure for Immunoprecipitation and Western Blotting



2.7 FLOW CYTOMETRIC ANALYSIS OF CELL CYCLING FOLLOWING IRRADIATION.

Cells were grown in 75cm² tissue culture flasks and irradiated in mid logarithmic growth. One hour prior to cell harvest 10µM of BrdU (5-Bromo-2-deoxyuridine; Sigma) in PBS was added to the culture medium for incorporation into cells undergoing active DNA synthesis. After harvesting of cultures with trypsin/EDTA as previously described cells were washed in PBS

fixed in 70% ethanol overnight at 4°C. Following washing in PBS cells were resuspended in 2.5mls of 2M HCl containing 0.1mg/ml pepsin (Sigma) for 20 mins at room temperature. After washing a further two times in PBS cell nuclei were now ready for antibody staining. Nuclei were resuspended in 0.5mls of PBS containing 0.5% goat serum (SAPU) and 0.5% Tween 20 (ICN Biochemicals) before addition of 25µl of anti-BrdU rat monoclonal antibody for 1 hour at room temperature (20mg/ml hybridoma culture media; Haddow Laboratories). Nuclei were then centrifuged at 2000rpm for 5 mins in 5 mls of PBS , resuspended in 0.5mls of PBS/goat serum/Tween 20. To this was added 10µl of goat anti-rat IgG FITC conjugate (Sigma; F-7250) for 1hour at room temperature. *Samples were washed in 5mls of PBS, resuspended in 1 ml PBS containing fresh RNase (BRL; 1mg/ml) for 30 mins at 37°C before addition of propidium iodide (Sigma; 4µg/ml). Samples were then analysed using a flow cytometer (FACScan; Becton Dickinson). The software associated with FACScan interpreted the data giving both a cell cycle profile (see figures 5.9 and 5.10) and information concerning the percentage of cells in G₁, S and G₂/M.

For simple, single channel staining of DNA only the procedure from * was followed after harvesting cells and fixation in alcohol.

FIG 2.6. Procedure for BrdU / antibody / propidium iodide staining in conjunction with the FACScan for cell cycle analysis following γ irradiation.

Cells incubated with 10 μ g/ml BrdU for 1 hour in culture media



Harvest cells with trypsin/EDTA, wash with PBS



Fix cells in 70% ethanol overnight at 4°C, wash with PBS



Remove cell cytoplasm using 2M HCl containing 0.1mg/ml pepsin (20 mins at room temperature). Wash twice with PBS.



Stain with anti-BrdU rat monoclonal for 1 hour at room temperature, wash with PBS.



Probe with goat anti-rat FITC conjugated IgG for 1 hour at room temperature, wash with PBS.



Treat with RNase (1mg/ml for 30 mins at 37°C).



Stain with propidium iodide (4 μ g/ml 30 mins at room temperature).



Analyse cell cycle profile using FACScan.

CHAPTER 3

CYTOGENETIC RESPONSES OF HUMAN UROEPITHELIAL CELL LINES AND A MALIGNANT BLADDER CARCINOMA CELL LINE TO X-RAYS.

3.1 Abstract.

3.2 Introduction.

3.3 Procedure.

Results

3.4 The effect of Cytochalasin B on Mn formation.

3.5 Mn frequencies in different cell lines following irradiation.

3.6 Staining of Mn.

Discussion

3.7 The optimal concentration of Cytochalasin B.

3.8 Relative radiosensitivities of human uroepithelial cells and the malignant carcinoma cell line T24.

3.9 The nature and significance of Mn as chromosomal aberrations in carcinogenesis

CYTOGENETIC RESPONSES OF HUMAN UROEPITHELIAL CELL LINES AND A MALIGNANT BLADDER CARCINOMA CELL LINE TO X-RAYS.

ABSTRACT

3.1

The response to X-rays of three Simian Virus 40 (SV40) immortalized but non-tumourigenic human bladder epithelial cell lines have been compared with a malignant bladder epithelial cell line using the micronucleus assay. An optimal concentration of Cytochalasin B was found to be 3 μ g/ml to induce binucleated cells for the scoring of micronuclei (Mn). A linear increase in radiation induced Mn was observed for all cell lines with increasing X-ray dose. The three SV40 immortalized lines were significantly less radiosensitive than the malignant cell line. Spontaneous levels of Mn indicate that certain cell lines within the SV40 immortalized lines have a higher genetic instability. This may be of importance when considering the generation of a fully transformed or tumourigenic phenotype after treatment with physical or chemical carcinogens.

INTRODUCTION

3.2

The effects of ionizing radiation on cells can be measured using a variety of different assays which vary greatly in terms of the end point used and the biological system chosen. Classically reproductive "cell death" as measured by colony type assays provided a lot of information concerning the cytotoxic or lethal nature of radiation. These assays were used largely on established cell lines such as rodent fibroblasts (eg. C3H 10 T¹/2), or cell lines generated from human malignancies. As mentioned in the main introduction these probably don't represent an accurate model for normal human epithelial tissues where a majority of human neoplasms originate.

Clonogenic assays give information about the toxic nature of ionizing radiation by measuring its effect on the "reproductive integrity" of the cell (Hall 1988), i.e. its proliferative capability following radiation treatment. This is usually expressed in terms of a survival curve. A prerequisite for using a colony type assay is the ability to generate a viable single cell suspension which itself can cause difficulties with certain cell types and lines. Clonogenic assays don't however provide data concerning the more immediate effects of ionizing radiations on the critical cellular target - nuclear DNA.

Cytogenetic analysis of chromosome and chromatid aberrations following radiation does provide data concerning the clastogenicity of ionizing radiation. However, scoring of these aberrations can be a lengthy and complex process requiring good quality metaphase spreads. The generation of these spreads can itself provide problems especially during long colcemid incubations for cells with long cell cycle times due to chromosome condensation and shortening (Sharma and Sharma, 1980).

Another method which scores aberrant acentric chromosome fragments that do not associate with the nucleus at nuclear division is that of measuring

micronuclei (Mn) formation. Although not as sensitive at measuring radiation induced chromosome damage as aberration scoring, Mn do provide an indicator of unrepaired damage at the chromosomal level, which are quicker and easier to score than the above mentioned aberrations. One reason for this disparity between Mn frequency and that of aberration frequency is that not all acentric fragments may be excluded from the nucleus at nuclear division. (Reviewed by Savage 1988).

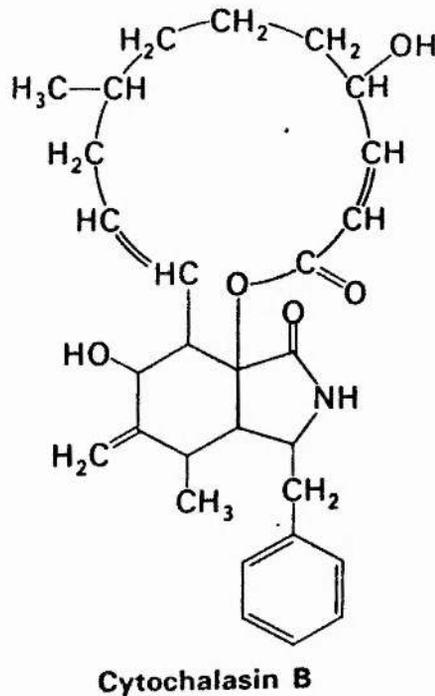


Fig. 1. Chemical structure of the alkaloid Cytochalasin B.

A modification of this technique was developed by Fenech & Morley 1985 using an agent that inhibits cytokinesis (cellular division) whilst still allowing nuclear division to occur. Cytochalasin B (Cyt B; Figure 3.1), an alkaloid which interferes with the assembly of actin filaments so preventing cellular division is such an agent, since it does not interfere with microtubules of the nuclear spindle so allowing nuclear division to take place (Stryer 1981). The use of this technique is now employed widely for studying a large range of genotoxic agents, both chemical and physical (Arlett *et al.*, 1989). The use of such a cytokinesis block method has two important

consequences when scoring Mn. Firstly Mn are far easier to score and secondly cells which have only been through one nuclear division can now be selected and scored. This allows cells from a similar "time window" to be scored i.e. they have cycled to the same extent following radiation, the significance of this will be commented on in the Discussion.

Another advantage of scoring Mn as a measure of radiation induced damage is that a viable single cell suspension or good quality metaphase spreads are not required. Both of these proved difficult to obtain for SV-HUC lines.

PROCEDURE

3.3

Irradiated cultures were incubated with Cyt B to obtain binucleate cells which were scored for Mn. The radiosensitivity of all four epithelial lines was assessed in this way. Spontaneous Mn levels were also examined in unirradiated samples to gain a measure of the genetic instability within each line. Two different methods of staining were employed to check the validity of Mn generated after Cyt B incubation.

The radiosensitivity as measured by surviving fractions in a clonogenic assay was also determined in T24 cells for which a viable single cell suspension could be obtained. Since the radiosensitivity for this cell line had now been measured by two different techniques this enabled approximate extrapolations to be made concerning the relative survival of SV-HUC lines following irradiation.

Finally Mn levels following irradiation were also used to determine the clastogenic effectiveness of two different radiation sources.

RESULTS

3.4 The effect of cytochalasin B on Mn formation.

A suitable working concentration of Cyt B was found to be 3 μ g/ml (final concentration in cell culture). The binucleate cell index plateaus at concentrations at and above 3 μ g/ml indicating that cells are no longer escaping cytokinesis blockage. There is little effect of increasing Cyt B concentration on the spontaneous Mn formation, and appears to actually be decreased slightly by higher doses of Cyt B (Figure 3.2).

3.5 Mn frequencies in different cell lines following irradiation.

In multiple sampling experiments (Figure 3.3) the effect of harvest time on Mn formation in irradiated cells blocked with Cyt B was investigated. These results indicate that there is no definite peak in Mn response following radiation at a given harvest time, but increases steadily with sampling time. NT11 cells were found to be considerably less sensitive than the bladder carcinoma cell line T24 in terms of Mn response to X-rays at comparable harvest times. This is also demonstrated by the X-ray dose-response curves obtained at a fixed harvest time of 48 hours (Figure 3.4). The three SV-HUC lines all show similar dose-response curves to one another, these being far less steep than that of the T24 cell line. At 6 Gy there was a significant difference between the Mn response of SV-HUC-1 (the most sensitive of the SV-HUC lines) and that of cell line T24 of $P < 0.01$, Student's *t*-test. all four lines showed a linear increase in frequency of induced Mn with X-ray dose at the 48 hour sampling time.

Although the cell cycling times of the cell lines in exponential growth were found to be approximately 39, 42, 43 and 20 hours for cell lines NT11, SV-HUC-1 (PC), BC16 and T24 respectively, a harvest time of 48 hours was found to be

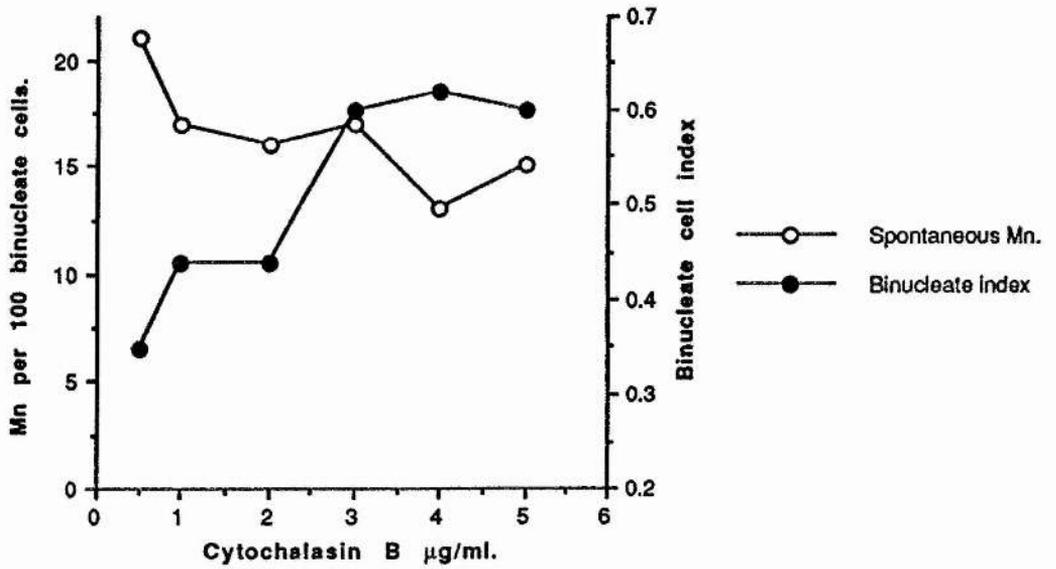


Fig. 3.2. Effect of Cytochalasin B on spontaneous Mn formation and cytokinesis blockage in SV-HUC-1 cells. Sampling time 48 hours after addition of Cytochalasin B.

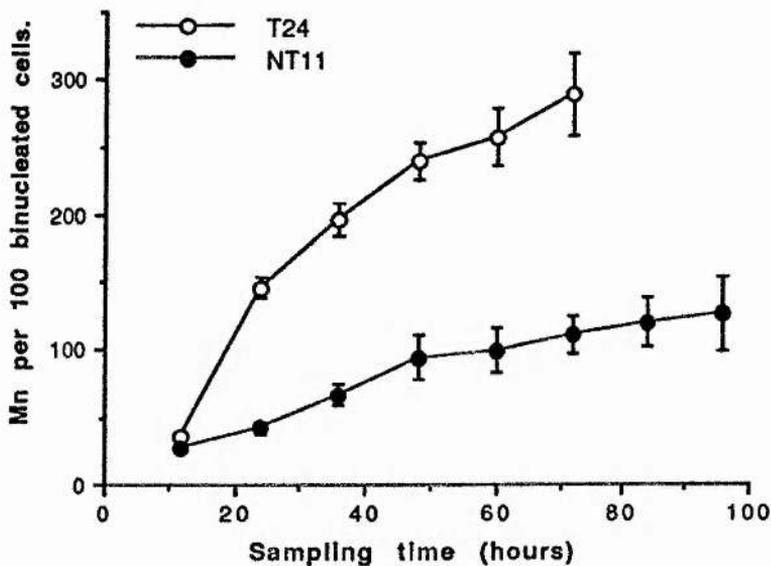


Fig.3.3. Frequency of Mn in binucleate cells as a function of time following X-irradiation (4.0 Gy). Error bars represent standard errors of mean (SEM) values from two separate experiments.

an optimal sampling time from experiments using multiple sampling times. At sampling times later than 48 hours the frequency of binucleate cells (binucleate index) fell to levels which made accurate scoring of Mn difficult. Conversely at sampling times much shorter than 48 hours the binucleate index is also low since not enough time has elapsed since the addition of Cyt B to allow a sufficient number of cell to accumulate in the binucleate cell compartment. Figure 3.5 shows that the maximum binucleate index occurred at approximately 48 hours for cell lines NT11 and T24.

The survival curve of T24 cells to X-rays (Figure 3.6) gave a point of reference for a comparison with the Mn dose-response curves. SV-HUC lines would be expected to show increased survival to X-irradiation when compared to T24 cells and is indicated by the dotted line.

Figure 3.7 shows the relationship between surviving fraction and the number of cells that do not contain Mn following radiation treatment for cell line T24. In general terms the increased generation of Mn can be seen to coincide with an increase in cell death (see Discussion for more detailed explanation).

The spontaneous level of Mn after Cyt B blockage alone (Figure 3.8) was found to be high for cell lines SV-HUC-1 and NT11 but far lower for that of BC16 and T24. The difference between BC16 and both SV-HUC-1 and NT11 was significant at the $P = < 0.05$ level, Student's *t*-test. The spontaneous Mn levels observed in cell lines BC16 and T24 fall within the range of values obtained for other human cells such as lymphocytes; 4.0-67.3 Mn/1000 binucleate cells (Fenech *et al.*, 1990).

3.6 Staining of Mn.

Acridine Orange (AO) staining was performed on selected slides to check for possible artifacts resulting from the Jenner/Giemsa staining. Cytospinning when coupled with the Jenner/Giemsa stain gave a very good contrast

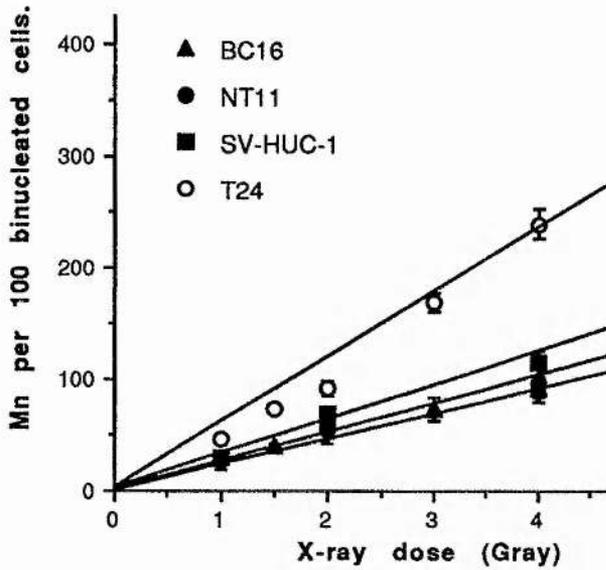


Fig.3.4. Dose - response relationship between frequency of Mn and X-ray dose. Sampling time was 48 h after irradiation. Error bars represent SEM values from at least three separate experiments. (Background Mn values have been subtracted from the data shown).

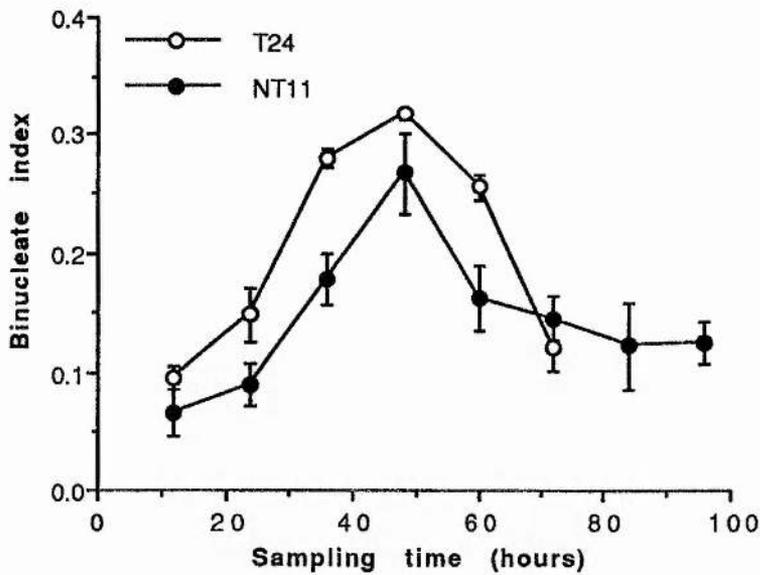


Fig.3.5. Binucleate cell index (frequency of binucleate cells/total number of cells scored as a function of sampling time following irradiation (4.0 Gy). Error bars represent mean values from two separate experiments.

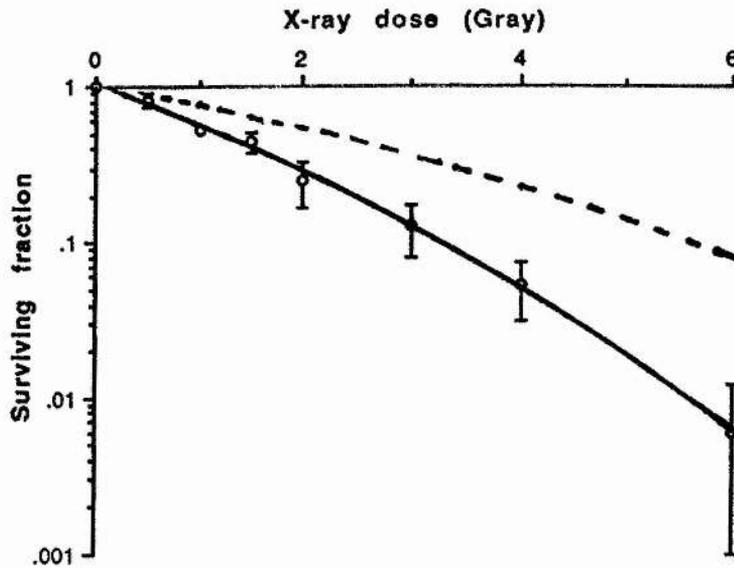


Fig.3.6. Cell survival curve for cell line T24. Error bars represent SEM values from at least three separate experiments. The dotted line represents the approximate survival curve for SV-HUC lines. Calculated from data in Fig. 3.7. and Table 3.1.

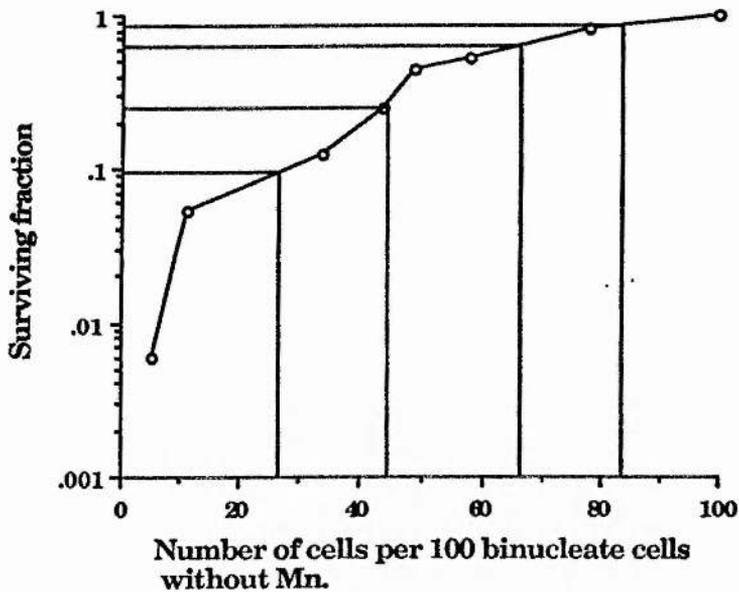


Fig.3.7. Plot of surviving fraction against cells without Mn following irradiation for T24 cells. The solid lines represent the corresponding values from an SV-HUC line taken from Table 3.1. All sampling times of 48h.

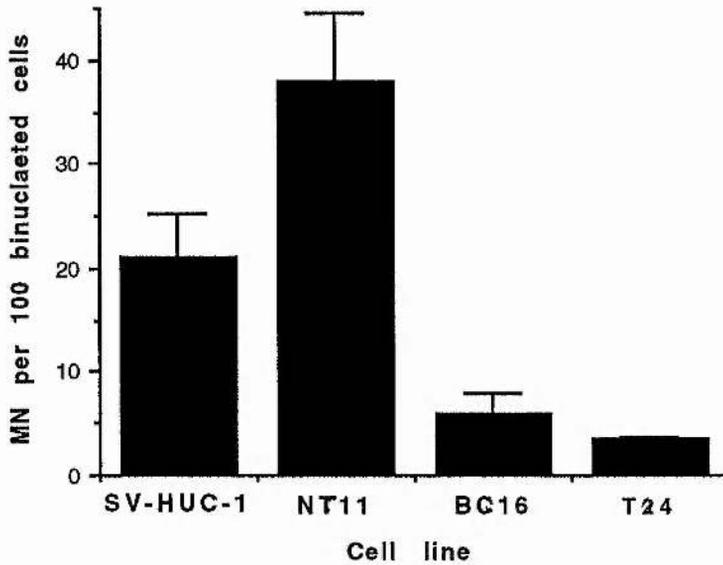


Fig.3.8. Spontaneous frequencies of Mn in binucleate cells 48 h after addition of Cyt B. Error bars represent SEM values from at least three separate experiments.

Radiation Dose in Gray	Surviving Fraction	Number of cells without Mn/100 binucleate cells
Cell line T24		
0	1.0	100
0.5	0.815	78
1.0	0.530	58
1.5	0.443	49
2.0	0.249	44
3.0	0.126	34
4.0	0.053	11
6.0	0.006	5
Cell line SV-HUC-1		
0	1.0*	100
1.0	0.85*	83
2.0	0.61*	67
4.0	0.25*	45
6.0	0.095*	27

Table 3.1. Data for cell lines T24 and SV-HUC-1 relating surviving fraction to the number of cells not containing Mn following radiation treatment.* Values were calculated from Fig. 3.7, these data provide the basis of the approximate survival curve for the SV-HUC lines shown in Fig. 3.6..

between cytoplasm (blue) and the nuclei or Mn (mauve; Figure 3.9). Another advantage of this method was that the distribution of cells on the slide was predictably in one area with cells in relatively close proximity. Thus, boundaries of cell cytoplasm could be easily identified. These factors allowed accurate scoring of slides much more rapidly than with standard spreading techniques. The staining with AO (Figure 3.10) indicated that the Mn produced are nuclear fragments and not artifacts of the Jenner/Giemsa stain.

Immunocytochemical staining for the SV40 T-antigen was positive for the SV-HUC lines (Figure 3.11) but no staining was observed with the malignant bladder cell line T24.

Finally, after failure of the X-ray source the "relative biological effectiveness; RBE" (Hall 1988) was checked between X-rays and Gamma rays for identical doses using the same end point, i.e. induced Mn following irradiation. The results indicate that there is no significant difference between X-ray and Gamma rays over the dose range used (0-6 Gray) in terms of induced Mn following radiation treatment (Figure 3.12).

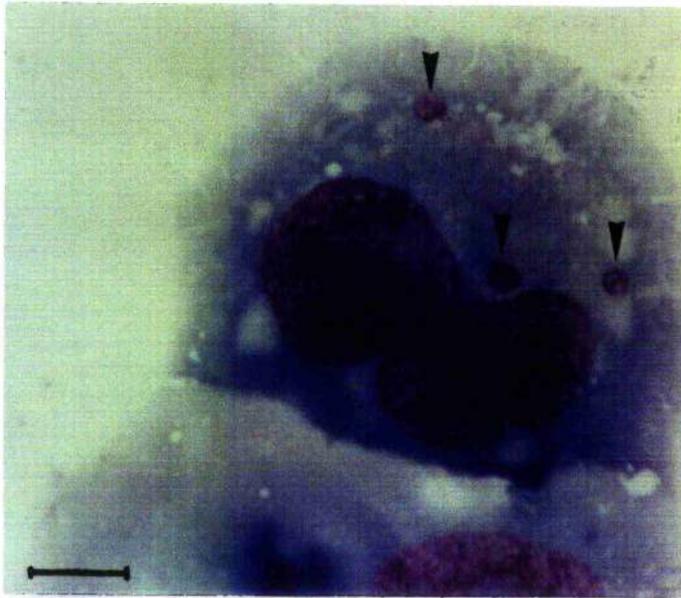


Fig.3.9. A Jenner/Giemsa stained binucleate NT11 cell 48 h after 4.0 Gy X-irradiation and cytokinesis blockage with 3 μ g/ml Cyt B. The arrows indicate Mn within the cell cytoplasm. Scale bar represents 10 μ m.

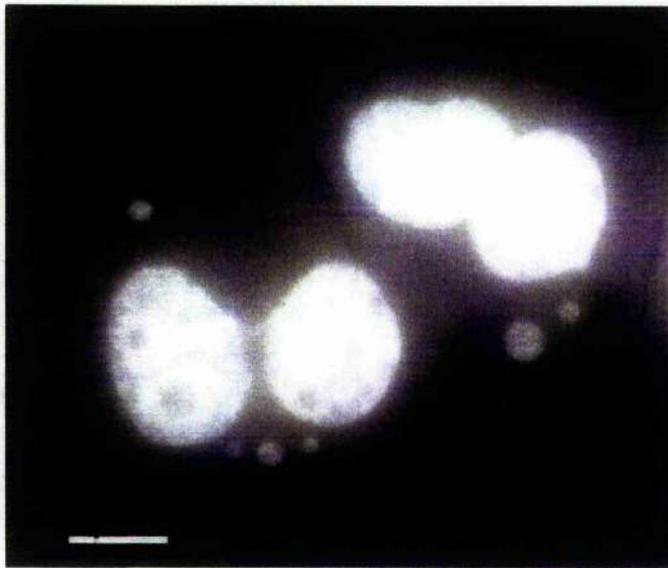


Fig.3.10. Acridine Orange fluorescence of binucleate NT11 cells 48 h after 4.0 Gy of X-rays and cytokinesis blockage with 3 μ g/ml Cyt B. Several Mn per cell are shown and the boundaries of cell cytoplasm can be clearly distinguished as with the Jenner/Giemsa stained samples. Scale bar represents 10 μ m.

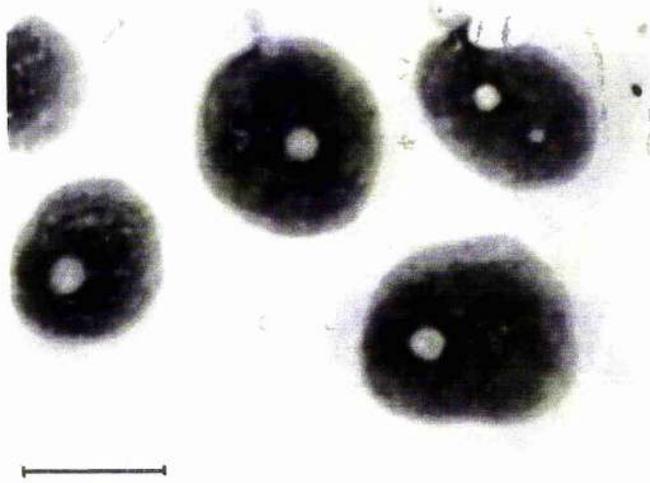


Fig.3.11. Immunocytochemical staining of SV-HUC-1 cells with antibodies against SV40 T-antigen. The cytoplasm & nucleolus have remained unstained, whilst the nucleus has stained darkly. Scale bar represents $10\mu\text{m}$.

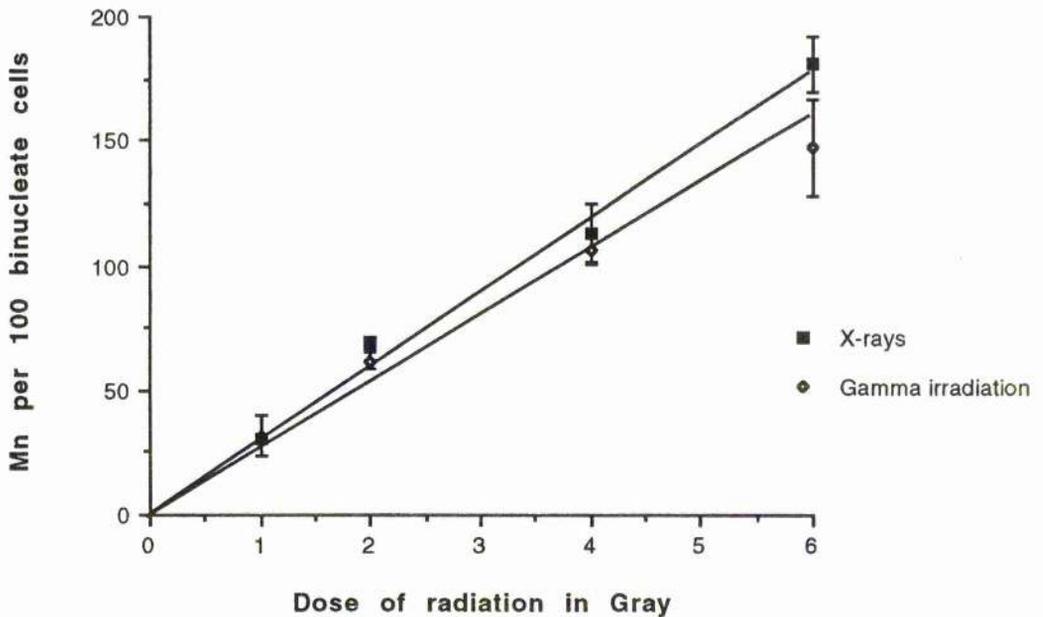


Fig. 3.12. Comparison of the effects of X-ray and Gamma radiation on Mn formation in SV-HUC-1 cells. Sampling time was 48 h and error bars represent SEM values from three separate experiments.

DISCUSSION

3.7 The optimal concentration of Cytochalasin B.

The original work of Fenech & Morley 1985 used Cyt B at a concentration of 3µg/ml in culture to induce binucleate cells. However more recent data (Weissenborn and Streffer, 1991, McMillan and Bush, 1991) has suggested that 3µg/ml of Cyt B itself can induce increased levels of spontaneous Mn but lower concentrations reduced this spontaneous Mn frequency whilst still permitting cytokinesis blockage. From Figure 3.2 it can be seen that for human uroepithelial cells an optimal working dose of Cyt B was found to be 3µg/ml and above since below that cells escape cytokinesis blockage as indicated by the reduced Binucleate cell index. Increasing concentrations of Cyt B were not found to generate a corresponding increase spontaneous Mn. Hence this supports the original findings of Fenech and Morley 1985.

3.8 Relative radiosensitivities of human uroepithelial cells and the malignant carcinoma cell line T24.

From Figure 3.3 it can be seen that the Mn frequency in binucleate cells increased with time following radiation treatment. The results show that the SV-HUC lines all had similar chromosomal radiosensitivities as estimated from Mn frequencies at 48 hours following X-irradiation (Figure 3.3). The data shown in Figures 3.3 & 3.4 indicates that the SV-HUC lines were significantly less radiosensitive than the T24 cell line (also of bladder epithelial origin). It is possible that the apparent radiosensitivity of T24 cells could be influenced by the shorter doubling time of T24 relative to the SV-HUC lines. This will lead to a more rapid accumulation of Mn with time, as seen in Figure 3.3 and as discussed by Savage (1989). This effect may however, to some extent, be offset by the more rapid exit of T24 cells from the binucleate into the quadranucleate

compartment (unscored cells) as shown in Figure 3.5. Hence comparisons of radiation induced Mn frequencies are only made between cells that have cycled to the same extent following radiation treatment and are in a similar "time window".

Tumourigenic T24 cells are themselves reported to be somewhat radioresistant when compared with certain other tumour cell lines (Parris *et al.*, 1988). The expression of the SV40 T-antigen within human fibroblasts has also been shown to decrease radiosensitivity (Arlett *et al.*, 1988). SV-HUC lines have been immortalized but are not fully transformed (i.e. non-tumourigenic as assayed by inoculation of cells into nude mice; Christian *et al.*, 1987). However, they do contain the SV40 T-antigen, Figure 3.11 and Christian *et al.*, 1987). This lack of a fully transformed phenotype but presence of the SV40 T-antigen supports the idea that it is this sequence of viral DNA that somehow confers increased radioresistance to the cell.

Due to the difficulty in obtaining a viable single cell suspension of the SV-HUC lines colony assays could not be used to determine survival curves directly. However, the Mn frequencies as shown in Figure 3.4 indicates an almost 2-fold lower induction of Mn in SV-HUC lines than in T24 cells. Because of the radioresistant nature of the SV-HUC lines in terms of X-ray induced Mn levels, a probable survival curve for the three SV-HUC lines is shown by the dotted line in Figure 3.6. This approximate survival curve is based on the Mn data from the SV-HUC lines and makes the assumption that most X-ray induced Mn will be lethal events (see Table 3.1). This approximate survival curve for the SV-HUC lines was calculated using the data in Table 3.1 and then reading the corresponding value of the number of cells without Mn against surviving fraction from Figure 3.7 (solid lines). Because the spontaneous level of Mn varies with cell line the background or 0 Gray radiation dose Mn value has been taken as zero since it is "induced" levels of Mn after irradiation that are being examined. 0 Gray was taken as 100%

survival. Spontaneous Mn values have been subtracted from the data shown in Table 3.1 and Figure 3.7. For actual values of spontaneous Mn see Figure 3.8.

The number of cells containing no Mn and the surviving fraction of cells after radiation treatment would not be expected to coincide exactly. This is because the induction of acentric fragments is by no means the only lethal lesion generated by ionizing radiation and not all acentric fragments are necessarily recovered as Mn in binucleate cells. In fact McMillan and Bush (1991) have reported that MGH-U1/T24 cells require approximately 1 Mn per lethal event as measured by clonogenic assays. However it does provide an indirect approximation of cellular survival for cell lines where clonogenic assays cannot be used.

3.9 The nature and significance of Mn as chromosomal aberrations in carcinogenesis.

The spontaneous or background level of Mn in binucleate cells (Figure 3.8) gives an indication of the genetic stability for each cell line. Cytogenetic instability has already been reported in the cell line SV-HUC-1 by Meisner *et al.*, (1988). Cell line NT11 having an even higher spontaneous Mn level than SV-HUC-1, suggests that NT11 cells have a greater genetic instability than the other SV-HUC lines.

Whereas X-ray induced Mn are known to be composed almost entirely of acentric fragments (Thomson and Perry, 1988; Littlefield *et al.*, 1989) it is not known if these spontaneous Mn are the result of acentric fragment formation or whole chromosome loss. However work by Meisner *et al.*, (1988) on the cell line SV-HUC-1 shows that this genetic instability resulted in chromosomal loss and duplications coupled with translocations and a reduction in polyploidy as passage number increased. These spontaneous

genetic changes alone did not result in the generation of a tumourigenic phenotype.

The genetic instability shown by two of the SV-HUC lines may allow a synergistic effect between this instability and other additional changes such as oncogene activation or tumour suppressor gene loss, both of which are known to occur by the types of genomic rearrangements mentioned above. These additional changes may be necessary stages in the multistep process of carcinogenesis.

CHAPTER 4

RADIATION AND CHEMICAL CARCINOGEN INDUCED TRANSFORMATION OF HUMAN UROEPITHELIAL CELLS.

4.1 Abstract.

4.2 Introduction.

4.3 Procedure.

Results

4.4 Cytotoxicity of carcinogen treatments.

4.5 Tumour formation following carcinogen treatments.

4.6 Transformation/dense type colony formation following carcinogen exposure.

4.7 Growth of transformed/dense type colonies in 0.3% agar.

Discussion

4.8 Mechanisms of action of carcinogens used.

4.9 Analysis of tumours - development and characteristics.

4.10 Generation of transformed/dense type colonies and their possible significance.

RADIATION AND CHEMICAL CARCINOGEN INDUCED TRANSFORMATION OF HUMAN EPITHELIAL CELLS.

ABSTRACT

4.1

The effects of ionising radiation and chemical carcinogen exposure on oncogenic transformation in human epithelial cell lines were examined. The criteria used to assess such a phenotype included; tumour formation in nude athymic mice, altered morphology in cell culture and reduced anchorage dependence in semi-solid media. The SV 40 immortalised epithelial cell lines were very resistant to both radiation and chemical induced transformation as assayed by tumour formation. Only 1 out of >80 carcinogen treated cultures generating a tumour in contrast to 4 out of 5 tumours being produced by the inoculation of human bladder carcinoma cells into athymic mice as positive controls. Altered morphology was observed in both radiation and chemical carcinogen treated cultures held at confluence with a much higher frequency than that of tumour formation. Such cultures also showed a strong correlation between altered cellular morphology (resembling Transitional Cell Carcinomas of the bladder when grown in culture) and an increased colony forming efficiency in 0.3% agar. However such cultures did not produce tumours when cells were inoculated into suitable host animals. The generation of such a phenotype in culture may represent an analogous situation to formation of benign papillomas *in vivo*.

INTRODUCTION

4.2

As with the earlier work on cellular radiosensitivities a majority of studies concerning *in vitro* radiation induced transformation, have to date, been carried out on rodent cell lines (Hall & Hei 1985). An over view of the different transformation systems which have previously been employed to study radiation induced transformation is given in the main introduction (sections 1.2 & 1.3).

The generation of a transformed phenotype from cells not exhibiting such a phenotype has been measured in several different ways. The formation of a tumour after inoculation of cells into athymic nude mice is normally viewed as the definitive test for neoplastic transformation, since several different human tumour types exhibit the same histopathology in such hosts as they do in the original patient (Giovanella *et al.*, 1978). However, this is costly and often requires long incubation periods in the animal. Other indicators of cellular transformation which can be more rapidly assayed include: morphological alterations (Reznikoff *et al.*, 1973a, Reznikoff *et al.*, 1983, Bryant & Riches 1989), increased colony forming ability in semi-solid media (0.3% Agarose; Trent *et al.*, 1990) or even the expression of a known marker of transformation for a particular model system such as the alkaline phosphatase 75 KDa protein tumour-associated antigen for HeLa x skin fibroblasts (Sun *et al.*, 1988).

Certain compounds such as phorbol esters whilst themselves alone are not capable of inducing neoplastic transformation can, when coupled with other carcinogen treatments, have a synergistic effect and lead to increased transformation frequencies (Kennedy *et al.* 1978). One such agent used in this study was TPA (12-O-tetradecanoyl-phorbol-13-acetate).

The effect of various carcinogen treatments both physical and chemical on the cellular transformation of SV40 immortalized human uroepithelial cells was studied. The cells used for transformation experiments were primarily SV-HUC-1 or PC stock for which transformation using chemicals has already been reported (Reznikoff *et al.*, 1988 and Bookland *et al.*, 1992). The usual end point for such experiments was the inoculation of cells into nude mice and subsequent checking for the formation of tumours. The effect of postconfluence growth inhibition following carcinogen treatment was also assayed by the formation of foci or "dense type colonies" and growth properties in 0.3% agarose.

PROCEDURE

4.3

The ability of radiation and chemical carcinogens to facilitate neoplastic transformation of human uroepithelial cells was assayed by tumour formation in nude athymic mice. Cells were exposed to different pre-determined doses/concentrations of the relevant carcinogen before a 6-8 week post-carcinogen growth regime. Cultured cells were then sub-cutaneously injected into test animals and followed for up to 6 months.

The cytotoxicity of such carcinogen treatments was assayed using a "grow back" technique. A known number of cells were, treated with the carcinogen and the cell number determined 96 hours after the end of treatment. Such carcinogen treated cultures were compared to control cultures having received no carcinogen treatment.

Morphological alterations and increases in anchorage independent growth were also studied following carcinogen exposure. Carcinogen treated cultures maintained at confluence were screened following staining with Giemsa for

foci or dense type colony formation. Increased anchorage independent growth as determined by the frequency of colony formation in 0.3% agar.

RESULTS

4.4 Cytotoxicity of carcinogen treatments.

The carcinogens used were:- X-irradiation, bleomycin, DMBA, MCA, MNNG and TPA. The cytotoxicity of these treatments is shown in Figures 4.1-4.5 and the dose used in transformation experiments indicated by arrows at the corresponding concentration/dose. Figure 4.1 shows the cytotoxicity of X-rays and agrees closely with the estimated survival curve of SV-HUC lines calculated from Mn data in chapter 3. Cytotoxicities of the chemical carcinogens Bleomycin, DMBA and MCA were not as great that of MMNG at the corresponding concentrations (Figures 4.2-4.6). TPA was found to increase cell survival at low concentrations (0.01 µg/ml and below) but was toxic at the higher doses used (Figure 4.6). The mechanisms of action for these mutagens will be considered in the discussion.

4.5 Tumour formation following carcinogen treatments.

In only one case did carcinogen treatment of SV40 immortalized human uroepithelial cells result in the generation of a tumour after passaging in cell culture and inoculation into nude athymic mice. One single dose of 2 Gy X-irradiation produced this transformed phenotype. Higher doses and multiple doses of radiation, as well as highly mutagenic chemical carcinogen treatments failed to produce any transformants as assayed by this technique (Tables 4.1 & 4.2).

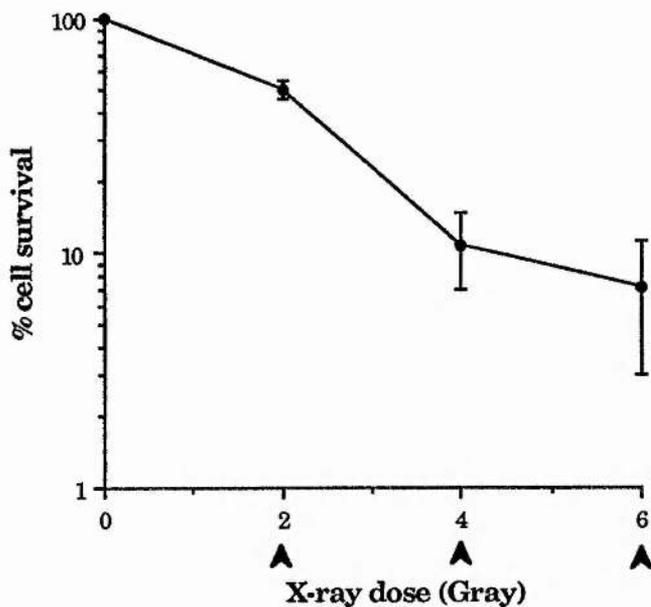


FIG 4.1. Cytotoxicity of X-rays (single treatment) on SV-HUC-1 cells. % survival is relative to controls 96 hours after irradiation. Error bars represent data from 3 samples. Arrows indicate the doses used in transformation experiments.

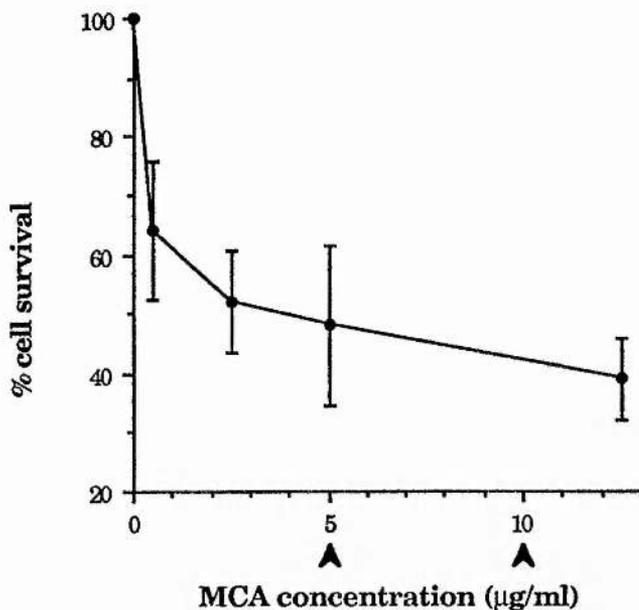


FIG 4.2. Cytotoxicity of 3-methylcolatherene(MCA; single 48 hour treatment) on SV-HUC-1cells. % survival is relative to controls 96 hours after addition of the carcinogen. Error bars represent data from 3 samples. Arrows indicate the concentrations used in transformation experiments.

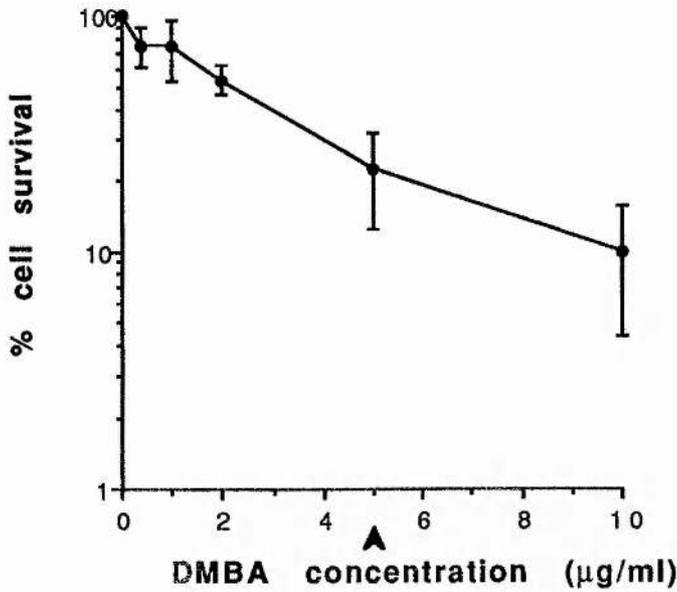


FIG 4.3. Cytotoxicity of 7, 12-Dimethylbenz-anthracine (DMBA; single 24 hour treatment) on SV-HUC-1 cells. % survival is relative to controls 96 hours after addition of the carcinogen. Error bars represent data from 3 samples. Arrows indicate the concentrations used in transformation experiments.

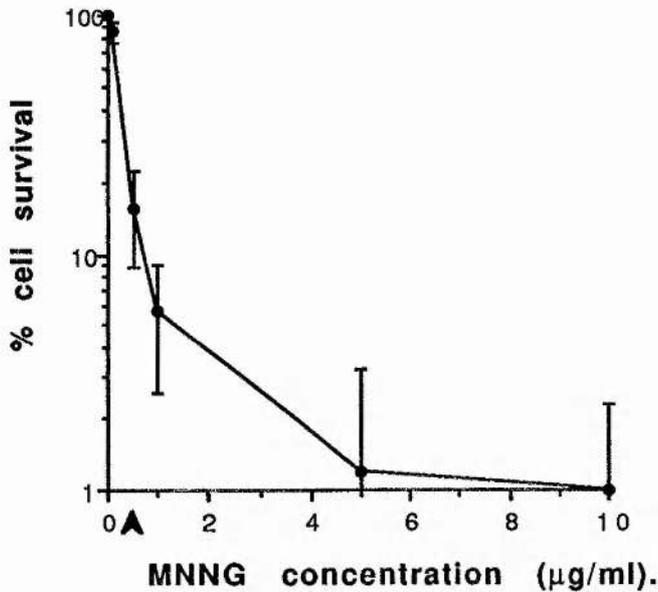


FIG 4.4. Cytotoxicity of N-methyl-N-nitrosoguanidine (MNNG; single 24 hour treatment) on SV-HUC-1 cells. % cell survival is relative to controls 96 hours after addition of the carcinogen. Error bars represent data from 3 samples. Arrows indicate the concentrations used in transformation experiments.

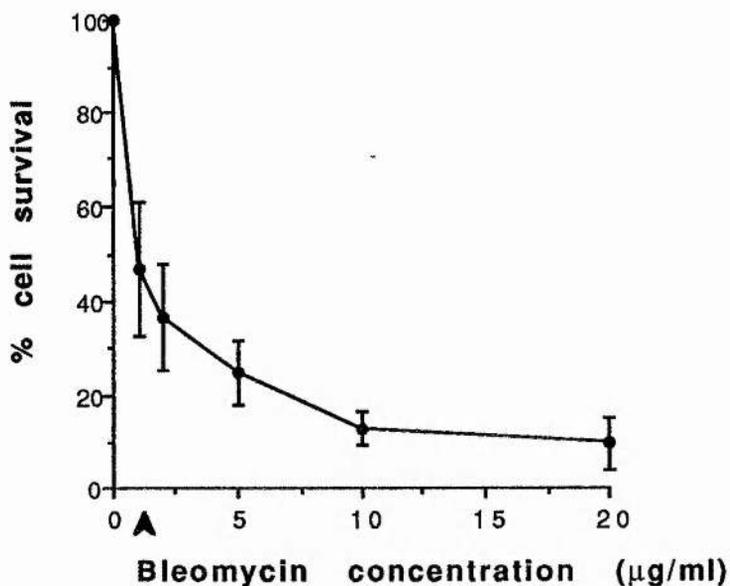


FIG 4.5. Cytotoxicity of Bleomycin (single 24hour treatment) on SV-HUC-1 cells. % cell survival is relative to controls 96 hours after the addition of the carcinogen. Error bars represent data from 3 samples. Arrows indicate the concentrations used in transformation experiments.

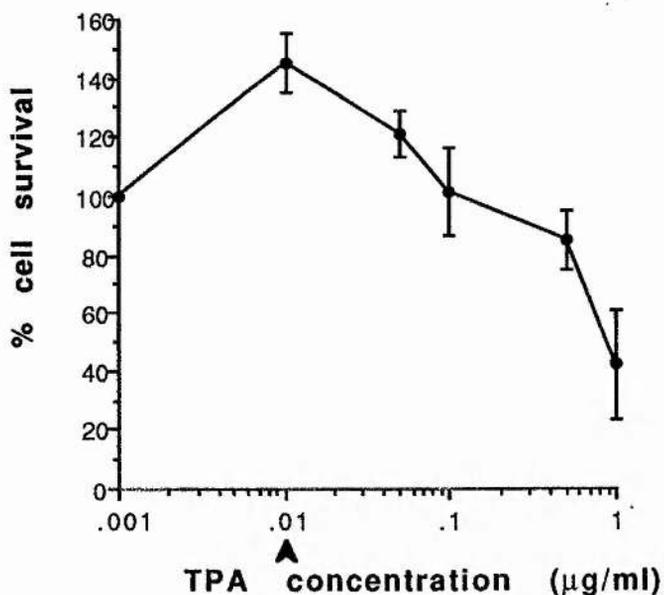


FIG 4.6. Cytotoxicity of 12-O-tetradecanoylphorbol-13-acetate (TPA; continuous exposure) on SV-HUC-1 cells. % survival is relative to controls 96 hours after the addition of the drug. Error bars represent data from 3 samples. Arrows indicate the concentrations used in transformation experiments.

The tumour formed after 2 Gy X-irradiation of SV-HUC-1 cells was biopsied after 160 days. Half the biopsy was fixed and sectioned in preparation for staining. The other half of the tumour biopsy was minced into 1mm x 1mm cubes and placed back into tissue culture. These however did not regenerate a cell line and no out growth from tumour explants was observed. The sectioned tumour biopsy was stained with haematoxylin and eosin (H & E) to enable histological examination (Figure 4.7) showing a small, undifferentiated carcinoma containing cords of tumourigenic cells in the surrounding stromal tissue. Staining with antibodies against SV40 T-antigen (Figure 4.8) and human cytokeratin was also carried out, both of which were positive.

		Number of tumours formed
Number of transformation assays on SV40 immortalized cells using radiation as the carcinogen.	54	1
Number of transformation assays on SV40 immortalized cells using chemical carcinogens	33	0
Number of bladder carcinoma cell line (T24) inoculations	5	4

TABLE 4.1. Simple breakdown of tumour formation data from nude athymic mice inoculated with human epithelial cells.

Four of the five bladder carcinoma cell line (T24) inoculations into nude athymic mice resulted in tumours which were biopsied after 34 days (Figure 4.9). Sections of biopsied tumours were placed back into tissue culture where they generated cell lines which were again found to be tumourigenic when

TREATMENT	NUMBER OF TUMOURS	COMMENTS
SV-HUC-1 (PC) cells		
Control	0/23	← Small tumour formed but failed to generate a cell line when tissue biopsy taken and reintroduced into tissue culture.
2Gy X-rays	1/8	
4Gy X-rays	0/10	
6Gy X-rays	0/6	
2x 4Gy X-rays	0/6	
2x 4Gy X-rays+TPA	0/6	
MCA(5µg/ml)	0/8	
MCA(10µg/ml)	0/6	
Bleomycin (1µg/ml)	0/3	
Bleomycin(1µg/ml)+ TPA	0/3	
MNNG (0.5µg/ml)	0/3	
DMBA (5µg/ml)+TPA	0/3	
Control + TPA	0/3	
2x 4Gy γ rays+ HAC	0/1	
2x MCA(5µg/ml)+ HAC	0/3	
8Gy γ rays + HAC	0/3	
Control HAC*	0/6	
T24 Bladder carcinoma cells.		
Positive Control	4/5	← Large rapidly growing tumours formed which did produce cell lines.
SV-HUC (NT 11) cells		
Control	0/3	
2Gy X-rays	0/3	
4Gy X-rays	0/3	
6Gy X-rays	0/2	
MCA (5µg/ml)	0/3	
SV-HUC (BC16) cells		
Control	0/3	
4Gy X-rays	0/3	
6Gy X-rays	0/3	
MCA (5µg/ml)	0/2	

TABLE 4.2. Tumour formation data from human epithelial cell lines injected s.c. into nude athymic mice. HAC (cultures Held At Confluence).

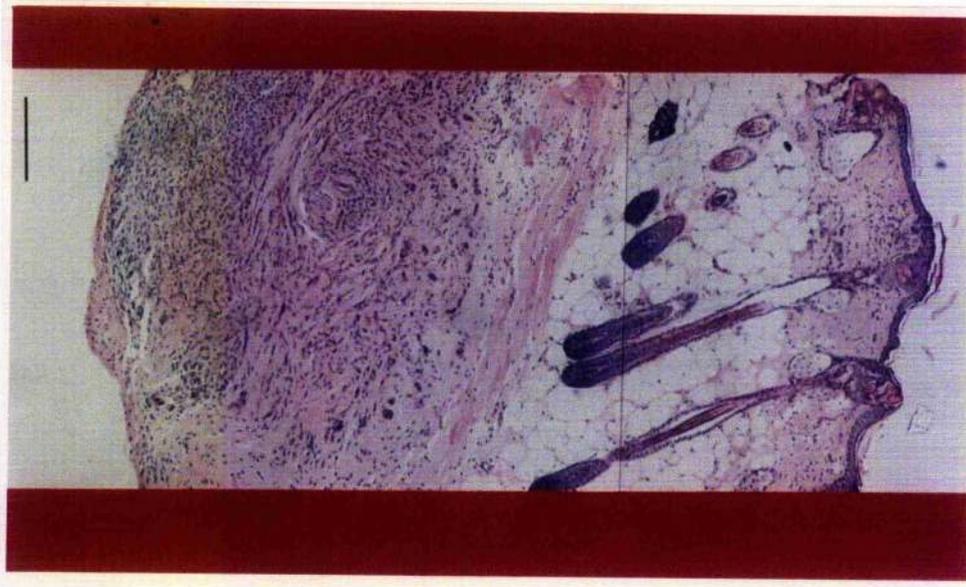


FIG 4.7. H & E stained section of the tumour generated after a single 2 Gy X-ray dose administered to SV-HUC-1 cells. The small tumour mass can be seen on the left of the photomicrograph. Connective tissue, mouse epidermis and hair follicles are also visible in the right half of the section. Scale bar represents 0.25mm.

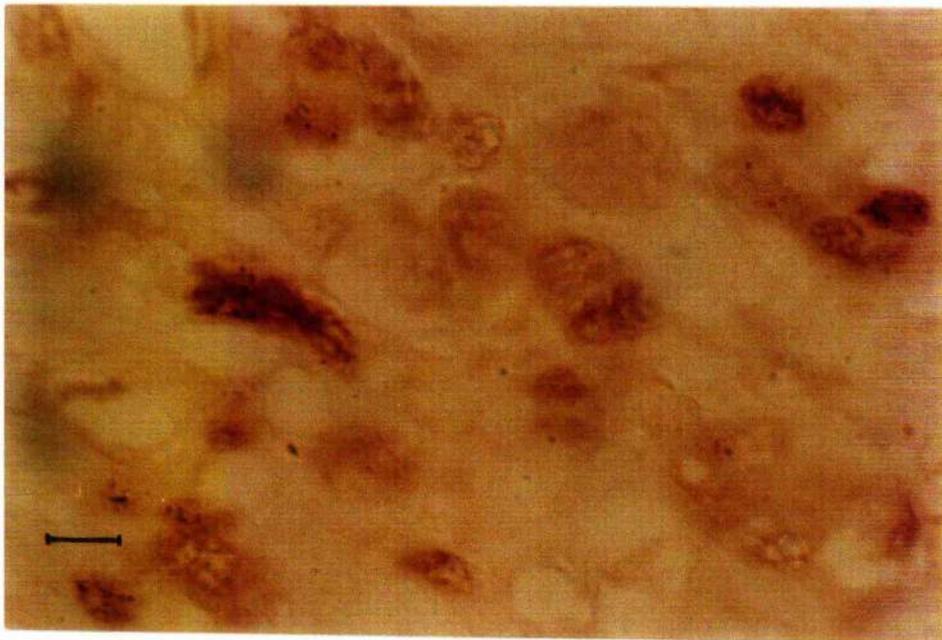


FIG 4.8. Monoclonal antibodies against SV40 T-antigen (antibody PAb 405) staining a fixed tumour section generated by 2 Gy X-rays on SV-HUC-1 cells (see Figure 4.7). Dark brown areas represent the staining of cell nuclei containing the SV40 T-antigen, i.e. SV40 transformed cells. Visualization carried out using DAB (Diaminobenzadine). Scale bar represents 10 μ m.

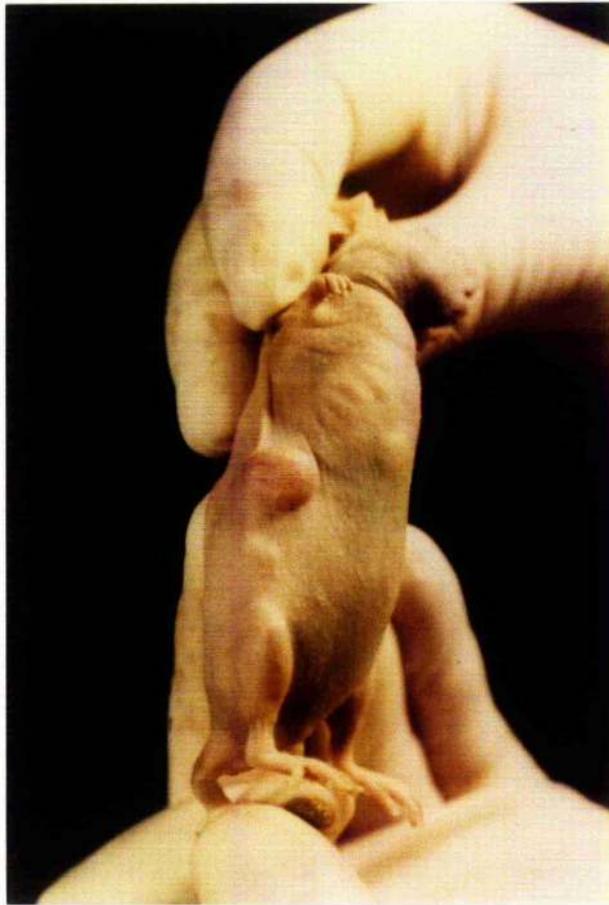


FIG 4.9. Tumour formed after s.c. inoculation of T24 bladder carcinoma cells into a nude athymic mouse. The site of tumour formation is consistent with the site of injection (mid way between fore and hind limbs in lateral position).

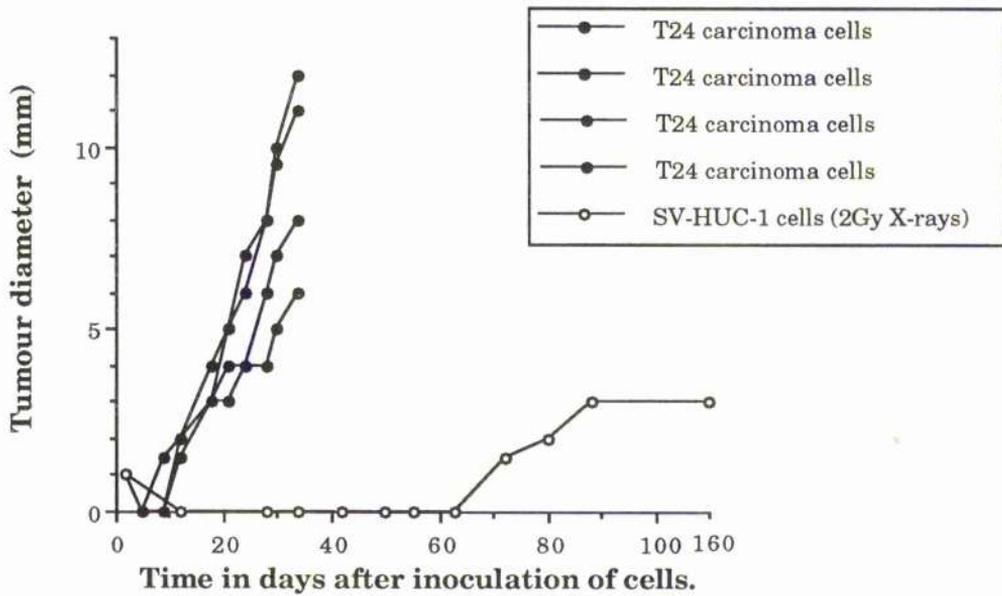


FIG 4.10. Tumour growth following inoculation of cells into nude athymic mice.

re-introduced into nude mice. It is unclear why only 4 of the 5 inoculated animals generated tumours, possibly due to a poor injection

The tumours generated by T24 cells were first observed 9-12 days after inoculation into nude mice and grew rapidly in size until they had reached 6-12 mm in diameter three weeks later when they were biopsied. The tumour generated from SV-HUC-1 cells following 2 Gy radiation took much longer to appear, 72 days after inoculation, and grew much more slowly resulting in a tumour mass of only 3 mm in diameter after 5¹/₂ months in the animal (Figure 4.10).

4.6 Transformed/dense type colony formation following carcinogen exposure.

"Transformed colonies" resulting from postconfluent growth inhibition following carcinogen treatment, were visible in tissue culture flasks even prior to staining as areas of high cell density on top of a confluent monolayer. Such colonies appeared 6-8 weeks after carcinogen treatment and confluent inhibited growth had been attained. However these could be more easily identified following fixation and staining of the flask with Giemsa (Figure 4.11). Such transformed colonies resulted from both radiation and chemical exposure in single and double treatment protocols (Table 4.3). This is in contrast to control cultures which did not show such morphological alterations but remained as a single confluent monolayer (Figures 4.12 - 4.14). Figures 4.12a and 4.13a show the typical appearance of non-transformed SV-HUC-1 cells at confluence, a small number of mitotic figures are visible in a background of non-overlapping "cobble stone" like cells. Figures 4.12b and 4.13b indicate the appearance of SV-HUC-1 cells at confluence and having undergone morphological alteration following carcinogen treatment coupled with appropriate culture conditions (see section 2.4 a).

FIG 4.11. Giemsa stained flask following 2x 4Gy gamma irradiations, passaging and maintenance at post confluent inhibited growth for 6 weeks prior to fixation and staining. The arrows indicate "transformed or dense" type colonies in a background of SV-HUC-1 cells.

With the double radiation exposure protocol and one passage between treatments, 4 Gray appeared to be an optimal dose for transformation, whereas with the single exposure protocol followed by no passaging 8 Gray was found to be optimal.

Upon transplantation of such colonies to a new tissue culture flask, cells only exhibited a limited outgrowth on the base of the flask. However, such colonies did continue to grow by cells piling up so generating a very tight and dense colony of considerable mass a further 6-8 weeks after transplanting (Figures 4.15-4.17). Some colonies become so large that they could be removed fixed, sectioned and H & E stained (Figure 4.18). Such sections showed a very dense accumulation of cells and cell debris with an active

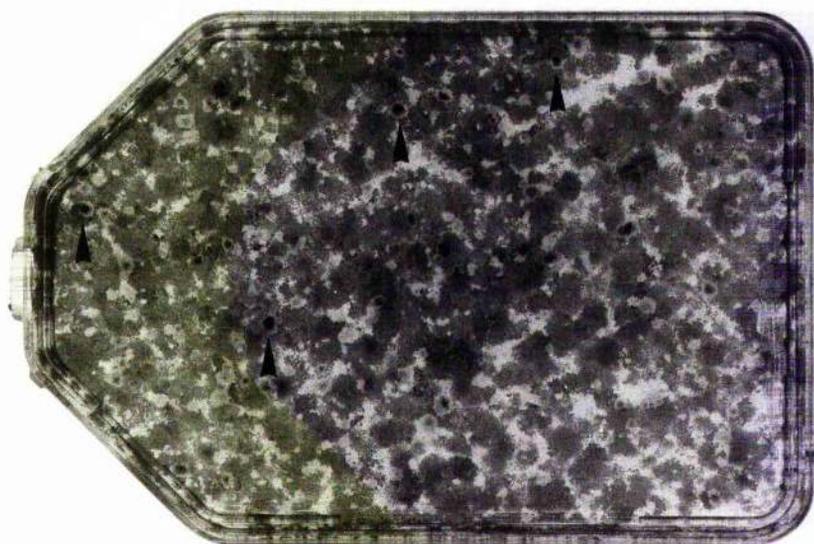


FIG 4.11. Giemsa stained flask following 2x 4Gy gamma irradiations, passaging and maintenance at post confluent inhibited growth for 6 weeks prior to fixation and staining. The arrows indicate "transformed or dense" type colonies in a background of SV-HUC-1 cells.

CARCINOGEN TREATMENT	"TRANSFORMED" PHENOTYPE WHEN HELD AT CONFLUENCE	INCREASED COLONY FORMATION IN 0.3% AGAROSE.
Double exposure protocol		
Control 1	-	-
Control 2	-	-
Control 3	-	-
2Gy γ rays 1	-	-
2Gy γ rays 2	-	-
2Gy γ rays 3	-	-
4Gy γ rays 1	+	+
4Gy γ rays 2	-	-
4Gy γ rays 3	+ (Fixed & stained)	ND
6Gy γ rays 1	-	-
6Gy γ rays 2	-	-
6Gy γ rays 3	-	-
MCA 5 μ g/ml 1	+	-
MCA 5 μ g/ml 2	+	+
MCA 5 μ g/ml 3	+	+
Single exposure protocol		
Control 1	-	-
Control 2	-	-
Control 3	-	-
2Gy γ rays 1	-	-
2Gy γ rays 2	-	-
2Gy γ rays 3	-	-
4Gy γ rays 1	-	-
4Gy γ rays 2	-	-
4Gy γ rays 3	-	-
8Gy γ rays 1	+	+
8Gy γ rays 2	+	+
8Gy γ rays 3	+	+

TABLE 4.3. "Transformed" colony generation data from SV-HUC-1 cultures held at post confluent inhibited growth for 6 weeks following carcinogen exposure. Double carcinogen exposure transformation experiments were performed with one passage between treatments. No passaging was carried out in single carcinogen exposure experiments. ND - Not determined.

FIG 4.12a. Phase contrast photomicrograph of normal, non- transformed SV-HUC-1 cells having obtained confluence. Cells maintain a "cobble stone" appearance and no piling up of cells is evident.

FIG 4.12b. Phase contrast photomicrograph of SV-HUC-1 cells from a transformed or dense type colony. Cells have changed their morphology and are more spindle like in appearance with cells now growing one on top of another i.e no longer exhibiting contact inhibition.

FIG 4.13a. Photomicrograph of Giemsa stained normal confluent SV-HUC-1 cells again exhibiting a "cobble stone" appearance.

FIG 4.13b. Photomicrograph of Giemsa stained cells from a transformed colony. Cells are more spindle like in appearance and considerable piling up of cells is evident in the bottom of the frame.

All scale bars represent 100 μ m.



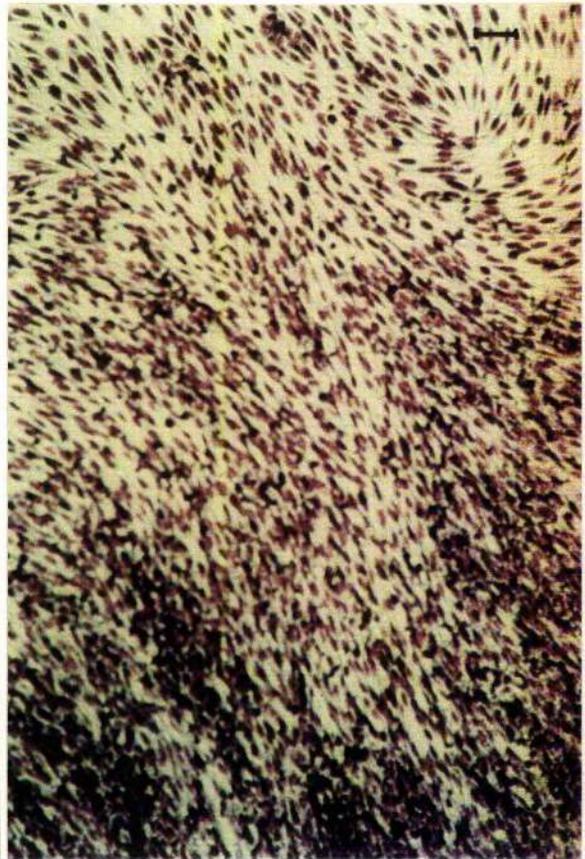
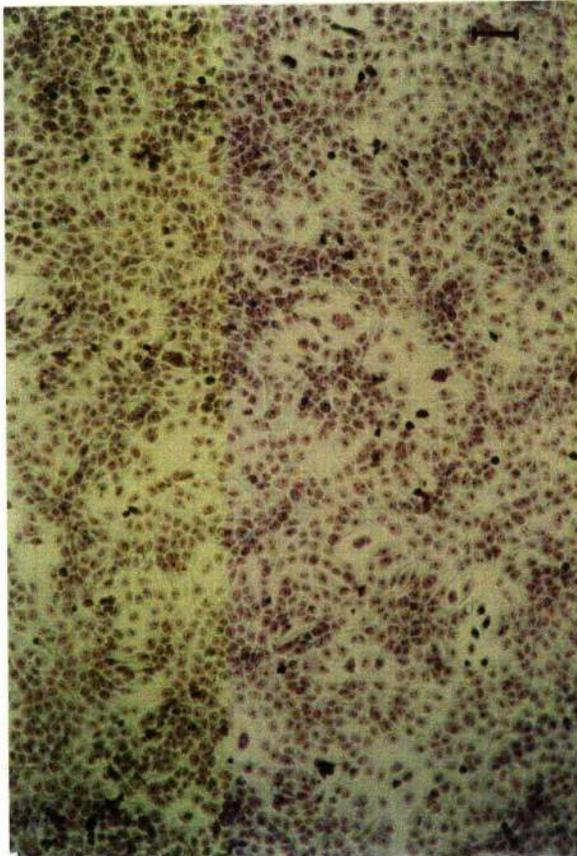
FIG 4.12a.

FIG 4.13a.



FIG 4.12b.

FIG 4.13b.



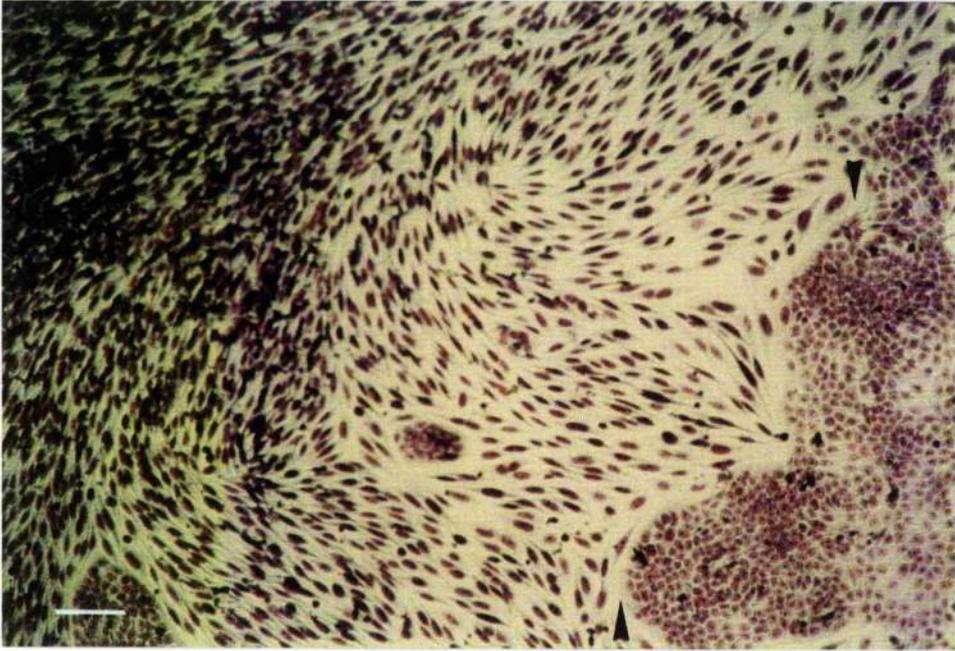


FIG 4.14. Photomicrograph of Giemsa stained cells from the junction between transformed cells (left) and non transformed cells (right). Piling up of cells is visible within the transformed area of growth and the non-transformed area of cells still shows the "cobble stone" appearance. The arrows indicate the boundary between transformed and non-transformed cell types where there is a clear demarcation point. i.e. The two cell populations do not appear to mix with each other. Scale bar 100 μ m.

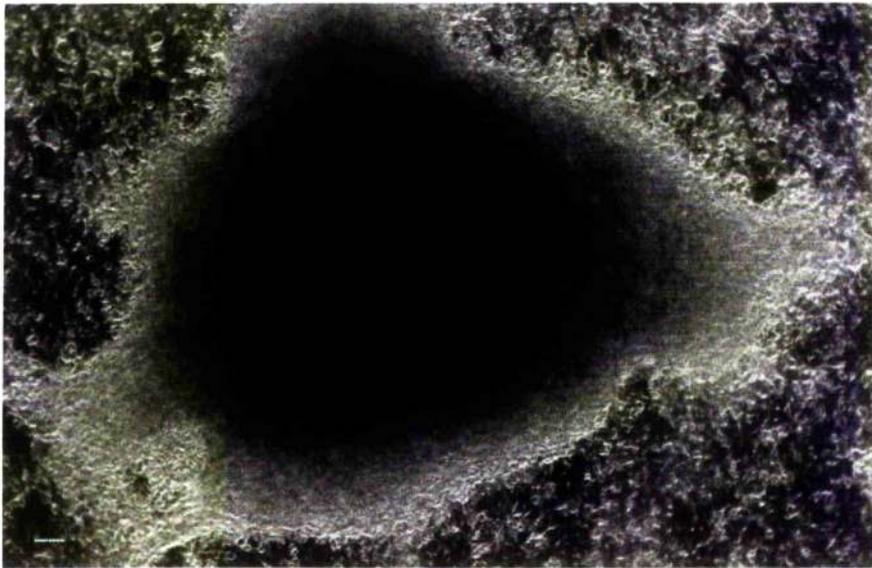


FIG 4.15. Phase contrast photomicrograph of a transplanted transformed colony. Cells continue to pile up whilst growing so generating a very dense, tight colony. Scale bar 100 μ m



FIG 4.16. Phase contrast photomicrograph from the edge of a transplanted transformed colony. The dense transformed phenotype can be seen on top of non-transformed "cobble stone" shaped cells. These non-transformed cells were probably transferred along with transformed cells when transplantation took place.



FIG 4.17. Gross overall appearance of transplanted transformed colonies growing on the base of a 25cm² tissue culture flask, approximately 8 weeks after transplantation.

margin of cell growth along the periphery of the colony. These colonies could be picked off and inoculated into nude mice as a solid mass of cells and checked for tumour formation.

Alternatively the initial flask exhibiting the transformed phenotype could be treated with EDTA/trypsin to remove cells and inoculations carried out as described previously. However no tumours were observed in any animals receiving cells from such transformed colonies. If transplatation into the mouse was from as a solid mass then a small lump persisted for 2-3 weeks before readsorption by the animal.

4.7 Growth of transformed/dense type colonies in 0.3% agar.

When cells were removed from cultures exhibiting a transformed or dense type colony, growth in semi-solid media (0.3% agar) was also examined. The EDTA/trypsin treatment generated close to a single cell suspension suitable for colony assays with low plating efficiency. Controls were non-carcinogen treated cultures held at confluence which did not show dense type colony formation. Only colonies greater than 50 cells were scored (Figure 4.19). In all cases where dense type colony formation had been observed in cultures maintained at confluence, there was a corresponding increase in colony formation above that of control samples (with the exception of one MCA treated culture; see Table 4.4).



FIG 4.18. H & E stained section of a transformed/dense type colony generated by a single 4 Gy γ radiation treatment of SV-HUC-1 cells, eight weeks after transplantation into a new culture flask. There is a dense accumulation of cells which no longer show the "cobble stone" morphology of the parental cell type. Scale bar 500 μ m.



FIG 4.19. A phase contrast photomicrograph of a colony of SV-HUC-1 cells growing in 0.3% agarose with Hams F12+ 1% FCS medium after a 21 day incubation period. A background of surrounding single cells which did not generate colonies can also be seen. Scale bar 100 μ m

Treatment	Colony Forming Efficiency $\times 10^{-4}$	Standard Error of Mean
Control 1	1.13	1.08
Control 2	1.69	0.56
Control 3	1.75	0.34
2 x MCA 1 †	1.53	0.37
2 x MCA 2 †	4.79*	0.14
2 x MCA 3 †	4.33*	0.37
2 x 4 Gy γ rays †	6.22*	0.31
2 Gy γ rays 1	1.30	0.59
2 Gy γ rays 2	2.40	0.23
4 Gy γ rays 1	2.19	0.59
4 Gy γ rays 2	2.63	0.91
4 Gy γ rays 3	2.07	0.45
8 Gy γ rays 1 †	6.03*	2.21
8 Gy γ rays 2 †	7.53*	0.82
8 Gy γ rays 3 †	11.68*	1.21

Table 4.4. Colony forming efficiency in 0.3% Agar. Data represents mean values from triplicate platings. * denotes a significant difference from control samples (student's *t*-test). † indicates cultures which formed dense type colonies when maintained at confluence.

DISCUSSION

4.8 Mechanisms of action of carcinogens used.

X-rays and other forms of ionizing radiation as with all carcinogens, achieve a mutagenic effect by direct or indirect interaction with nuclear DNA. In the case of ionizing radiation this is often via the induction of highly reactive free radicals, two thirds of X-ray damage to DNA is estimated to be caused by OH· radicals (Hall 1988, Schans *et al.*, 1982). Base modification and rupture of the phosphodiester backbone in the DNA molecule via single & double strand breaks and other local multiple damaged sites have all been shown to result following treatment of mammalian cells with ionizing radiations (reviewed by Ward 1986, Teoule 1987). As with all carcinogens it is the non-repair or misrepair of sublethal damage within cells that leads to the fixation of such genetic alterations, mutations and possible oncogenic activation (Elkind 1984, Hanawalt *et al.*, 1979). Due to the nature of certain types of damage i.e. chain rupture, a large fraction of radiation induced DNA damage will be manifested as deletions rather than single base substitutions of the type induced by many chemical carcinogens. Hence these changes tend to be of a more lethal nature and not as oncogenic as many potent chemical carcinogens (Harris 1991).

Bleomycin a radiomimetic drug also damages DNA by free radical mechanisms and has a reported mutational spectrum similar to that of X-rays (Povirk & Goldberg 1987). DMBA (7, 12- Dimethylbenzanthracene) known to be a potent carcinogen for over 40 years, causes primarily A:T->T:A transversions (Harris 1991). The mechanism of action for MCA (3 Methylcholatherene) is not clearly known (Reznikoff *et al.*, 1998). MNNG (N-methyl-N-nitrosoguanidine) is an alkylating agent which induces a large number of O⁶-methylguanosine residues so causing mispairing with

thymine during DNA synthesis (Bridges & Lehmann 1982). TPA (12-O-tetradecanoylphorbol-13-acetate) a phorbol ester is a tumour promotor and has been shown to enhance the transformation frequency of mammalian cells following radiation treatment (Kennedy *et al.* 1978). This is probably due to an increased cell saturation density being obtained. TPA is reported to interfere with cell-to-cell communication, recent work suggests this is mediated by activation of protein kinases (such as protein kinase C), so leading to further phosphorylation of proteins which act as growth/cell cycle control regulators (Meek and Street 1992, Tauchi *et al.* 1992).

4.9 Analysis of tumours - development and characteristics.

Inoculation of control cells (unirradiated or no carcinogen treatment) into nude mice shows that these immortalized human epithelial cells are non-tumourigenic (0 tumours out of > 25 inoculations into host animals). This agrees with work previously reported by Christian *et al.*, 1987.

The low transformation frequency after both radiation and chemical carcinogen treatments (1 out of > 80 carcinogen exposure experiments generating a tumour in host animals) indicates that these human cell lines are very resistant to full oncogenic transformation. The generation of a tumourigenic phenotype by chemical carcinogens (MCA and 4-Aminobiphenyl) which have been reported by Reznikoff *et al.*, 1987 and Bookland *et al.*, 1992 using the above cell line (SV-HUC-1), could not be demonstrated here. The enhanced transformation frequency shown by the synergistic effects of TPA and carcinogen treatment as reported in rodent cell lines (Kennedy *et al.*, 1978, Elkind *et al.*, 1985) was also not evident in such experiments. This is not entirely surprising considering the very low number of transformants obtained and also in the view of the evidence that TPA obtains its effect by increasing saturation densities, since the experimental protocol used did not allow cultures to reach

saturation/confluence. Due to the low number of transformants generated by chemical or physical agents as assayed by tumour formation using SV-HUC lines, the calculation of any transformation frequencies or risk assessments was not possible.

The staining of the radiation induced tumour with antibodies to SV40 T-antigen and human cytokeratin indicated that the tumour obtained was of human epithelial origin and from cells immortalized by SV40 virus, i.e. derived from the SV-HUC-1 parent cell line and not a contaminant or spontaneous mouse neoplasm.

There was a long incubation time *in vivo* before manifestation of the tumour coupled with a slow and indolent growth pattern i.e. producing a tumour of small diameter which neither progressed or regressed. This suggests the generation of a non-aggressive tumour phenotype after the inoculation of a relatively small number of transformed cells in the initial inoculum when compared to the carcinoma cell line T24. Unfortunately, biopsy explants of this tumour did not generate a cell lines in tissue culture so limiting the characterization of the transformants to fixed material. This inability to produce a cell line after tumour explantation from a suitable host animal has also been reported by Craise *et al.*, 1991 after irradiation of immortalized but nontumorigenic human keratinocytes (using multiple radiation treatments and an *in vitro* protocol of > 1 year in tissue culture). However full onocgenic transformation of human epidermal keratinocytes including the regeneration of a cell line from tumour biopsies has now been reported (Thraves *et al.*, 1990).

4.10 Generation of transformed/dense type colonies and their possible significance.

The transformed or dense type colonies formed after carcinogen treatment in SV-HUC cultures which were allowed to remain in a state of post

confluence inhibited growth, share some similarity to the colonies formed in rodent systems eg. $10T^{1/2}$. Although these transformed colonies do not produce tumours when inoculated into suitable host animals the morphology of these transplanted transformed colonies i.e. into new tissue culture flasks, resembles closely that shown by transitional cells carcinomas (TCC's) of the urinary bladder when growing in tissue culture (Reznikoff *et al.*, 1986b).

These "transformed" colonies whilst not showing full oncogenic transformation (i.e. tumour formation) probably represents the generation of a phenotype similar to a benign papilloma in *in vivo* studies, although progression from this state to a more aggressive/oncogenic phenotype was not studied here. The progression from an indolent *in vivo* phenotype in nude mice using SV-HUC-1 cells to a more aggressive, rapid growing, neoplastic phenotype has been reported by Wu *et al.* (1991). This resulted after combined chemical carcinogen treatments with MCA initially and subsequently N-hydroxy-4-acetylaminobiphenyl. In this present study there was also a corresponding increase in colony formation in 0.3% agarose for cultures exhibiting transformed/dense type colonies (except one MCA culture). This supports the suggestion of a "transformed" phenotype, evidence provided by both an altered morphology and a reduced anchorage dependence in such cultures.

The single dose of 8 Gy radiation that produced transformed colonies will have had a lethal effect on a large fraction of the initial irradiated population. This means that whilst no passaging was carried out, the surviving fraction will have undergone a considerable amount of "grow back" going through many cell doublings before reaching confluence and growth inhibition. Conversely with the double exposure protocol 4Gy appeared to be optimal for induced transformation. However, passaging was

performed so allowing all surviving irradiated cells to progress through several cell doublings before confluence was attained.

Several factors influencing the frequency of radiation induced oncogenic transformation of rodent 10T¹/2 cells have been reported. These include dose rate, if fractionation of the dose was administered, cell density (both initial and confluent, which can be altered by agents such as TPA) and linear energy transfer (LET) of the radiation given (reviewed by Elkind *et al.*, 1985). More recent evidence also indicates that the phase of the cell cycle when the irradiation is carried out may be important, G₂ appearing as a sensitive window for oncogenic transformation (Cao *et al.* 1992, Miller *et al.* 1992).

Although such a diverse range of different conditions was not examined here, it appears that the generation of a transformed phenotype in radiation induced transformation studies using SV-HUC cultures depends strongly on the ability of cells to undergo several cell divisions before reaching post confluent inhibited growth.

This need for cell division following radiation treatment may reflect a fixation of cellular damage and a proliferation of "initiated" but not yet transformed cells. A selection pressure must then be applied if these "initiated" or altered cells are to proliferate relative to surrounding cells. In cultures maintained at or near optimal growth conditions there is little chance for a selective pressure to be applied since all cells are proliferating at or close to a maximal rate. The maintenance of NIH 3T3 mouse cells at low cell density in high serum conditions (i.e. optimal growth conditions) has been shown to reduce transformation frequencies (Yao *et al.*, 1990). Also reduction/removal of serum in human keratinocyte cultures has been shown to increase transformation (Hill & Hillova 1990). However, if cultures are maintained at confluence then reductions in growth contact inhibition and other selective growth advantages can be visualized by colony formation, i.e. proliferation relative to surrounding cells. This is also more

analogous to cancer formation *in vivo* where cells are kept in check by their position within a tissue and relative to surrounding cells, thus aberrant growth relative to other more strictly controlled cells constitutes the generation of a neoplasm. Hence growth conditions that constrain rather than encourage multiplication of already initiated cells may lead to the increased detection of transformed phenotypes *in vitro*.

"Thus, growth stimulation *in vivo* and growth inhibition *in vitro* may converge on the optimal conditions for transformation" Faber and Rubin 1991.

CHAPTER 5

INDUCTION OF p53 PROTEIN AND CELL CYCLE DELAY FOLLOWING IRRADIATION OF HUMAN EPITHELIAL CELLS.

5.1 Abstract.

5.2 Introduction.

5.3 Procedure.

Results

5.4 Expression of wild type and mutant p53 protein in unirradiated cells.

5.5 Expression of wild type p53 protein in irradiated cells.

5.6 Cell cycle changes following irradiation.

Discussion

5.7 The effects of viral oncoproteins on cellular immortalisation and p53.

5.8 Extended sampling times to monitor cell cycle changes.

5.9 Radiation induced p53 protein and cell cycle delay.

5.10 Possible controlling mechanisms of p53 and cell cycle delay.

5.11 To what extent can this model for p53 function be relied upon?

INDUCTION OF p53 PROTEIN AND CELL CYCLE DELAY FOLLOWING IRRADIATION OF HUMAN EPITHELIAL CELLS

ABSTRACT

5.1

Mutations in the p53 gene which lead to the expression of mutant protein sharing a common conformational effect, have been identified as the most frequent genetic alterations in human cancer. Until recently however, the normal cellular function and mechanism of activation of wild type p53 has remained unclear.

Changes in the level of wild type (wt) p53 protein following irradiation was assessed in three human epithelial cell lines from different origins. As a result of treatment with ionizing radiation one cell line HPV (human papilloma virus transformed) exhibited an approximate two fold increase in p53 levels. This induction of wt p53 protein could be achieved with relatively low doses of gamma radiation (1 Gray). The induction of p53 in other cell lines following irradiation could not be detected with increasing doses of radiation nor with extended sampling times.

Again only cell line HPV showed any form of cell cycle delay following irradiation with cells accumulating in G₂ and not G₁. This result suggests that the E6 protein of the human papilloma virus is not capable of blocking the induction of wt p53 following irradiation although its function may be modified or abrogated, i.e. a G₁ arrest is not observed.

INTRODUCTION

5.2

Mutations in the gene coding for the 393 amino acid nuclear phosphoprotein p53 are the most frequently observed genetic alterations so far identified in human cancer (Vogelstein 1990). The p53 gene located on chromosome 17p13.1, now termed a tumour suppressor gene (genes for which loss-of-function mutations result in oncogenic transformation), largely due to the initial observation that this gene is mutated or deleted in 70-80% of colorectal carcinomas (Baker *et al.*, 1989). However, alterations of the p53 gene have now been identified in a wide range of human cancers including:- breast, bladder, brain, colon, lung, ovarian and prostate neoplasms (Nigro *et al.* 1989, Isaacs *et al.* 1991, Jones *et al.* 1991 and Eccles *et al.*).

The p53 protein was first identified bound to the large T-antigen of SV40 transformed cells (Lane and Crawford 1979). More recently the wild type p53 protein (wt p53) has also been found to bind proteins from several other viruses such as adenovirus and human papilloma virus (Werness *et al.* 1990, Yew and Berk 1992). This association with viral proteins appears to alter the DNA sequence specific binding of wt p53 in a similar way that the MDM2 protein does. The MDM2 gene is often found amplified in human sarcomas where no p53 mutations have been observed and codes for a protein which binds wt p53 (Oliner *et al.* 1992). Hence this protein may be a normal human cellular counter part acting in a similar way to the DNA tumour virus oncoproteins.

The mutant p53 proteins have a much longer half life (4-8 hours) than that of the wild type (6-20 min; Lane and Benchimol 1990) so leading to the accumulation of the mutant protein in transformed cells (Levine *et al.* 1991). A wide range of point mutations within the p53 gene have been identified that are capable of facilitating oncogenic transformation, however all such mutations produce a common conformational change within the protein (Gannon *et al.* 1990). Cells expressing mutant p53 protein or cells that contain no endogenous

p53 protein due to the complete deletion of this gene, can have an oncogenic phenotype suppressed by the transfection of a wild type cDNA copy (Chen *et al.* 1990). Further evidence as to the involvement of mutant p53 in the generation of human neoplasia came from families affected by Li-Fraumeni syndrome, who show a high incidence of tumour formation in a variety of different organs. Germline p53 mutations have been identified in such patients who already contain one wild type and one mutant p53 allele (Wienberg 1991). Hence mutation or loss of the one remaining wild type allele is a far more likely event than loss/mutation of two alleles in individuals not affected by this syndrome.

How then can the p53 protein play such an important role in carcinogenesis?

The inhibition of mammalian cell cycling by radiation induced DNA damage has been well documented (Little 1968, Painter and Young 1980, Kilmer *et al.* 1981, Zampetti-Bosseler *et al.* 1981). This mitotic delay/inhibition of DNA synthesis appears to allow an increased repair of damaged DNA prior to progression through the cell cycle and so reduce the chance of fixing damaged DNA at S-phase or mitosis which could generate mutation. Ultraviolet irradiation of mouse 3T3 cells (Maltzman and Czyzyk 1984) and X-irradiation of human haematopoietic progenitor cells (Kastan *et al.* 1991) have been reported to show a corresponding increase in wt p53 levels with a cell cycle delay occurring predominantly at G₁. More recently Kuerbitz *et al.* (1992) have shown that cell lines expressing mutant p53 fail to arrest at G₁ following γ irradiation.

The wild type p53 protein is thought to be a transcription factor binding to specific DNA sequences as a tetrameric complex which modulates the transcription of downstream genes controlling cell cycle determining factors (Lane 1992a, Lane 1992b). Wt p53 is stabilized by a post-translational mechanism which is induced by damaged DNA. This causes an alteration in the level/activity of the cell cycle determinant(s) so leading to an increased cell cycle delay and time for repair of damaged DNA. If successful repair is not carried out then wild type p53 has the ability to induce apoptosis (Yonish *et al.*

1991, Ryan *et al.*, 1993). Both these strategies employed by the cell ensure that clones with mutations do not survive and so act as a source of future neoplastic or preneoplastic cells. Since the genome of any cell is constantly at risk from mutations both spontaneous and induced, a mechanism which keeps a check on the fidelity of the genome is of vital importance. Hence individuals who lack such a process leave their cells open to a much greater risk of accumulating potential oncogenic genomic alterations.

Linked to these observations is a "dominant negative" model of tumour suppressor gene inactivation (reviewed by Oren 1992). When one p53 allele becomes mutated the protein produced from that gene undergoes a conformational change and ceases to function as the wild type protein does. However in this situation protein of both wild type and mutant conformation is present, this may lead to an increased proliferative advantage for such cells so generating a large pre-neoplastic subpopulation from which only one wild type p53 allele has to be lost or mutated in order that full oncogenic transformation take place. Such a model can be further explained by the observation that mutant p53 protein binds to the wild type (Levine *et al.*, 1991) so rendering it physiologically inactive. This leads to a sharp decrease in the level of functionally active wt p53 since the protein in its mutant conformation is far more stable than that of the wild type. In such cells there will be very low levels of wt p53 that is not bound to the mutant protein, hence the growth advantage. However, there will be enough residual wild type function to inhibit full oncogenic transformation until the loss or mutation of the second p53 allele. Although this model is not without its limitations, it does serve to help explain some, if not all the observations concerning mutant, wild type or complete lack of p53, both at the genetic and biochemical level.

Since a vast majority of adult human tumours originate in epithelial tissues (Cairns 1975) the role of p53 after DNA damage in such cells types may well play an important part in the development of many human cancers. The

induction of wild type p53 protein in three human epithelial cell lines of different origins (SV40 transformed, human papilloma virus transformed and a carcinoma cell line) was studied following treatment with γ radiation. Cell cycle delay was also examined in order to assess the relationship between wt p53 induction and cell cycle changes in human epithelial cells following radiation induced DNA damage.

PROCEDURE

5.3

Levels of wild type p53 protein in different human epithelial cell lines was analysed using ELISA tests with antibody DO-1 as the primary specific antibody. The presence of mutant p53 protein was determined by immunoprecipitation by antibody PAb240 which is specific for mutant forms of p53. Following irradiation of exponentially growing cells changes in the level of wt p53 were examined also using ELISA tests.

Immunostaining of irradiated and unirradiated samples using HRP and FITC detection techniques were also performed. This was coupled with light and laser confocal microscopy respectively to indicate the location of wt p53 protein within the cell.

Cell cycle changes following irradiation of cultures were analysed using flow cytometry after staining with appropriate DNA dyes/uptake of base analogues.

RESULTS

5.4 Expression of wild type and mutant p53 protein in unirradiated cells

The SV40 immortalized cell line SV-HUC-1, BC16 & NT11 showed a characteristic high level of wt p53 due to its interaction and stabilization with the SV40 T-antigen (Lane and Crawford 1979). The human papilloma virus immortalized cell line HPV and the carcinoma cell line T24 both expressed low but detectable levels of wt p53 (Figure 5.1). The staining pattern of wt p53 within unirradiated cells is also shown in Figures 5.3a, 5.3b, 5.8a and 5.8b showing strong nuclear staining of the nucleus in SV-HUC-1 cells and HPV cells exhibiting no nuclear but slight cytoplasmic staining.

Immunoprecipitation of p53 proteins (Figure 5.2) showed that all SV-HUC lines gave a positive result for p53 in both the wild type and mutant conformations (antibodies DO-1 and PAb 240 respectively). However since DO-1 recognises both mutant and wild type p53 protein, any cell line expressing mutant p53 protein as determined by positive PAb 240 immunoprecipitation will automatically be positive for DO-1 as well. The Human Osteosarcoma cell line HOS also gave a positive result for mutant p53. This cell line was used as a positive control, having a known p53 point mutation of an Arginine to Proline change at codon 152 (Romano *et al.*, 1989).

Cell lines T24 and HPV were negative for mutant p53 protein. HTori- cells (Human Thyroid Epithelial Cells immortalized by SV40 virus; Lemoine *et al.*, 1989) gave a positive result with respect to the wild type p53 due to the stabilization of p53 by the T-antigen. However, these cells were negative for mutant p53. HTori(T) denotes a tumour cell line generated after irradiation of the parent cell line. Lanes probed with Anti-Rb antibody IF8 stained negative, indicating no non-specific binding by CM-1 serum (anti-p53 antibodies). The

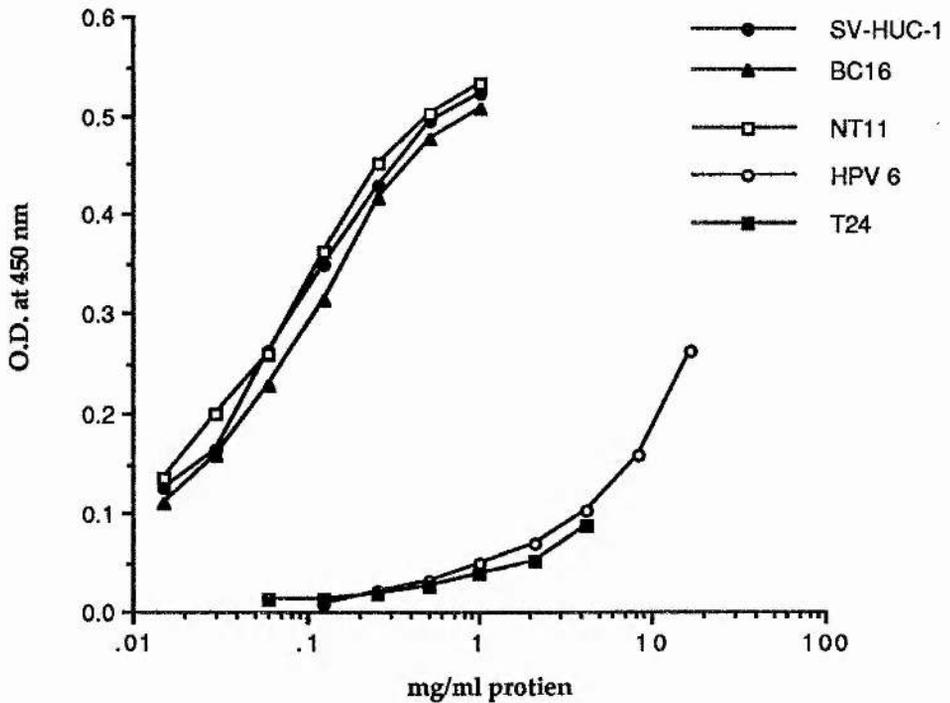


FIG 5.1 Two site immunoassay with epithelial cell extracts using DO-1 as coating antibody to detect wild type p53 protein. Plates were probed with rabbit anti-p53 serum CM1. Optical density at 450nm is proportional to the amount of bound antibody and hence p53. Data is representative of three repeat experiments.

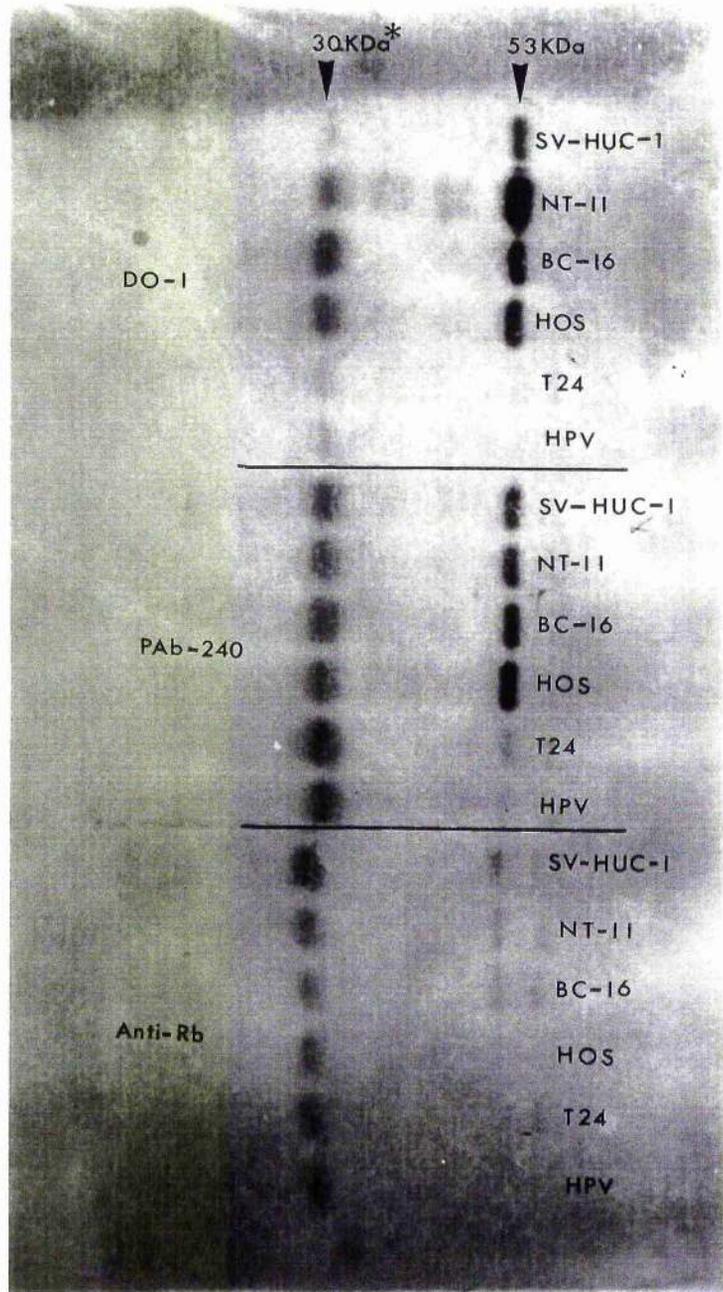


FIG. 5.2a.

Figures 5.2a & 5.2b. Immunostaining of a Western Blot containing p53 proteins immunoprecipitated by antibodies DO-1 (wild type and mutant conformation), PAb 240 (mutant only) or Anti-Rb (irrelevant antibody). Blots were probed with anti-p53 serum (CM1) and visualized using HPR conjugated swine anti-rabbit immunoglobulins. Cell lines are listed on the right hand side. Molecular weights of the proteins are given at the top of the blot. * Protein G.

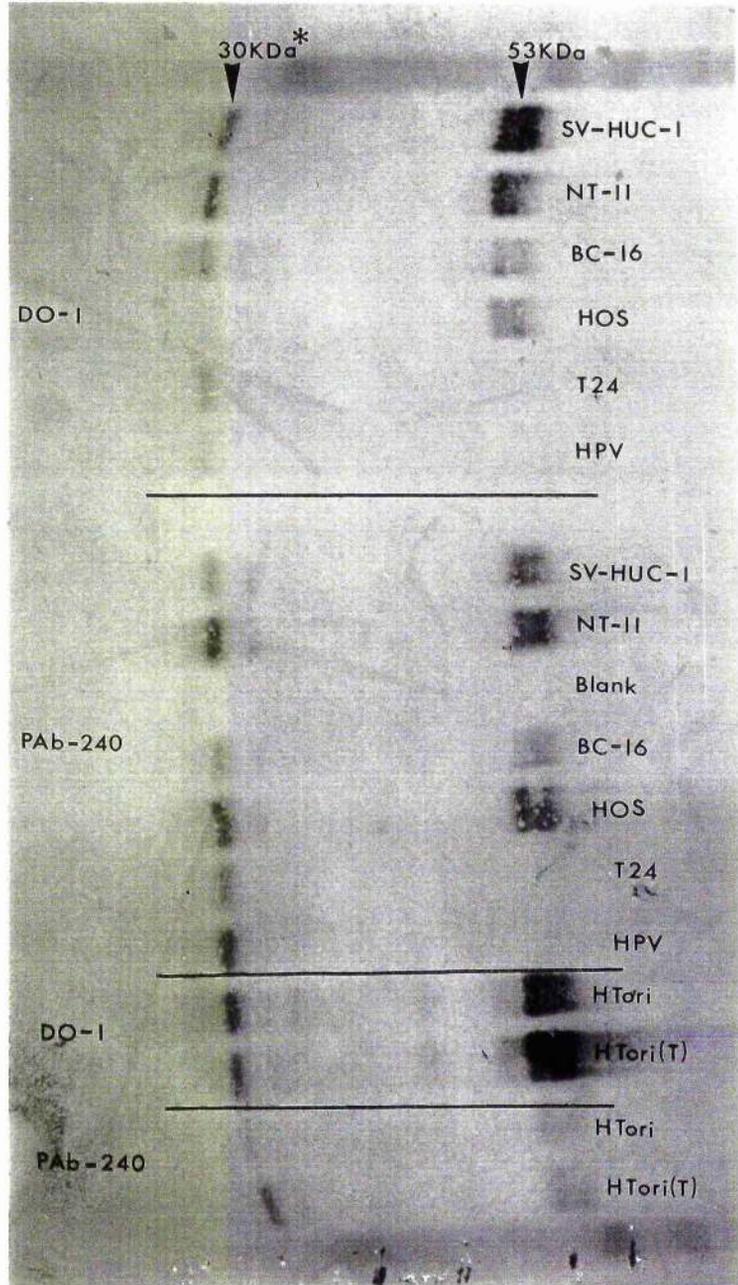


FIG. 5.2b.

FIG 5.3a



FIG 5.3b

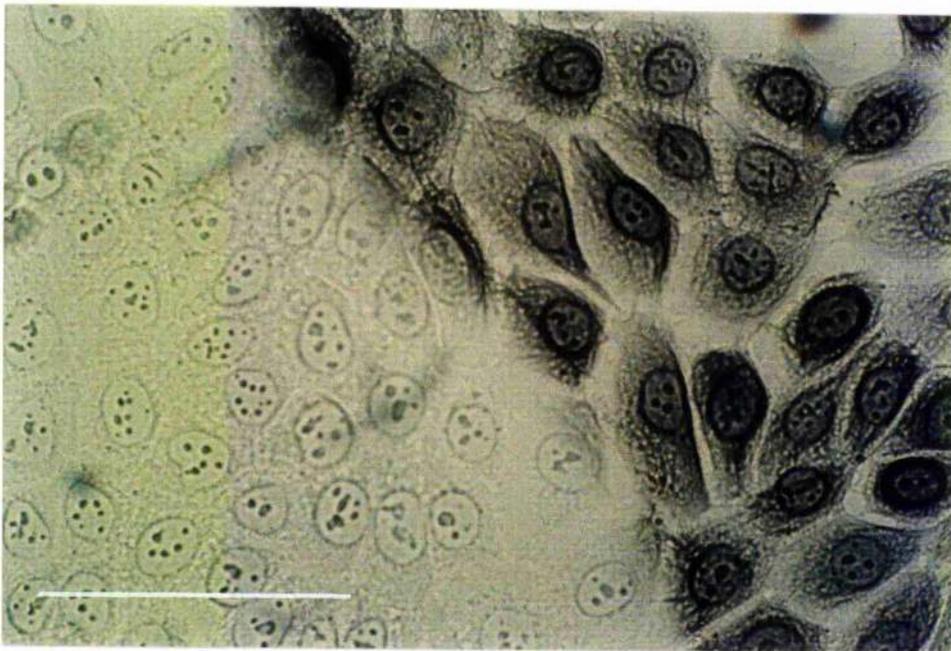


FIG 5.3 Immunoperoxidase staining with DO-1 as the primary antibody and a rabbit anti-mouse HRP conjugate as the secondary antibody to detect wt p53 protein. **a** SV-HUC-1 cells showing strong nuclear staining. **b** HPV cells exhibiting no nuclear but slight cytoplasmic staining. Cells to the right of the frame represent a negative control (i.e. primary antibody omitted). Scale bars represent 100 μ m.

band at 30,000 Daltons is due to protein G which was used to couple the antibody-p53 protein complex.

5.5 Expression of wild type p53 protein in irradiated cells.

Only one cell line HPV showed a increase in wt p53 protein levels following a single 2 Gy γ radiation dose, rising rapidly within six hours of irradiation and remaining elevated for 48 hours (greatest sampling time used), exhibiting a maximal two fold increase in optical density (Figure 5.5). Cell lines SV-HUC-1 and T24 did not show any increase in p53 protein levels at any of the post irradiated sampling times (2 to 48 hours; Figures 5.4 and 5.5). Similarly only in cell line HPV did increasing doses of γ radiation produce an increase in wt p53 expression up to 4 Gy and falling slightly at 10 Gy. Increased expression of wt p53 above control/unirradiated samples was observed even at the lowest doses used (1 Gy; Figure 5.7). Again cell lines SV-HUC-1 and T24 did not show any similar corresponding increase in wt p53 protein levels following increased radiation doses. This lack of any inducible p53 protein expression by radiation treatment in SV40 immortalized human epithelial cells is consistent with previous reports of a similar lack of response in SV40 transformed mouse fibroblasts following treatment with the U.V. mimetic 4-nitroquinoline-1-oxide (Maltzman and Czyzyk, 1984). Due to the high levels of T-antigen bound wild type p53 protein present in SV-HUC-1 cells no significant increase in p53 was observed following γ irradiation.

Following radiation treatment p53 protein could also be detected in the nuclei of HPV cells by immunochemical staining with antibody DO-1 when compared to unirradiated cells (Figures 5.8c and 5.8b respectively). Not all irradiated HPV cells showed such a pattern of p53 protein induction within the nucleus, this may reflect cell cycle differences or differing extents of DNA damage within the HPV

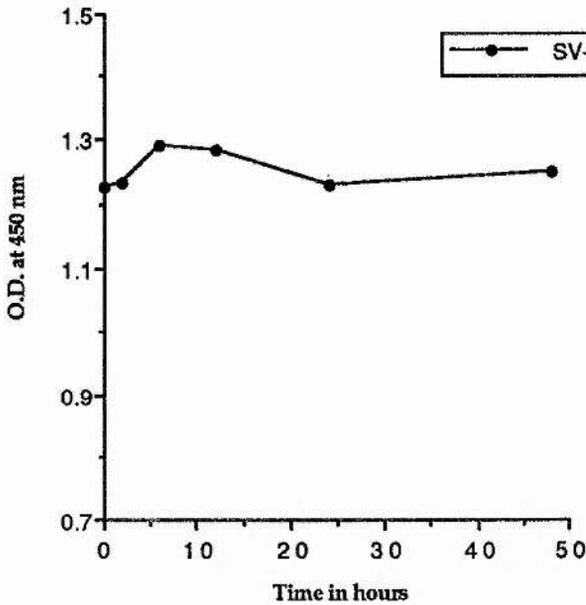


FIG. 5.4 Two site immunoassay with SV-HUC-1 cell extracts using DO-1 as coating antibody. All cultures received a 2 Gy γ radiation treatment prior to the generation of cell extracts at the sampling times indicated. Plates probed with CM1 serum. Data is representative of two repeat experiments.

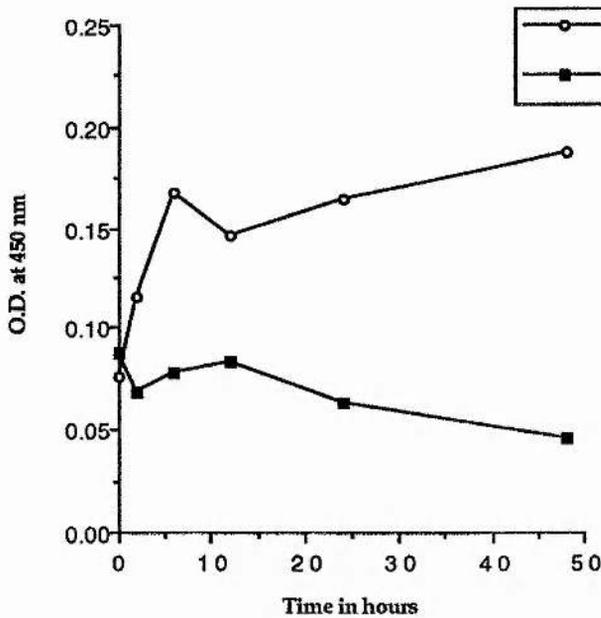


FIG 5.5 Two site immunoassay with HPV 6 and T24 cell extracts using DO-1 as coating antibody. All cultures received a 2 Gy γ radiation treatment prior to the generation of cell extracts at the sampling times indicated. Plates probed with CM1 serum. Data is representative of two repeat experiments.

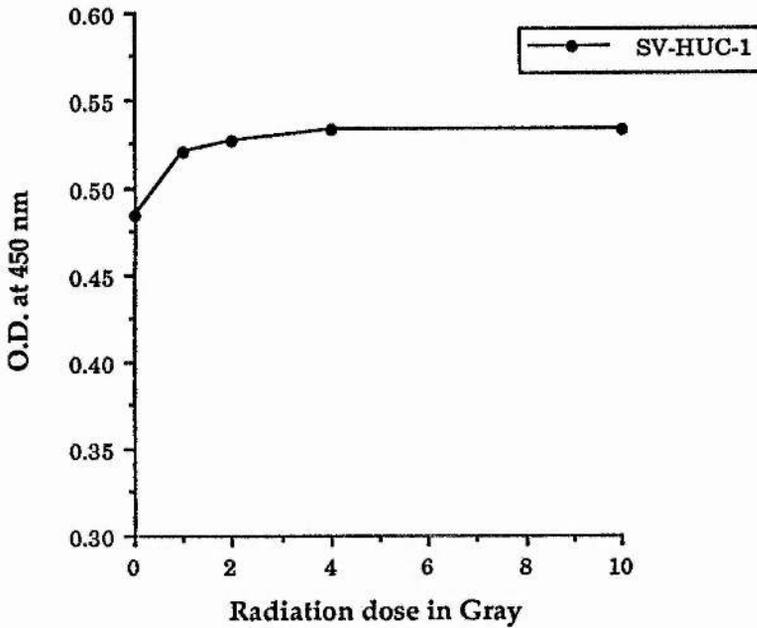


FIG 5.6 Two site immunoassay with SV-HUC -1 cell extracts using DO-1 as coating antibody. All cultures were sampled six hours after radiation treatment. Plates were probed with CM1 serum. Data is representative of three repeat experiments.

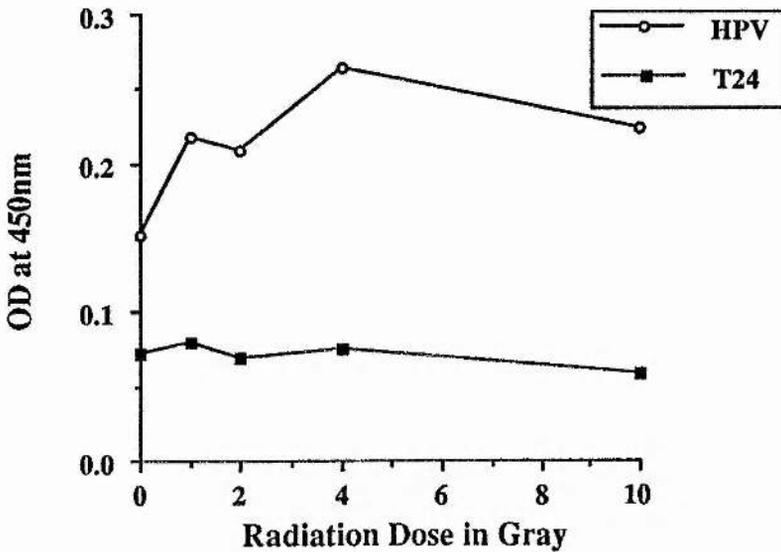


FIG 5.7 Two site immunoassay with HPV and T24 cell extracts using DO-1 as coating antibody. All cultures were sampled six hours after radiation treatment. Plates were probe with CM-1 serum. Data is representative of three repeat experiments

FIG 5.8 a

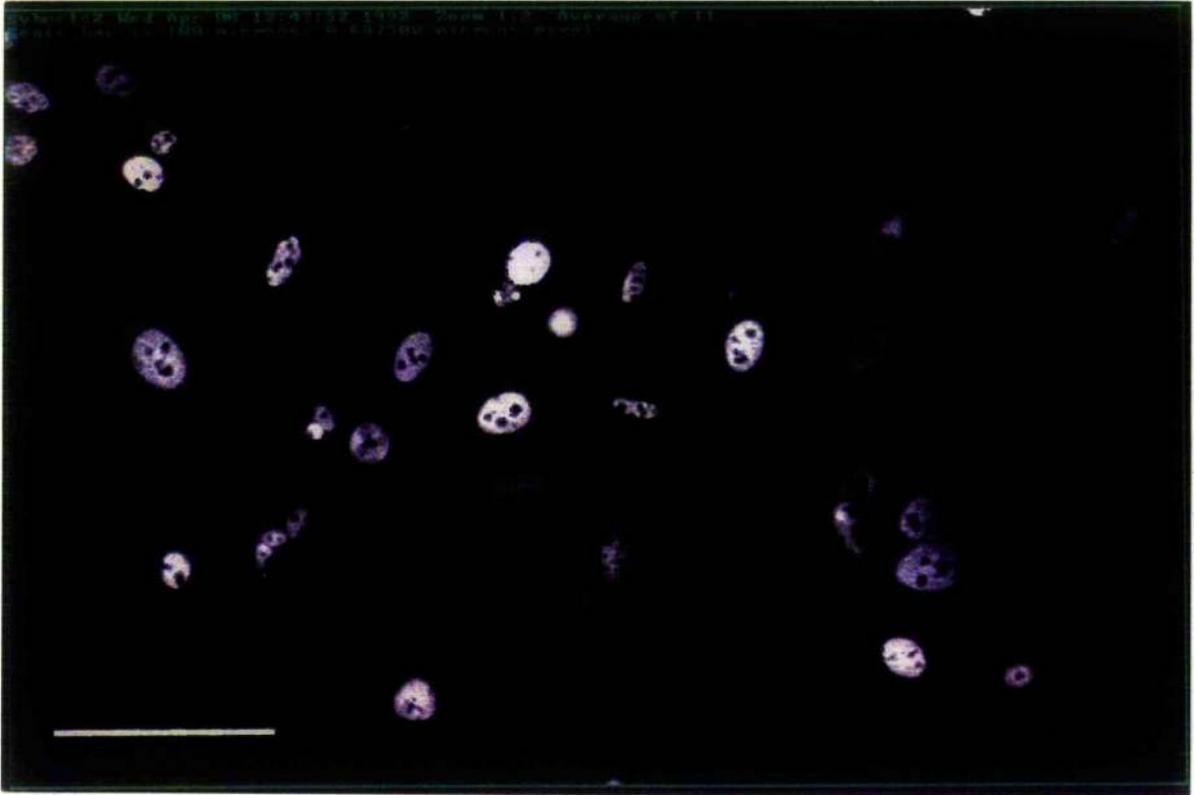


FIG 5.8 Immunochemical staining of wild type p53 protein with DO-1 antibodies in **a.** control (no irradiation) SV-HUC-1 cells: strong nuclear staining. Scale bar represents 100 microns **b.** control HPV cells: no nuclear but limited cytoplasmic staining. Scale bar 25 microns **c.** HPV cells 6 hours following a 2 Gy γ radiation treatment: showing localised nuclear staining. Scale bar 25 microns. Visualization was with FITC conjugated sheep anti-mouse immunoglobulins and viewed by a confocal laser scanning microscope, laser wavelength 488nm.

FIG 5.8 b

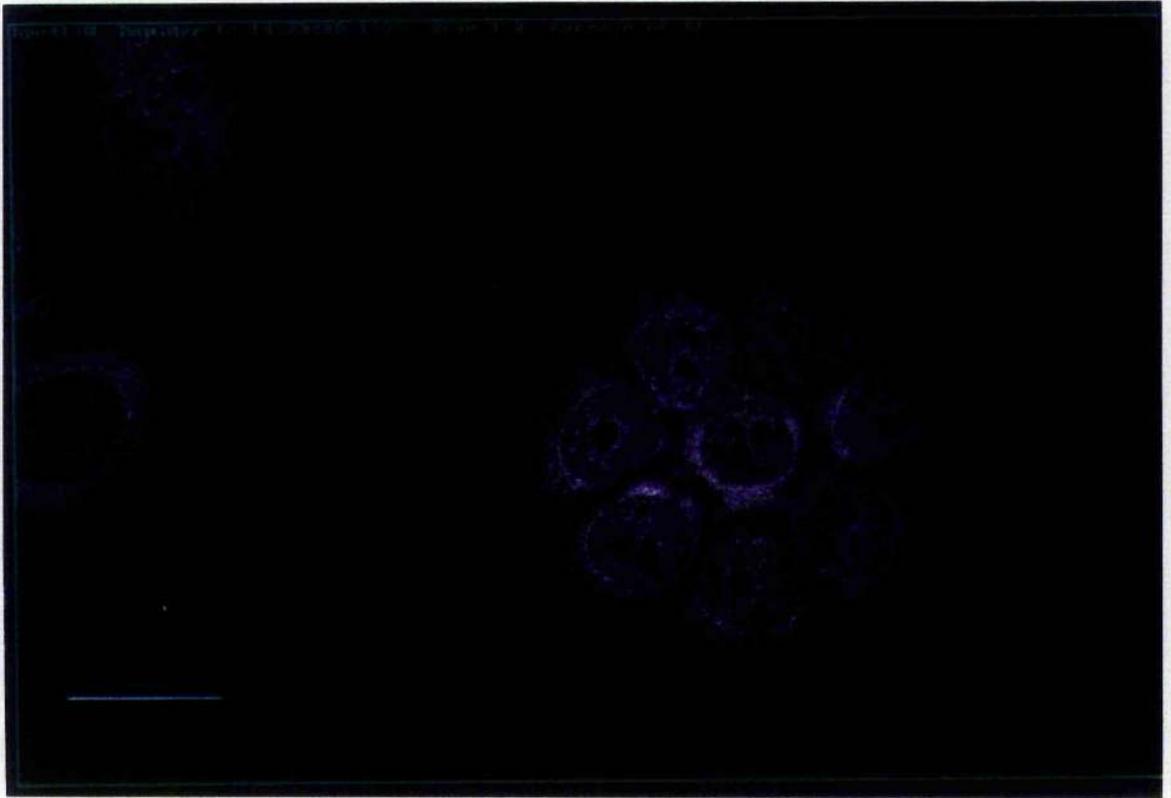
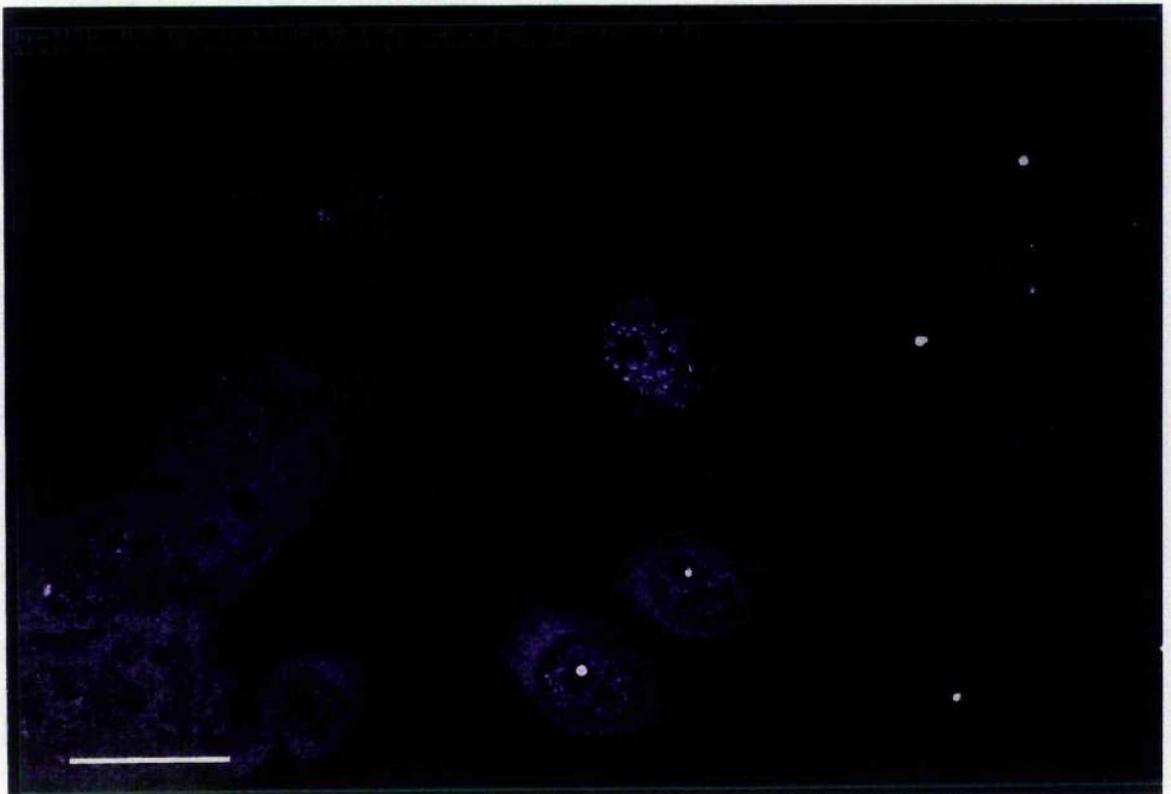


FIG 5.8 c



cell population following radiation treatment. No HPV cells exhibited the same intensity of staining as SV-HUC-1 cell nuclei (Figure 5.8a).

5.6. Cell cycle changes following irradiation.

Both single channel staining with propidium iodide and double channel staining using both propidium iodide and FITC conjugated antibodies in conjunction with BrdU uptake, were utilized to study cell cycle changes (Figures 5.9). However only single channel staining provided data which could be meaningfully interpreted. This was for two reasons ; a. incomplete separation of the different cell populations b. inability of the FACScan software to fit an appropriate model to the data generated. Therefore only the results of single channel staining will be shown.

Cell cycle changes are shown in Figures 5.10 and 5.11 for all cell lines used. However, the results shown in Figure 5.11 represent the computer's interpretation of data assimilated using the FACScan. From Figure 5.10 it can be seen that there was a considerable disparity between actual data (solid line) and model/theoretical data (dotted line) for certain radiation treatments. Therefore the data represented in Figure 5.11 should only be treated as approximate values.

None of the cell lines studied showed a significant accumulation of cells in the G₁ compartment compared to control samples following different radiation doses (Figures 5.11a, b, c). Only the HPV cells exhibited either a G₁ or G₂ delay following radiation treatment with an increasing proportion of cells accumulating in the G₂/M compartment following increasing radiation doses up to 4 Gy and falling slightly at 10Gy. The proportion of cells in G₁ fell in all cell lines following radiation treatment. Hence, of the three human epithelial lines studied only cell line HPV appeared to have a functioning cell cycle delay mechanism following radiation damage which arrests cells predominantly at G₂ and not G₁.

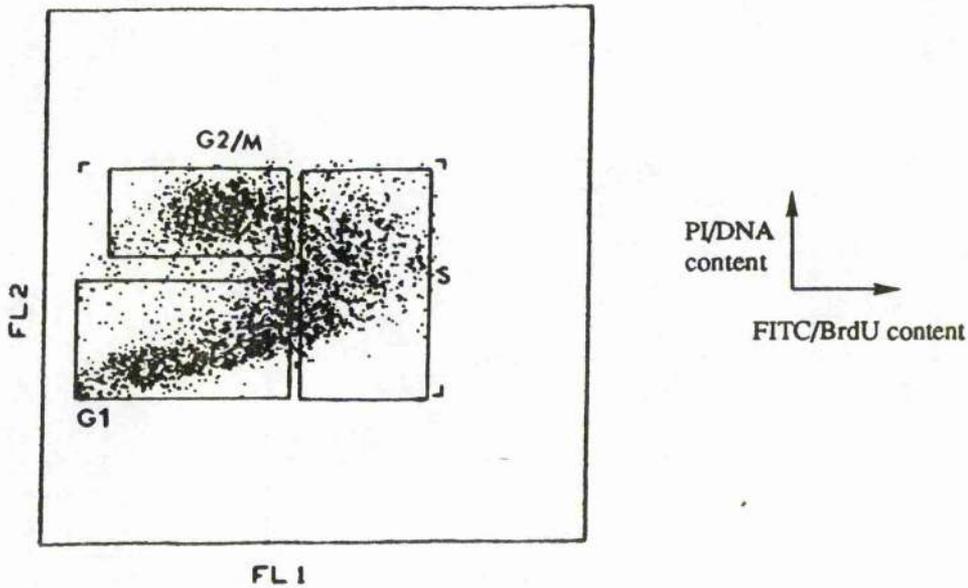


FIG. 5.9a. Dot plot of a cell cycle profile from unirradiated SV-HUC-1 cells using dual channel staining and analysed on a Fluorescence Activated Cell Scanner (FACScan). Note the incomplete separation of the G₁ and S phase components. PI-Propidium Iodide. FITC/BrdU- Fluorescein Isothiocyanate /5-Bromo-2-deoxyuridine.

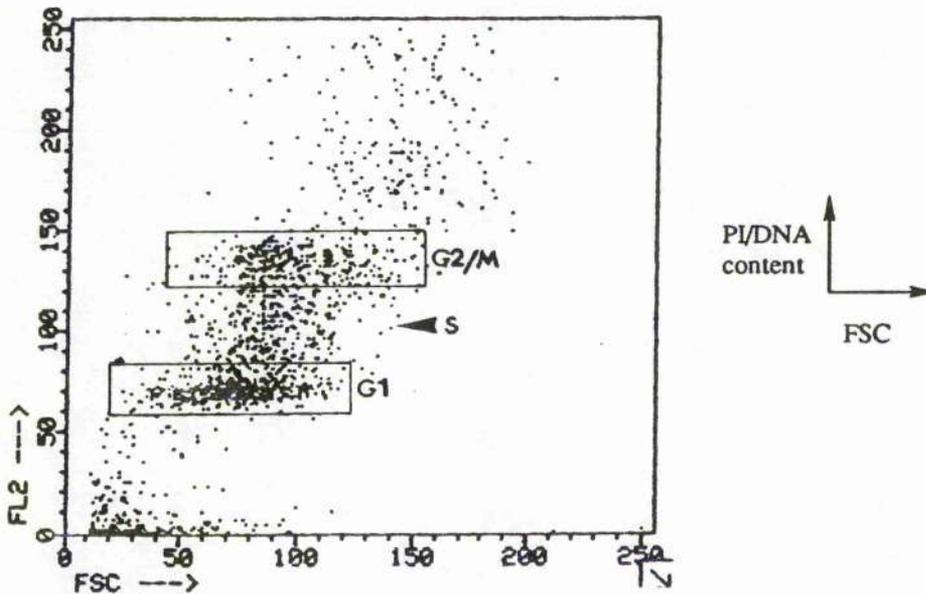


FIG. 5.9b. Dot plot of a cell cycle profile from unirradiated SV-HUC-1 cells using single channel staining and analysed on a FACScan. Data of this type could be successfully analysed using the software available on the FACScan. FSC- Forward Scatter.

0 Gray

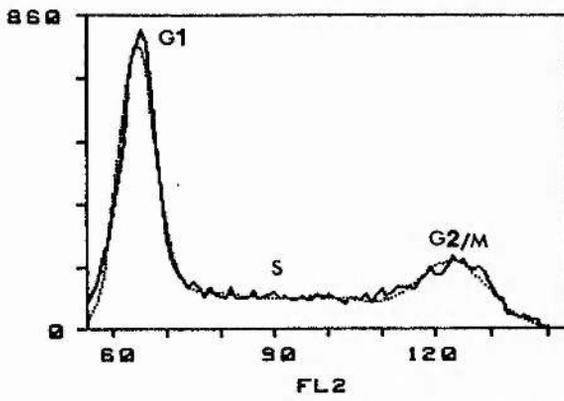
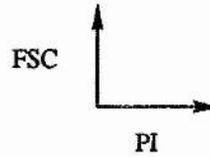
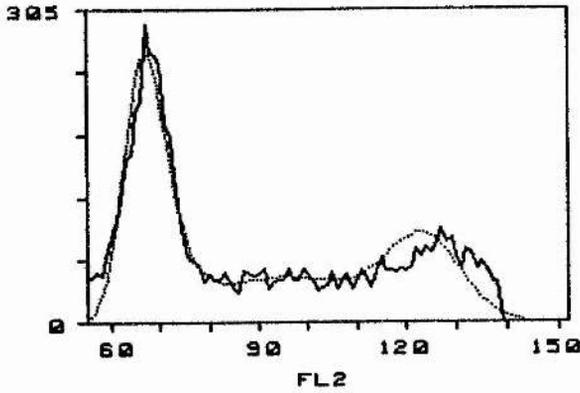


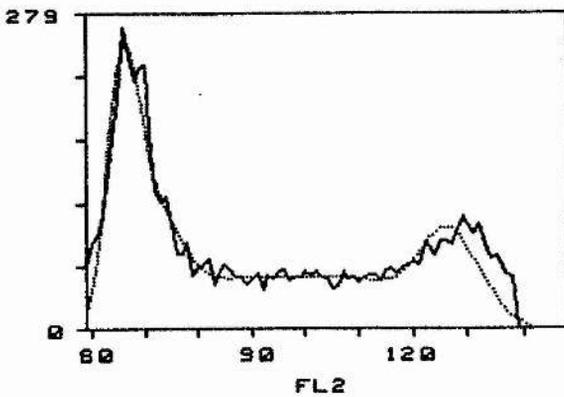
FIG. 5.10a. Cell cycle changes in SV-HUC-1 cells 10 hours following various doses of γ radiation. The solid line represents actual data collected and the dotted line the computers closest model data set.



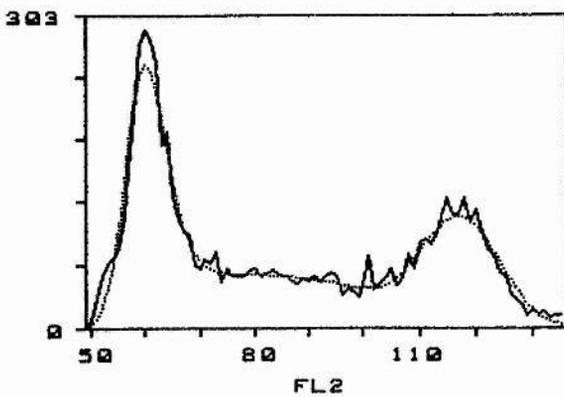
1 Gray



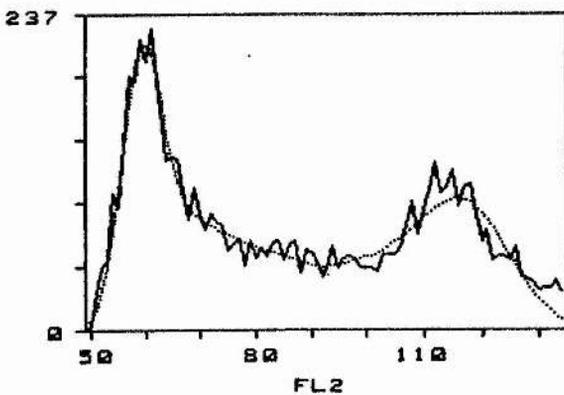
2 Gray

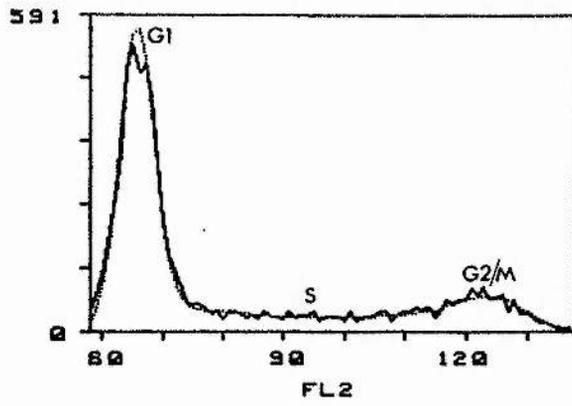


4 Gray



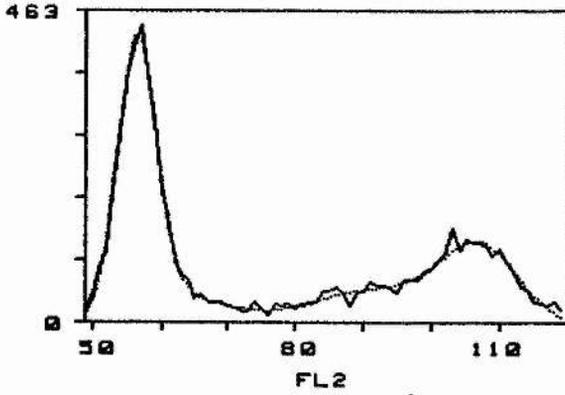
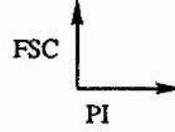
10 Gray



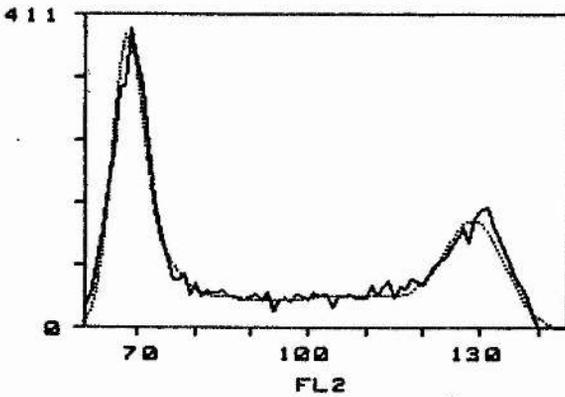


0 Gray

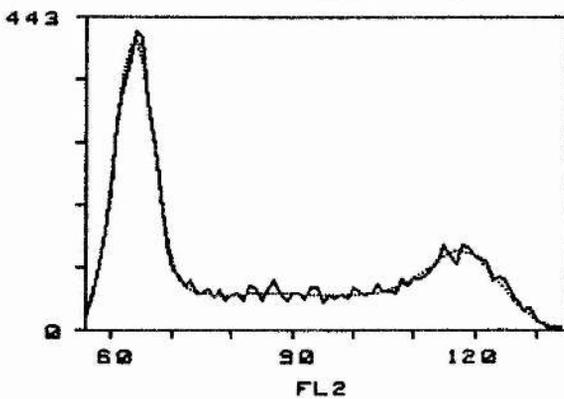
FIG. 5.10b. Cell cycle changes in T24 cells 10 hours following various doses of γ radiation.



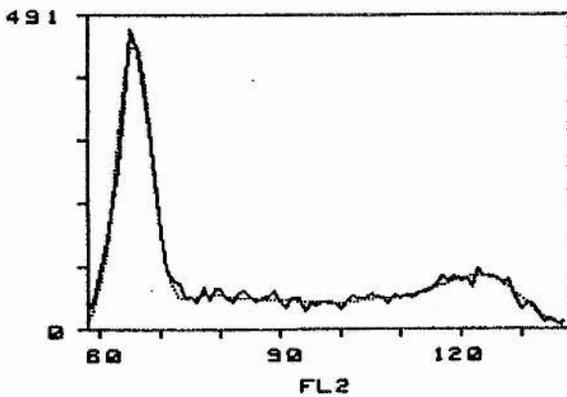
1 Gray



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10 Gray

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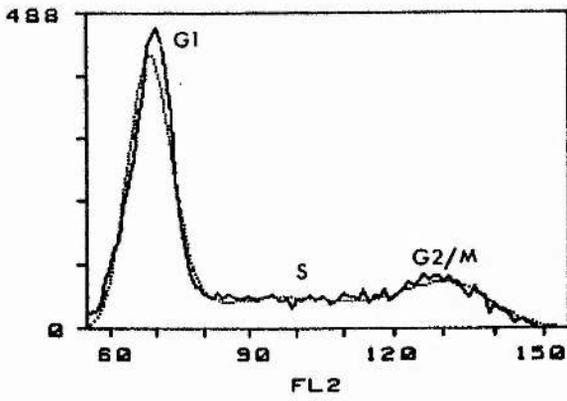
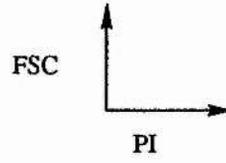
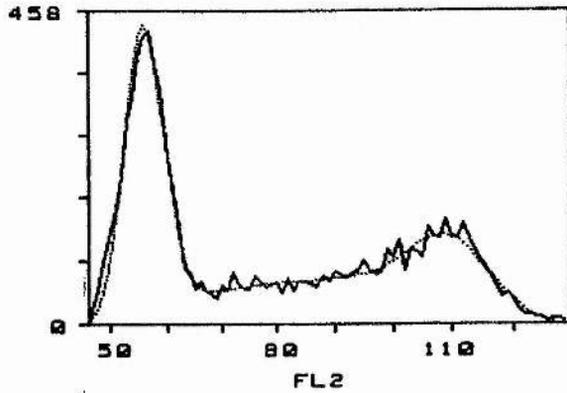


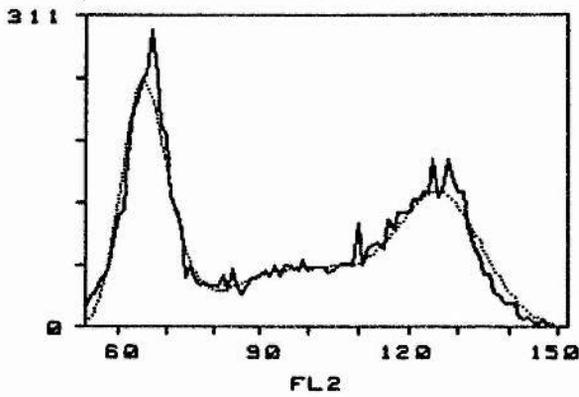
FIG. 5.10c. Cell cycle changes in HPV cells 10 hours following various doses of γ radiation.



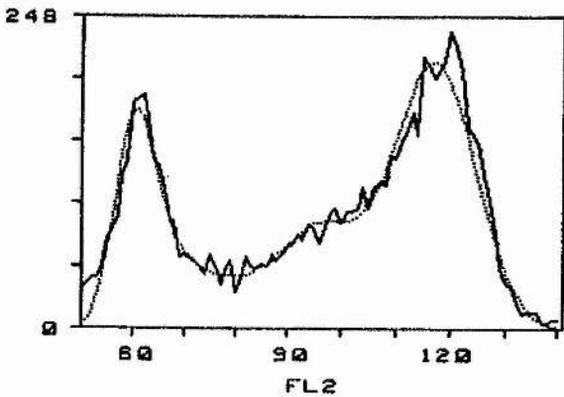
1 Gray



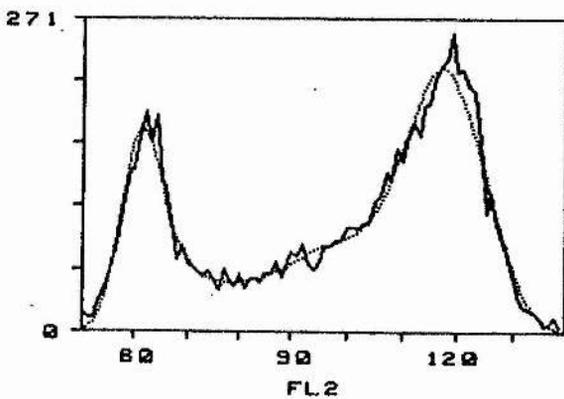
2 Gray



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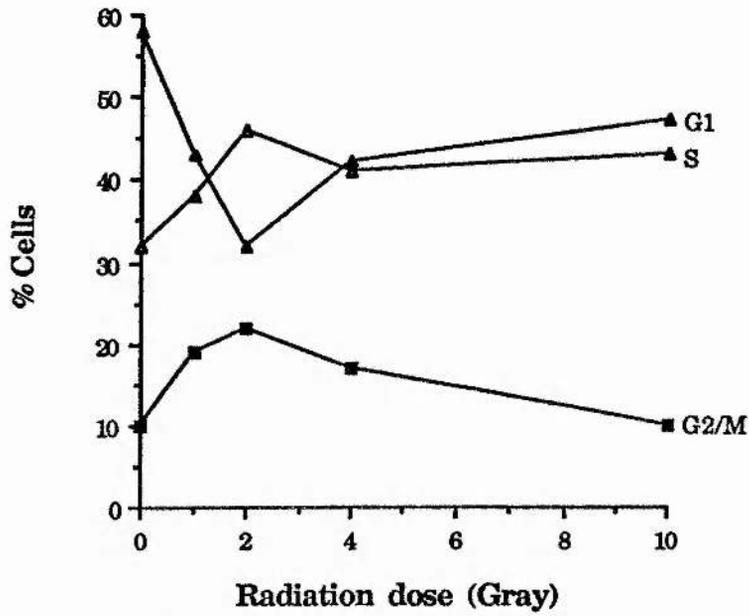


FIG 5.11a. Cell cycle distribution data for SV-HUC-1 cells determined using flow cytometric analysis. Cultures were sampled 10 hours following radiation treatment.

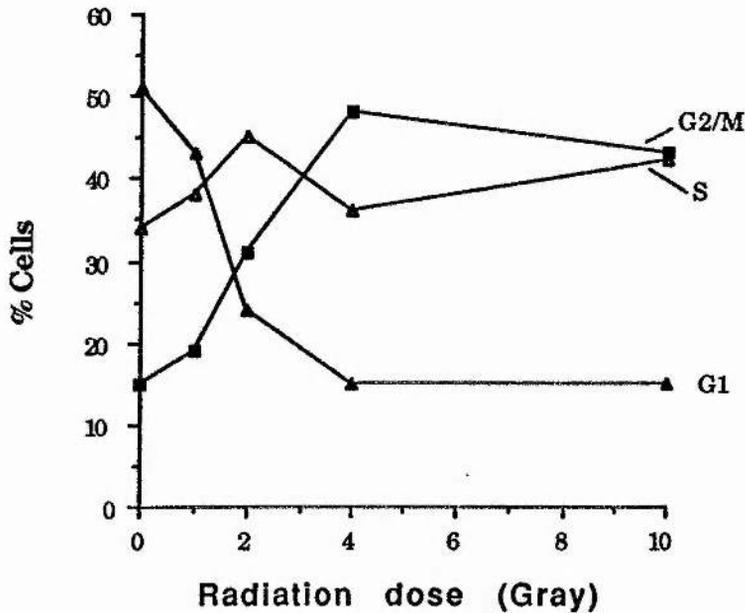


FIG 5.11b. Cell cycle distribution data for HPV cells. Cultures were sampled 10 hours following radiation treatment.

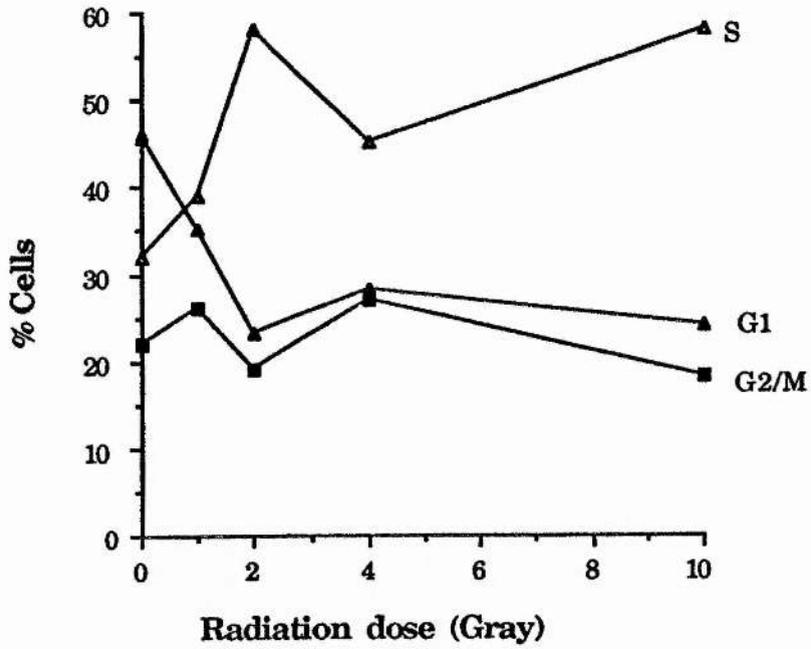


FIG. 5.11c. Cell cycle distribution data for T24 cells. Cultures were sampled 10 hours following radiation treatment.

DISCUSSION

5.7 The effects of viral oncoproteins on cellular immortalisation and p53.

Immortalisation of human epithelial cells is usually achieved by infection of tissue explants with DNA tumour viruses, or by transfection with vectors containing sequences derived from tumour viruses (Christian *et al.*, 1988, DiPaolo *et al.*, 1989, Thraves *et al.*, 1990). Both these techniques leave the now immortal cell with oncoproteins that bind or interact with the wild type p53 protein (Lane and Crawford 1979, Werness *et al.*, 1990, Scheffner *et al.*, 1990, Bargontti *et al.*, 1991, Yew and Berk 1992).

Cell lines HPV and T24 both contain levels of wild type p53 protein similar to that expressed in other normal (nontransformed, noncancerous) human tissues as measured by similar ELISA tests using DO-1 antibody (Barnes *et al.*, 1992). Hence both HPV and T24 cells must contain at least one functioning copy of the wild type p53 gene since they are not without any p53 of the wild type conformation. Also HPV cells do not appear to have their p53 protein degraded by the E6 oncoprotein of the human papilloma virus, which has been reported to interact with and break down wild type p53 *in vivo* (Mietz *et al.*, 1992). However, it is probable that the transforming/immortalising ability of human papilloma virus E6 protein, SV40 T-antigen and adenovirus E1B protein is linked to their ability to modulate p53-mediated transcriptional activation (Lechner *et al.*, 1992, Mietz *et al.*, 1992, Yew and Berk 1992 and Bartek *et al.*, 1993).

SV-HUC lines immortalised with SV40 gave a positive result with antibody DO-1 which detects both mutant and wild type p53 due to the stabilisation of p53 by the T-antigen. However, a positive result was also obtained with antibody PAb 240 (Figure 5.2) which detects mutant p53 and

does not recognise p53 when complexed to SV40 T-antigen since the epitope needed for binding of this antibody to the protein is then concealed (amino acids 156-214; Gannon *et al.*, 1990). This phenomenon is not solely as a result of immortalisation by the SV40 T-antigen, HTori- cells also immortalised using a plasmid construct of SV40 containing the T-antigen did immunoprecipitate with DO-1 but not PAb 240 antibodies i.e. they contain stabilized wt but not mutant p53 protein. Therefore two possible explanations can be proposed to account for the results obtained. a. Two distinct populations of p53 protein; One wild type in nature which binds to the T-antigen and is stabilized, the other consisting of mutant protein which due to the presence of the mutation is also stabilized. b. That only mutant p53 is present but this is unlikely since the cells are known to be non-tumourigenic (see Table 4.2) and very few species of mutant p53 bind to SV40 T-antigen (Bartek *et al.*, 1993).

All SV-HUC lines are in fact sub-clones of different infection/transfection experiments using the same individual's uretral tissue. If this individual was in fact heterozygous for p53, or alternatively if during subsequent passaging following infection/transfection a mutation in one p53 allele resulted, then this too could account for the results observed. The situation is also complicated by the observation that SV-HUC lines are pseudo-diploid. That is they contain a bi-modal karyotype distribution, most cells being near diploid and the rest being near tetraploid (Christian *et al.*, 1988, also see section A.4). A single p53 mutation in one of four possible chromosome 17's would make interpretation of the data difficult. Fortunately the effect of mutant p53 or wt p53 bound to SV40 T-antigen is reported to be the same in terms of cell cycle arrest/delay following DNA damage i.e. it is abrogated (Kastan *et al.*, 1991, Oren 1992).

5.8. Extended sampling time to monitor cell cycle changes.

The sampling time used to determine cell cycle changes following radiation treatment was 10 hours, 4 hours after the maximal induction of p53 in HPV cells following a single 2 Gy γ radiation treatment (Figure 5.5). Kuerbitz *et al.* (1991) have proposed that p53 is a specific transcription factor altering the progression of damaged cells through the cell cycle. Therefore the accumulation of cells in any particular compartment of the cell cycle will not be observed instantaneously, but be dependant on the initial rate of cell cycling versus the extent of inhibition of cell cycling in the now damaged cells, hence the later sampling times.

5.9. Radiation induced p53 protein and cell cycle delay.

The lack of any radiation induced p53 protein in SV-HUC-1 cells was not unexpected due to the presence of the SV40 T-antigen and/or the mutant protein leading to the accumulation of high levels of non-functional p53. In addition there was an apparent corresponding absence of any radiation induced cell cycle delay, (neither G₁ nor G₂/M arrest) as measured by flow cytometry in SV-HUC-1 cells. Although this agrees with the previously reported observations of Maltzman and Czyzyk (1984), cell lines containing the T-antigen due to immortalisation by the SV40 virus are often more radioresistant than the corresponding non-immortalised cells from which they were derived (Arlett *et al.*, 1988). In view of the proposed function of wild type or non-oncoprotein bound p53, i.e. as a specific transcriptional protein regulating down stream events that controls regulation of the cell cycle in response to DNA damage, it is difficult to reason why such cell lines would have an increased radioresistance conferred to them.

The lack of any inducible p53 protein or cell cycle delay in T24 cells containing no viral sequences and normal levels of wt p53, may reflect differences between normal and cancer cell types even when mutant p53 protein is not involved. Any protein which binds to wt p53 so abolishing its normal function could achieve this goal. Such a protein has recently been found in the form of the MDM2 protein reported in human sarcomas by Oliner *et al.* (1992). The examination of a greater number of such cell lines will provide more conclusive data.

The only cell line in this study which did show both a radiation induced p53 protein increase and a cell cycle delay was HPV. The increased level of wt p53 (approximately two fold) in HPV cells following irradiation was not as great as the 3-8 fold increase reported to occur at G₁ arrest by Maltzman and Czyzyk (1984) and Kastan *et al.* (1991). An increase in the G₂/M population and not G₁ was observed with HPV cells following irradiation.

5.10. Possible controlling mechanisms of p53 and cell cycle delay.

It is possible that this G₂/M delay is not directly caused by elevated p53 levels but as a consequence of a control mechanism affecting a number of other factors which act to delay the cell cycle not only at G₁ but also at G₂ following radiation induced DNA damage. Wt p53 protein may be expressed at an increased level, due to increased stabilization via phosphorylation events, interaction with other proteins or oligomerization (Lane and Crawford 1979, Finlay *et al.*, 1998, Hupp *et al.*, 1992). However, this protein must also be in an active form to achieve its regulatory function. A possible candidate for such a control mechanism are the changes in phosphorylation of p34^{cdc2} kinase which alters its kinase activity. γ irradiation of Chinese hamster ovary cells has been shown to cause a rapid inhibition of p34^{cdc2} kinase

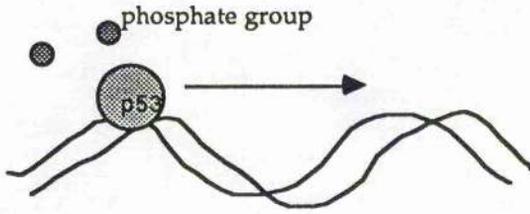
activity and subsequent G₂ arrest (Lock and Ross, 1990). The wild type p53 protein is also affected by p34^{cdc2} kinase activity, becoming more phosphorylated in S phase (Bischoff *et al.*, 1992). Hence a reduction in p34^{cdc2} kinase activity in response to radiation damage would lead to a less phosphorylated p53 protein molecule which in turn would inhibit progression of the cell into or through S-phase. Alternatively if such a pathway was no longer functioning then the same kinase also appears to have the ability to regulate cell cycling via a control mechanism acting at G₂. (See Figures 5.13 and 5.14 for an overview of possible control mechanisms acting at G₁ and G₂ respectively). Finally it should be noted that this increase in p53 protein is reported to occur through a post transcriptional mechanism since mRNA levels do not change significantly following irradiation (Kastan *et al.*, 1991). Also that the mechanism of control of this protein is probably not a simple one since it is acted upon by other kinases/ phosphatases such as protein phosphatase 2A (PP 2A) which dephosphorylates p53 (Scheidtman *et al.*, 1991).

5.11. To what extent can this model for p53 function be relied upon?

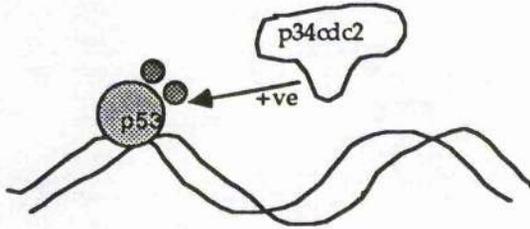
The overwhelming body of evidence now supports the theory of wt p53 as a tumour suppressor gene. The protein which is coded for by this gene, although not essential for development does appear to be needed to limit damage to the DNA and maintain the integrity of the genome. (Mice having no p53 genes develop normally but suffer from a high incidence of tumours in a variety of organs early in life, Donehower *et al.*, 1992).

However, caution should be exercised when interpreting the data concerning DNA damage induced cell cycle delay, both in this study and others which have used flow cytometry to analyse such changes. Although

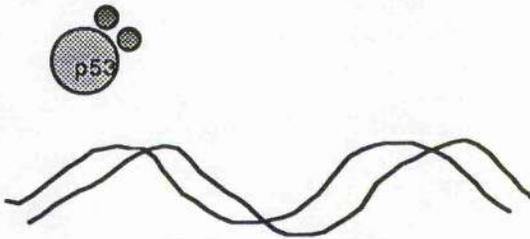
FIG. 5.13. G1



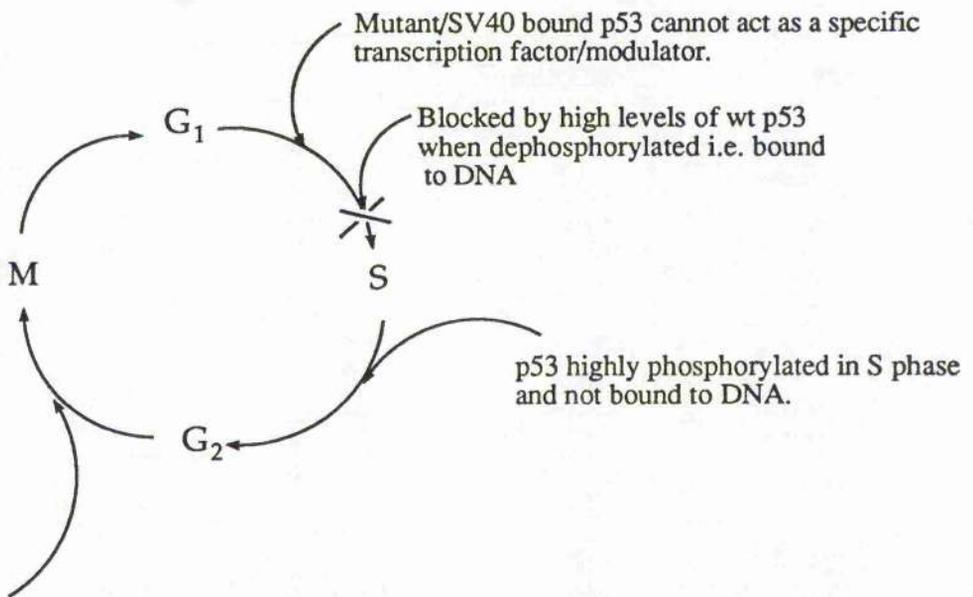
p53 bound to DNA at C-terminal domain resulting in transcriptional modification of downstream genes (possibly DNA polymerase α) so leading to G₁ arrest/inhibition of DNA synthesis.



Active/functional kinase activity of p34^{cdc2} and phosphorylation of p53.

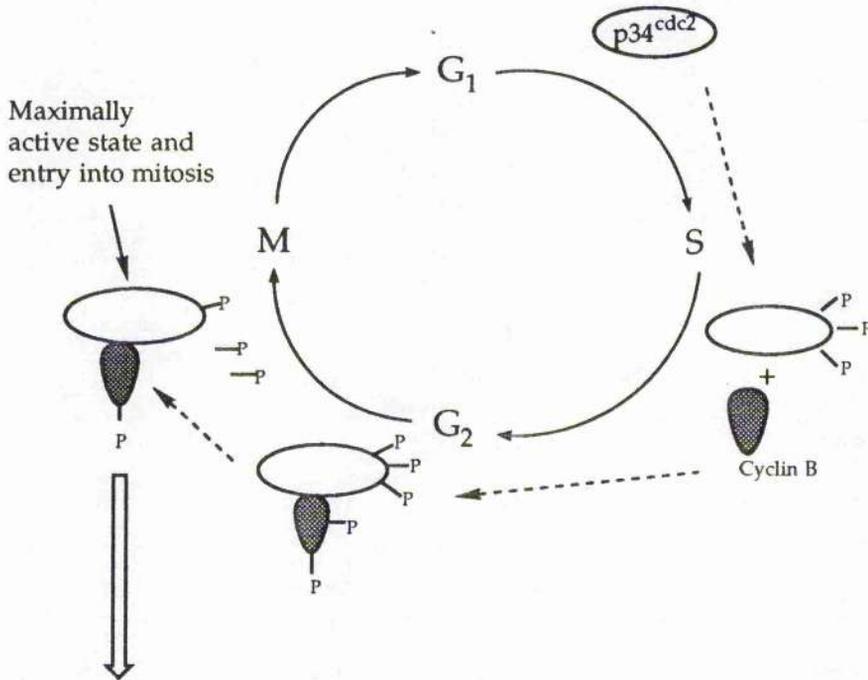
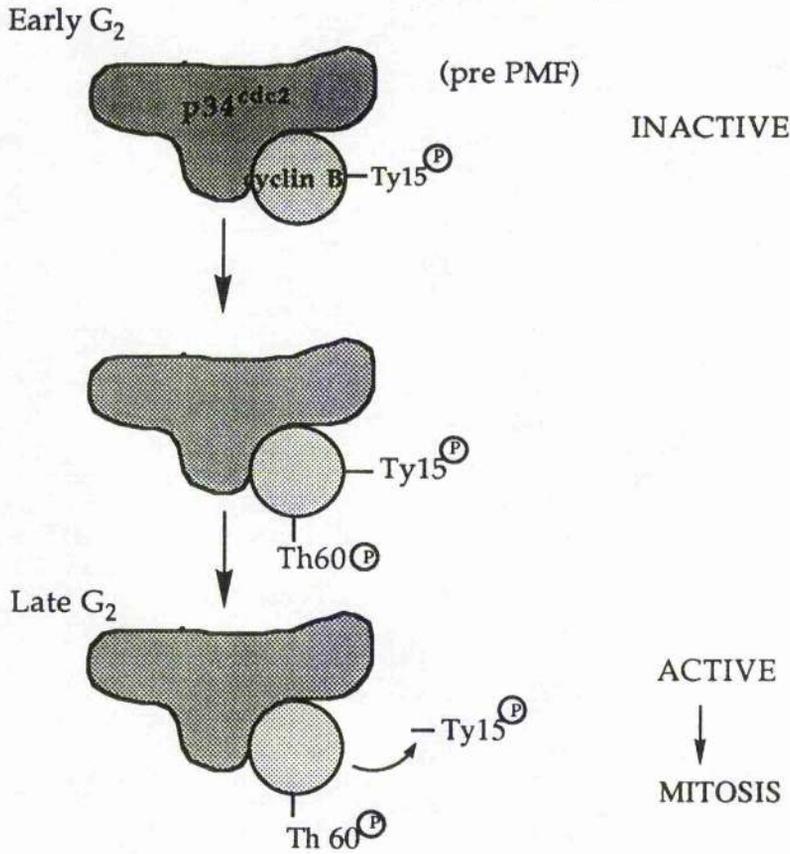


p53 no longer binds to specific DNA sequences, no modification of downstream genes and progression through the cell cycle.



p34cdc2 also found in association with cyclin B as maturation promotion factor (MPF). Acts to delay mitosis by interaction with Histone H1 (see Figure 5.13)

FIG. 5.14. G₂



p34cdc2 kinase activity reduced - cell cycle blocked in G₂.
 Perhaps due to phosphorylation status of Histone H1.
 H1 phosphorylated - dissociation with DNA- chromosome condensation and mitosis.

Adapted from Meek and Street 1992, Murray 1992.

flow cytometry does provide a rapid means of measuring the cell cycle distribution of a large cell population, the distinction between a G₁ arrest and inhibition of DNA synthesis may not always be clear. A G₁ arrest implies that cells do not enter into S phase but accumulate in G₁, probably in response to DNA damage. Due to the technique of dual channel staining using an S-phase specific marker such as BrdU in flow cytometry, the G₁, S and G₂/M compartments of the cell cycle can easily be separated (Kastan *et al.*, 1991a, Kastan *et al.*, 1991b, Kastan *et al.*, 1992, Kuberitz *et al.*, 1992). Painter and Young (1980) reported that the cell cycle can in fact be halted by inhibition of replicon initiation within S phase following irradiation of human cells. If the cell cycle is perturbed in such a way then flow cytometry will not be able to distinguish between this S phase arrest and a G₁ arrest. It will simply be manifested as a failure to take up the S phase specific marker with a corresponding lack of FITC fluorescence.

Nethertheless, it is clear from the high incidence of p53 mutations or cellular alterations that act to abrogate wt p53 function, which are found in human cancer, that this gene plays a vital role in the development of many human neoplasias. The most likely mechanism for which is via a G₁ or S phase arrest following DNA induced damage so allowing cellular repair processes time to rectify such damage before progression through the cell cycle.

CHAPTER 6

GENERAL CONCLUSIONS

6.1 Cytogenetic responses to radiation.

Urinary epithelium immortalised by SV40 was approximately two fold more radioresistant in terms of the clastogenicity of radiation treatment than malignant cells from the same tissue. However, it is difficult to make any overall comparisons between SV40 immortalised cells and their normal counterparts from the same tissue type with the data available. T24 cells are not essentially normal because they were derived from a malignant source and only this one other cell line was available for study. A greater number of cell lines would need to be examined to make more general conclusions concerning the effects of the SV40 T-antigen on radiosensitivity with any great degree of certainty.

All SV40 immortalised human uroepithelial cell lines (SV-HUC-1, NT11 and BC16) were derived from different transfection/infection experiments with the same individual's tissue and are not sub-clones from the same infection experiment. These cell lines exhibited quite marked differences in genomic stability as measured by spontaneous micronuclei frequencies, despite similar morphological appearances and cell cycling times. Therefore it is not solely the mere fact of the SV40 T-antigen within the a cell which leads to increased genomic instability but may also depend upon its site of integration, number of copies of the viral DNA sequence per host genome and secondary events following infection as an immortal clone is generated.

6.2 Radiation induced p53 protein.

Induction of the nuclear phosphoprotein p53 following irradiation of human epithelial cells could only be detected in one of the cell lines studied; HPV a human papilloma virus immortalised cell line (HPV) which exhibited an approximate two fold p53 induction above unirradiated cells. The study of cell cycle delay following irradiation where proposed DNA repair mechanisms gain an increased period for genomic repair revealed the following:-

No accumulation of any of the cell lines, SV40 immortalised, HPV and a malignant bladder carcinoma (T24), was observed in the G₁ compartment. Only cell line HPV exhibited any cell cycle delay which occurred at the G₂/M border following irradiation. The reason for this G₂/M delay may not in fact be due to p53 induction, since the p53 of HPV cells will be complexed with the E6 protein of human papilloma virus and hence probably already have usurped its wild type p53 function. However, another control mechanism which induces a number of proteins in response to genomic damage to allow prolonged cell cycling and extended DNA repair could have been activated. A possible candidate for this is the p34^{cdc2} complex (see section 5.10).

If mutant p53 is present or wild type p53 has had its function abrogated this then leads to greater genetic instability since p53 is acting as a "guardian of the genome" (Lane 1992b). Therefore cells having SV40 T-antigen which interferes with the normal function of p53 maybe expected to have altered genomic stabilities. This is reflected in the data presented in Figure 3.8 and discussed in section 6.1. Recently Lowe *et al.*, 1993, Clarke *et al.*, 1993 (and reviewed by Lane 1993) have shown that p53 is needed for radiation induced apoptosis. Thymocytes from null mice containing no p53 protein are 20 times more resistant to cell death as thymocytes containing two wild type alleles after irradiation. Cells which are heterozygous for p53 are intermediate in their response to radiation induced apoptosis. This provides

support for the observation that SV40 immortalised cells (with altered p53 function) were estimated to be approximately two fold more resistant to the lethal effects of gamma radiation than the bladder carcinoma cells T24 (section 3.8)

The induced p53 radiation response of T24 cells is at first a little puzzling since they do not have any mutant p53 nor viral sequences that may act to abrogate its function, but T24 cells did not show an increase in the level of p53 protein following irradiation. From chapter 3 T24 cells have been shown to have a relatively high radiosensitivity and exhibit a high yield of micronuclei following radiation and cytokinesis blockage with cytochalasin B. Also from chapter 5 it can be seen that T24 cells do not display any significant cell cycle delay in G₁ or G₂/M following irradiation, which again is unexpected given the p53 status of this cell line.

The above data could be accounted for if instead of the p53 status being changed, the proximal part(s) of the cell cycle delay were no longer functioning correctly. eg. If S of figure 6.1 was abrogated, and the sensory mechanism for detecting DNA damage which then leads to an increased stability of p53 protein was no longer functioning. This would result in no increase in the induced level of p53, no cell cycle delay and increased levels of DNA damage i.e. p53 function is altered because it is no longer able to be induced/stabilised at the correct time, such as in response to genotoxic agents.

Further evidence for this type of mechanism as opposed to a DNA repair defect being the fault and accounting for increased radiosensitivity, could be to check for unscheduled DNA synthesis (UDS - DNA synthesis outside of S-phase). If the sensory mechanism(s) were no longer functioning correctly the UDS would still occur, although to a more limited extent since there would be little cell cycle delay to permit increased DNA repair when compared to cells with a normal sensory mechanism and wild type p53

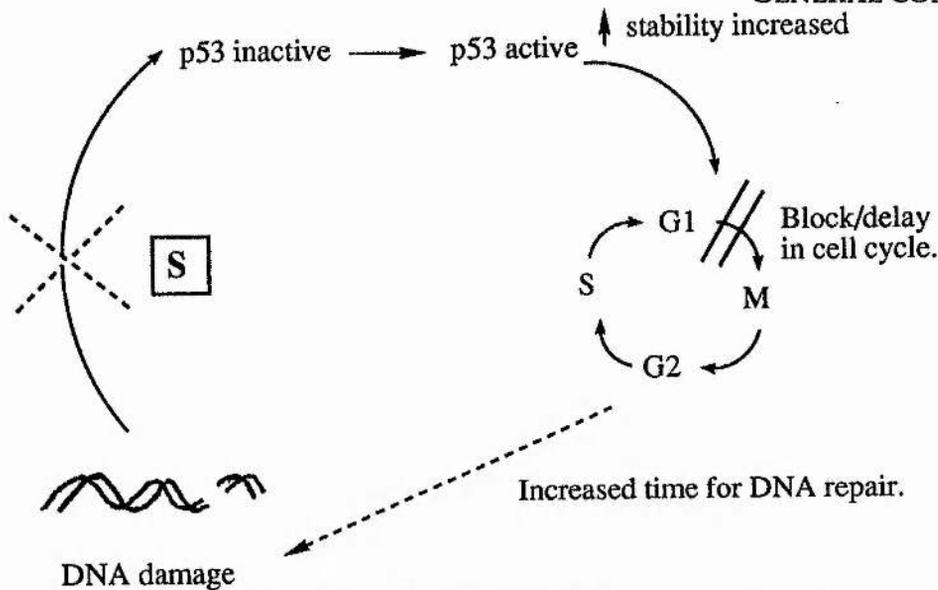


Fig. 6.1 Possible defects in the p53 response to radiation of cell line T24. See text for details.

status. If however, the defect was due to a repair enzyme/complex deficiency then UDS would not occur. Yet another approach to checking for this idea of DNA repair defect v. a fault with sensing DNA damage and relating this to p53 protein modulation, might be to use U.V. radiation as opposed to gamma radiation. U.V. radiation is known to induce p53 and cell cycle delay in normal mouse cells (Maltzman and Czyzyk 1984) but the type of DNA lesion generated (largely thymidine dimers) requires different repair enzyme complexes than those needed for gamma radiation induced DNA damage. T24 cells are unlikely to be deficient in both types of repair pathway and any lack of cell cycle delay/p53 induction would in that case be due to an inability to sense/recognise such damage and couple this to cell cycle delay.

Such approaches would not be without their problems, eg. different mechanisms may be responsible for sensing different types of DNA damage. However, it does give some suggestion of what future work in this area might perhaps try to elucidate i.e. the identification of other cellular components that interact directly or indirectly with p53 to mediate its function, such as the recently identified MDM2 gene product (Oliner *et al.*, 1992).

6.3 Radiation Induced Oncogenic Transformation.

Despite the genetic instability shown by two of the SV40 immortalised lines, such cell lines proved very resistant to full oncogenic transformation using a variety of different treatment protocols and culture regimes. Single and double radiation exposure experiments as well as maintaining cells as a confluent monolayer or continued passaging following carcinogen exposure all failed to produce such transformants. However, phenotypic alterations (morphological and decreased anchorage dependant growth) of human uroepithelial cells could be achieved when cells were maintained in a state of post confluent inhibited growth following both chemical and radiation carcinogen treatments. But, the progression from this "altered" phenotype to a tumourigenic one (i.e. tumour formation of inoculated cells in athymic mice) was not observed. From this it may be concluded that for SV40 immortalised human uroepithelial cells

- a. cell culture conditions are of substantial importance in selecting for these phenotypically altered cells to be visualised
- b. Relatively infrequent events are probably required for the generation of a fully tumourigenic cell type from an "altered/initiated" phenotype *in vitro*, which by comparison occur with a much higher frequency.

This difficulty in obtaining fully oncogenic transformants when compared to the relative ease with which they are generated in rodent cell lines may reflect mechanistic differences in the progress of oncogenesis between human and rodent cells. Alternatively as suggested by Faber and Rubin 1991, rodent cell lines may require as few as two events (genetic or otherwise, see section 1.4d) to generate neoplastic transformants, whereas human cells may require up to seven independent events to generate a similar phenotype.

Any data generated concerning radiation induced *in vitro* oncogenic transformation using either rodent cell lines or human cells immortalised with viral sequences must be treated with a degree of caution if direct comparisons/extrapolations are to be made to the *in vivo* human situation. It is highly unlikely that neither of these two experimental systems exactly mimic tissues within the human body. However, we are faced with a dilemma, exposure limits, risk assessments etc... must be based on the data available. Rodents cells do not appear to make an accurate a model as human cells but even such human lines have been drastically altered by the viral sequences used to immortalise them when compared to normal human tissues. Although human keratinocytes have been reported to be capable of being maintained in culture using serum free medium with a mixture of growth factors and pituitary cell extract, similar reports with epithelial cells have not so far appeared. This problem will unfortunately remain unless alternative methods of immortalisation for human epithelial cells can be found.

APPENDIX

OPTIMISATION OF CULTURE CONDITIONS AND GENERAL CHARACTERIZATION OF HUMAN UROEPITHELIAL CELLS (HUC).

A.1 Culture media.

HUC lines have previously been grown in continuous culture since 1987 by Dr. Catherine Reznikoff's group (Department of Human Oncology, University of Madison, Wisconsin, USA). However, it was decided to assess the effect of different culture media on the growth/cell survival of HUC lines as measured by "grow back" assays since this data was not previously available.

Briefly; almost confluent cultures of SV-HUC-1 cells grown in Hams F12 medium supplemented with 7% fetal calf serum (FCS) were dispersed using 0.1% EDTA for 20 mins at 37°C and 1×10^6 cells plated into 75 cm² tissue culture flasks with 10 mls of the appropriate medium. 48 hours after plating the medium was changed and then again a further 72 hours later. After a total of eight days incubation in the different media, cultures were harvested using 0.1% EDTA and cell counts obtained. All platings were done in triplicate. See Materials and Methods (section 2.1a) for a detailed description of culture media and the supplements used.

Figure A.1 shows the effect of the different culture media on cell growth for SV-HUC-1 cells. There is no significant difference between F12 7%FCS, F12+ 1%FCS (supplemented with Fe free human transferrin) and F12+ 1% FCS (supplemented with Fe saturated human transferrin) in terms of differential growth rates. However, the growth of SV-HUC-1 cells was significantly reduced if cells were grown in either F12+ or F12 alone ($P = 0.05$,

student's *t*-test). Hence cells grew at an optimal rate in medium containing FCS even if this was reduced to 1%. FCS probably contains a growth factor that is not supplied by F12 medium which is only required at a low concentration for the growth of SV-HUC-1 cells if other necessary growth supplements are also provided i.e. "+" medium. Therefore the medium used to routinely grow HUC cells was F12+ 1%FCS (supplemented with Fe free human transferrin).

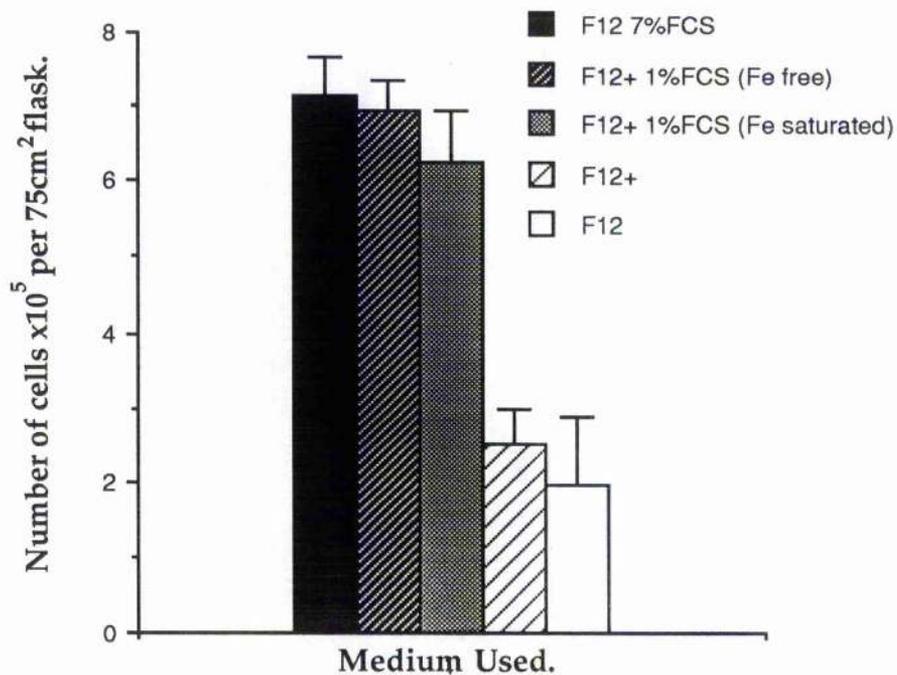


FIG A.1 Effect of different culture media on the growth of SV-HUC-1 cells. Cells were harvested eight days after plating 1×10^6 cells (plating efficiency of approx 25% see table A.2). Error bars represent SEMs from triplicate platings.

A.2 Dispersal Methods.

A crucial procedure for the successful culture of any cell line which grows as an attached monolayer, is the ability to remove cells from the surface of a

culture container and replate into a new flask/dish (passaging). If the viability of cells after such a procedure is greatly reduced, or only a small fraction of attached cells are removed, then maintaining such cells in culture will become increasingly difficult. Therefore different dispersal methods were assayed in order to elucidate the least toxic and most efficient removal conditions for HUC lines.

Briefly; 25 cm² culture flasks were seeded with 5x10⁵ SV-HUC-1 cells after being removed using 0.1% EDTA for 20 mins at 37°C followed by tapping the flask (medium used was F12 7%FCS). After 4 days cultures were dispersed using the procedures outlined in table A.2. The "plating efficiency (P.E.)" data was calculated after replating 2.5 x10⁵ cells in 25 cm² flasks using F12 7% FCS medium. These cultures were left for a further 4 days (approximately two cell doublings, see section A.3) when the theoretical number of cells should be 1x10⁶ if 100% plating efficiency. The P.E. values shown are expressed as a fraction of this figure, again cells were removed using 0.1% EDTA for 20 mins at 37°C followed by tapping the flask. "% cells removed" was calculated relative to certain trypsin treatments(*) for which the method of dispersal removed all attached cells. All cell counts were obtained using a haemocytometer

The collagenase treatments removed very few of the cells (less than 5% of the total number of cells in a flask). Therefore another flask could not be replated with 2.5 x10⁵ cells and accordingly a P.E. value has not been recorded. Shorter collagenase treatments (20 and 30 mins) removed even fewer cells, the data from which has not been shown. No single treatment produced a complete single cell suspension although that produced by EDTA coupled with trypsin treatment did produce the greatest number of single cells. Trypsin treatment alone greatly reduced cell viability even though a high proportion of attached cells were usually removed. When EDTA alone was used the plating efficiency varied between 18 and 27%. The

DISPERSAL METHOD	Single cells	Double cells	Double + cells	% cells removed	Plating Efficiency (P.E.)
EDTA 0.1% 20 mins at 37°C - tapping flask	22%	17%	61%	64.9% ±14.2	22% ± 5.7
EDTA 0.1% 30 mins at 37°C - tapping flask	41%	15%	44%	71.3% ±8.7	18% ± 6.0
EDTA 0.1% 40 mins at 37°C - tapping flask	42%	12%	46%	74.8% ±21.4	21% ± 11.2
EDTA 0.1% 60 mins at 37°C - tapping flask	67%	7%	26%	82.0% ±17.5	18% ± 10.1
EDTA 0.1% 20 mins at 37°C - ppt up and down	21%	10%	69%	78.5% ±32.9	27% ± 7.9
Collagenase 0.1% 40 mins at 37°C - tapping	47%	25%	28%	1.7% ±3.3	N.D.
Collagenase 0.1% 60 mins at 37°C - tapping	40%	46%	14%	1.5% ±2.3	N.D.
Trypsin 0.01% 5 mins at 37°C - tapping	61%	11%	28%	62.7% ±12.8	5% ± 4.3
Trypsin 0.01% (x3C) 5 mins at 37°C - tapping	40%	38%	22%	100%*	8% ± 7.5
Trypsin 0.01% (x3C) 10 mins at 37°C - tapping	52%	12%	36	100%*	4% ± 5.9
Trypsin 0.01% 10 mins at 40°C + 5 mins at 37°C - tapping flask	55%	22%	33%	100%*	5% ± 6.7
Trypsin 0.05% 5 mins at 37°C - tapping flask	63%	26%	11%	59.7% ±21.9	3% ± 5.0
EDTA 0.1% 20 mins at 37°C + 0.01% trypsin added to EDTA soln for 2 mins at room temperature no tapping of flask.	71%	18%	11%	89% ±24.0	64% ± 17.3

Table A.1 Effect of different cell dispersal methods on SV-HUC-1 cells. * indicates cultures in which the method of dispersal removed all attached cells, the other values for % of cells removed were calculated relative to this value. N.D. not determined. x3C three times re-crystallized trypsin. ± represent standard deviations from triplicate platings.

use of EDTA coupled with trypsin treatment resulted in a cell suspension with the greatest viability. This method of cell dispersal/removal was routinely used to passage HUC cells.

A.3 Growth Kinetics of Epithelial Cell Lines.

The rate at which cells divide i.e the length of cell cycle, is of great importance when carrying out several assays utilized in this study such as the generation of binucleate cells for Mn analysis or the time taken for passaged cells to reach confluence in carcinogen treated cultures.

Briefly; 1×10^5 cells were plated in 25 cm² culture flasks in appropriate medium (see section A.1a) which was changed every three days, harvested at 24 hours intervals and a cell counts taken. Table A.3 gives the cell doubling times in logarithmic phase of growth for the different epithelial lines used.

CELL LINE	CELL DOUBLING TIME in logarithmic phase of growth (hours)
SV-HUC-1	42
NT11	39
BC16	43
T24	20
HPV	31

Table A.3 Cell doubling times for different human epithelial cell lines.

Cell cycling times for the SV40 immortalized lines (SV-HUC-1, NT11 and BC16) were all similar (approximately 40 hours), where as that of the carcinoma cell line T24 was much shorter only 20 hours. The human papilloma virus transformed line HPV, had an intermediate cell cycling time of 31 hours. The cell cycling time for H Tori-3 cells was not determined

because these cells were not used for any cell cycle dependant experiments, only for mutant or wild type p53 evaluation.

A.4 Chromosome Analysis

Cell line SV-HUC-1 had a range of chromosomes from 43 to 78 per scored metaphase. Within this spread of chromosome numbers there appeared to be two sub-populations, one being at or near diploid and the other exhibiting a highly aneuploid karyotype ranging in chromosome number from 62 to 78 per scored metaphase (Figure A. 2)

No chromosome 15 was observed (Figure A. 3) this agrees with the data of Wu *et al.*, 1998 and Klingelhutz *et al.*, 1991 who also reported no chromosome 15 in some SV-HUC lines.

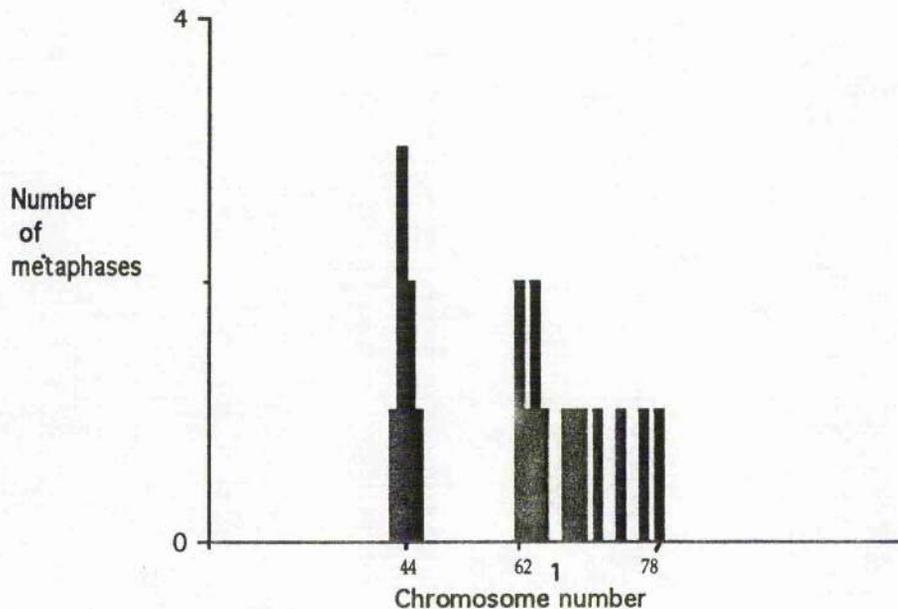


Fig. A.2 Ploidy of SV-HUC-1 cells at passage 37.

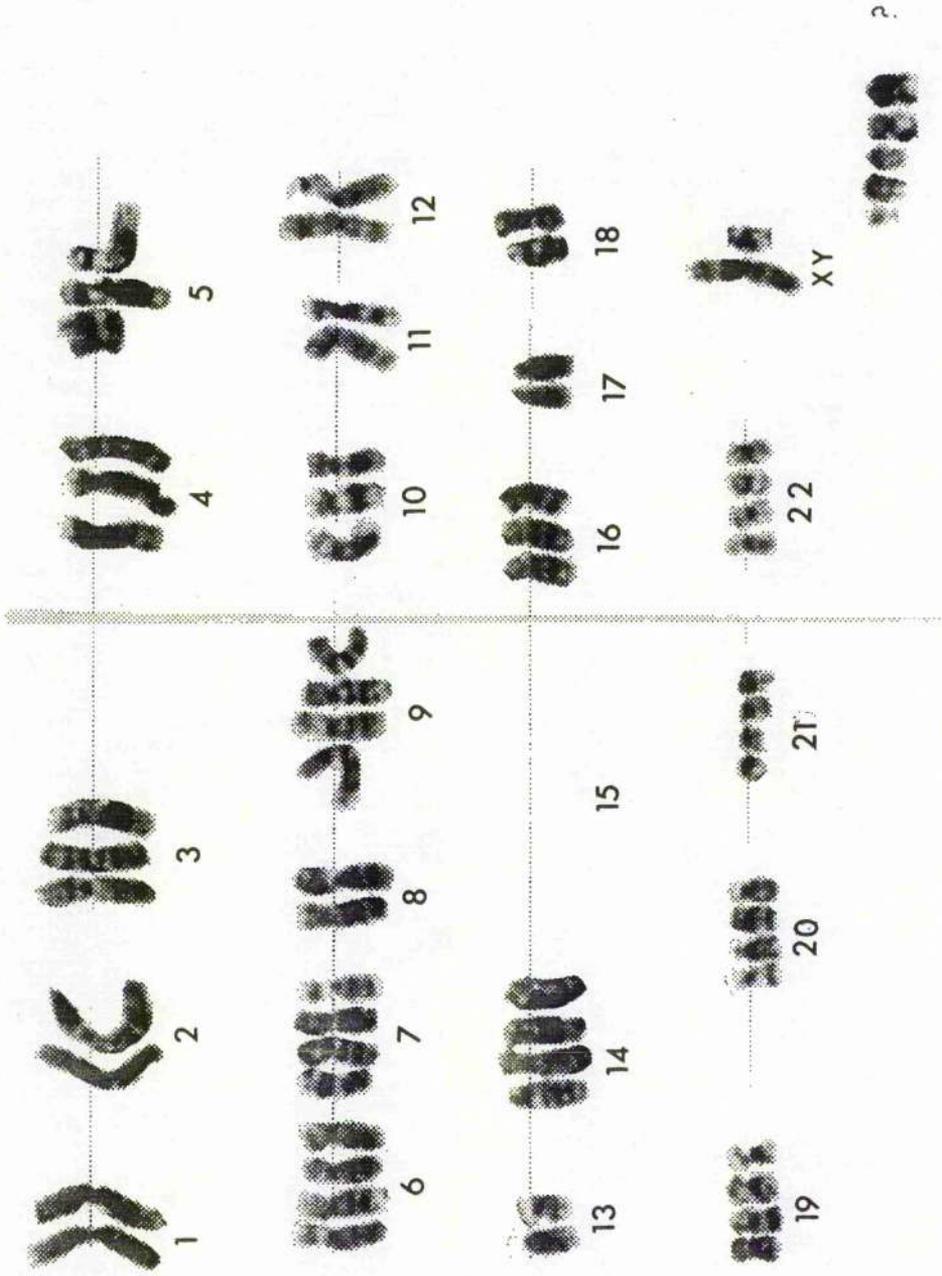


Fig. A.3. Karyotype of an SV-HUC-1 colcemid blocked metaphase. Slides were digested with 0.25 % Trypsin for 5 second before Giemsa staining. The metaphase contains 70 chromosomes.

Cytogenetic responses of human uroepithelial cell lines and a malignant bladder carcinoma cell line to X-rays

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The responses to X-rays of three simian virus 40 (SV40) immortalized but non-tumourigenic human bladder epithelial cell lines have been compared with a malignant bladder epithelial line using the micronucleus assay. A linear increase in induced micronuclei (MN) was observed for all four cell lines with increasing X-ray dose. The three SV40 immortalized lines were found to be significantly less sensitive than the malignant cell line. Spontaneous levels of MN indicate that certain cell lines within the SV40 immortalized lines have a higher genetic instability. These cell lines may have a predisposition towards the generation of a fully transformed phenotype when treated with carcinogenic agents.

Introduction

Present knowledge concerning radiation induced neoplastic transformation *in vitro* has relied heavily on the use of rodent embryonic fibroblast cell lines such as C3H10T $\frac{1}{2}$ (Terzaghi and Little, 1976; Elkind *et al.*, 1985; Hall and Hei, 1986; Hei *et al.*, 1988). The responses of rodent cell lines although useful, probably cannot be directly compared to that of human cells due to interspecies differences (Reznikoff *et al.*, 1986). Moreover, the majority of human cancers (>80%) originate in epithelial cells (Cairns, 1975). Thus fibroblastic lines may not represent an appropriate model for human carcinogenesis.

Christian *et al.* (1987) have reported the use of human uroepithelial cell lines (SV-HUC) immortalized with simian virus 40 (SV40) as a possible model system for studying the oncogenic effect of chemical carcinogens. The use of the chemical 3-methylcholanthrene on a non-tumourigenic but immortal SV-HUC line resulted in transformation to a fully neoplastic phenotype (Reznikoff *et al.*, 1988).

In this paper we present data on the clastogenic response to radiation of three such SV-HUC lines as a prelude to an investigation of the carcinogenic response of these lines to ionizing radiation. Due to the difficulty in obtaining viable single cell suspensions the use of clonal assays proved an unreliable measure of radiation response when assayed by cell survival in SV-HUC lines. The clastogenic response of these cells was therefore chosen as an alternative to a clonal assay as a way of assessing their radiosensitivity. The clastogenic effect of X-rays has been measured in these cell lines using the cytokinesis-block technique of Fenech and Morley (1985). The cytogenetic response of these SV-HUC lines has been compared with that of a urothelial carcinoma line (T24) for which we were able to obtain a survival curve using a clonogenic assay.

Materials and methods

Cell culture

SV-HUC lines were derived by Dr C.A.Reznikoff using SV40 to infect human ureteral explant tissue. This gave rise to a number of immortalized but non-tumourigenic clonogenic cell lines (Christian *et al.*, 1987). SV-HUC lines

(designated SV-HUC 1, NT11 and BC16) were cultured in Hams F12 medium (Gibco) supplemented with 7% fetal bovine serum (FBS; Globepharm), penicillin 100 units/ml (Glaxo) and streptomycin 100 μ g/ml (Evans). Cultures were maintained at 37°C in an atmosphere of 5% CO $_2$ in air using 75 cm 2 culture flasks. Cell densities ranged from $\sim 4 \times 10^5$ cells/flask when initially seeded into a flask to 7×10^6 cells/flask at confluence. Medium was changed every 2-3 days. Removal of cells from flasks for routine passaging was achieved using 0.1% ethylenediamine tetraacetic acid (EDTA; Sigma) in Hanks' balanced salts solution. When just subconfluent, cells were removed from the flasks surface with EDTA and seeded in 25 cm 2 flasks at a density of $\sim 1.5 \times 10^5$ cells/flask. These cultures were incubated for ~ 1 week until cells were in mid-exponential phase of growth before irradiation.

Bladder carcinoma cells used were MGH-U1 and have actually been shown to be T24 cells by DNA fingerprinting tests (Masters *et al.*, 1988). These cells were cultured in a similar way except the medium used was RPMI (Northumbria Biologicals Ltd) supplemented with 5% FBS, antibiotics and L-glutamine 2.0 mM (Flow Laboratories). Cells were removed from flasks using 0.01% recrystallized trypsin (Cooper Biomedical) with 0.2% EDTA in phosphate-buffered saline (PBS). Cultures were again passaged just prior to confluence, replated at 1×10^5 cells/flask in 25 cm 2 flasks and incubated for 3-4 days before irradiation, again in mid-exponential phase of growth.

Passage numbers used were: SV-HUC 1: P32-P36; NT11: P20-P25; BC16: P28-P31. The passage number of T24 cells is unknown.

Colony assays after X-ray treatment of T24 cells were carried out in 90 mm plastic petri dishes. The number of cells plated was adjusted so that 100-200 colonies per dish would result after a 6 day incubation period. Dishes were then fixed in methanol for 10 min, stained in 10% Giemsa in water for 10 min and rinsed in water. Colonies of >50 cells were scored.

X-irradiation

Cells were irradiated whilst attached to flasks. Cells were given various doses of 250 kv (0.5 mm Cu) X-rays. The dose rate was 0.74 Gy/min. Doses were checked using a ferrous sulphate technique (Frankenburg, 1969).

Micronucleus assay

Micronuclei (MN) were assayed using the cytokinesis block technique. The method was essentially that of Fenech and Morley (1985). Briefly, immediately after irradiation cytochalasin B (Cyto B; Sigma; stock solution 3 mg/ml in DMSO) was added to give a final concentration of 3 μ g/ml. Cultures were then incubated for a 48 h before harvesting with EDTA or trypsin.

Preparation of slides

Slides were prepared by centrifugation using a Shandon Cytospin 2 (800 r.p.m. for 10 min) and fixed in methanol (10 min). Slides were then stained in 56% Jenners in water for 5 min, rinsed in water, 25% Giemsa in water for 10 min and again rinsed in water. Acridine Orange staining was performed on some slides using the method described by Tinwell and Ashby (1989) after destaining Jenner/Giemsa stained slides in methanol:acetic acid (3:1) overnight. Fluorescence microscopy was performed using 450-490 nm wavelength light. A total of 200 binucleate cells were scored per sample per experiment.

Immunocytochemical staining for SV40 T-antigen

Cells were grown overnight on glass slides in a humidified incubator in a 5% CO $_2$ in air atmosphere. Slides were then washed in PBS, fixed in ice cold acetone:methanol (1:3) and rehydrated in PBS containing 1% sheep serum (1% SSPBS), 37°C for 30 min. Mouse antiserum antibodies (IgG $_1$) prepared against SV40 T-antigen was diluted 1:25 in 1% SSPBS and added to the fixed cells which were then incubated for 1 h as above. (Cells incubated in 1% SSPBS without the primary antibody served as a negative control.) After rinsing in PBS to remove the primary antibody, HRP-conjugated sheep antimouse IgG antibodies diluted 1:20 in 1% SSPBS were added and incubated for a further 1 h. Following rinses in PBS and staining in 3,3'-diamino benzidine tetrahydrochloride (DAB; Sigma) for 5 min, cells were finally counter stained in haematoxylin, dehydrated and clear mounted.

Results

In multiple sampling experiments (Figure 1) we investigated the influence of harvest time on MN formation in Cyto B blocked

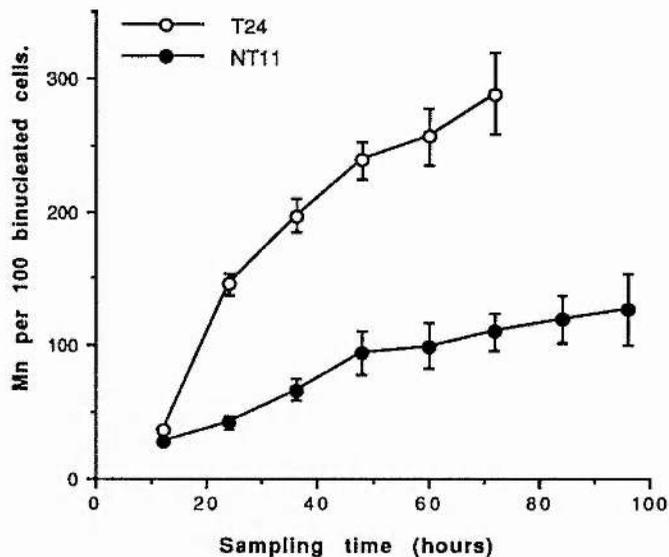


Fig. 1. Frequency of MN in binucleate cells as a function of time following X-irradiation (4.0 G). Error bars represent standard errors of mean (SEM) values from two separate experiments.

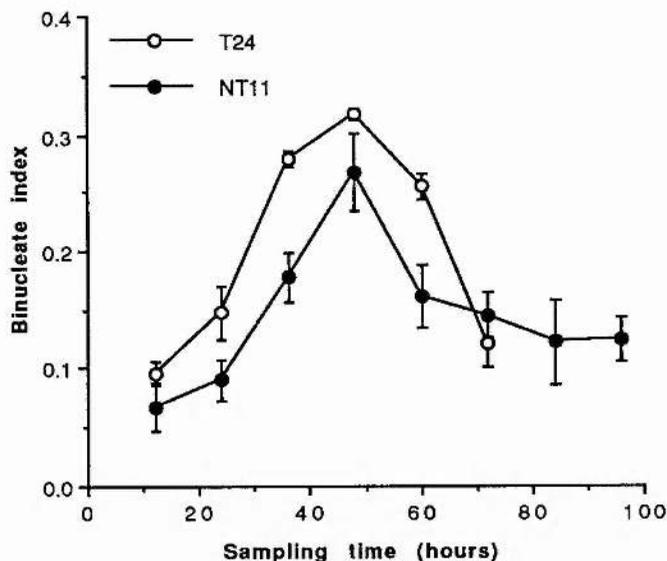


Fig. 3. Binucleate cell index (frequency of binucleate cells/total number of cells) as a function of sampling time following irradiation (4.0 G). Error bars represent mean values from two experiments.

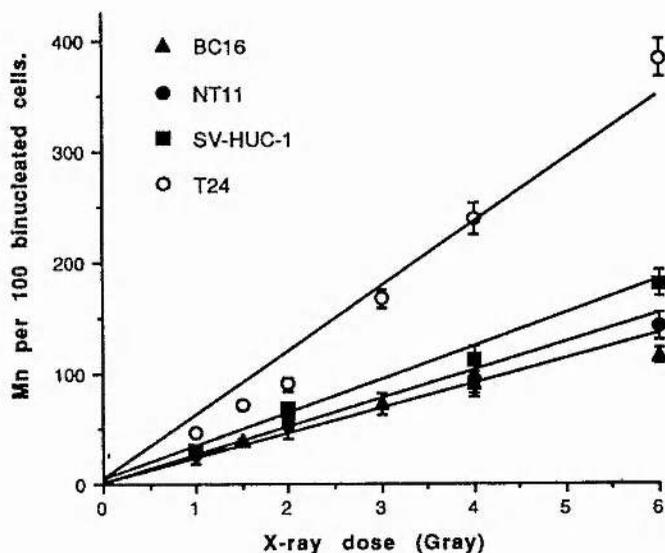


Fig. 2. Dose-response relationship between frequency of MN and X-ray dose. Sampling time was 48 h after irradiation. Error bars represent SEM values from at least three separate experiments. (Background MN values have been subtracted from the data shown.)

irradiated cells. These results showed that there is no definite peak in MN response at a given harvest time, but that the MN frequency increases steadily with sampling time and appeared to plateau in the cell line NT11. NT11 cells were found to be considerably less sensitive than the bladder carcinoma line (T24) in terms of its MN response to X-rays at comparable harvest times. This is also demonstrated by the X-ray dose-response curve obtained at a fixed harvest time of 48 h (Figure 2). The three SV-HUC lines showed similar dose-response curves to one another. These lines were much less steep than that of the T24 line. At 6 Gy there was a significant difference between the MN response of SV-HUC 1 and that of cell line T24 of $P < 0.01$, Student's *t*-test. All four lines showed a linear increase in frequency of induced MN with X-ray dose for the 48 h sampling time.

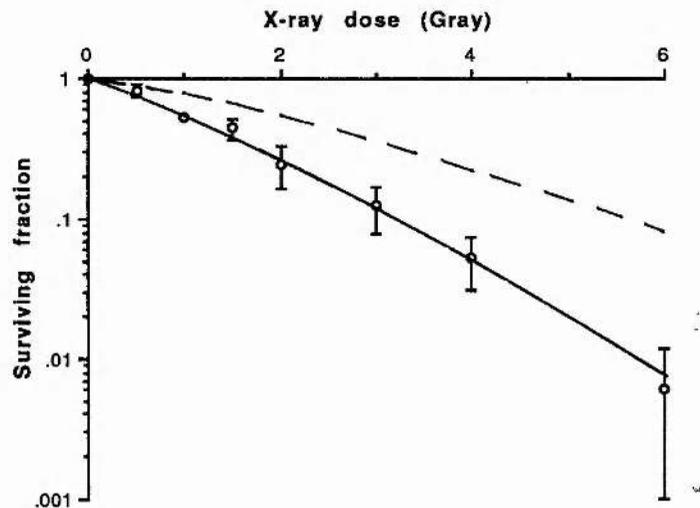


Fig. 4. Cell survival curve for cell line T24. Error bars represent SEM values from at least three separate experiments. The dotted line indicates the approximate survival curve for SV-HUC lines.

Although the cell cycling times of the cell lines in exponential growth were found to be ~39, 42, 43 and 20 h for cell lines NT11, SV-HUC 1, BC16 and T24, respectively (data not shown), 48 h harvest time in multiple sampling time experiments was found to be an optimal sampling time. At sampling times later than 48 h the frequency of binucleate cells (binucleate cell index) fell to levels which made accurate scoring of MN very difficult. Figure 3 shows that the maximum binucleate index occurred at ~48 h for the cell lines NT11 and T24.

The survival curve of T24 cells to X-rays (Figure 4) gave a point of reference for a comparison with the MN dose-response curves. SV-HUC lines would be expected to show increased survival to X-rays when compared to T24 cells and is indicated by the dotted line.

The spontaneous level of MN after Cyto B blockage alone (Figure 5) was found to be high for cell lines SV-HUC 1 and NT11 but low for BC16 and T24. The difference between BC16

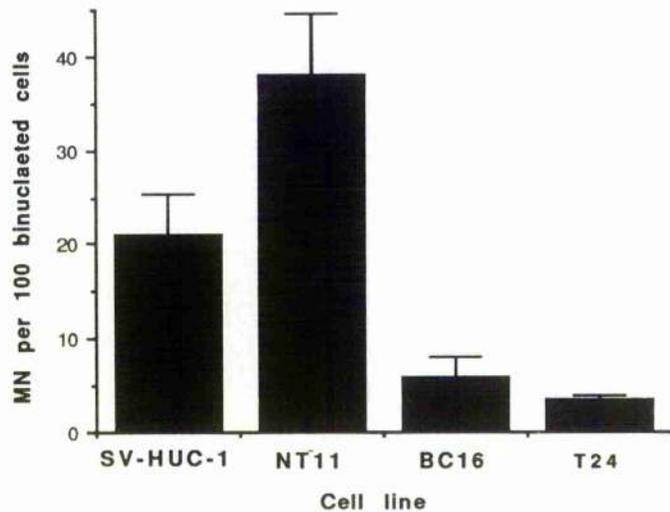


Fig. 5. Spontaneous frequencies of MN in binucleate cells 48 h after addition of cytochalasin B. Error bars represent SEM values from at least three separate experiments.



Fig. 6. Acridine Orange fluorescence of binucleated NT11 cells 48 h after 4.0 Gy of X-rays and cytokinesis blockage with cytochalasin. Several MN per cell are shown and the boundaries of cell cytoplasm can clearly be distinguished. The bar represents 10 μ m.

and both SV-HUC 1 and NT11 was significant ($P < 0.05$), Student's *t*-test. The spontaneous MN level observed for cell lines BC16 and T24 fall within the range of values observed for other human cells such as lymphocytes; 4.0–67.3 MN/1000 binucleated cells (Fenech *et al.*, 1990).

Acridine Orange staining was performed on selected slides to check for possible artifacts resulting from the Jenner/Giemsa staining. Cytospinning when coupled with Jenner/Giemsa staining gave a very good contrast between cytoplasm (blue) and the nuclei or MN (mauve). Another advantage of this method was that the distribution of cells on the slide was predictably in one area with cells in relatively close proximity. Thus, boundaries of cell cytoplasm could be easily identified. These factors allowed accurate scoring of slides much more rapidly than with standard spreading techniques. The staining with Acridine Orange (Figure

6) indicated that the MN produced are nuclear fragments and not artifacts of the Jenner/Giemsa stain.

Immunocytochemical staining for SV40 T-antigen DNA was positive for the SV-HUC lines but no staining was observed with the malignant bladder cell line T24.

Discussion

From Figure 1 it can be seen that the frequency of MN increased with time following irradiation. The results show that the SV-HUC lines all had similar chromosomal radiosensitivities as estimated from MN frequencies at 48 h following X-irradiation (Figure 2). Data shown in Figures 1 and 2 indicates that these SV-HUC lines were significantly less radiosensitive than the T24 cell line (also of bladder epithelial origin). It is possible that the apparent relative radiosensitivity of T24 cells could be influenced by the shorter doubling time of T24 relative to the SV-HUC lines. This will lead to the more rapid accumulation of MN with time, as seen in Figure 1 and as discussed by Savage (1989). This effect may however to some extent be offset by the more rapid exit of T24 cells from the binucleate into the quadrinucleate compartment (unscored cells) as shown in Figure 3. Tumourigenic T24 cells are themselves reported to be somewhat radioresistant when compared with certain other tumour cell lines (Parris *et al.*, 1988). The expression of the SV40 T-antigen within human fibroblasts has also been shown to decrease radiosensitivity (Arlett *et al.*, 1988). SV-HUC lines have been immortalized but are not fully transformed. However, they do contain the SV40 T-antigen (Christian *et al.*, 1987) and immunocytochemical staining in our laboratory. This lack of a fully transformed phenotype but presence of the SV40 T-antigen supports the idea that it is this sequence of viral DNA that somehow confers increased radioresistance to the cell.

Due to the difficulty in obtaining a viable single cell suspension of the SV-HUC lines colony assays could not be used to determine survival curves. However, the MN frequencies as shown in Figure 2 indicates an almost 2-fold lower induction of MN in SV-HUC lines than in T24 cells. Because of the radioresistant nature of the SV-HUC lines in terms of X-ray induced MN levels, a probable survival curve for the three SV-HUC lines is shown by the dotted line in Figure 4. This approximate survival curve is based on the MN data and makes the assumption that most X-ray induced MN will be lethal events.

The spontaneous or background level of MN in binucleated cells (Figure 5) gives an indication of the genetic stability for each cell line. Cytogenetic instability has already been reported in cell line SV-HUC 1 by Meisner *et al.* (1988). Cell line NT11, having an even higher spontaneous MN value than cell line SV-HUC 1, suggests that NT11 cells have a greater genetic instability than the other SV-HUC lines.

Whereas X-ray induced MN are known to be composed almost entirely of acentric fragments (Thomson and Perry, 1988; Littlefield *et al.*, 1989) it is not known if these spontaneous MN are the result of acentric fragment formation or whole chromosome loss. However, work by Meisner *et al.* (1988) on cell line SV-HUC 1 shows that this genetic instability resulted in chromosomal loss and duplications coupled with translocations and reduction in polyploidy as passage number increased. These spontaneous genetic changes alone did not result in the generation of a tumourigenic phenotype.

The genetic instability shown by two of the SV-HUC lines may allow a synergistic effect between this instability and other additional genetic changes such as oncogene activation or tumour

suppressor gene loss, both of which are known to occur by the types of genomic rearrangements mentioned above. These additional changes may be necessary stages in the multistep process of carcinogenesis. Transformation experiments using the SV-HUC lines exposed to X-rays are under way in our laboratory.

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