RADIOSENSITIVITY STUDIES OF LYMPHOCYTES AND PROSTATE CELLS DERIVED FROM PROSTATE CANCER PATIENTS.

A thesis presented for the degree of Doctor of Philosophy

University of St Andrews

By

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ABSTRACT.

Radiosensitivity of peripheral blood lymphocytes (PBLs) from prostate cancer patients was investigated using the G2 assay and cytokinesis block micronucleus (CBMN) assay. The G2 assay evaluates chromosomal damage caused by irradiating the cells in the G2 phase of the cell cycle. The CBMN assay quantifies the post mitotic micronuclei, which are the expression of damage incurred during G0.

Data indicates there is an identifiable group of men with increased chromosomal radiosensitivity within the prostate cancer population. Using the G2 assay and the 90^{th} percentile of the controls as a cut off point for sensitivity, no significant difference between the control and patient population was found with 13.33% of the controls being sensitive and 27.27% of the prostate cancer patients. However, using the CBMN assay and the 90^{th} percentile, 6.25% of the control group were sensitive compared with 32.68% of the carcinoma cases.

The implications of this increased radiosensitivity are unclear, but it is indicative of increased chromosomal fragility and therefore, possibly associated with malignant transformation. Hence, it might prove useful in identifying individuals at increased risk of developing prostatic carcinoma or in predicting disease progression.

The effects of dihydrotestosterone (DHT) the active androgen in the prostate, on radiosensitivity were investigated using PC3 (hormone insensitive) and LNCaP

(hormone sensitive) prostate cell lines and PBLs. The MTT assay showed that the presence of DHT had no affect on the radiation dose response curves of the cell lines. Conversely, using the CBMN assay, the presence of DHT significantly reduced the expression of chromosomal damage in both of the cell lines and PBLs.

Additionally, attempts were made to immortalise human prostate epithelial cells using a retroviral vector containing human telomerase (hTERT). The goal was to generate continuously proliferating human prostate epithelial cell lines and to use ionising radiation to induce neoplastic transformation and study the molecular and genetic changes. Unfortunately, immortalisation was not achieved, but life extension was, suggesting that hTERT alone was insufficient to overcome senescence in prostate epithelium.

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LIST OF ABBREVATIONS.

ALT alternative mechanism of telomere maintenance

ANOVA analysis of variance

AR androgen receptor

A-T ataxia-telangiectasia

BN binucleate

BPE bovine pituitary extract

BPH benign prostate hyperplasia

BSA bovine serum albumin

CBMN cytokinesis blocked micronucleus

CDK cyclin dependent kinase

CGH comparative genomic hybridisation

CK cytokeratin

CT computer tomography

DAB 3,3'-diamenobenzidine peroxidase substrate

DCC deleted in colon cancer

dH_2O distilled water

DHT dihydrotestosterone

DMEM dulbeccos modified eagle medium

DMSO dimethyl-sulphoxide

DNA deoxyribonucleic acid

DPX distrene dibutyl phthalate xylene

DRE digital rectal examination

ds double stranded

EBV Epstein Barr virus

EDTA ethylene diamine tetra acetic acid

EGF epidermal growth factor

EGFR epidermal growth factor receptor

FCS foetal calf serum

FGF fibroblast growth factor

FISH fluorescence in situ hybridisation

Gy Gray of ionising radiation

HBSS hepes buffered saline solution

HGF hepatocyte growth factor

HIV human immunodeficiency virus

HNPCC hereditary non-polyposis colorectal cancer

HPV human papilloma virus

hr(s) hour(s)

hTERT human telomerase catalytic subunit

hTR telomerase associated RNA template of reverse transcriptase

IGF insulin like growth factor

IR ionising radiation

IU international units

KCl potassium chloride

KGF keratinocyte growth factor

LHRH lutenising hormone releasing hormone

LOH loss of heterozygosity

LTR long terminal repeat

min(s) minute(s)

MN micronucleus

MNU N-methyl-N-nitrosurea

MoMLV moloney murine leukaemia virus

MTT 3-(4,5-dimethylthylthizyl)-2,5-diphenyl tetrazolium bromide

NBS Nijmegen breakage syndrome

No. number

OD optical density

OH hydroxy radical

PAP prostatic acid phosphatase

PBL peripheral blood lymphocyte

PBS phosphate buffered saline

PDGF platelet derived growth factor

pen penicillin

PHA phytohaemagglutinin

PIN prostatic intraepithelial neoplasia

polybrene hexadimethrine-bromide

PrEBM prostate epithelial basal medium

PrEGM prostate epithelial growth medium

PSA prostate serum antigen

Rb retinoblastoma

RNA ribonucleic acid

RPE retinal pigment epithelium

rpm rotation per minute

RPMI roswell park memorial institute

RT radiotherapy

strep streptomycin

SV40 simian virus 40

TGF transforming growth factor

TNM tumour node metastasis

TNS trypsin neutralising solution

TRUS transrectal ultrasound

TURS tranurethral resection

VSV vesicular stomatitis virus

3D-CTR three-dimensional conformal radiotherapy.

CHAPTER 1

GENERAL INTRODUCTION.

CHAPTER 1

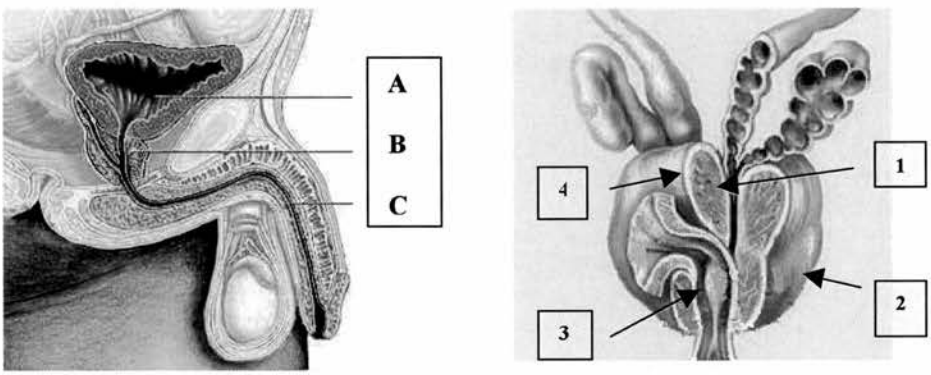
GENERAL INTRODUCTION.

1.1. Structure and function of the prostate gland.

1.1.1. Structure.

The prostate constitutes part of the male reproductive system. It is a small gland located at the base of the bladder encircling the urethra comprising of four lobes, anterior, posterior, medial and lateral (see Figure 1.1.), within which are histologically identifiable zones, central, transitional and peripheral. The prostate is a complex tubuloalveolar gland composed of an epithelial parenchyma embedded in a connective tissue matrix, all of which is enclosed by a dense fibrous outer capsule.

Figure 1.1.



a) b)

Figure 1.1. The prostate gland.

Panel a) shows the location of the prostate (B). A is the bladder, B is the prostate and C is the urethra (image adapted from http://www.phoenix 5.org/prostate01.html). Panel b) shows the prostate. 1 is the medial lobe of the prostate, 2 the lateral lobe, 3 the urethra and 4 the fibrous capsule (image adapted from http://www.bartleby.com).

There are three main types of cells discernible within the prostatic epithelium, basal (proliferative), luminal (secretory) and neuroendocrine (majority of which contain serotonin and thyroid stimulating hormone) (Collins and Maitland 2003). Basal cells are the least common, their function is not fully understood, but they are known to secrete components of the basement membrane and a subset might constitute the stem cell compartment for the luminal epithelial cells (Feldman and Feldman 2001). The luminal cells secrete components of the prostatic fluid, express androgen receptor (AR) and secrete prostate specific antigen (PSA) in an androgen dependent manner (Feldman and Feldman 2001). These epithelial cells are arranged in ducts that branch out from the urethra and terminate into acini. Figure 1.2. shows some of the prostatic components described.

The stroma or connective tissue matrix is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating cells such as, mast cells and lymphocytes. Some stromal cells are androgen responsive and produce growth factors that act on epithelial cells in a paracrine fashion. The stromal-epithelial cross talk is an important regulator of the growth, development and hormonal responses of the prostate (Feldman and Feldman 2001).

Figure 1.2.

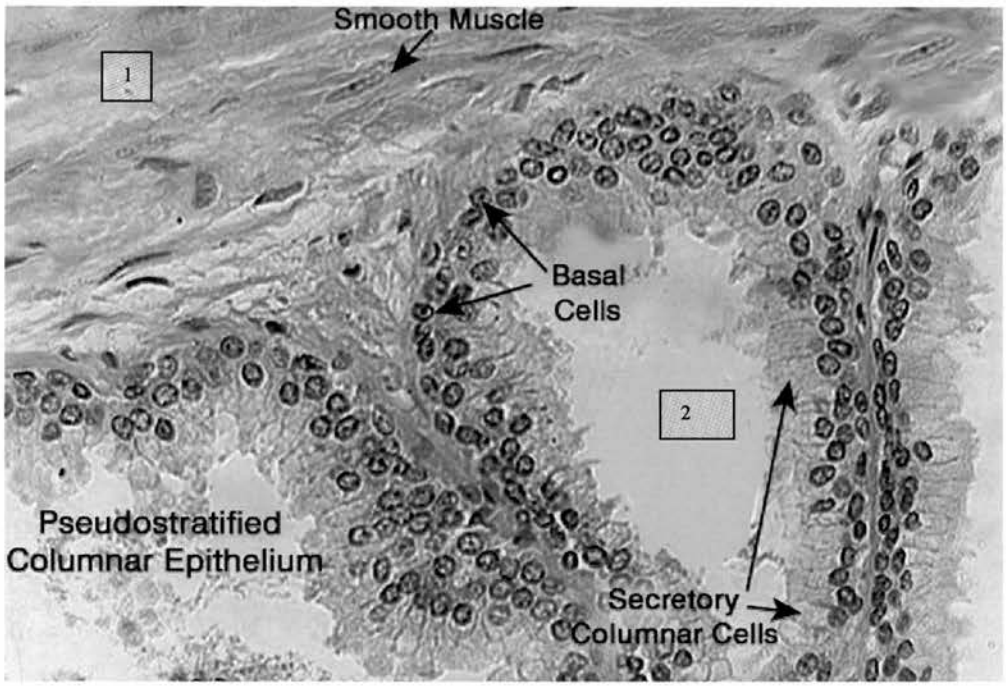


Figure 1.2. Histological section of the prostate gland.

This is a section of the prostate stained with haemotoxylin and eosin showing prostate tissue arrangement into epithelial and stromal layers. 1 indicates the stromal area and 2 indicates the lumen. Image adapted from http://www.asb.aecom.yu.edu.

1.1.2. Function of the prostate gland.

Prostatic function has not been clearly defined, but secretions comprise approximately 30% of the components of the seminal fluid and may provide nutrients for sperm. One component, PSA, is a form of chymotrypsin, it functions as an anticoagulant, maintaining the fluidity of semen (Hudson 2002). It also provides alkaline substances (bicarbonate), enzymes (phosphatase,

fibrinolysin), proteins (fibrinogen) and minerals (zinc) to the semen in order to enhance sperm motility and fertility and penetration (Pentyala *et al* 2000).

1.1.3. The role of androgens in prostate function.

The prostate grows and functions in a multihormonal environment responding to a variety of regulatory factors. The functional activity of the prostate primarily depends on the maintenance of normal concentrations of plasma testosterone. Testosterone enters the prostate soon after its secretion from the Leydig cells of the testes. The active androgenic steroid hormone within the prostate gland dihydrotestosterone (DHT) is then derived from testosterone by the 5-α reductase enzyme. DHT binds to the AR with an affinity two hundred times greater than that of testosterone and elicits the androgenic affects within prostate cells. The interactions of the DHT-AR complex allows it to modulate or initiate the transcription of proteins like PSA, growth factors intimately concerned in the control of cellular growth processes and other important genes.

In the stroma, DHT mediated regulatory mechanisms induce the production of several growth factors that modulate the signal transduction pathways within epithelial cells to promote growth and differentiation. Activation of mesenchymal androgen receptors stimulates, epithelial proliferation, ductal branching, morphogenesis and cytodifferentiation. These processes signify an induction of the secretory phase. Androgens also stimulate stromal smooth muscle cells inducing differentiation; these in turn maintain prostatic epithelium in the differentiated state (Kondylis and Klotz 2003).

The prostate is a gland with a high tendency to develop diseases later in life, for example, benign prostatic hyperplasia (BPH), a proliferative disorder of the transitional region that surrounds the urethra and prostate cancer, which originates mainly from epithelial cells in the peripheral region. Both of these conditions are disorders of cell proliferation, possibly a result of an imbalance between cell renewal and cell death. Androgens are the main regulators of these processes; hence they have a potential role in disease development.

1.2. Prostate Cancer.

1.2.1. An overview of the disease.

Prostate cancer is a commonly diagnosed malignancy, the second leading cause of cancer deaths in men in the western world, exceeded only by lung cancer (Bostwick 1995, Naguib *et al* 1998 and Pentyala *et al* 2000). Despite its high incidence, understanding of the underlying molecular and genetic mechanisms remains limited, especially in comparison with other cancers like colon, lung and breast.

Prostate cancer is a multistep progressive disease with a typical onset later in life. The increased longevity of the western population means that the incidence and mortality of prostate cancer is rising (Philip and Millikan 1995). It occurs almost exclusively in men over the age of forty and can be divided into two categories, early onset (patient under the age of fifty five) and late onset. The average age of diagnosis is seventy-two (Pentyala *et al* 2000).

1.2.2. Behaviour of prostate cancer.

Prostate cancer is an adenocarcinoma, unpredictable in its behaviour; many tumours will remain quiescent and clinically unimportant, whereas others will progress to advanced and metastatic disease resulting in considerable mortality and morbidity (Eeles1995 and Naguib *et al* 1998). Clinical presentation can be asymptomatic, with an elevated PSA and/or a palpable nodule on digital rectal examination (DRE), or it can present symptomatically with complaints ranging in severity from urinary frequency to severe bone pain as a result of metastasis. The majority of cancers that arise in the prostate are in the peripheral zone and hence, when metastasis occurs, either via lymphatic or circulatory routes, structures in close proximity are affected. This accounts for the symptoms manifested by the patients. Skeletal metastases are more common than metastases at other distant sites possibly because of a reciprocal interaction between the tumour and bone marrow. It has been demonstrated that bone cells produce selected growth factors, which stimulate the proliferation of prostate cells and that prostate cells produce factors that stimulate bone formation (Pentyala *et al* 2000).

All men are at risk from prostate cancer and the majority of older men develop at least microscopic evidence of disease. This prevalence of latent disease is characteristic of the prostate gland. *Post mortem* examinations of men over the age of fifty have revealed microscopic foci of well differentiated adenocarcinoma (histologically almost all prostate cancers are adenocarcinomas) (Philip and Millikan 1995) in at least 30% of prostates removed from men who have died of other causes. These carcinomas are

clinically indistinguishable from active cancers yet they have not grown or killed the patient (Franks 1995).

1.2.3. Risk factors.

Many risk factors for prostate cancer have been identified, but with inconclusive results. These include many dietary factors. Definitive risk factors include age, family history, race and insulin like growth factor (IGF)-1 level.

Numerous studies have shown that after the age of fifty both the incidence and mortality rate of prostate cancer increases at a near exponential rate (Pentyala *et al* 2000). Every decade of ageing nearly doubles the incidence of microscopic prostate cancer: from 10% for men in their fifties to 70% for men in their eighties (Philip and Millikan 1995). Approximately 90% of men who eventually develop clinically recognised prostate cancer had dormant cancer that remained undetected for decades (Gittes 1991).

1.2.3.1. Familial risk.

Also men with a family history have significant risk of developing the disease, thus suggesting a genetic factor in the disease aetiology and the possibility of inheritance of predisposition (Carter 1991). The clustering of prostate cancer in families could be because of genetic susceptibility, exposure to common environmental factors, or chance alone since the prevalence of this cancer is so high. 10-15% of cases are familial i.e. have at least one relative affected (Narod 1998, Grönberg 2003). The risk of developing prostate cancer in relatives increases with the number of individuals affected and with a decrease in age of

diagnosis. It is estimated that men from families with two or more affected first or second degree relatives have as much as an eight times higher risk of developing the disease than the average male (Philip and Millikan 1995). However, it is possible that a number of sporadic cases may occur in one family and that they may exist alongside hereditary ones.

Men who have a brother with the disease are more likely to develop prostate cancer than those with an affected father suggesting the disease is recessive or linked to the X chromosome (Grönberg 2003).

Additionally, familial prostate cancer might be a more aggressive form with a higher incidence of recurrence after treatment. The hereditary form appears to be transmitted by autosomal dominant inheritance of rare yet highly penetrant susceptibility gene/s (Pentyala *et al* 2000).

Familial clustering of prostate cancer has been attributed to the dominant inheritance of a rare high risk allele. This appears to be analogous to predisposing alleles associated with inherited cancers of the breast (Grönberg 2003). Family studies indicate that a region on the long arm of chromosome 1 (specifically 1q24-25) contains a susceptibility gene (locus has been named HPC 1) (Smith *et al* 1996 and Cooney *et al* 1997).

1.2.3.2. The role of ethnicity.

The incidence of clinically diagnosed prostate cancer is also significantly affected by geographical and racial factors. Despite the incidence of clinically

detected prostate cancer, the prevalence of latent prostate cancer is quite similar across ethnic groups. This suggests that there may be differences in factors required to cause progression to a clinically detectable stage (Philip and Millikan 1995). Thus mortality varies widely across ethnic groups intimating that race is a risk factor for the development or progression of the disease (Lara *et al* 1999).

This ethnic difference is illustrated by the risk of prostate cancer development being 50% higher in African-American men than in European-Americans and two to three fold lower in native Japanese and Chinese men (Borg 2001). Differences in ethnic populations are real and not explained only by the discrepancies in health care and registration (Grönberg 2003). These differences may be partly attributable to factors such as, the ethnic differences in circulating levels of free testosterone (it has been shown that black men have, on average, a 15% higher serum testosterone level than white men (Philip and Millikan 1995)), and genes associated with androgen synthesis or differences in AR-related signalling pathways (Borg 2001). Whatever the reasons, the incidence of prostate cancer is increasing in both the high risk and low risk populations.

1.2.3.3. Role of other growth factors.

Recent studies have shown that people with high IGF-1 concentrations (those within the highest quartile) are at increased risk of prostate cancer. IGF-1 is a peptide growth factor that is easily measured in the circulation; it is a mitogen for prostate epithelial cells (Grönberg 2003). IGF-1 is critical for maintaining cell survival through its anti-apoptotic activity, particularly in transformed cells.

The interactions between the glandular epithelium, the muscle cells and fibroblasts of the stromal compartment appear to be regulated by the availability of IGF (Kondylis and Klotz 2003).

The IGF system might be the link between the sedentary western lifestyle and prostate cancer: consumption of large amounts of fat result in raised production of insulin that in turn increases production of IGF, thus explaining how IGF could be a risk factor for prostate cancer (Grönberg 2003).

Other growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), platelet derived growth factor (PDGF), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) have been demonstrated to contribute to the control of normal and cancerous prostatic growth through paracrine, auotcrine and intracrine factors (Kondylis and Klotz 2003).

1.2.3.4. Dietary factors and prostate cancer risk.

The association between dietary factors and prostate cancer has been a feature of many different studies. The results of these studies have been conflicting and mostly negative, but some dietary components are consistently linked with prostate cancer. These studies were recently reviewed by Grönberg, who reported that high intakes of α-linolenic acid and calcium increased the risk of prostate cancer development. Whereas, lycopene (a carotenoid found in tomatoes) consumed in large amounts lowered the risk by 16%. Other dietary components that were found to decrease the risk had an effect on disease

initiation and progression. For example, phyto-oestrogens, especially flavenoids, have a prophylactic effect on prostate cancer, micronutrients such as selenium (a non-metallic trace element) have an inhibitory effect on tumourigenesis and vitamin E (fat soluble) has antioxidant affects with particular activity in oxidative induced DNA damage (Grönberg 2003).

1.3. Symptoms of prostate cancer.

Prostate cancer may remain asymptomatic for many years, however, as the malignancy spreads, it might constrict the urethra causing urinary problems. Later stage symptoms typically include: frequent urination (especially at night), weak urinary stream, inability to urinate, trouble holding back the flow of urine, painful or burning urination, a weak interrupted flow of urine, blood in the urine or semen, painful ejaculation, frequent (chronic) pain or stiffness in the spine (lower back), pelvis, hip bones or upper thigh. (These chronic pains may herald the occurrence of bony metastases). The symptoms may also be accompanied by significant weight loss. However, these symptoms are not always indicative of cancer, but may often be caused by a non-cancerous enlargement of the prostate related to diseases such as BPH and prostatitis (Philip and Millikan 1995 and Pentyala *et al* 2000). Thus increasing the difficulty of prostate cancer diagnosis.

BPH generally affects men about their mid forties when the prostate begins to enlarge through a process of cell multiplication. The overgrowth occurs in the central area or transitional zone of the prostate gland, the area closest to the urethra, hence, the production of symptoms similar to those experienced in late stage prostate cancer. BPH usually begins with microscopic nodules in younger

men with approximately 25% eventually growing large enough to cause symptoms (Bostwick 1995).

There are a number of similarities between BPH and cancer: both display a parallel increase in prevalence with age; both require androgens for growth and development. Most cancers arise in prostates with concomitant BPH and cancer is found incidentally. It should be noted that prostate cancer commonly occurs in the outer area of the prostate, the peripheral zone where most glandular tissue is located and that BPH is neither a premalignant lesion nor a precursor of carcinoma (Bostwick 1995).

1.4. Detection and diagnosis of prostate cancer.

Prostate cancer can be detected before symptoms occur, in its early stages, before it has metastasised and the cancer is localised (confined) to the gland, this is when treatment is easiest and more likely to be successful. Two tests are commonly used to detect prostate cancer in the absence of symptoms, digital rectal examination (DRE), and a blood test for PSA. These two tests are complimentary.

1.4.1. Digital rectal examination.

DRE is a test which is relatively non-invasive; a physician palpates the prostate through the rectum to detect abnormalities such as masses or hard lumpy areas. The success of this technique is dependent on the skill and experience of the examiner.

1.4.2. PSA blood test.

The blood is tested for PSA, which is a serine protease with chymotrypsin like activity. PSA is the product of a member of the kallikrein gene family. It is synthesised and secreted by the prostatic epithelial cells to help keep the ejaculate in a liquid form and is a good serological marker. Small amounts normally leak into the circulation; certain disorders and interventions such as BPH, prostatitis, prostatic infarction, vigorous massage and biopsy may cause a rise in blood concentrations. Thus, PSA is not cancer specific, but the levels of PSA have been shown to correlate with the extent and existence of cancer; higher levels indicate a larger tumour burden, including metastatic disease (DeFeo-Jones *et al* 2000). PSA has primarily been used in the monitoring of the disease and the prediction of relapse after treatment. However, more recently, it is increasingly being used for early detection and screening.

PSA exists in different molecular forms in the serum and small amounts leak out into the circulation. PSA can be bound to α1-antichmotrypsin and α_2-macroglobulin or be free (Frydenberg *et al* 1997). The distinction and ratio of different forms may prove useful in distinguishing adenocarcinoma from BPH. It may also help in the differentiation between latent and malignant forms of prostate cancer and to decide which cancers are going to progress to clinical significance (Frydenberg *et al* 1997).

PSA although a useful indicator in clinical diagnosis of prostate cancer is not definitive, a potential complication is that although it is specific to the prostate in benign tissues, recent studies have shown other human malignancies such as

breast and ovarian cancer may also express low levels of PSA (Philip and Millikan 1995), but prostate tissue secretes at least a hundred times more PSA than breast tissue (DeFeoJones *et al* 2000).

1.4.3. Transurethral ultrasound and biopsy

If the results of either the DRE or the PSA are abnormal the next step is a transrectal ultrasound (TRUS), it has minimal morbidity, but up to 30% of prostate lesions easily palpable by DRE can be missed (Philip and Millikan 1995). None of the screening techniques previously mentioned are infallible, and many cancers are missed. The only way that the diagnosis of prostate cancer can be confirmed is by biopsy. Needle biopsies are the preferred method they provide more specific information about the grade and extent of the tumour (DeMarzo *et al* 2003). The extent of the tumour is the best predictor of prognosis (Frydenberg *et al* 1997). The presence of high grade prostatic intraepithelial neoplasia (PIN) is sometimes detected on biopsy, this is indicative of an increased risk of cancer somewhere in the prostate, but not necessarily at the site of the neoplasia (DeMarzo *et al* 2003). Following biopsy and diagnosis, prostate cancer is classified using stage and grading systems, these are discussed in section 1.5.

1.5. Classification of prostate tumours.

Prostate cancer is characterised by stage and grade. Histological sections of tumour tissue are taken and microscopically examined. Comparisons are made between tumour appearance and normal tissue. Grade is the term used to describe how closely a tumour resembles normal tissue. Pathologists may

describe it as a low, medium or high grade tumour. The most commonly used systems to define prognosis and therapeutic strategy are, the Whitmore-Jewitt staging system (see Appendix I, section I.2.), the Tumour, Node, Metastasis (TNM) staging system (see Appendix I, section I.3.) and the Gleason score (see Appendix I Table I.) (Cussenot 2003).

Clinical TNM classification is based on DRE and TRUS prostate imaging or magnetic resonance imagery (MRI) to assess extracapsular extension of the disease, Computer Tomography (CT) or MRI in the pelvis and reteroperitoneal space to assess lymph node metastasis and bone scintigraphy to assess bone metastasis. A combination of PSA levels, Gleasons score and pathological stage on prostate biopsy can also be useful in predicting the probability of extraprostatic extension of the disease using Partins table (see Appendix I section I.4.). Partins tables predict the results obtained on pathological examination of the prostate and lymph nodes after surgery. They can be used to combine data on the PSA value, the Gleason score and the clinical stage of a specific patient in order to try and predict the risk of that patient.

The Gleason score is the most frequently used grading system for prostate cancer. The overall grade is not based on the highest grade within the tumour because it has been shown that prognosis of prostate cancer was intermediate between that of the most predominant and the second most predominant pattern. These patterns are identified and graded from 1 (most differentiated) to 5 (least differentiated) and the two grades are added. If a tumour only had one histological pattern, the primary and secondary scores are the same. The

combined Gleason grade ranges from 2 (for tumours uniformly of pattern 1) to 10 (for undifferentiated tumours). The Gleason score is a powerful prognostic indicator and does influence treatment (DeMarzo *et al* 2003). For a more comprehensive explanation of the Gleason system refer to Appendix I, section I.1.

1.6. Treatment of prostate cancer.

Treatment options for prostate cancer depend primarily on grade and stage of tumour, however, the general health and age of the patient also plays a role. Also the ability of the proposed therapy to ensure disease free survival and its associated morbidity are taken into consideration when deciding a course of action.

Treatment of prostate cancer provides prolonged disease free survival for many patients with localised disease, but is rarely curative in patients with locally extensive tumours. Even when the cancer appears clinically localised to the prostate gland, a substantial proportion of patients will develop disseminated tumours after local therapy with surgery or irradiation. Metastatic tumours are currently not curable. Asymptomatic patients of advanced age or with concomitant illness may require only careful observation without immediate active treatment, especially those with low-grade and early stage tumours.

1.6.1. Treatment strategies.

There are three main management strategies employed for localised prostate cancer: watchful waiting, radiotherapy, and radical prostatectomy.

Watchful waiting is based on the premise that prostate cancer is a minor disease that most people diagnosed with clinical disease die from other causes. The patient is followed and assessed regularly and a decision to treat may be based on a rising PSA or a change in DRE. It is usually recommended for patients with well-differentiated tumours of low volume. Problems arise when trying to identify patients in whom this treatment deferred approach can be safely employed without jeopardising the survival or adversely affecting the quality of life.

1.6.2. Radiotherapy.

Radiotherapy (RT) is an effective treatment for localised prostate cancer, but it is not without its side effects and risks. It involves the delivery of radiation energy to the prostate. Usually it is administered on an outpatient basis using external beam radiation from high energy linear accelerators. The treatment involves up to 50Gy of wide field radiation that includes the pelvic lymph nodes followed by an additional booster to the prostate and surrounding tissues for a total dose of 70Gy. Complications such as delayed impotence and radiation induced rectal damage may occur, however, incontinence is relatively uncommon. Throughout the RT treatment, PSA levels are monitored and should fall to normal within six to twelve months after RT, persistent elevated levels may indicate residual disease and poor prognosis. The energy can also be delivered by placing radioactive seeds in the prostate during a surgical

procedure. Another option is high precision three-dimensional conformal radiotherapy (3D-CTR). This uses a sophisticated computer to accurately conform the distribution of a prescribed radiation dose to the anatomic boundaries of the prostatic target, hence, avoiding the problems associated with exposure of normal tissues to external beam radiation (Philip and Millikan 1995, Pentyala *et al* 2000).

1.6.3. Surgical resection.

Radical prostatectomy is a surgical procedure in which, the prostate, seminal vesicles and ampullae of the vasa are removed and a direct anastomoses of the bladder neck to the membranous urethra is made. Sometimes lymph nodes of the pelvic area are also removed. There are a few potential side effects such as impotence and incontinence due to nerve damage during the surgical procedure. Additionally there is the possibility of haemorrhage, but the risk is small. PSA must by definition become undetectable after curative radical prostatectomy and its presence after this procedure indicates residual prostatic cancer cells.

1.6.4. Endocrine therapy.

In the advanced and metastatic stages of the disease, treatment is based on endocrine therapy, castration (medical or surgical) or complete androgen blockade (administration of an antagonist of the AR in association with medical or surgical castration). Androgen ablation is not a curative therapy in patients with lymph node metastases, but is an effective method of disease control. The duration and response to this therapy is variable and most patients will develop hormone refractory disease (i.e. androgen independent prostate cancer) within

one to two years. For this type of treatment to be effective, the sources of the hormones must be taken into account i.e. the testes and the adrenal cortex. Bilateral orchidectomy (removal of the testes) remains the most effective anti testosterone treatment. Oestrogenic preparations are also used, they exert their anti-testosterone effects by inhibiting the secretion of pituitary lutenising hormone through a negative feedback loop (Philip and Millikan 1995, Frydenberg *et al* 1997). This could bring the hormonal balance back to normal and hence prevent or retard tumour growth.

Another class of anti-androgens includes receptor agonists such as flutamide, which inhibits the negative feedback of androgens on the pituitary. However, the most commonly used approach involves the use of hypothalamic lutenising hormone-releasing hormone (LHRH). These are synthetic hormones administered by parenteral injection to achieve reversibly suppressed levels of testosterone (Philip and Millikan 1995).

Hormone refractory disease is defined by biological progression (increased PSA levels) or clinical progression under androgen withdrawal (testosterone levels below 0.3ng/ml) (Philip and Millikan 1995). Some patients whose disease has progressed on combined androgen blockade may respond to anti-androgen withdrawal or a variety of second line hormonal therapies. Many strategies are used for painful bone metastases, including pain medication, external beam irradiation, corticosteroids, bone seeking radionuclides and bisphosphonates. Chemotherapy can provide some palliative benefit to some patients. Various

chemotherapy regimens have been reported to produce subjective improvement in symptoms and reduction in PSA levels.

1.7. Aetiology of prostate cancer.

The stepwise transition from benign tissue to malignant metastatic prostate cancer is characterised by a multitude of genetic and molecular changes. It is now apparent that a series of genetic alterations transforms normal glandular epithelium from a putative pre-neoplastic state to prostate intraepithelial neoplasia (PIN) and invasive carcinoma (Lara *et al* 1999). Further molecular insults in hormone insensitive disease promote the development of androgen independence. Prostate cancer like all other cancers and unlike normal tissue is characterised by molecular and genetic aberrations that confer proliferative advantage or resistance to programmed cell death (apoptosis). These molecular aberrations will not only provide prognostic information, but also potential therapeutic targets. The diagnosis of prostate cancer relies on the morphological assessment of a tissue specimen by an experienced histopathologist. It is thought that invasive prostate cancer evolves through certain histopathological stages such as hyperplasia, metaplasia, dysplasia and in situ carcinoma. Candidates for putative preneoplastic lesions include intraductal dyspalsia, PIN and atypical adenomatous hyperplasia. PIN is considered as the most likely precursor lesion and its presence has been shown to increase the risk of developing overt prostate cancer (Lara *et al* 1999).

Despite the increasing incidence of prostate cancer, knowledge of the molecular and cellular biology of prostatic adenocarcinoma remains limited compared

with other neoplasms. Also the mechanism of progression from latent to clinically significant cancer remains an enigma suggesting that tumours differ in basic aspects of their biology. The aetiology of prostate cancer is not fully understood. Epidemiological and basic research studies have failed to implicate a single factor possibly because of its multifactorial aetiology. Over the last few decades, basic research in cell and molecular biology has failed to produce any translational breakthroughs for the diagnosis and treatment of prostate cancer or any clues to alter the progression of prostate cancer.

1.7.1. Progression of prostate cancer.

Transition from latent or histological prostate cancer is mediated by germ line mutations. This development seems to be mediated by a variety of processes including loss of heterozygosity (LOH), methylation of promoter regions of certain genes and the mutation of tumour suppressor genes such as P53 and retinoblastoma (Rb) 1. It is difficult to form a conclusion concerning their specificity in the aetiology of prostate cancer based on these observations.

1.7.2. Genetics of prostate cancer.

Loss of heterozygosity studies, the use of comparative genomic hybridisation techniques and allelotyping has identified previously undetected regions of chromosome loss or gain. These techniques have suggested that chromosomes 6q, 8p, 9p, 10q, 13q, 16q, 17p, and 18q are potential sites for genes associated with the initiation of prostate carcinoma. In one study three quarters of the primary prostatic tumours showed evidence of DNA sequence copy number change. Losses were more common than gains. The most common

abnormalities affected 8p and 13q. Chromosome 8p is of interest because it is shown to have loss of heterozygosity in other cancers such as hepatocellular, colorectal and lung. It has also been suggested that loss of 8p may be related to the development of androgen independence. Deletions on both the long and short arm of chromosome 10 have been associated with advanced stages and rapidly progressive disease. Among the genes located on 10q is the PTEN tumour suppressor gene, which modulates signal transduction through its protein phosphatase and its lipid phosphatase gene product. Furthermore, the genetic changes seen in recurrent tumours with frequent gain of chromosomes 7, 8q and X suggest that the progression of prostatic cancer and the development of hormone independent growth may have a distinct genetic basis (Philip and Millikan 1995 and Lara *et al* 1999). Familial clustering of prostate cancer has been attributed to the dominant inheritance of rare high risk allele. This appears to be analogous to predisposing genes associated with inherited cancers of the colon and the breast. Family studies indicate that a region on the long arm of chromosome one (specifically 1q24-25) contains a prostate cancer susceptibility gene (the locus has been called HPC1) (Smith *et al* 1996 and Cooney *et al* 1997).

Although several oncogenes (Ras, Myc and Sis) are expressed with a higher frequency in prostatic cell lines, their overexpression in localised (i.e. early stage) prostatic tumours is uncommon. The role of expression or amplification of oncogenes in prostate cancer remains unclear although some evidence suggests that it contributes to the transformation of preneoplastic lesions into overt carcinoma. In contrast, the loss of function of tumour suppressor genes

appears to play a significant role in prostatic carcinogenesis. The known tumour suppressor genes Rb on 13q and p53 on 17p may play an important role in the progression of prostate cancer. Tumour suppressor gene alterations are the most frequent change observed in prostate carcinoma. Mutations in p53 are considered late events associated with advanced stage and the conversion from a hormone dependent state to a hormone refractory state. In contrast the loss of Rb (a key determinant of cell cycle progression through its interactions with transcription factors) appears to be an early event in prostate carcinogenesis. The tumour suppressor DCC (deleted in colon cancer) encodes a protein that is homologous to the neural cell adhesion molecules and is involved in cellular processes such as wound healing, embryogenesis, immunoreaction and tumour progression or metastasis. It has been found to have decreased expression in both prostate cancer cell lines and patient tissue; this low level expression or complete deletion has been found in oesophageal, gastric, pancreatic and colorectal carcinomas (Philip and Millikan 1995 and Lara *et al* 1999).

1.7.3. Role of growth factors in disease progression.

Peptide growth factors influence cellular proliferation through their autocrine and paracrine actions. As prostate cancer achieves androgen independence, these growth factors may assume critical roles in tumour progression. The abnormal expression of peptide growth factors and their receptors may contribute to the growth and development of both local and metastatic prostate cancer. The TGF, EGF and IGF families are the most widely investigated. EGF and epidermal growth factor receptors (EGFR) have roles in normal prostatic development and differentiation. EGF is known to be mitogenic in normal and

malignant cancer cells (Philip and Millikan 1995, Lara *et al* 1999). There is enhanced EGF and co-expression of EGFR in human prostatic tumours. TGFβ inhibits epithelial growth through a heteromeric complex of two kinases (TGFβ receptor I and II), which in turn are modulated by the extracellular matrix, androgens and other growth factors (Philip and Millikan 1995). Either may be inactivated or downregulated which may lead to cancer development. Tissue containing BPH and several prostate cancer cell lines express higher levels than normal of TGFβ as well as TGFα and EGFR. The fibroblast growth factors (FGFs), which have been isolated from prostatic tissue, have also been implicated in prostate cell growth. Recently FGF-2 was found to be significantly increased in localised prostate cancers when compared with uninvolved prostate tissue. Also there was an increase in the expression of the FGF receptor. This receptor overexpression was also shown to correlate with poorer tumour differentiation (Philip and Millikan 1995 and Lara *et al* 1999) meaning that the resultant tumour is more likely to be malignant and more aggressive. The members of the IGF family have also been shown to have mitogenic and anti-apoptotic effects on normal and transformed prostate epithelial cells. IGF-1 has been implicated as an important factor during the initiation and early progression of prostate cancer, whereas advanced disease seems to be IGF-1 independent (Wolk *et al* 2000). As mentioned in section 1.2.3.3. increased IGF-1 levels have also been shown as a definitive risk factor.

The effects of IGF are mediated through the IGF-1 receptor, which is also involved in cell transformation induced by tumour virus protein and oncogene products. Six IGF binding proteins (IGFBPs) can inhibit or enhance the actions

of IGFs. It has been shown that IGFs exerts strong and anti-apoptotic actions on various cancer cells. IGFs also act synergistically with other mitogenic growth factors and steroids and antagonise the effect of anti-proliferative molecules on cancer growth (Yu and Rohan 2000).

Other members of the tyrosine growth factor receptor family related to EGFR include the HER-2/neu (c-erbB-2) (amplification of this gene in breast cancer has been shown to be a significant predictor of relapse or inferior overall survival) and c-erbB-3 oncogenes. The amplification of expression of HER-2/neu has been demonstrated in 30 to 70% of prostate carcinomas and is associated with androgen independent prostate cancer growth and correlates with increased probability of disease recurrence. Increased PSA levels have been observed in prostate cancer cells expressing HER-2. It thus appears that the HER-2 family of receptors, which plays a significant role in the progression of breast cancer, may also participate in prostate cancer (Lara *et al* 1999). The increased expression of HER-2/neu and c-erbB-3 has also been demonstrated in PIN and primary prostate cancer and in matching metastases from the same patients. Within the group of primary prostate tumours there is a positive correlation between stage and Gleason grade (see section 1.5.) of tumour and immunohistochemical expression of HER-2/neu.

1.7.4. Oncogenes.

The proto-oncogenes MET and RET have tyrosine kinase products that have been implicated in renal and prostatic development (foetal). Met has been described in both prostate cancer and PIN, it has been proposed as a possible

tumour marker for the early progression of human prostate cancer. RET has been demonstrated to be overexpressed in high grade PIN and prostate cancer as compared to normal prostate epithelium.

The progression of prostatic tumours to a hormone refractory state is frequently associated with the expression of the anti-apoptotic gene bcl2 (Philip and Millikan 1995).

The expression of the surface adhesion molecule crucial for normal differentiation, E-cadherin, has been mapped to 16q and was found to be absent in almost fifty percent of prostatic tumours, which correlated with stage and grade and overall survival. Loss or methylation of the promoter leading to lack of E-cadherin expression has been described in both prostate cancer cell lines and tumour tissue.

Tumour induced angiogenesis is an essential step in the progression of malignant neoplasms and the development of metastases.

Both benign and malignant prostatic diseases are influenced by interactions between cells in the stromal and epithelial compartments. It has been shown that the presence or absence of mesenchymal cells affects the *in vivo* and *in vitro* behaviours of prostatic epithelial cells.

Recognition of premalignant lesions in the prostate may permit the identification of a high risk population that might benefit from early screening. Because PIN is much more common in prostate glands with invasive carcinoma, this type of growth has been designated a premalignant lesion. Another

premalignant lesion is atypical adenomatous hyperplasia or prostatic adenosis (Philip and Millikan 1995).

A number of genetic changes have been documented in prostate cancer ranging from allelic loss to point mutations and changes in DNA methylation patterns. The most consistent changes are those of allelic loss events with the majority of tumours examined showing a loss of alleles from at least on chromosomal arm. The short arm of chromosome 8 followed by the long arm of chromosome 16, seem to be the most frequent regions of loss suggesting the presence of novel tumour suppressor genes. Deletions of one copy of RB and TP53 genes are less frequent as are the mutations of the TP53 gene and accumulating evidence suggests the presence of an additional tumour suppressor on chromosome 17p which is frequently inactivated in prostate cancer. Alterations in E-cadherin/α catenin mediated cell to cell adhesion mechanism appear to be present in almost half of all prostate cancers and may be critical to the acquisition of metastatic potential of aggressive prostate cancers (Isaacs *et al* 1995).

The prostate grows and functions in a multihormonal environment responding to a variety of regulatory factors (some of which have been mentioned above in sections 1.1.3. and 1.2.3.3.). The functional activity of the prostate depends on the maintenance of normal plasma testosterone concentrations. This is usually derived from the Leydig cells of the testes and converted to DHT within the prostate these steroids can also be derived from the adrenals. This particular source of DHT has been implicated in the progression of disseminated prostatic carcinoma after primary endocrine therapy. The interactions of the DHT-AR

complex allows it to modulate or initiate the transcription of proteins like PSA, growth factors intimately concerned in the control of cellular growth processes and also the proto oncogenes such as Fos Jun and Myc.

The role of androgens in the development of prostate cancer is obvious. Early prostate cancer is endocrine dependent and arises from those epithelial cells capable of being stimulated by DHT but it does not appear to arise from atrophic cells. More support for the influence of androgens on prostate cancer comes from the observance that eunuchs and patients with 5α-reductase deficiency do not develop prostate cancer and that castration produces a dramatic regression of cancer growth. Advanced metastatic cancers treated with androgen ablation therapy often continue to progress, which suggests that these cells can become hormone independent and resume growth, invasion and metastasis. The transition from androgen sensitive to androgen insensitive is poorly understood. Figure 1.3. shows a possible multistep model for prostate cancer including some of the aetiological factors mentioned in section 1.7.

Figure 1.3.

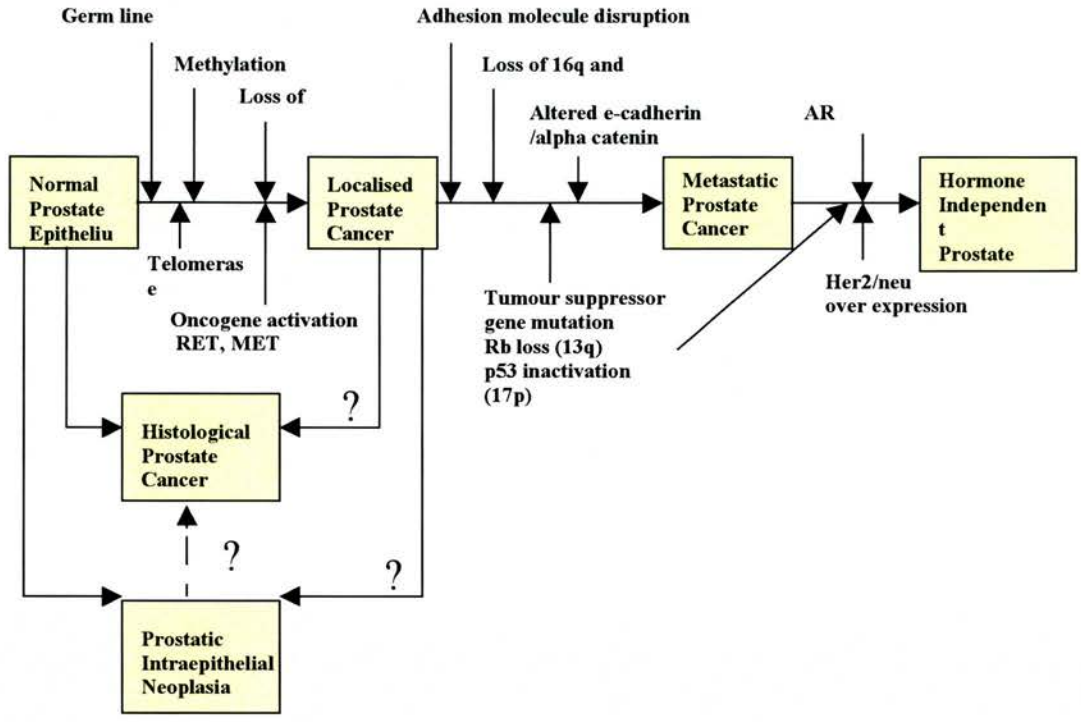


Figure 1.3. Multistep carcinogenesis model for prostate cancer.

A possible sequence of events involved in the development of prostate cancer showing a progression from normal prostate epithelium to metastatic and hormone independent disease (Adapted from Isaacs et al 1995 and Lara et al 1999).

1.8. Cancer and the cell cycle.

The cell cycle involves a step by step progression through a series of molecular events, each of which is required to allow the cell to move on to the next phase. Abnormalities in the molecules that regulate the cell cycle are common in cancer and offer the opportunity both for therapeutic targets and for prediction.

The cell cycle consists of an organised series of controlled events ultimately leading to cell division and is induced by a diverse number mitogens for example, cell type specific cytokines. It can be divided into four distinct phases: G1, where a succession of molecular events occur committing the cell to progression through the cycle (this generally lasts for approximately twelve hours); S-phase where DNA replication is undertaken (six to eight hours in length); G2 where replication is checked and preparations are made for division (three to six hours in duration); M-phase (mitosis) culminates in cell division (lasts approximately thirty minutes). The timings mentioned vary with cell type and growth conditions. When cells are metabolically active but not progressing to or through the cycle they are referred to as quiescent or in G0 (Shackelford *et al* 1999). Figure 1.4. is a simple diagrammatic representation of the steps mentioned above.

Figure 1.4.

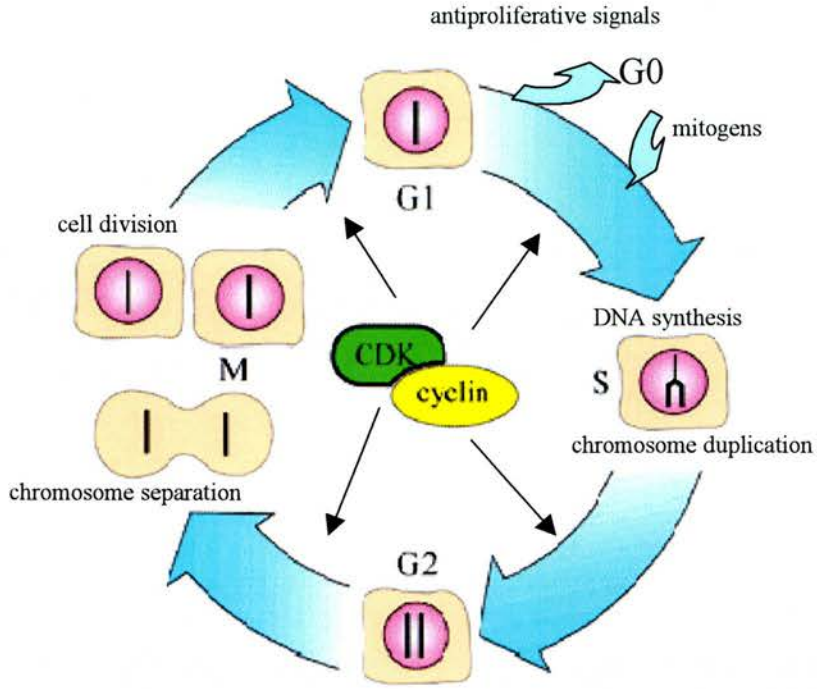


Figure 1.4. Diagram of the cell cycle.

The diagram shows the four distinct phases of the cell cycle and some of the important events involved in cell division. (Adapted from http://www.nobel.se/medicine/laureates/2001/press.html)

In mammalian cells, progression through the cycle is driven by a series of cellular phosphorylations mediated by cyclin dependent kinases (CDKs), which are activated by association with cyclins, (these can be associated by phosphatase dephosphorylation and by proteolysis). Cyclins expression levels change during different cycle phases. These associated complexes are continuously being formed and activating or inactivating a series of biochemical pathways. Until the checkpoint machinery receives the appropriate signals, transition from one phase of the cycle to the next is halted. The major role of

checkpoint control is to minimise somatic genetic alterations and or events affecting cellular survival (Nojima 1997). Understanding defects in the status of the cell cycle and its checkpoints in tumours is crucial in determining their progression and for designing appropriate therapies.

Multiple genetic changes occur in the development of cancer. The evolution of normal cells to cancer cells is facilitated by a loss in fidelity of DNA replication and maintenance. If one or more of the cell cycle checkpoint components are mutated the chances of genetic instability during one round of the cell cycle increase accordingly with consequent acceleration of cellular evolution to the cancerous state. Cancer cells may acquire independence from mitogenic signals normally necessary for cell cycle progression; they do not require adherence to specific surfaces in order to divide. The cycle of cancerous cells may also be faster than their normal counterparts; this uncontrolled proliferation can be explained by gain or loss of function of proteins that constitute the cell cycle machinery.

1.9. Radiosensitivity assays and cancer predisposition.

The study of DNA damage at the chromosome level is important, as chromosomal mutation is a major event in carcinogenesis. Physical and chemical agents can cause this DNA damage, for example, exposure to ionising radiation can induce major alterations. Ionising radiation was first demonstrated to be mutagenic by Muller in 1927. Since then it has been demonstrated to induce mutations and cause cancer in a dose dependent manner. Ionising radiation damages DNA through direct and indirect mechanisms. Direct damage

to DNA occurs as a result of the interaction of radiation energy with DNA. (Shackelford 1999). Evidence suggests that chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level (Evans 1977, Savage 1993, and Fenech 2000). These structural chromosomal changes can lead to activation of proto-oncogenes and elimination of tumour suppressor genes (Heim and Meitelman 1996, Scott *et al* 1998).

Both exogenous and endogenous damaging agents are continually assaulting the DNA of mammalian cells. Radiations from the sun and other external sources, chemical mutagens in the atmosphere, elements contained in food or drugs, as well as the body's normal metabolites such as, hydrogen peroxide and its derivative •OH, can produce DNA lesions. These lesions if not repaired, can have serious consequences, resulting in infidelity of replication, mutations, neoplastic transformation and even cell death. Multienzymatic cellular repair mechanisms have evolved to deal with these lesions. Monitoring and repair of DNA damage sustained during the G2 phase, just before mitosis and distribution of chromosomes to daughter cells, appears to play an important role in carcinogenesis (Parshad *et al* 1996).

Techniques such as the G2 assay and the cytokinesis block micronucleus (CBMN) assay have been developed as a way to induce and visualise these lesions and make inter and intra-individual comparisons. They enable the measurement of chromosome damage incurred at different phases of the cell cycle. Both techniques have been used successfully in population monitoring and early detection of groups susceptible to cancer development.

1.9.1. G2 assay.

In the G2 assay cells are subjected to ionising radiation as they approach mitosis, (the G2 phase) the cells are then arrested at metaphase, and chromosome spreads made, where the damage incurred appears as visible discontinuities of chromatid structure. These discontinuities/aberrations are then scored in metaphases where all forty-six chromosomes are present and unobscured.

Figure 1.5.

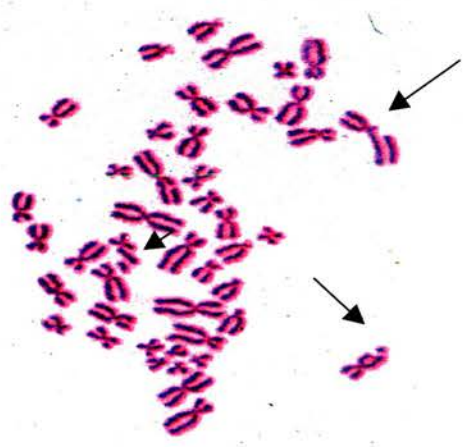


Figure 1.5. A metaphase spread.

Metaphase spread from human peripheral blood lymphocyte culture, showing damage incurred (some of which is indicated by the arrows) as a result of irradiation (0.4Gy) in the G2 phase of the cell cycle. Picture x63 magnification. (Image courtesy of Dr Bryant).

1.9.2. The cytokinesis block micronucleus assay.

The CBMN assay quantifies the post mitotic micronuclei (MN) (an expression of damage incurred at G0) (see Appendix III, Figure III.1. for the various

possible fates cytokinesis blocked cells). MN require one cell division to be expressed, they consist of small amounts of DNA that arise in the cytoplasm when chromatid and chromosomal fragments or whole chromosomes are not incorporated into the daughter nuclei during mitosis (see Appendix III, Figure III.2. for the origin of micronuclei). Thus the CBMN provides a reliable measurement of both chromosome loss and chromosome breakage. Hence, it has emerged as one of the preferred methods for assessing chromosomal damage.

The use of micronuclei to measure chromosome damage was first proposed by Countryman and Heddle (1976) and has subsequently improved with development of the CBMN assay, which allowed micronuclei to be scored specifically in cells, which had completed nuclear division. It was originally described for cultures of isolated lymphocytes (Fenech and Morely 1985 and 1986) and then subsequently adapted for whole blood cultures and applied to and adapted for other cell types. An absolute value for the micronucleus frequency can only be obtained in cells that have divided only once (Fenech 1997). This is because with subsequent divisions following DNA damage, the micronucleus frequency declines.

For the CBMN assay, the cells are irradiated in G0 and stimulated to divide by mitogens such as phytohaemagglutinin (PHA). Cytokinesis is blocked by agents such as cytochalasin B a fungal metabolite of Helminthosporium dematioideum which acts by inhibiting actin polymerisation preventing the formation of the cytokinesis ring thus, cytoplasmic division. Binucleate (BN) cells are

accumulated in their first division cycle. These are scored for micronuclei; this provides an assessment of chromosomal damage. Scoring of micronuclei is predicted to provide consistent results under all culture conditions (Fenech *et al* 2000).

Figure 1.6.

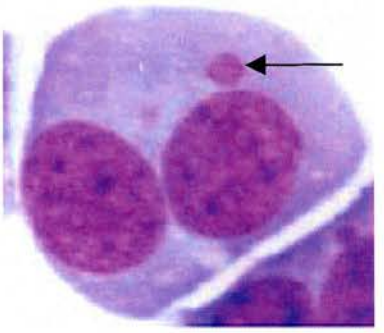


Figure 1.6. Cytokinesis blocked binucleate lymphocyte with micronucleus. *Picture of a binucleated cell exhibiting chromosomal damage in the form of a micronucleus. The micronucleus is indicated by the arrow. (Image adapted from http://www.bio.2001.csiro.au/Biomarkers.htm.)*

1.9.3. Uses and implications of the radiosensitivity assays.

This evaluation of DNA damage induced by radiation in the cell cycle is of potential significance. It can be used to assess individuals genetically predisposed to cancer and also in the identification of radiosensitive individuals within the normal population and highlighting those that are more susceptible to damage or who do not repair it as efficiently.

These assays mentioned can detect small differences in chromosomal radiosensitivity. In addition, there are several different mechanisms leading to chromosomal radiosensitivity, including defects in DNA repair (Preston 1980, Parshad *et al* 1983), cell cycle checkpoint control (Little and Nagasawa 1985, Wang *et al* 1996), differences in chromatin structure (Mozdarani and Bryant 1989, Hittelmann and Pandita 1994) and in the premitotic elimination of potentially clastogenic damage by apoptosis or premature cell senescence (Schwartz *et al* 1995, Wang *et al* 1996, Williams *et al* 1997).

An association between the hypersensitivity of cells to ionising radiation and cancer predisposition was first demonstrated for patients with ataxia-telangiectasia (A-T) a recessively inherited multisystems disorder (Taylor *et al* 1975). Radiation induced chromosome damage assays such as those mentioned in sections 1.9.1 and 1.9.2. showed the greatest discrimination between A-T patients and normal controls (Taylor 1983). Elevated sensitivity to the chromosome damaging effects of ionising radiation is a feature of a number of heritable conditions and many cancer prone conditions. Evidence has been accumulating that cells (including, peripheral blood lymphocytes, fibroblasts and epithelial cells) derived from individuals with heritable cancer prone conditions exhibit significantly higher incidence of chromosome damage following irradiation in comparison with normal healthy controls. Sanford *et al* (1989) reported that G2 phase cells from fifteen of these heritable cancer prone conditions exhibited higher yields of induced chromosome damage (aberrations two or three fold higher). This has been further expanded and demonstrated more recently in twenty other inherited cancer prone conditions (listed in Scott

et al 1996, Scott *et al* 1998). This suggests that defects in the processing DNA damage of the type induced by ionising radiation could contribute to cancer predisposition in these rare conditions.

In 1994, Scott *et al* reported that using PHA stimulated peripheral blood lymphocytes they could discriminate between A-T heterozygotes and normal controls according to the number of chromosome aberrations induced by low doses of radiation (the G2 assay). They then conducted this assay on bloods obtained from breast cancer patients and established that they exhibited G2 sensitivity similar to the A-T heterozygotes. On average, the lymphocytes of breast cancer patients are more sensitive to the induction of chromosome damage than normal healthy controls, whether the cells are irradiated in either the G2 or G0 phase of the cell cycle (Scott *et al* 1994, 1998 and 1999).

Using the G2 assay and the 90^{th} percentile of the control population as a cut off point for sensitivity 42% of untreated breast cancer patients exhibited sensitivity. Various researchers (Parshad *et al* 1996, Helzlsouer *et al* 1996, Patel *et al* 1997, Terzoudi *et al* 2000, Riches *et al* 2001) have confirmed these findings in breast cancer patients. Using the G0/CBMN assay and the same criteria for sensitivity, 27% of patients showed elevated radiosensitivity (Scott *et al* 1999).

No correlation between the aberration yields of the G2 or G0 assay was found when the assays were performed on the same patients. This suggests that cellular defects leading to enhanced sensitivity are different in these cell cycle

stages and that different mechanisms are in operation. Each patient appears to be defective in only one mechanism, possibly via mutations (or polymorphisms) of a single gene in a substantial proportion of patients (Scott *et al* 1999).

The degree of chromosomal radiosensitivity displayed by the G2 assay is an inherited characteristic within breast cancer families. Those with the radiosensitive phenotype have an associated increased lifetime of developing breast cancer. This characteristic could be attributed to the segregation of one or two genes in each family (Roberts *et al* 1999, Scott *et al* 2000). There is also preliminary evidence that elevated sensitivity in the G0/CBMN assay is a heritable trait in first-degree relatives of breast cancer patients (Burrill *et al* 2000).

These observations lead to the suggestion that such chromosomal radiosensitivity may be a marker for cancer predisposing genes. This hypothesis is supported by the exhibition of this type of elevated radiosensitivity in many inherited cancer prone conditions (aforementioned reviewed in Scott *et al* 1999). There is a contrasting situation in the breast cancer studies, because these rare syndromes have highly penetrant gene defects. However, it has been proposed that the defects leading to the enhanced radiosensitivity seen are associated with low penetrance genes, therefore a lesser risk of cancer. There is good epidemiological evidence that the inherited risk of breast cancer is greater than can be accounted for by mutations in the highly penetrant genes BRCA1, BRCA2 and TP53 (Teare *et al* 1994, Chen *et al* 1995, Houlston and Peto 1996, Lichtenstein *et al* 2000, Peto and Mack 2000, Papworth *et al* 2001). This

chromosomal radiosensitivity has also been more recently demonstrated in a significant proportion of patients with no obvious family history (Terzoudi *et al* 2000, Burrill *et al* 2000). There is also indirect evidence for the existence of low penetrance, inherited, predisposing factors for cancers other than breast. The assays have been used to investigate head and neck cancer (Papworth *et al* 2001), hereditary non-polyposis colorectal cancer (HNPCC) (Franchitto *et al* 2001), glioma (Bondy *et al* 2001) and Terzoudi *et al* (2000) tested various types of cancer including 20 prostate cancer patients.

Other uses for applications have been proposed for these chromosomal radiosensitivity assays. Riches *et al* (2001) correlated the G2 scores of untreated breast cancer patients with the Nottingham prognostic indicator and results implied that besides influencing the risk, the genetic factors determining G2 sensitivity also influence tumour characteristics and patient prognosis. Results of a study by Rothfuβ *et al* (2000) supports the usefulness of induced micronucleus frequencies as biomarkers for cancer predisposition and suggests its application as a screening test for carriers of BRCA1 mutation in breast cancer families. The CBMN assay has already been thought applicable for use in determining individual intrinsic radiosensitivity in prostate cancer patients before the start of radiotherapy treatment (Lee *et al* 2000 and 2003) this may enable the individualisation of dose.

Individualisation of cancer radiotherapy based on predictive radiosensitivity assays could lead to an improvement of in the results of treatment and may help to identify those patients at risk from complications. The CBMN has been

proposed as for use as part of a work up (Catena *et al* 1996, Widel *et al* 2003). Lee *et al* (2000) noted that MN frequency induced by irradiation was considerably higher in two of three prostate cancer patients with unusually severe acute side effects. Widel *et al* (2003) study presents a significant correlation between the radiosensitivity of lymphocytes from cervical carcinoma patients (as determined by the CBMN) and the severity of acute and late normal tissue reaction in patients treated with radiotherapy. However, would not recommend the method for routine clinical use, as the predictive value is limited. The overlap of the MN frequencies for people with differing reactions is too great. Ozen *et al* (2000) conclude from their research into non-random chromosomal aberrations in peripheral blood lymphocytes (PBL) from prostate cancer patients that they can correlate these aberrations with specific clinical parameters and that these correlations can be used to identify a prostate cancer patient's risk response to therapy.

1.9.4. Cellular responses to ionising radiation and defects leading to enhanced chromosomal radiosensitivity.

Enhanced chromosomal radiosensitivity is a trait of several defined human disorders. The best characterised of these are ataxia-telangiectasia (A-T) and Nijmegen breakage syndrome (NBS). They arise from single gene defects involving mutation in DNA repair or checkpoint pathways. The genes involved, the ATM gene in A-T and the NBS gene in Nijmegen breakage syndrome are known to be involved in cellular signalling of damage. There is also considerable inter-individual heterogeneity within the general population in reaction to ionising radiation (IR) (examples of this can be found in Scott *et*

*al*1994, 1999 and Riches *et al* 2001). Part of this variation is thought to be due to genetically determined intrinsic differences in cellular or chromosomal response to IR damage (Fernet and Hall 2004).

Exposure to IR results in several different types of DNA damage such as specific base damage and DNA strand breaks. DNA double strand breaks are considered to be the most critical lesion for cell death (Dikomey *et al* 2003) and chromosomal aberrations (Bryant *et al* 2004). Mammalian cells possess several enzymatic processes for repairing double strand breaks. Non-homologous end joining (NHEJ) is the major mechanism. This involves the binding of the heterodimeric Ku proteins Ku70/Ku80 to the double stranded DNA ends. DNA bound Ku recruits the DNA dependent protein kinase catalytic subunit (p450) and activates its kinase activity. The XRCC4 gene product (p38) is also involved in the holoenzyme complex and DNA ligase IV seals the double strand break. Cell lines with mutations in any of these genes are radiosensitive and show marked deficiencies in double strand break after IR (Kühne *et al* 2004). Homologous recombination (HR) also contributes to double strand break repair rejoining in the mammalian cell. This mechanism requires the homology of the sister chromatid or chromosome homologue and possibly also DNA sequences that possess extensive homology to the damaged sequence. HR has generally been regarded as a minor pathway, but recent evidence has demonstrated that it might be important in maintaining chromosome stability in the absence of exogenous damage (Li *et al* 2001).

Enhanced chromosomal radiosensitivity derives from and inability to recover from DNA damage either because of a reduction in damage detection or repair processes (as proposed by Parshad *et al* 1996), this leads to a higher level of residual chromosomal damage (Kühne *et al* 2004). The importance of these damage response mechanisms to the maintenance of genomic integrity is highlighted by an array of heritable disorders attributable to defects in proteins that function in these mechanisms (O'Driscoll *et al* 2004).

In addition to these repair pathways, DNA lesions are also recognised by components of the cell cycle checkpoint pathways. Together DNA repair and DNA damage cell cycle checkpoints constitute the main defence system for maintaining genetic integrity in the event of DNA damage. Chromosomal aberrations (chromatid breakage induced during the S- and G2-phase of the cell cycle and chromosome breakage at G0) provide the link between primary damage and the end points of cell death and cellular transformation (Bryant 2004).

1.10. Epithelial cell culture.

1.10.1. Senescence.

Cell culture is essential to many biological studies. Isolating a specific cell type and examining it *in vitro* opens up many research avenues and may be advantageous in answering fundamental questions. However, the use of normal human cells is restricted in part by their limited proliferative potential. Normal somatic cells cease proliferation after a predetermined number of divisions, a phenomenon termed replicative senescence (an irreversible phase of growth

arrest from which the cells might be directed to apoptosis). This determination of life span was first identified by Hayflick and Moorhead (1961) and is still not clearly understood. They also proposed that each cell contained a timing mechanism, (or retained memory) to measure its lifespan. Hence, bringing the conclusion that senescence is a function of the cumulative number of cell division cycles and that the replicative limit is directly related to the number of population doublings and or more precisely DNA replications. More recently, this idea has been supported by the discovery of the capped ends of the chromosomes known as telomeres. At each round of cell division these telomeres are eroded, inducing senescence when a critical length is reached. The erosion is due to the physical constraints of the DNA copying machinery, the end replication problem.

1.10.2. Telomeres and telomerase.

The chromosome ends in all eukaryotic species have specialised tracts of repetitive non coding DNA sequences that together with associated proteins are known as telomeres. They act to stabilise and protect the ends of chromosome from a range of catastrophic consequences including degradation by endogenous exonucleases, end to end fusion and abnormal recombination events, they anchor chromosomes within the nucleus and assist the replication of linear DNA (Cerni 2000, Neidle and Parkinson 2002). Telomeric DNA is typified by simple tandem repeats of guanine rich sequences, human telomeres possess over a thousand copies of TTAGGG hexanucleotide repeats (that are conserved across the vertebrates). These repeats are not fully replicated during

S-phase, which ultimately leads to a loss of telomeric DNA during a cells life (Blackburn *et al* 1978, Wright *et al* 1997).

The 3' GT rich terminus of eukaryotic telomeric DNA is typically a hundred to two hundred bases long and is single stranded (Neidle and Parkinson 2002) (this is classed as an overhang). This is produced by the inability of the DNA replication machinery to copy the final bases of the lagging strand (the end replication problem) and also contributed to by the action of 5'-3' exonuclease that degrades the CA rich strand. The result of this strand recession is telomere shortening in the next round of DNA synthesis, because of the reduced template for leading strand synthesis (Colgin and Reddel 1999). Typically, fifty to two hundred bases are lost at each round of division (Neidle and Parkinson 2002). This shortening has been proposed as the timing mechanism for cellular ageing. Once the telomeres have reduced to a predetermined critical length, (usually after twenty to thirty generations) senescence ensues (Huschtscha and Reddel 1999). However, the telomere repair plays a role in development and also their maintenance is useful (essential for) in stem cells and germ cell lineages, so mechanisms have evolved to counteract the replication associated loss of telomeric DNA, an example is telomerase.

Telomerase is the most studied and important of the telomere repair/maintenance systems. The enzyme was first identified in ciliates (Greider and Blackburn 1985) it is not detectable in most normal human tissue, but is present in at least 80 to 85% of tumour cells (Yasumoto *et al* 1996, Neidle and Parkinson 2002). Although its activity has been identified in mouse and primate

models during brain development (Klapper *et al* 2001) and also earlier in embryogenesis during differentiation of embryonic stem cells (Hiyama *et al* 2001, Armstrong 2000). Telomerase is a multisubunit ribonucleoprotein enzyme complex generated from two principal genes and a host of ancillary proteins (Nugent and Lundblad 1998). It is a novel reverse transcriptase comprised of a catalytic subunit TERT which uniquely carries its own associated RNA template (termed hTR in humans) with this it can catalyse the addition telomeric DNA repeats onto the G-rich 3' single strand, thus extending the overhang. The catalytic subunit of human telomerase (hTERT) was identified in 1997 (Colgin and Reddel 1999) and has been mapped to chromosome 5p15.33 (Cerni 2000). (hTERT also has sequence homology with the viral reverse transcriptases with an active site containing a triad of aspartate residues). The RNA template captures the terminal 3' end of the telomere by hybridising it to the templates complimentary sequence (Neidle and Parkinson 2002).

1.10.3. Immortalisation.

Immortalisation is a process that interferes with the mechanisms associated with cellular ageing enabling cells to escape normal constraints thereby extending their lifespan. Hence, it is crucial to carcinogenesis as it allows cells to undergo more cycles of cell division, thus providing the time necessary for accumulation of mutations. Experimentally, many of the same genetic changes that lead to abnormal cell proliferation conspire to confer replicative immortality upon cells in culture (Price *et al* 1999). The generation of an immortalised phenotype is not dependent upon single events but is brought about by a combination of changes.

Three phases define the development of an immortalised phenotype, prolonged proliferation, crisis, then the rare event of escape (Colgin and Reddel 1999).

In terms of research it would be ideal to have unlimited numbers of carefully characterised cells with the desired phenotype. The most commonly used methods to achieve this objective so far have been the establishment of tumour derived cell lines or the transduction of normal cells with genes from DNA tumour viruses such as simian virus 40 (SV40), a human papilloma virus (HPV) or Epstein-Barr virus (EBV). Although tumour derived cell lines are useful and have yielded a great deal of information on senescence and immortalisation as well as generating numerous useful lines, they have limitations. Transformation induced by DNA tumour-virus often results in loss of aspects of the differentiated phenotype, the proteins they encode disrupt many cellular processes and may cause undesirable changes in the immortalised line including loss of differentiated properties and loss of normal cell cycle checkpoint controls. Therefore there is a need for the development of methods that allow normal cells to avoid senescence without losing other normal properties (Yeager and Reddel 1999).

Nevertheless, studying these cell lines has elucidated genetic mechanisms that restrict the proliferative potential of normal cells in culture. For example the p53 tumour suppressor gene is important in normal senescence, the retinoblastoma (Rb) gene and $p16^{INK4a}$, (an inhibitor of the phosphorylation of the Rb protein (pRb)) are involved in restricting proliferative potential.

According to the telomere hypothesis of senescence, the cell division counting mechanism that determines when a cell should permanently stop dividing is the progressive shortening of the telomeres. This happens in normal cells with each cell division, that is, that senescence occurs when the telomeres have shortened below a preset length. Cells that permanently escape from all limitation on proliferative potential have been found to evade this normal telomere shortening by either activating the enzyme telomerase or by an alternative mechanism of telomere maintenance (ALT). Following the cloning of genes encoding components of telomerase, it has been shown that expression of exogenous telomerase at least in some normal cells may result in a remarkable increase in their ability to proliferate in culture (Yeager and Reddel 1999).

It now seems possible that some of those unwanted changes caused by other immortalisation protocols might be avoided by the induction of telomerase activity. The ability to do this resulted from cloning the human gene for the telomerase subunit hTERT. Although the telomerase holoenzyme contains multiple subunits, the availability of hTERT appears to be limiting in many normal cells, such that expression of exogenous hTERT is sufficient to induce telomerase activity.

The generation of an immortalised phenotype and particularly the escape from the inevitable onset of senescent crisis is closely linked with an active telomerase maintenance system. Although adult human cells do not have active telomerase, the expression of hTER (RNA component) is retained. Co-expression of hTERT and hTER in rabbit reticulocyte lysates resulted in

telomerase activity demonstrating that these proteins were key to telomere length (Weinrich *et al* 1997). This concept was transferred to cell models, such as human fibroblasts where the mechanism was reactivated simply by exogenous expression of the catalytic subunit hTERT (Beattie *et al* 1998, Nakayama *et al* 1998). The presence of an active telomerase system is sufficient to rescue the cells from the crisis phase associated with immortalisation (Wen *et al* 1998) it is also able to produce an immortalised phenotype in rodent and human epithelial cells.

Figure 1.7.

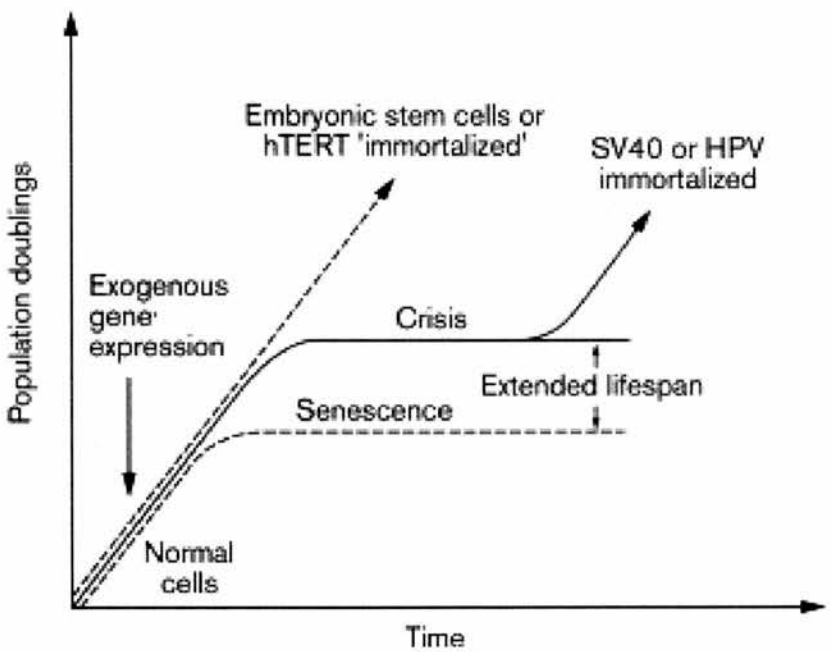


Figure 1.7. Graph showing normal proliferation and lifespan extension. *Graphical illustration of the proliferation of normal cells. Lifespan extension by transformation, and rare crisis escape. Increase in lifespan by forced hTERT expression and possible immortalisation. (Graph from Yeager and Reddel 1999).*

The first reports of lifespan extension by activating telomerase in normal cells described the expression of hTERT in both BJ foreskin cells (Varizi and Benchimol 1998) and retinal pigment epithelial cells (RPEs) (Bodnar *et al* 1998). Follow up reports indicate that some of these cells are able to continue to proliferate at least for two or three fold more population doublings than their normal counterparts. They have stable telomeres and do not display any of the karyotypic and phenotypic alterations characteristic of oncogenic immortalisation. Subsequently, hTERT was also shown to extend the lifespan of some breast epithelial cells. They also proved to be non-tumourigenic and free from phenotypic abnormalities (Yaswen and Stampfer 2002). Determining whether these cells have truly unlimited proliferative potential will have major implications for our understanding of the molecular genetics of immortalisation, but the magnitude of the increase in lifespan in the BJ fibroblasts is sufficiently great that for most practical purposes they may be regarded as immortal cell lines. Many other cell types are currently being transduced with hTERT expression vectors, this will hopefully provide a wide spectrum of normal somatic cells from which much information can be gleaned. However, it appears that for some normal cell types that hTERT alone is insufficient for extension of proliferative lifespan (Yeager and Reddel 1999).

1.10.4. Retroviral mediated immortalisation.

Retroviruses integrate into the genomic DNA of cells where they replicate and are transmitted to all the progeny. This means that they can be used as vectors to gain entry into the cell and insert genes of interest. For successful retroviral transfer dividing cells are required. Retroviral vectors have advantages when

compared to many other gene transfer systems, a wide range of cell types from different species can be transduced, genetic material carried by the vector can be precisely integrated into the recipient cells and the transduced genes are expressed at high levels. Retroviral transfer also has another advantage over transfection, the expression of retrovirally transduced genes only occurs after the integration of the viral genome.

1.10.5. Retroviruses.

Retroviruses comprise a large diverse family of enveloped RNA viruses, which includes lentiviruses such as human immumodeficiency virus (HIV) and traditional retroviruses such as Moloney Murine Leukaemia virus (MoMLV). The single strand RNA genome is encapsulated into the virion, this mediates the transfer of the viral genome to the hosts cell. After penetration, the genome is transcribed to double stranded (ds) DNA and subsequently integrated into genome of the cell.

The viral life cycle proceeds in discrete phases, defined by the expression of different viral genes. The wild retroviral genome consists of a promoter region called the 5' long terminal repeat (LTR) a packaging signal known as ψ, the structural viral proteins gag and pol, envelope proteins and at the end, the 3' LTR, which contains a polyadenylation signal. The LTRs regulate viral gene expression and therefore replication and pathogenesis. The main enhancer of viral transcription is the 5'LTR however, the 3'LTR may function as an enhancer when the 5'LTR is deleted or damaged. The viral genome is packaged into the capsid with a tRNA, which functions as a template for the replicative

phase of the viral life cycle. During this replicative phase, the viral genome is reverse transcribed into the dsDNA molecule known as the provirus, which is integrated into the host cell genome. The viral LTR contains signals involved in integration of the viral genome at random sites within the host genome. After integration of the wildtype genome, the expression of the viral proteins begins for the new generation of viral progeny.

The ability of viruses to infect different species is called tropism. The tropism of the virus is conferred by the envelope, which mediates the entry of the virus to the host cell usually via a specific cell surface receptor (Miller and Chen 1996, Uckert *et al* 1998). The amphoteric enveloped retroviruses infect human and rodent cells, however, the envelope is quite fragile and does not allow long term storage of viral supernatants. Substituting the vector carrying the envelope gene can easily change the envelope protein in the recombinant retrovirus. The MoMLV virus used was pseudotyped with the vesicular stomatitis virus (VSV)-G (protein) envelope, which has two advantages over the natural MoMLV. The VSV-G envelope protein does not use a specific vector for entry into the host cell, instead its docking and uptake is mediated by general components of the plasma membrane common to all species (Roberts *et al* 1999, Picard-Maureau *et al* 2003). The VSV-G envelope is extremely stable, which allows the concentration of retroviral supernatants by ultracentrifugation and their storage at -80°C with minimal loss of titre (Yang *et al* 1995).

Most recombinant viral technology available is based on the MoMLV and has been developed over the last twenty years. The different components of the

wildtype retroviruses are separated in to three different vectors. The gag-pol proteins and the envelope are removed from the viral genome and placed under the control of a constitutive promoter. The viral genome then contains the packaging signal and cDNA of interest. This separation of the different viral components prevents the production of a replication competent virus as only the viral genome containing the packaging signal and cDNA is packaged into the viral capsid. The viral capsid is transported via the exocytotic pathway to the cell surface where it buds off and is released into the tissue culture medium. This medium is also called the viral supernatant. This supernatant from the packaging cells is then used to infect the target cells.

1.10.6. Prostate epithelial cell lines.

Increasing interest in research into the causes of prostatic diseases has lead to the development of culture systems for prostate cells. Model systems using other species for example rodents have been found to be incomparable to humans. Therefore it became essential to develop ways of culturing human prostatic epithelial and mesenchymal cells (Hudson 2002)

In vitro cell models are critical for defining the mechanism of prostate cancer progression and for testing preventative and therapeutic regimens. The generation of immortalised human prostate epithelial cell culture that accurately reflects *in situ* characteristics of both normal and malignant prostate epithelium is imperative. The majority of available commonly used cell lines are derived from biopsies of metastatic cancer and are more appropriate for studies of on advanced progression and treatment. These cell lines include the well

characterised PC3, LNCaP and DU145, which were all derived from distant metastatic sites. Hence, the elucidation of molecular and genetic events involved in prostate cancer progression remains poorly understood.

Webber *et al* (2001) have developed a family of prostate epithelial lines from RWPE1 cells (HPV immortalised) by exposing the cells to N-methyl-N-nitrosourea (MNU). The derived cell lines show progressive changes from a pre-neoplastic to non-invasive to increasing invasive tendencies. This *in vitro* model may mimic and be a representation of the continuum of changes observed in human prostate carcinogenesis.

To study early genetic and molecular lesions, cell lines derived from primary tumours are needed. However, such are not presently available. Successful generation of immortal malignant prostate cell culture from primary tumours by HPV and SV40 has been described. These models have limited value because they frequently contain oncogenic viral DNA and accompany major cytogenetic alterations and growth degradation. Yasunaga *et al* (2001) have produced a telomere immortalised cell line derived from primary tumour of a patient with family history. Which as expected retained normal phenotype and genotype and may prove to be a useful tool in furthering understanding.

1.11. Project aims.

Increased chromosomal radiosensitivity has been shown by previous studies to be prevalent in the cancer patient population and may be indicative of a predisposition to cancer development. The aim of this project is to establish if there is an identifiable radiosensitive group within the prostate cancer

population. To do this, the radiosensitivity assays discussed, (the cytokinesis block micronucleus (CBMN) assay and the G2 assay), were employed to examine peripheral blood lymphocytes of both control subjects and patients with prostate cancer. If there is an identifiable radiosensitive subpopulation of prostate cancer patients, is there any correlation between the two assays and between prognosis, determined by PSA levels and Gleason score.

Hormonal status has been shown to affect radiosensitivity, but the only the hormones involved in the normal menstrual cycle and pregnancy have been studied. Does testosterone, or more importantly its more active form within the prostate, DHT, affect radiosensitivity? To study effects of exposure to DHT on the radiosensitivity, a hormone responsive cell line (LNCaP) and non-responsive cell line (PC3) are being used. Is the radiosensitivity of the cell of choice for the assays, the peripheral blood lymphocyte, affected by the presence of DHT?

The final aim is to attempt to establish prostate epithelial cell lines derived from non-tumour tissue through selection and immortalisation by means of hTERT transfection and to demonstrate that the resulting cells are morphologically representative of epithelial cells of the human prostate gland. Once this has been established these cell lines, can be used in conjunction with radiation to induce transformation and investigate the genetic changes involved. Hopefully providing clues to the changes involved in the stepwise progression to a cancerous state.

CHAPTER 2
MATERIALS AND METHODS.

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2.1. Chemicals and buffers.

Most basic chemicals were supplied by BDH or Sigma unless otherwise stated. Solutions were made with MilliQ distilled water.

2.2. Patient samples and clinical data.

Blood and tissue samples were obtained with permission from the patients and Tayside and Fife Committees on Medical Research ethics. The bloods from untreated prostate cancer patients were taken at the urology clinic at Ninewells Hospital Dundee and the controls were from various sources, the University of St Andrews, Ninewells Hospital Dundee and St Andrews Memorial Hospital. Normal epithelial tissue was obtained from Benign Prostatic Hyperplasia patients undergoing transurethral resection (TURS).

2.3. Cytokinesis block micronucleus assay for human peripheral blood lymphocytes.

Fresh peripheral venous blood was collected by venepuncture using sterile lithium heparin vacutainers (Becton Dickinson) and stored (for a maximum of 24hrs) or transported at room temperature. 1ml of whole blood was added to 9mls of pre-warmed, gassed (in a humidified atmosphere of 5% CO_2 at 37°C (in a Heraeus incubator)) RPMI 1640 (Roswell Park Memorial Institute) culture medium (Biowhittaker) supplemented with 10% foetal calf serum (FCS (SeraQ, batch tested)), L-glutamine (2mM), penicillin (50IU/ml) and streptomycin (50μg/ml) in a 25cm^3 flask. (Two flasks were set up for each sample). Flasks

were replaced in a humidified atmosphere for 1hr. One flask was then exposed to 3.5Gy gamma irradiation from a caesium 137 gamma source (IBL437C). The other flask was mock irradiated (0Gy).

Immediately after radiation exposure, 135μg/ml (150μl of ~9mg/ml stock in PBS) phytohaemagglutinin (PHA; HA 15; Abbott Murex) was added to each culture. The flasks were then placed flat in a humidified and gassed incubator (5% CO_2, 37°C).

44hrs after irradiation and PHA stimulation 6μg/ml cytochalasin B (30μl of 2mg/ml stock in DMSO) was added to allow accumulation of binucleate cells. Samples were then replaced in incubator for a further 28hrs.

Cultures were harvested after a total incubation of 72hrs. The cells and medium were transferred to a 50ml falcon tube and the flasks rinsed with a further 10mls medium this was added to the 50ml falcon and centrifuged at 4°C 800rpm for 5mins.

The supernatant was aspirated and discarded. The pellet was gently resuspended in 5mls 0.075 KCl (diluted in dH_2O) at 4°C and left on ice for 2mins. Centrifugation was repeated and supernatant aspirated. The pellet was resuspended in 20mls fixative (50% 10:1 methanol: acetic acid and 50% 1x Phosphate Buffered Saline (PBS) (Gibco BRL)) and centrifuged again, supernatant aspirated. This time pellet was resuspended in fixative (10:1 methanol:acetic acid) centrifuged and aspirated as before and previous

resuspension and centrifugation repeated, supernatant was aspirated and pellet resuspended in ~500μl of 10:1 methanol:acetic acid or volume required for good spreading.

The cell suspension was then dropped onto glass slides and air dried. Slides were stained with Rapi Diff (Raymond Lamb) and mounted using DPX. The slides can be stored indefinitely until required for scoring.

2.3.1. Criteria for scoring micronuclei.

Slides were coded for analysis so that the scorer is unaware of diagnosis. The same scorer should be used throughout experiments, as there could variability in the way that scoring criteria are interpreted. Slides were examined under light microscope using x40 lens. The frequency of micronuclei in 1000 binucleated cells and the proportions of binucleated tri-nucleated and tetra-nucleated cells in the total cell count were evaluated (see Appendix II, section II.1. for an example of the score sheet used).

The cytokinesis blocked cells scored were binucleated, both nuclei were of approximately the same size, had intact nuclear membrane, were situated within the same cytoplasmic boundary, which was clearly distinguishable from adjacent cells and had equal staining patterns and intensities. Nuclei attached by a nucleoplasmic bridge a quarter of the nuclear diameter were included. Cells with overlapping nuclei were scored only if nuclear boundaries were clear (see Appendix III, Figure III.3).

Micronuclei (MN) are morphologically identical to, but smaller than the main nuclei and have the same or occasionally darker intensity of staining. Their diameter in human lymphocytes varies between $1/16^{th}$ and $1/3^{rd}$ of the mean diameter of the main nuclei, corresponding to $1/256^{th}$ and $1/9^{th}$ of one of the nuclei in a binucleated cell respectively. MN are round or oval in shape and are not linked to the main nuclei, they are scored if they are touching the main nuclei, but not if they are overlapping, their boundary should be clearly distinguishable (Fenech 2000, Fenech et al 2003). For examples of the typical appearance and relative size of micronuclei in binucleate cells see Appendix III, Figure III.4.

2.3.2. Cytokinesis block micronucleus assay for cells.

$1x10^5$ cells of choice were suspended in 2mls of appropriate medium in flat bottomed tissue culture tubes (Nunclon), two tubes were set up for each experiment. One flask was mock irradiated and the other irradiated at 3.5Gy using the caesium gamma source (IBL473C). Cells were then placed in a humidified atmosphere 37°C, 5% CO_2 (making sure that the lid of the tube was loose so gas could access as filter cap not present) and left to attach for 24hrs. Then 2μg/ml cytochalasin B was added (2μl of 2mg/ml stock) and tubes replaced in incubator for a further 48hrs. After 48hrs, cells were trypsinised using 0.05% trypsin in PBS/ 0.01%EDTA. Cells were rinsed with 2mls of trypsin that was removed and then a further wash with 1ml trypsin performed. This time a small amount of trypsin was left in the tubes, which were then placed in 37°C incubator for no more than 5mins and monitored under microscope until all the cells were detached. Trypsinisation was stopped by

resuspending cells in 1ml of medium. After extraction from the tissue culture tubes, slides were created by cytospinning suspension at 800rpm for 10mins in a Shandon cytospin. The resulting cell concentrate was air dried, then stained and fixed using the Rapi Diff kit (Raymond and Lamb). Slides were mounted using DPX, these slides can be stored until required for scoring. Slides were scored using the same criteria outlined above.

2.4. G2 assay.

The blood was collected in the same manner as described above for the CBMN assay. Two flasks were set up for each blood sample; 1ml of whole blood was added to 9mls of pre-warmed and gassed medium (RPMI 1640 containing 10% FCS, L-glutamine and antibiotics) in a $25cm^3$ flask. 150µl PHA (Murex HA15) was added to each sample. The flasks were placed flat in a humidified gassed incubator (5% CO_2 37°C) for 72hrs. One flask was then exposed to 0.4Gy gamma irradiation from a caesium 137 gamma source (IBL437C). The other flask was mock irradiated. The flasks were then returned to the gassed humidified incubator for 30 minutes. 150µl of colcemid (demecolcine solution 10µg/ml) was added to each flask and then they were returned to the incubator for another hour. Meanwhile the centrifuge was cooled to 0°C, 15ml falcons cooled on ice and slides placed in dH_2O in coplin jar in fridge. Cells and medium transferred to pre-cooled centrifuge tubes and kept on ice. Centrifuged at 0°C 1200rpm for 10 minutes. Supernatant was removed and discarded. Pellet was resuspended in 5mls cold hypotonic (0.075M KCl) and left on ice for 20mins.The centrifugation step was repeated, again the supernatant was discarded. Pellet was resuspended in cold fixative (3:1 methanol: acetic acid) by

flicking the tube until no trace of red. This was then centrifuged at 1200rpm 0°C for 10 minutes. Supernatant was discarded and replaced by fresh fixative. (Samples can be stored at this point at -20°C if necessary.) Then fixation steps are repeated twice using fresh fixative. Pellet finally resuspended in ~500µl of fix and placed on ice. Slides were taken from fridge and drained of dH_2O then flooded with cold 50% acetic acid. The excess acetic acid was drained off and ~50µl of sample dropped on the slide and allowed to spread. Slides were air dried then stained with 4% Giemsa. Slides were stained in Giemsa for 10mins and then rinsed in Gurrs buffer pH 6.8 and air dried. Slides were coded and metaphase spreads scored for aberrations under x100 oil immersion lens.

2.4.1. Scoring criteria for the G2 assay.

Metaphase spreads were located by scanning the slide using light microscopes x16 lens. Once a metaphase spread was found, a drop of immersion oil (Zeiss) was placed over it and x100 oil immersion lens used. The number of chromosomes in each metaphase was counted. To ensure an accurate score, all 46 chromosomes had to be completely visible, with no overlaps. The aberrations were then counted and recorded for fifty suitable metaphase spreads for each sample (see Appendix II, section II. for an example of the scoring sheet).

2.5. Tissue culture.

The culture of mammalian cells was carried out under standard asceptic operating procedures in a class II cabinet. Most reagents were sourced from Sigma and Clonetics and the plastics were supplied by Nunc. The medium was supplied by Biowhittaker, GibcoBRL and Clonetics unless otherwise stated.

2.5.1. Standard cell culture.

Cells were cultured in the presence of 50U/ml of antibiotics penicillin and streptomycin. Cells were cultured in a humidified atmosphere at 37°C with 5% CO_2. The media conditions used for various cell lines are outlined in Table 2.1.

Table 2.1.

Cell Line	Growth Medium
LNCaP	RPMI 1640, 10% FCS, L-gluatmine, penicillin/streptomycin
PC3	RPMI 1640, 10% FCS, L-glutamine, penicillin/streptomycin
J2 3T3	DMEM, 10% FCS, L-glutamine, penicillin/streptomycin.

Table 2.1. Media conditions for cell lines.

Table showing cell lines and their maintenance medium and required additives.

2.5.2. Passaging of cell lines.

Most cell lines were passaged using 0.05% trypsin, 0.01% EDTA in PBS. Cells were washed twice with trypsin and incubated with a small amount of the trypsin solution at 37°C for a maximum of 5 minutes. The trypsin was neutralised by adding the appropriate maintenance medium. The cells were then seeded in $75cm^3$ (T75) flasks at correct density with fresh medium.

2.5.3. Cryopreservation and resuscitation of cell lines.

For cryopreservation cells were detached as described above in section 2.5.2. and pelleted by centrifugation at 800 rpm for 5mins, the supernatant was removed. The cell pellets were resuspended in the appropriate maintenance medium with 10% dimethylsulphoxide (DMSO). The cell suspensions were aliquoted into cryotubes (Corning Incorporated) placed in a 'Mr Frosty' container (Nalgene) and held at -70°C overnight. The container uses the gradual freezing process of isopropanol to reduce the internal temperature by 1°C per hour. This slow reduction in temperature is optimal for the recovery of mammalian cells. The next day the cells were transferred into liquid nitrogen for long-term storage.

In contrast to the slow freezing process cells were thawed quickly. Vials were removed from liquid nitrogen and then plunged into a waterbath at 37°C until thawed. The cells were then placed in a T75 flask and 10mls of pre-warmed fresh medium was added dropwise. The next day after the cells had attached, medium was refreshed in order to remove DMSO used in the freezing process as this can inhibit cell growth.

2.6. Primary culture of human prostate cells.

Primary prostate epithelial cells were extracted from variable sized 'chips' of prostate tissue removed from patients with BPH undergoing prostate resections (received from Mr K. Baxby Consultant Urologist, Ninewells Hospital). Immediately after removal from the patient, the prostate chips were placed in Falcon tubes containing 30mls of transport medium (RPMI 1640 with 20mM

Hepes (GibcoBRL), 5/6% FCS, penicillin/streptomycin and 5mls Fungazone (250μg/ml stock GibcoBRL)) (if none available, tissue was wrapped in sterile saline soaked swabs and placed in sterilin specimen jars) and into the fridge to await collection. Tissue was transported in a cool box and kept at ~ 4°C until processed.

2.6.1. Preparation of primary prostate cells.

Tissue was washed of all traces of blood using transport medium (constituents outlined above in section 2.6.), then tissue was placed in a petri dish and weighed. Tissue was cut into ~ 2mm cubed pieces under sterile conditions using a scalpel whilst being kept moist with transport medium. Minced tissue was placed in a 50ml falcon tube and washed with approximately 10-20mls of transport medium. This transport medium was discarded then 5mls of transport medium and 1500IU of collagenase per gram of tissue was added. The falcon tube sealed with Nescofilm (Azwell Incorporated) and mixture (tissue, medium and collagenase) was then placed on Rotator (Stuart) and rotated gently at 37°C for 20hrs. After this incubation, the digest was titurated by repeated pipetting with a 10ml pipette. The mixture was decanted into sterile falcon and centrifuged at 2000rpm for 10mins at room temperature to sediment cells. Supernatant was discarded and cells resuspended in 25mls of RPMI 1640 cells were shaken well to mix then centrifuged 2000rpm for 10mins. This wash was repeated and supernatant decanted. Cells were resuspended in 10mls of RPMI 1640 then allowed to settle under gravity for 15mins (alternatively centrifuge for 20secs 800rpm at room temperature). The epithelial cells settle out at the bottom of the tube and the supernatant contains aggregates of fibroblasts

(stromal cells). The sedimented deposits of epithelial cells are collected using a sterile Pasteur pipette and placed into another sterile falcon tube. This process is repeated twice then the cells collected are suspended in 5-6mls of PrEGM per 2g of original tissue and placed in a T75 flask. PrEGM (prostate epithelial growth medium) is made up of PrEBM (prostate epithelial basal medium) and singlequots comprising 2mls Bovine Pituitary Extract (BPE), 0.5ml insulin, 0.5ml hydrocortisone, 0.5ml GA-1000 (gentamycin), 0.5ml retinoic acid, 0.5ml transferrin, 0.5ml T3 (Triiodothyronine), 0.5ml epinephrine and 0.5ml hEGF (human epidermal growth factor) (BioWhittaker). One T75 flask per 2g of tissue used. The flask(s) were then placed in gassed humidified incubator (37°C, 5% CO_2) for 48hrs then supplemented with 9mls PrEGM. When cells have attached (after approximately 3-4days) medium is changed.

2.6.2. Collection of prostate fibroblasts.

For the collection of fibroblasts, the remaining supernatant (after the removal of epithelial deposits) was spun at 2000rpm for 10mins at room temperature. The supernatant was discarded and resulting cell pellet resuspended in 15mls medium (RPMI 1640, 10% FCS, pen/strep and L-glutamine). The cell suspension was then placed in a T75 flask and incubated at 37°C, 5% CO_2 for 48hrs (One flask per 2g of original tissue). After 48hrs medium removed and cells washed in RPMI 1640 and fed with fresh medium.

2.6.3. Secondary culture of epithelial cells.

After approximately 5 days, when there were good cell outgrowths or the flask is subconfluent the medium was discarded and the cells rinsed in 5mls Hepes

Buffered Saline Solution (HBSS) (Clonetics), 5mls of Trypsin (Clonetics) was added and the flask placed at 37°C for 5-6mins until cells were detached. When cells were detached the trypsin was neutralised with 5mls of Trypsin Neutralising Solution (TNS) (clonetics) the cells were then titurated in the flask to give a single cell suspension. The cell suspension was then placed in sterile 15ml falcon and centrifuged at 2000rpm for 10mins at room temperature. Supernatant was removed and the resulting cell pellet resuspended in PrEGM this cell suspension was split into two T75 flasks with 15mls of medium in each and replaced in incubator.

2.6.4. Cryopreservation of primary epithelial cells.

For cryopreservation, cells were detached as described above in section 2.6.3, pelleted by centrifugation at 800rpm for 5mins and resuspended in an appropriate volume of PrEGM. An equal volume of freezing mixture (16% glycerol, 24% FCS, and 60% PrEGM) was added to the cell suspension this was transferred to cryotubes, these were then placed in a 'Mr Frosty' and in -70°C freezer overnight. Cells were then placed in liquid nitrogen for long term storage.

2.6.5. Preparation of feeder layers for epithelial growth.

J2 3T3s were maintained in culture until required. Cells are grown in Dulbeccos Modified Eagle's medium (DMEM) with 10% FCS, L-glutamine, pen/strep until semi-confluent. The medium is then removed and replaced with fresh medium containing 2μg/ml mitomycin C (200μl of 100μg/ml stock in 10mls medium). The cells are incubated in the presence of mitomycin C for 2hrs at

37°C, 5% CO_2 (once treated cells must be used within 48hrs). The medium and mitomycin C was removed and flask trypsinised as described in section 2.5.2. Cells were resuspended in 5mls DMEM and centrifuged at 1000rpm for 5mins. Supernatant was removed and discarded, pellet was resuspended in an appropriate volume of PrEGM. 1-2x10^5 cells seeded per T75 flask. The flasks were incubated at 37°C, 5% CO_2 for a minimum of 2hrs, but if possible overnight so that cells have attached, before the addition of the epithelial cells either from frozen aliquots or fresh after collagenase treatment. Cells were then maintained as described above insection 2.6.1. and 2.6.3.

2.7. Retroviral infection.

The virus enters the cell by fusing with the host cell membrane, during which the viral envelope is lost. This is followed by the loss of the capsid, after which the replicative phase of the life cycle begins. The single stranded RNA genome is reverse transcribed, by the viral reverse transcriptase, (which was contained within the viral capsid) to DNA. The single stranded DNA is then copied forming a double stranded viral genome that is then integrated into the host cell genome during cell division. After integration, the host cell's RNA polymerase transcribes the viral genome, making the next generation. Simultaneously to viral genome replication, the host cell makes viral proteins for the production of the capsid. The final stage of infection is the lytic stage where the encapsulated viral genome is released from the host cell surface. It is possible to make replication competent retroviral vectors, by the addition of sequences to existing viruses, but more commonly the design involves the replacement of retroviral

sequences to create replication defective vectors (see chapter 1 sections 1.10.4. and 1.10.5. for more details).

2.7.1. Retroviral constructs.

ψCRIPpBABEpurohTERT (ψCRIP is the amphoteric retroviral producer line, pBABE retroviral expression vector, puromycin antibiotic selection agent hTERT construct for insertion) and ψCRIPpBABE were kindly provided by Dr Christopher Jones (University of Wales). A vector map can be found in Appendix IV.

2.7.2. Collection of retroviral supernatant.

ψCripBABEpurohTERT cells were grown to 95% confluence in Dulbecco Minimum Eagle Medium (DMEM), 10% FCS, pen/strep, L-glutamine and 2.5μg/ml puromycin. Selective medium was removed and cells washed in pre-warmed HBSS or medium. 10mls of pre-warmed medium suitable for the target cell population containing fresh glutamine but no puromycin was added to a T75 flask and incubated at 37°C 5% CO_2 for 12-18hrs. The medium was collected and centrifuged at 1000rpm room temperature for 5mins. It was then filtered using a pre-equilibrated 0.45μm Millipore filter. The retroviral supernatant was then aliquoted into 3ml cryotubes and snap frozen in liquid nitrogen and stored at -70°C until required.

2.7.3. Amphoteric retroviral infection of human target cells with hTERT.

Target cells were seeded at $1x10^5$ in 60mm dishes, (two dishes per procedure, one for control which is mock infected). Dishes were incubated overnight at

37°C, in 5% CO_2 to allow cell attachment. Medium was removed from plates and replaced with fresh medium containing 8μg/ml polybrene (Sigma). Plates were replaced in the incubator for 1hr. After 1hr the medium containing polybrene was removed and replaced by 3mls retroviral supernatant containing 8μg/ml polybrene in one plate and 3mls medium containing polybrene in the other control plate (mock infection), these plates were then incubated for 4hrs. Then, 2mls of medium (without polybrene) was added and this diluted the viral particles, and left the cells in a total volume of 5mls. The plates were then returned to the incubator overnight. After overnight incubation, the retroviral supernatant plus medium was removed and replaced with 5mls fresh medium (without polybrene). The plates were once again returned to the incubator overnight. Cells were then trypsinised and resuspended in an appropriate volume of fresh medium (without polybrene) and seeded into 90mm dishes. The retrovirally infected cells at 1/2, 1/10, 1/50, 1/250, 1/500 fold dilutions (with a final volume of 10mls) and the mock infected cells of the control, two plates at 1/2 and two plates at 1/500. These plates were incubated overnight to allow the cells to attach. After incubation, the selection agent, puromycin was added at a final concentration of 1μg/ml to all of the plates except for 1/2 and 1/500 dilutions of the control cells (+ve controls). These plates were incubated for 14 days at 37°C, 5% CO_2 then the resulting colonies selected (using cloning rings) in order to generate immortalised clones. Each of the selected colonies was placed in a well of 24 well plate and fed with fresh medium without the presence of the selection agent, the 24 well was then placed in the incubator and cells allowed to grow.

2.7.4. Measurement of lifespan extension.

The definitive test of whether telomerase insertion has an effect on target cells was to determine if they become immortal. This was ascertained counting the number of population doublings that the hTERT expression clones would undertake and making comparisons with cultures of non-infected primary cells.

2.8. Immunocytochemistry.

2.8.1. Slide preparation.

Immunocytochemistry was performed using the Vectastain Elite ABC kit immunoperoxidase system (Vector laboratories, California). All primary antibodies were sourced from Novacastra, Vector labs. Cells were extracted from culture flasks and slides were created by spinning approximately $5x10^4$-$1x10^5$ cells per slide at 800rpm for 10mins in a Shandon Cytospin II. The resulting cell concentrate was allowed to air dry, circled using a PapPen (wax pen) then fixed in cold acetone for 20mins (fixative may differ for different cell types and antibodies). Following fixation, the slide was washed twice in 10mM PBS at pH 7.5, each time for 10mins. Slides were wiped dry around the wax circle.

2.8.2. Immunostaining.

In order to reduce background staining the slide was incubated in a wet chamber with horse blocking serum (vectastain) in PBS buffer for 20mins. The slide was washed twice in fresh PBS as previously described and then incubated with anti mouse monoclonal primary IgG antibodies. Antibodies were used against cytokeratin 5 (CK-5 1:150), cytokeratin-18 (CK-19 1:60), cytokeratin-14 (1:20),

p63 (1:40), hTERT (1:40). For each sample, one slide received primary antibody diluted in 0.1% bovine serum albumin (BSA); a further slide received BSA only, as a negative control. The primary antibodies were incubated for 1hr or overnight at room temperature.

The primary antibody was flicked off and the slides were washed twice in PBS buffer and then incubated in a wet chamber with an anti-mouse biotinylated secondary IgG antibody (vectastain) for 30mins. Whilst the secondary antibody was incubating, the ABC solution from the vectastain kits was prepared as it required 30mins to mature. Following the washes in PBS, fresh Vectastain ABC solution (a complex of avidin and biotinylated secondary horse radish peroxidase) was added to the slide and incubated for 30mins; avidin of the ABC complex binds irreversibly with the biotinylated secondary antibody, localising horse radish peroxidase enzyme.

During the 30mins of ABC solution incubation DAB solution was prepared. The slides were washed twice in PBS before the localisation of peroxidase by the addition of DAB solution (0.7mg/ml 3,3'-diaminibenzidine tetrahydrochloride, 0.2mg/ml urea hydrogen peroxidase and 0.06M tris buffer in deionised water) in the absence of light: this solution is the substrate for the peroxidase enzyme. The slide was incubated with the DAB solution in the dark for 6-8mins and then rinsed in tap water.

Counterstaining was performed using the Vector haemotoxylin nuclear counterstaining method (Vector laboratories, California). The slide was

immersed in undiluted haemotoxylin for 2mins then washed under tap water until the water was colourless. The slides were air dried then mounted with a coverslip using DPX. They were then viewed under the light microscope at x40 magnification. Eight fields of view were picked randomly for each slide and scored. For every field of view, the total number of cells present was counted and out of those, the positively staining cells recorded, a percentage of positive cells were obtained for each slide.

2.9. Cell counts.

The cellularity of a single cell suspensions produced by trypsinisation was quantified using a Coulter Z1 cell and particle counter (Beckman Coulter Inc). A known cell volume was added to 20mls isotone, which was analysed by the Coulter counter set to measure particles of a certain volume, for example 350fl was the size established for primary prostate epithelial cells. The cell counter measured the number of particles in 0.5ml isotone solution; this number was then used to calculate the cellularity of the original suspension.

2.10. Cellular survival and proliferation.

LNCaP and PC3 cells were extracted from culture and diluted to a cellularity of $2x10^4$ cells/ml. Then 200µl aliquots of cell suspension were plated in 96 well flat bottomed tissue culture plates (Nunclon), these plates were incubated in a 5% CO_2, 37°C humidified incubator. Each sample was plated in at least four wells of a plate creating at least four replicates for each culture condition studied. A series of negative control wells containing medium only were included in each plate.

2.10.1. The MTT assay

Cellular survival and proliferation were measured by a spectrophotometric method, first described by Mosmann (1983), 50µl of 5mg/ml tetrazolium salt (MTT) (3-(4,5-dimethylthylthiozyl-2yl)-2,5-diphenyl tetrazolim bromide; Sigma) in PBS was added to each well of the plate (protected from light, the plate was then placed in a 5% CO_2, 37°C humidified incubator for 4hrs. The tetrazolium ring of MTT is cleaved by active mitochondria, leaving a blue-purple formazan product. This reaction occurs in living cells, with proportionality to cell activity, thus measuring cellular survival and proliferation. After the 4hr incubation period wells were emptied of medium/MTT, leaving the formazan product attached to the bottom of each well. This was dissolved by the addition of 200µl DMSO (Sigma) and 50µl Sörensens buffer (0.1M glycine and 0.1M NaCl at pH10.5). The optical density of the solution in each well was measured using an MRX-ELISA multiwell scanning spectrophotometer (Dynex Technologies, Virginia) recording a test wavelength of 570nm with reference wavelength of 690nm.

2.11. Data Analysis.

Data from the G2 and CBMN assays was reported as absolute counts per patient. In the case of the G2 assay, the number of chromosomal aberrations per 50 metaphases was stated and for the CBMN assay, the number of micronuclei per 1000 binucleate cells. These counts were then grouped and graphically represented.

Data from MTT assays was reported and graphically represented as the mean optical density for each sample (measured in 8 identical wells n=8) with standard error bars or as a percentage of the control optical density.

2.11.1. Statistical Analysis.

Measurements were summarised as the mean and standard errors as mentioned above in section 2.11. Measurement of analysis of variance (ANOVA) was used to examine the relationship between hormone addition and dose response of the prostate cell lines. Data sets with unequal numbers were analysed using the General Linear Model.

Comparisons between patient groups for the CBMN and G2 assays were made using Fishers exact test, which allowed a comparison of proportions and Mann Whitney U test, used to compare continuous variables. The student T-test was used for the assessment of group difference regarding the production of micronuclei in PBLs treated with DHT or untreated. Spearmann Rank correlations were used to examine the relationship between age and radiosensitivity.

2.11.2. Clinical Data.

All of the clinical data, the patient diagnoses, the PSA levels and the Gleason scores were obtained from patients records or urology clinics by Mr Keith Baxby and Dr Justine Royle and other members of staff.

2.11.3. Figure compilation.

Images were taken using a Nikon digital camera and a Zeiss light microscope and processed using Adobe Photoshop 7.0 or Microsoft Photo Editor 3.0. Graphs were drawn using Microsoft Excel from Office 2000 and statistical analyses were carried out using Minitab.

CHAPTER 3

RADIOSENSITIVITY OF PERIPHERAL BLOOD LYMPHOCYTES FROM PROSTATE CANCER PATIENTS.

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RADIOSENSITIVITY OF PERIPHERAL BLOOD LYMPHOCYTES FROM PROSTATE CANCER PATIENTS.

3.1. Introduction.

3.1.1. Chapter Rationale.

A wide variety of biological behaviour is implicated in the development of prostate cancer, including both exogenous (e.g. diet) and endogenous (e.g. hormonal imbalance, family history and genetic makeup) factors (Ozen *et al* 2000). All men are at risk of developing prostate cancer and to date many potential risk factors have been identified, but the results of many studies have proved inconclusive. A positive family history is a definite risk factor, approximately 10-15% of all prostate cancer cases are familial (Pentyala *et al* 2000), suggesting that certain predisposing genes exist and play an important role in the progression and development of prostate cancer. Additionally, the prognosis depends on the stage of the disease at diagnosis. Therefore, identification of genetic changes that predispose individuals to prostate cancer is very important for early diagnosis and treatment (Ozen *et al* 2000) whether these changes are inherited or accumulated. The ability to distinguish between cases of prostate cancer that are destined to progress rapidly and those with little likelihood of causing morbidity and mortality would improve the clinical outcome, because the scale and type of treatment could be tailored to the individual patients requirements.

3.1.2. Biomarkers and cancer risk.

The possible use of biomarkers representing intermediate steps in the pathway to disease are being investigated as they have the potential to estimate individual risk and may provide a means for early detection of cancer in the general population. Chromosomal aberrations are among the biomarkers most commonly considered for such a purpose, as they are a direct representation of chromosomal damage. A relationship between chromosomal damage and cancer development has been suggested since the beginning of the 20th century (Bonassi *et al* 2000).

3.1.3. Assessment of susceptibility to chromosomal damage.

One way to assess a person's susceptibility to chromosomal damage is to use ionising radiation (IR) as a tool to induce damage and to assess it. IR damages all components of the cell and in particular damages DNA through direct and indirect mechanisms (Shackelford *et al* 1999). Direct damage to DNA occurs as a result of the interaction of the radiation energy with DNA components. The consequences of this could be the generation of a variety of lesions, including the destabilisation of the N-glycosidic bond, and the production of single and double strand DNA breaks. Indirect damage comes from the interaction of DNA with reactive species formed by IR (Shackelford *et al* 1999). Despite these damaging effects, IR has been used for nearly a century to treat human cancer (Hall 2000) and recently explorations into using it as a tool for the identification of individuals predisposed to cancer and those patients with specific clinical parameters to aid in the tailoring of specific treatment regimes for particular patients, have been undertaken.

A range of cell types and cells derived from various individuals display different responses to IR exposure. Quantification of the generation of chromosomal abnormalities can be used as a means of assessing how the DNA damage incurred has been handled. An association between hypersensitivity of cells to IR and cancer predisposition has been clearly demonstrated in a number of heritable conditions (Sanford *et al* 1989). Hypersensitivity was first established for patients with ataxia-telangiectasia (A-T) a recessively inherited multi-systems disorder (Taylor *et al* 1975). Radiation induced chromosome damage assays (such as the G2 assay (see section 1.9.1. and 2.4.) and the CBMN assay (see sections 1.9.2. and 2.3.)) showed the greatest discrimination between A-T patients and normal controls (Taylor *et al* 1983). This intrinsic link between hypersensitivity and predisposition has been further demonstrated in 20 other inherited cancer prone conditions (as listed in Scott *et al* 1996 and 1999).

The Scott et al (1994) in vitro study using PBLs stimulated with PHA, clearly discriminated between A-T heterozygotes and normal controls according to the number of chromatid aberrations induced by low doses of radiation (the G2 assay). They demonstrated that 42% of untreated breast cancer patients but only 9% of the controls exhibit sensitivity similar to that of the A-T heterozygotes (Scott *et al* 1994 and 1998). Subsequently, other groups have confirmed these findings in breast cancer patients (Parshad *et al* 1996, Patel *et al* 1997, Terzoudi *et al* 2000, Riches *et al* 2001). This association between enhanced intrinsic radiosensitivity measured by the level of chromosome damage induced in either the G2 or G0 phases of the cell cycle and the risk of breast cancer has also been conclusively demonstrated (Roberts *et al* 2003).

3.1.4. Aims.

The concept of a causal association between chromosomal aberrations and cancer is based on the hypothesis, that genetic damage demonstrated in PBLs reflects similar damage in the cells undergoing carcinogenesis. Therefore, an association between enhanced radiosensitivity and prostate cancer is possible. The purpose of this research was to investigate radiosensitivity particularly in relation to prostate cancer. The main aims were to identify a radiosensitive subpopulation of prostate cancer patients, to test whether there were any correlations between the results of the two radiosensitivity tests and to see if there was any connection between PSA levels or Gleason score which define specific clinical parameters and radiosensitivity.

3.2. Experimental procedures and results.

The G2 and CBMN assays measure chromosomal radiosensitivity of PBLs irradiated in either the G2 or G0 phases of the cell cycle. Both of these experimental procedures were described in materials and methods sections 2.3 (CBMN assay) and 2.4 (G2 assay). For the G2 assay, the numbers of chromosome aberrations were assessed in a cytogenetic examination of metaphase cells and expressed as the number of chromosome aberrations per 50 cells. For the G0 assay (CBMN), the technically similar endpoint of micronucleus formation was used as the indicator expressed as the number of micronuclei per 1000 cells. For both endpoints the small number of spontaneous aberrations or micronuclei were subtracted from the total number observed in the irradiated cells to give an estimate of the aberration yield induced by the radiation. For the purposes of comparison, sensitive patients were initially

defined using an arbitrary cut-off of the 90th percentile of the distribution in a population of apparently normal individuals (Scott *et al* 1999, Roberts *et al* 2003). The 90th percentile was initially decided upon as a cut off value after an examination of the distribution of the results from an apparently normal population. This arbitrary value appeared reasonably satisfactory for defining sensitivity and as a way of expressing the assay results (Scott *et al* 1999). This can now be argued as approximating the cut off between those carrying the sensitive genotype and those who do not (Roberts *et al* 1999 and 2003).

3.2.1. Radiosensitivity of peripheral blood lymphocytes of untreated prostate cancer patients measured by the G2 assay.

Whole blood cultures were set up as described in materials and methods section 2.4. Two samples were used for each subject, one was mock irradiated and the other exposed to 0.4Gy gamma irradiation. After 30mins incubation to re-equilibrate, colcemid was added to each flask and then they were incubated at 37°C for another hour. The cells were then harvested and metaphase spreads made using standard protocols (materials and methods section 2.4.). Slides were coded for scoring and 50 metaphase spreads per patient were analysed for chromatid breaks (materials and methods 2.4.1.). This assay was optimised for a previous study into the radiosensitivity of breast cancer patients (Riches *et al* 2001).

Figure 3.1 illustrates the distribution of G2 scores for patients and controls (see Appendix V for all the scores and patient details). The mean overall G2 score for the prostate cancer patients (71.0 ± 17.6) was greater than that for the control

group (42.8 ± 25.1) (see Table 3.1), this was confirmed statistically by performing the Mann Whitney U test which gave $p = 0.0001$ showing that the medians were significantly different. Using the 90^{th} percentile as the cut off point, the proportion of prostate cancer patients with a high G2 sensitivity was 27.27% compared to 13.33% for the controls (since scores were grouped, 13.3% approximates the closest to the 90^{th} percentile). These proportions were not significantly different ($p = 3.03$) indicating that there was not a substantial sub-group of prostate cancer patients in this population exhibiting increased lymphocyte radiation sensitivity (see Table 3.1). The significant difference between the medians when the Mann Whitney U test was applied suggested that a larger study population is required.

Figure 3.1.

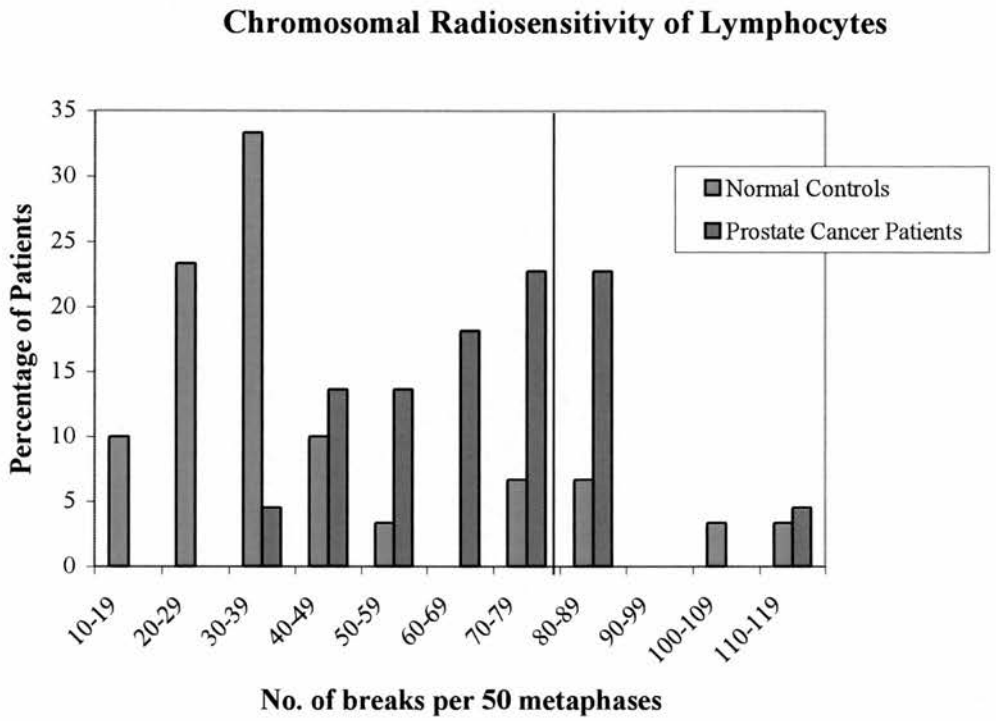

Figure 3.1. Chromosomal radiosensitivity of lymphocytes as measured by the G2 assay.

Comparison of the G2 scores (number of chromatid breaks per 50 metaphases) measured in irradiated peripheral blood lymphocytes from prostate cancer patients and normal controls. With the 90th percentile cut-off from the control population indicated approximately by the line.

Table 3.1. is an expansion and clarification of the data presented graphically in figure 3.1. It shows the range of scores for both the prostate cancer patient population and the control population, the range of ages and the percentages of each population deemed to be sensitive by the 90th percentile cut off. Also the statistical tests carried out on the scores are included.

Table 3.1.

	Normal Controls	Prostate Cancer Patients	
Mean G2 score ± SD	42.8 ± 25.1	71.0 ± 17.6	p=0.0001[a]
Range	17-111	42-110	
Number of subjects	30	22	
Sensitive sub-group	4/30 (13.33%)[c]	6/22 (27.27%)[c]	p=0.303[b]
Age range	23 - 75	39 – 90	
Median age	43	74	

Table 3.1. Comparison of G2 scores in prostate cancer patients and normal controls.

Mean G2 scores (number of chromatid aberrations per 50 metaphases scored in peripheral blood lymphocytes), population standard deviations, ranges and proportions of sensitive prostate cancer patients and normal controls in the G2 assay. [a]Mann-Whitney U test; [b]Two tailed Fishers exact test; [c]The 90th percentile was selected as the cut-off point (as the results were grouped, more that 10% of the patients had scores equal to or greater than this figure).

3.2.1.1. Comparison of patient age and radiosensitivity as measured by the G2 assay.

For the purposes of this study it was important to show that there was no correlation between age and radiosensitivity. In particular that the variation observed is not a result of the control population not being age matched with the patient population. This is necessary because if radiosensitivity increases with

age, then it is not a useful biomarker for prostate cancer predisposition. The age ranges and medians for both the control and patient populations can be seen in Table 3.1. The individuals within the patient population were on average older than those in the control population.

Figure 3.2. shows a scatter plot of age versus the number of breaks per 50 metaphases (chromosomal damage as measured by the G2 assay) that includes the data for both groups. It can be seen that there is no correlation between age and radiosensitivity with the sensitive individuals being found across the range of ages. This was confirmed statistically by the Spearmann Rank correlation (this statistical test has been used to successfully show that there were no significant differences in individual radiosensitivity associated with age in other studies using this assay (Scott *et al* 1999)), for the control population, $r = -0.025$, $p = 0.898$ and for the prostate cancer population $r = -0.034$, $p = 0.880$. Both p values are >0.05, this means that there is no correlation between age and radiosensitivity. Therefore, for this assay, there is no problem caused by not age matching the controls and patient population.

Figure 3.2.

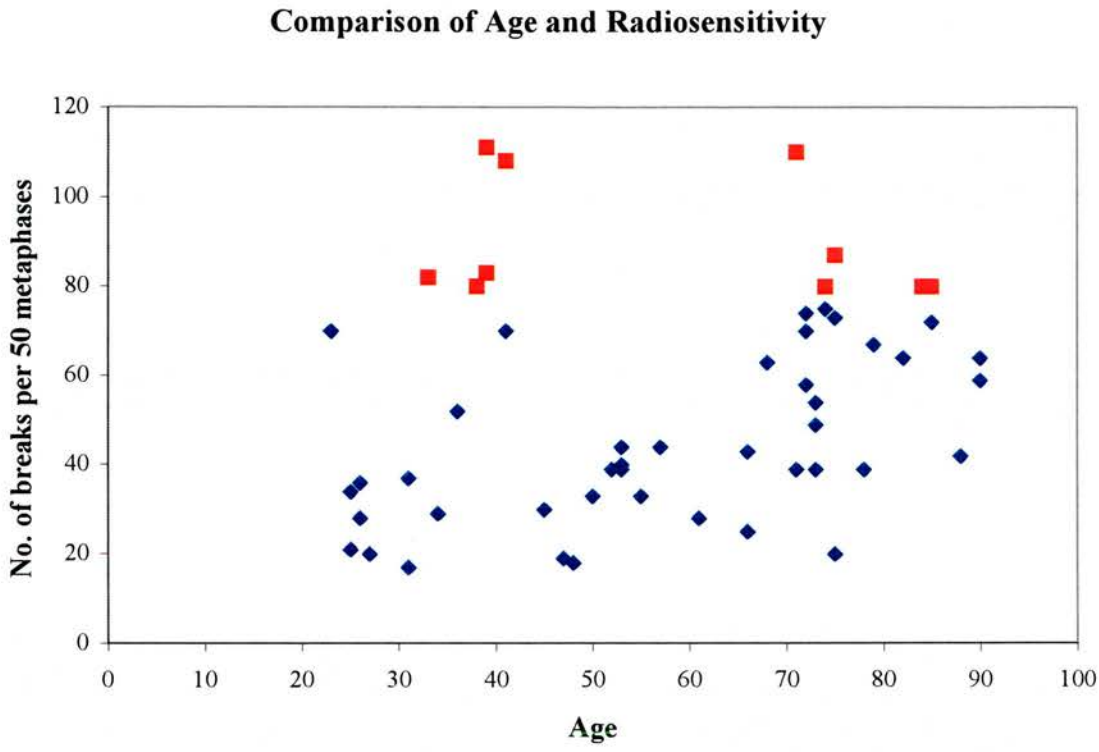

Figure 3.2. Comparison of patient age and radiosensitivity as measured by the G2 assay.

Graph showing the scatter plot of G2 scores in relation to the age of the individual. Each marker represents one person; the red square markers represent the sensitive individuals and the blue rhomboid markers the non-sensitive individuals.

3.2.1.2. Comparison of G2 radiosensitivity with specific clinical parameters.

The PSA levels and Gleason scores of the recently diagnosed prostate cancer patients included in this study were obtained from the clinic after scoring and decoding had been completed, although it proved difficult to track down information close to the date of the sample for PSA or find Gleason scores within the patients' records. Out of the 22 patients, it was possible to obtain

serum PSA levels for 15 and Gleason scores for 5, therefore the already small patient population was reduced further making any meaningful comparison of clinical parameters with radiosensitivity measured by the G2 assay difficult. However, the data obtained is presented below in figures 3.3 and 3.4.

3.2.1.2.1. Protein specific antigen (PSA) levels compared with G2 radiosensitivity.

PSA, as already mentioned in section 1.4.2, is a serine protease with chymotrypsin like activity involved in liquefication of the seminal gel and is a member of the kallikrein gene family (Sheehan 1998, DeFeo-Jones *et al* 2000). It is a single chain glycoprotein that has been shown to be a useful tumour marker, for prostate cancer, that circulates in free and complexed forms (Sheehan 1998). The measurement of PSA has a number of potential uses. It could be used for screening purposes, but at the moment this is a controversial issue. Currently, it is used for the diagnosis and staging of prostate cancer and for monitoring the patient after treatment.

Diagnostic evaluation of serum PSA levels improves early detection and the likelihood of identifying organ-confined disease. The greater the concentration of PSA, the less likely it is that the cancer is confined to the prostate gland. Total PSA levels between 2 and 4ng/ml are considered normal (Uzzo *et al* 2003). However, 20-30% of men with prostate cancer and PSA >4 have disease which has spread beyond the prostate. Fifty-nine percent of men with a PSA>10 have organ confined disease. A PSA >20 makes the presence of bony metastases extremely likely. 80% of cancer patients with a PSA >50 have bone

metastases (Sheehan 1998). Large scale studies have shown that DRE and PSA are complimentary as some tumours are detectable by one and not the other.

The PSA levels of the 15 patients whose radiosensitivity has been determined by the G2 assay ranged from 0.2 to 47.2 ng/ml and the number of breaks per 50 metaphases ranged from 43 to 110.The G2 scores are representative of the range seen in the study population (see table 3.1).

Figure 3.3. shows the PSA levels versus the number of breaks, as mentioned before it is difficult to reach meaningful conclusions with such a small number of patients, but from the graph, there doesn't appear to be any correlation between radiosensitivity and PSA. There is no obvious pattern on the graph although three of the four sensitive patients have PSA scores close to 30 ng/ml. However, for this to be established conclusively, the size of the study needs to be expanded.

Figure 3.3.

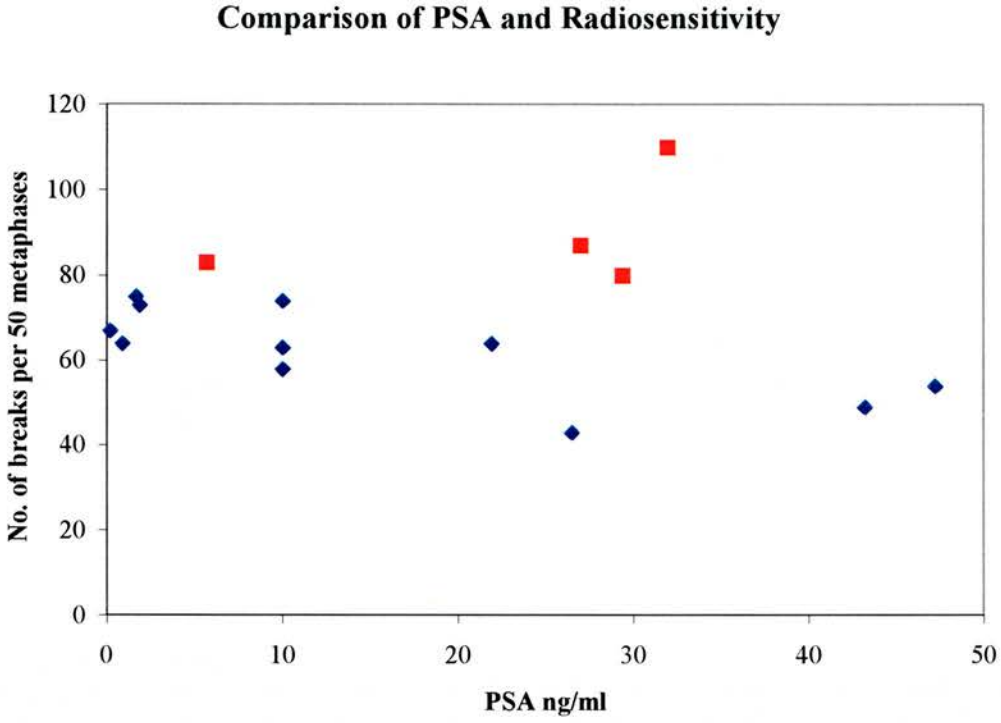

Figure 3.3. Comparison of PSA and radiosensitivity measured by the G2 assay.

Graph showing scatter plot of the G2 scores in relation to individual PSA levels. Each marker represents one patient, the red square markers indicating the sensitive and the blue rhomboid markers representing those within the normal range.

3.2.1.2.2. Gleason score compared with G2 radiosensitivity.

Gleason score is a powerful prognostic indicator that is reached through histological assessment of the tumours. The two most common histological patterns observed are assigned a grade from 1 to 5 with 1 being highly differentiated tissue and 5 the tumour tissue shows little or no differentiation. The grades are then added together to give a score. The lower the score, the

better the prognosis. A Gleason score of 2-4 is usually indicative of a slow growing cancer, which is likely to remain within the confines of the gland, whereas tumours with scores of 8-10 are likely to grow rapidly and metastasise (see Appendix I).

It has only been possible to obtain the Gleason scores for 5 of the 22 prostate cancer patients. The Gleason score range from 2-4 to 8-9, a score of 7 or above is classed as a high score, the number of breaks per 50 metaphases ranged from 39 to 80. Figure 3.4 is a histogram of Gleason scores plotted against number of breaks per 50 metaphases. It shows that the only individual classed as sensitive by the G2 assay has the lowest Gleason score of 2-4 whereas the person with the lowest G2 score has a high Gleason score of 7.

Figure 3.4.

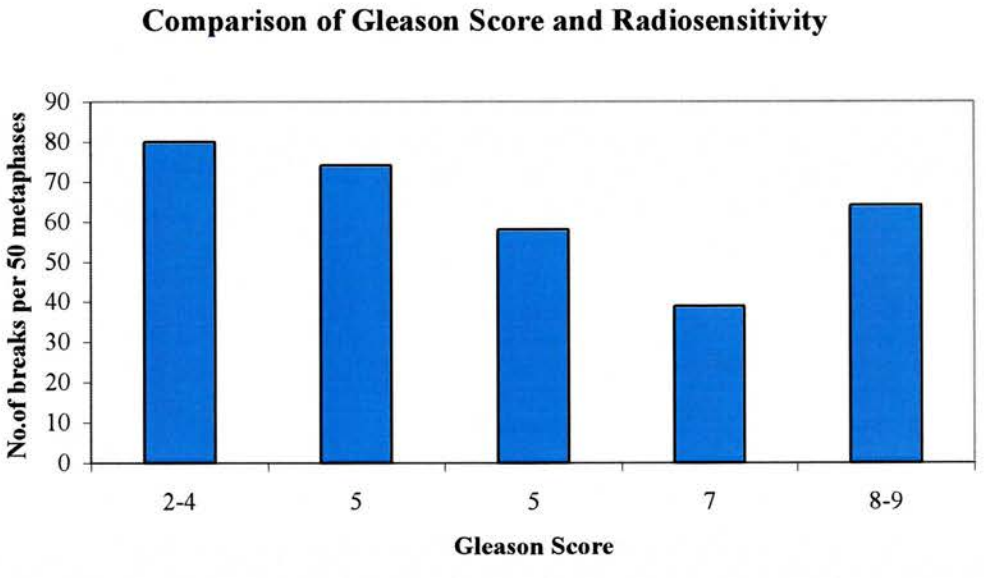

Figure 3.4. Comparison of Gleason score and G2 radiosensitivity.

Histogram showing the Gleason score of five of the prostate cancer patients and the number of breaks per 50 metaphases. Each bar represents one patient.

3.2.2. Radiosensitivity of peripheral blood lymphocytes of untreated prostate cancer patients measured by the cytokinesis block micronucleus (CBMN) assay.

Whole blood cultures were set up as described in material and methods section 2.3. Two flasks were set up for each sample one mock irradiated to provide a control and the other exposed to 3.5Gy gamma radiation. Cytochalasin B, which affects cytokinesis ring formation, was added to the culture to prevent cytokinesis and accumulate binucleate cells. Lymphocytes were harvested, spread on slides and scored according to the criteria outlined in materials and methods section 2.3.1.

3.2.2.1. Optimisation of the CBMN assay.

3.2.2.1.1. Time.

In order to ascertain if the time between the blood sample being taken and the assay being started affected the outcome, multiple 5ml samples of the same individuals blood were taken and left in the lithium heparin vacutainers for different lengths of time at room temperature. As the samples had to be transported and were sometimes uplifted by a courier service it would have been difficult to eliminate this variable if it had any affect on the outcome. Table 3.2. shows the total number of cells counted, the binucleate index and the number of micronuclei per 100 binucleate cells for three patients with assays set up at three different time point, directly after sample being taken, 6hrs after and 24hrs later. As you can see there is no significant difference between the number of micronuclei generated or the binucleate index observed in any of the three subjects. These figures were statistically analysed by using two-way analysis of

variance (ANOVA). For the comparison between different patients $p = 0.071$ and for the different time points $p = 0.079$ confirming the null hypothesis, thus proving that the differences seen are insignificant. This means that there is no significant difference in the outcome of the assay as long as the blood is used within 24hrs.

Table 3.2.

Sample	Total Number of cells	Binucleate index	MN per 100 BN cells
F1T0	433	23.09%	69
F1T1	597	16.75%	70
F1T2	680	14.70%	55
F2T0	390	25.64%	84
F2T1	377	26.53%	79
F2T2	369	27.10%	67
F3T0	397	25.19%	100
F3T1	463	21.60%	115
F3T2	376	17.36%	65

Table 3.2. Comparison of the CBMN assay started at different times after sampling.

Table shows binucleate indexes and number of micronuclei per 100 binucleate cells for three different people, (F1, F2 and F3 anonymous codes for samples) at three different time points. T0 assay set up immediately after blood was taken. T1 assay set up 6 hrs after blood was taken. T2 assay set up after blood left at room temperature overnight (~24hrs). All samples were irradiated at 3.5Gy. It shows that there is no significant affect on the binucleate index or on the number of micronuclei generated by blood samples being left at room temperature for 24hrs.

Other issues included how long to culture the lymphocytes in total and when to add the cytochalasin B (the cytokinesis blocking agent). Three different incubation times were set up, 48hrs (24+24), 72hrs (44+28) and 96hrs (44+52). Table 3.3. shows the binucleate indexes and MN scores and total cell numbers for two individuals whose blood was assayed at the different incubation times. As can be seen from the table, there is little difference between the number of micronuclei expressed at any of the incubation times, with a range from 79-100 for F3 and 62-75 for M01. However, as expected the binucleate index varies greatly from 2.58% to 25.19% for F3 and from 1.99% to 14.56% for M01. The total number of cells that had to be counted to include 100 binucleates varied from 297 to 3883 for F3 and 474 to 5015 for M01 the numbers of multinucleate cells observed (data not shown) also vary greatly increasing in number with the length of the incubation time. From this evidence it was decided to incubate the blood cultures for a total of 72hrs as this gave a reasonable binucleate index.

Figure 3.5 shows the number of micronuclei expressed a different radiation doses ranging from 1Gy to 5Gy and the effect of the addition of cytochalasin B after 12hrs and 44hrs. The graph shows an increase in the number of micronuclei with the increase in radiation dose. This is expected as the greater the dose the more chromosomal damage caused. It can also be seen that there is little difference between the numbers of micronuclei at either time of cytochalasin B addition.

Table 3.3.

Sample	Total number of cells	Binucleate Index	MN per 100 BN cells	Total incubation time
F3	3883	2.58%	79	48 hrs (24 + 24)
F3	397	25.19%	100	72hrs (44 + 28)
F3	819	12.21%	83	96hrs (44 + 52)
M01	5015	1.99%	62	48 hrs (24 + 24)
M01	474	21.10%	64	72hrs (44 + 28)
M01	687	14.56%	75	96hrs (44 + 52)

Table 3.3.Comparison of CBMN assay scores and different incubation times.

Table shows the total number of cells, binucleate index and number of micronuclei expressed per 100 cells at varying incubation times. F3 and M01 are the anonymous codes for samples. All samples were exposed to 3.5Gy gamma radiation. There is little difference in micronucleus number, but a large variation in the binucleate index and the total number of cells.

Figure 3.5.

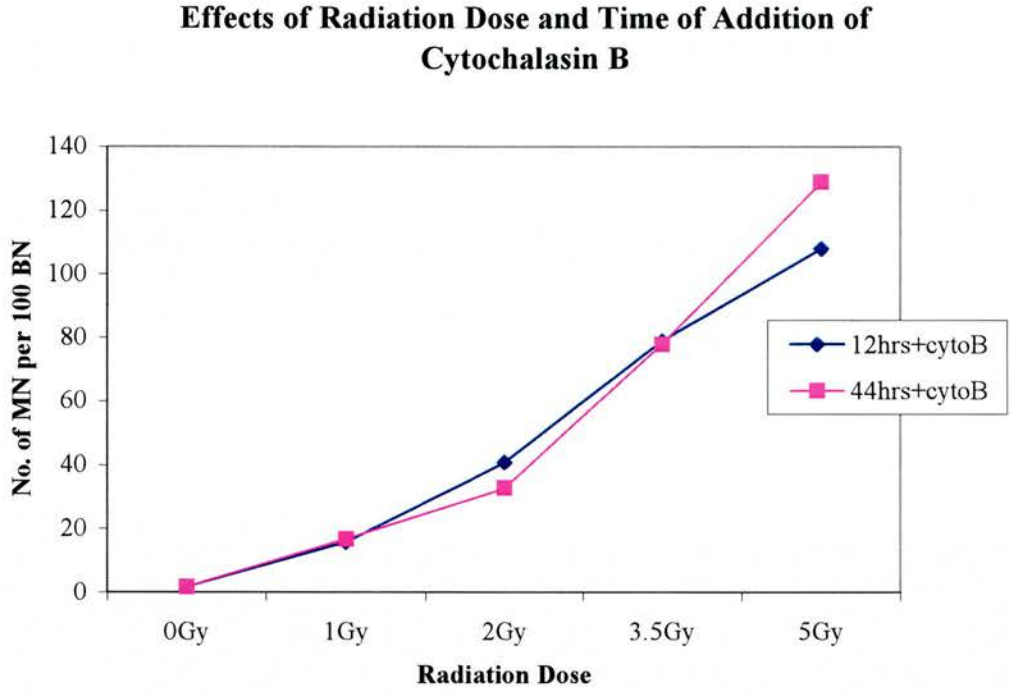

Figure 3.5. Effects of radiation dose and time of addition of cytochalasin B on the number of micronuclei expressed per 100 binucleate cells.

Graph shows the number of micronuclei increase with radiation dose, but that the time of addition of cytochalasin B makes no difference. Both lines represent the number of micronuclei in lymphocytes taken from one person and cultured under different conditions.

For all future experiments it was decided that the cultures should be grown for a total of 72hrs and that cytochalasin B should be added after 44hrs and that the cultures should be irradiated at 3.5Gy based on the results displayed in Table 3.2 and 3.3 and Figure 3.5. These findings are in accordance with those of other groups (Fenech 2000).

3.2.2.1.2. Assessment of the number of binucleates scored.

In all of the above experiments, 100 binucleates were counted to assess the number of micronuclei expressed in each culture this number was chosen as it was fairly quick to score and it made analysis easy. However, in order to improve the accuracy of the assay larger numbers of binucleates were counted on the same slide, this allowed the assessment of how many binucleates to count to facilitate reproducibility. The CBMN assay was carried out as described in materials and methods section 2.3. and scored according to the criteria in material and methods section 2.3.1.

Figure 3.6. shows the variation seen in the number of micronuclei when the number of binucleates scored ranges from 100 to 1000. The graph shows micronucleus scores for five different people and the variations observed. The variation was large when less than 500 binucleate cells were scored, when larger numbers of binucleates were scored, the variation seemed to lessen. For ease and accuracy of analysis 1000 binucleate cells were counted. Figure 3.7 shows the coefficient of variation when increasing numbers of binucleates were counted. The greater the number of binucleates counted the smaller the variation, therefore the greater the degree of accuracy of the endpoint expressed as number of micronuclei per 1000 binucleates. Also the more likely it is that if the same count is repeated on the same slide the numbers will be similar, or the difference between them statistically insignificant. From this data it was decided that in all subsequent experiments to score 1000 binucleate cells.

Figure 3.6.

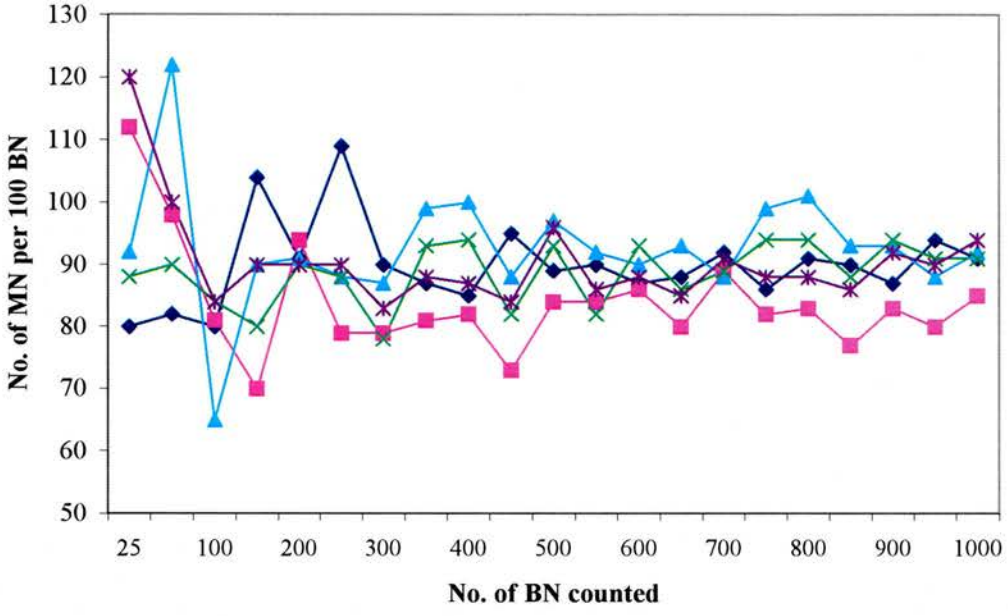

Figure 3.6. Differences in the micronuclei expressed when increasing numbers of binucleate lymphocytes scored.

Graph showing the variation in the number of micronuclei (MN) per 100 binucleates (BN) when increasing numbers of binucleates scored. As expected the larger the number of binucleates counted the smaller the variation seen. Each line represents one person.

Figure 3.7.

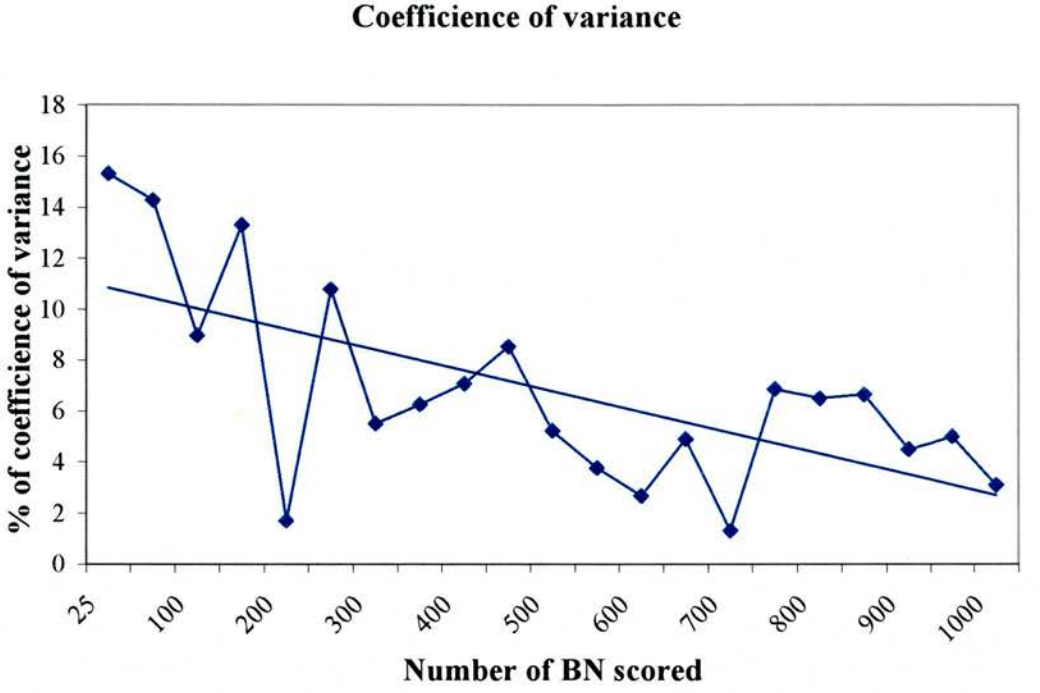

Figure 3.7. Coefficience of variance of the number of binucleate lymphocytes scored.

Graph shows a calculation of the coefficience of variance of the scores shown in Figure 3.6. The trend line shows the decline in variance with the increase in the numbers of binucleates scored.

Having optimised the CBMN assay and established scoring reproducibility, the assay was performed on blood samples taken from a normal control population, untreated prostate cancer patients, and benign hyperplasia (BPH) patients.

3.2.3. The number of micronuclei expressed in the peripheral blood lymphocytes of untreated prostate cancer patients.

Whole blood cultures were set up for the CBMN assay as described in materials and methods section 2.3. and above in section 3.2.2. Lymphocytes were

extracted, dropped onto slides and 1000 binucleate cells were assessed for micronucleus frequency.

Figure 3.8 illustrates the distribution of micronucleus scores for prostate cancer patients and normal controls (see Appendix VI). The mean overall MN score for the prostate cancer patients (895.52 ± 243.44) was greater than that for the control group (753.99 ± 168.96) (see Table 3.4.). This was confirmed statistically by performing the Mann Whitney U test which gave $p = 0.0011$, showing that the medians were significantly different. Using the 90^{th} percentile as the cut-off point, the proportion of prostate cancer patients with a high G0 sensitivity was 32.68 % compared with 6.25 % for the controls (since scores were grouped 6.25 % approximates the closest to the 90^{th} percentile). These proportions were significantly different ($p = 0.002$) indicating that there was a substantial sub-group of prostate cancer patients in this population exhibiting increased lymphocyte radiosensitivity in G0 (see Table 3.4.).

Figure 3.8.

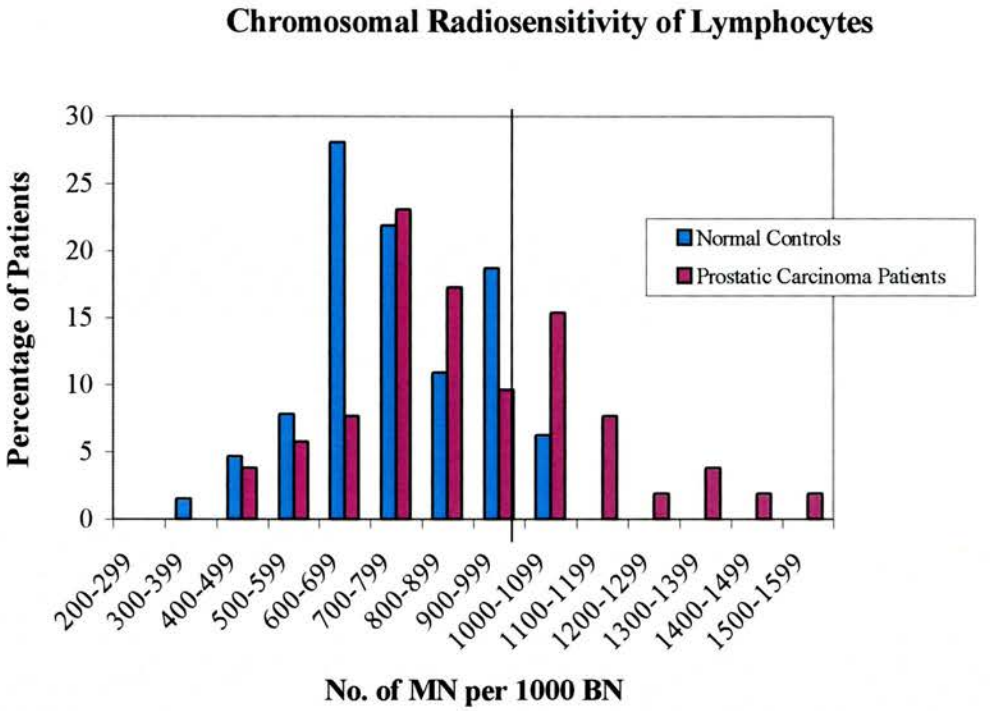

Figure 3.8. Chromosomal radiosensitivity of PBLs measured by the CBMN assay.

Comparison of MN scores measured in peripheral blood lymphocytes from prostate cancer patients and normal controls irradiated in the G0 phase of the cell cycle. Line indicates the 90th percentile cut-off from the control population.

Table 3.4. is an expansion and clarification of the data presented graphically in figure 3.8 (see Appendix VI). It shows the range of scores for the prostate cancer population and the normal control population, the age ranges and the percentage of each group deemed to be sensitive by the 90th percentile cut off point. Also the statistical tests performed on the data are included.

Table 3.4.

	Normal Controls	Prostate Cancer Patients	
Mean MN score ± SD	753.99 ± 168.96	895.52 ± 243.44	p=0.0011[a]
Range of MN scores	309-1087	438-1518	
Number of subjects	64	52	
Sensitive sub-group	4/64 (6.25%)[c]	17/52 (32.69%)[c]	p=0.002[b]
Age range	18-63	61-91	
Median age	22	73	

Table 3.4. Comparison of MN scores in prostate cancer patients and normal controls.

Mean MN scores (number of micronuclei per 1000 binucleate peripheral blood lymphocytes), population standard deviations, ranges and proportions of sensitive prostate cancer patients and normal controls in the CBMN (G0) assay. *[a]Mann-Whitney U test;* *[b]Two-tailed Fishers exact test;* *[c]The 90th percentile was selected as the cut-off point (as the results are grouped, less than 10% of the patients had scores greater than or equal to this figure).*

3.2.3.1. Comparison of age and radiosensitivity of untreated prostate cancer patients and normal controls measured by the CBMN (G0) assay.

The comparison of age and radiosensitivity was necessary as it was difficult to obtain age matched controls for this study. It was important to rule out any correlation between age and radiosensitivity and to show that the differences in sensitivity were due to intrinsic variation. The age ranges and medians for both

the control and patient populations are shown in Table 3.4 . The individuals within the patient population were significantly older than those in the control population.

The ages (obtained from the various sample sources) and micronucleus scores for both populations were plotted as a scatter graph shown in Figure 3.9. Looking at the graph it can be seen that there is no correlation between age and G0 radiosensitivity as the sensitive individuals are found across the entire range of ages (18-91). This was confirmed statistically by Spearman rank correlation, for the control population $r = 0.182$, $p= 0.148$ and for the prostate cancer population $r = 0.282$, $p= 0.054$. Therefore for this assay, not having age matched controls did not cause a problem.

Figure 3.9.

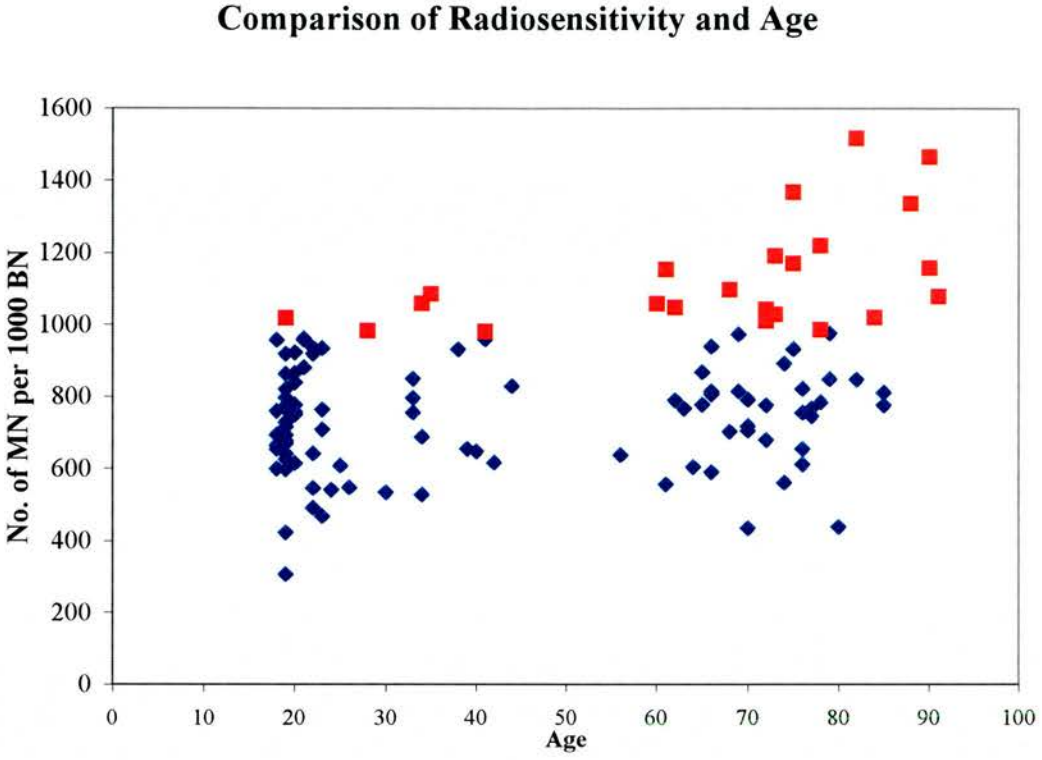

Figure 3.9. Comparison of age and radiosensitivity measured by the CBMN assay.

Scatter plot of MN scores in relation to the age of the individual. Each marker represents one individual. The red square markers indicate the sensitive individuals and the blue rhomboid markers the non-sensitive individuals.

3.2.3.2. Comparison of G0 radiosensitivity with specific clinical parameters.

The levels of PSA found in the patients and their Gleason scores were the clinical assessors that were of interest as these are crucial for diagnosis of prostate cancer and decisions on appropriate treatment regimes. PSA levels and Gleason scores were obtained for the prostate cancer patients after scoring and decoding had been completed. In some cases it proved difficult to track down information close to the date of the clinic when the sample was taken. Out of the

52 patients, it was only possible to obtain serum PSA levels for 24 and Gleason for 16. This reduces the patient population making any meaningful comparison of clinical parameters with radiosensitivity measured by the CBMN assay difficult. However, the data obtained is presented in figures 3.10 and 3.11.

3.2.3.2.1. PSA levels compared with G0 radiosensitivity.

The PSA levels of the 24 patients whose radiosensitivity was determined by the CBMN assay, ranged from 1.3 to 47.2 ng/ml (levels were assessed at the urology clinic) and the number of micronuclei expressed ranged from 438 to 1518. The MN scores are representative of the range seen in the study population (see Table 3.4.); 8 of the 24 patients whose PSA values were deemed sensitive by the CBMN assay.

Figure 3.10 shows the PSA levels plotted versus the number of micronuclei per 1000 binucleate lymphocytes. There doesn't appear to be any correlation between G0 radiosensitivity and PSA as sensitive patients (indicated by the red markers on the graph) are spread across the range of PSA values (see sections 1.4.2. and 3.2.1.2.1.for explanation of the range). However, for this to be established conclusively, the size of the study needs to be expanded.

Figure 3.10.

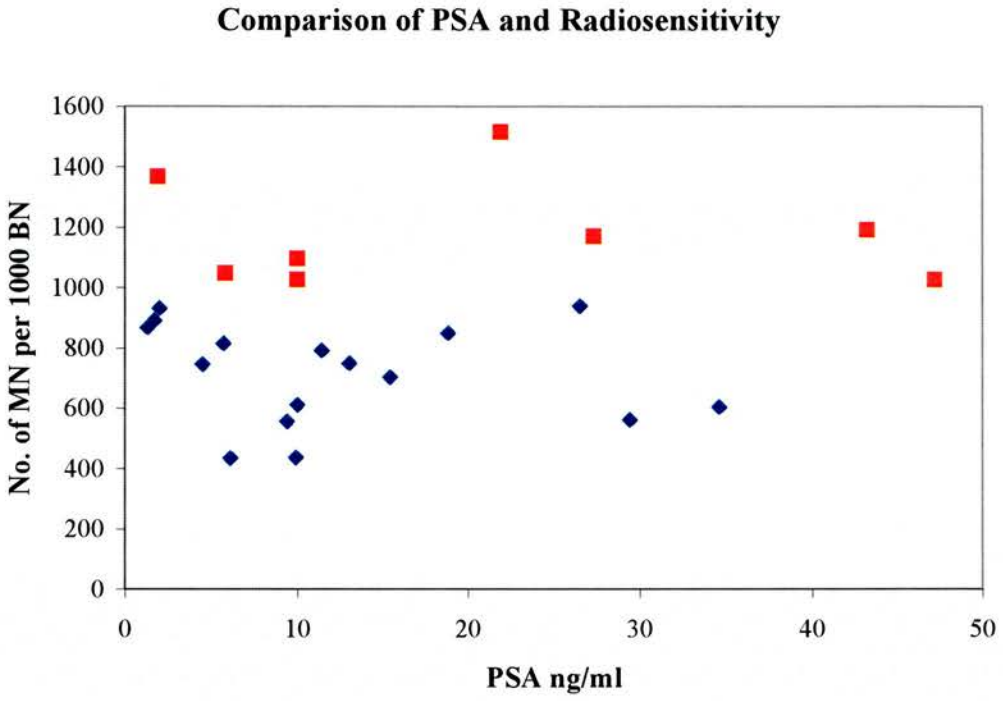

Figure 3.10. Comparison of PSA and radiosensitivity measured by the CBMN assay.

Scatter plot of the micronucleus scores in relation to PSA levels. Each marker represents one individual, the red square markers indicating the sensitive and the blue rhomboid markers representing those within the normal range.

3.2.3.2.2. Gleason score compared with radiosensitivity.

The Gleason scores for 16 of the 52 prostate cancer patients were obtained from patient records. The Gleason scores range from 2-4 to 8-9 with 4 out of the 16 patients classed as having a high Gleason score of 7 or above (see Appendix I, Table I). The number of micronuclei per 1000 binucleate lymphocytes ranges from 438-1518 again representative of the population range (see table 3.4.). 4 out of the 16 patients whose Gleason score is known were found to be sensitive.

Figure 3.11. is a histogram of the Gleason score plotted against the number of micronuclei per 1000 binucleate lymphocytes. It shows that the individuals classed as sensitive by the CBMN assay all have a Gleason score of 5 or above and the person with the highest Gleason score of 8-9 also has the highest MN score. However, it is impossible to tell whether there is any correlation between Gleason score and G0 radiosensitivity.

Figure 3.11.

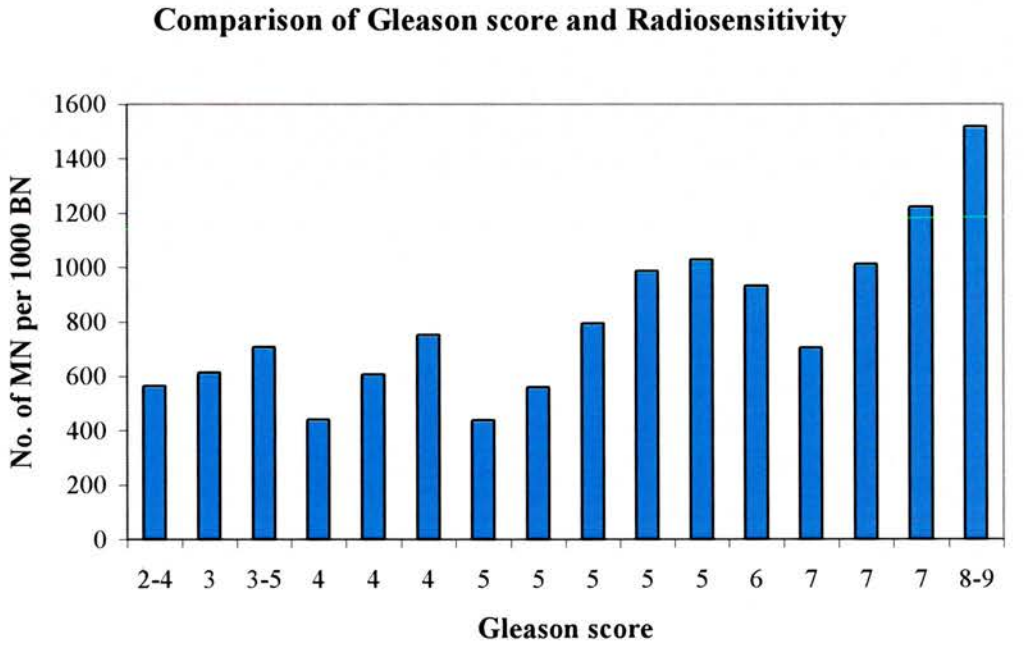

Figure 3.11. Comparison of Gleason score and G0 radiosensitivity.

Histogram showing the Gleason score of prostate cancer patients and the number of micronuclei per 1000 binucleate lymphocytes. Each bar represents one patient.

3.2.4. The number of micronucleus expressed in the peripheral blood lymphocytes of benign prostatic hyperplasia (BPH) patients.

Whole blood cultures were set up for the CBMN assay as described in materials and methods section 2.3 and in section 3.2. Lymphocytes were separated from the blood cells by lysis and centrifugation and dropped onto slides. A thousand binucleate lymphocytes were examined for micronucleus frequency. Figure 3.12. illustrates the distribution of scores for BPH patients and normal controls. The mean overall score for the BPH patients (738.73 ± 199.99) was less than that for the control group (753.97 ± 168.96) (see Table 3.5.). The difference was found to be statistically insignificant by performing the Mann Whitney U test which gave a $p = 0.9571$, thus proving the null hypothesis that the medians were the same. Using the 90^{th} percentile as a cut-off point, the proportion of BPH patients with a high G0 sensitivity was 6.67% compared with 6.25% for the controls (since the scores were grouped 6.25% approximates the closest to the 90^{th} percentile). These proportions were essentially the same indicating that there is no difference between this population of BPH patients and the normal control population (see Table 3.5.). However, this is a small population of BPH patients and needs to be enlarged before any definite conclusions can be reached.

Figure 3.12.

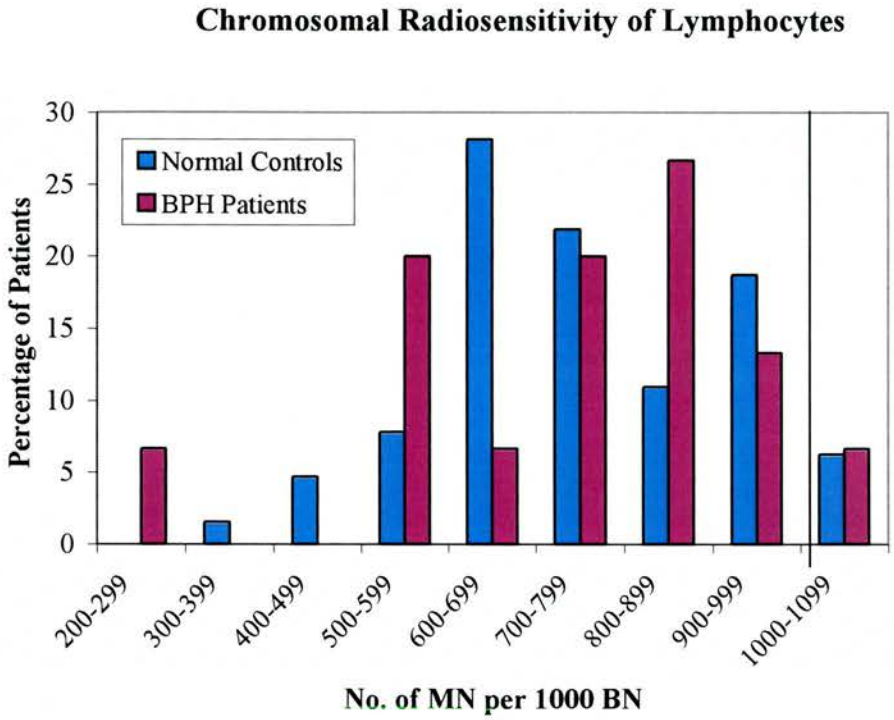

Figure 3.12. Chromosomal radiosensitivity of PBLs measured by the CBMN assay.

Comparison of MN scores measured in peripheral blood lymphocytes from benign hyperplasia patients and normal controls. Line indicates the 90th percentile cut-off from the control population.

Table 3.5. is an expansion and clarification of the data presented graphically in Figure 3.12. It shows the range of scores for both the BPH population and the normal control population, the range of ages and the percentages for each population of those deemed to be sensitive by the 90th percentile cut off point. The statistical tests used to assess the data are also included.

Table 3.5.

	Normal Controls	BPH Patients	
Mean MN score ± SD	753.97 ± 168.96	738.73 ± 199.99	p=0.9751[a]
Range	309-1087	278-1062	
Number of subjects	64	15	
Sensitive sub-group	4/64 (6.25%)[b]	1/15 (6.67%)[b]	p=?[c]
Age Range	18-63	62-78	
Median Age	22	71	

Table 3.5. Comparison of MN scores in BPH patients and normal controls. *Mean MN scores (number of micronuclei per 1000 binucleate lymphocytes), population standard deviations, ranges and proportions of sensitive BPH patients and normal controls in the CBMN assay. [a]Mann-Whitney U test; [b]Two tailed Fishers exact test could not be performed as numbers less than 5; [c]The 90th percentile was selected as the cut-off point (as results were grouped less than 10% of the patients had scores greater than this figure).*

3.2.4.1. Comparison of age and radiosensitivity of BPH patients and normal as controls measured by the CBMN assay.

The age ranges and medians for both the control population and the BPH patient population can be seen above in Table 3.5. the individuals in the patient population were on average older than those in the control population. Figure 3.13. shows a scatter plot of age versus the amount of chromosomal damage measured by the CBMN assay (number of MN per 1000 BN PBLs) and includes all the data for both groups. It can be seen that the sensitive individuals are

spread over the entire age range (see table 3.5. for values). G0 radiosensitivity was not correlated with age; Spearman rank correlation r = 0.500, p= 0.058.

Figure 3.13.

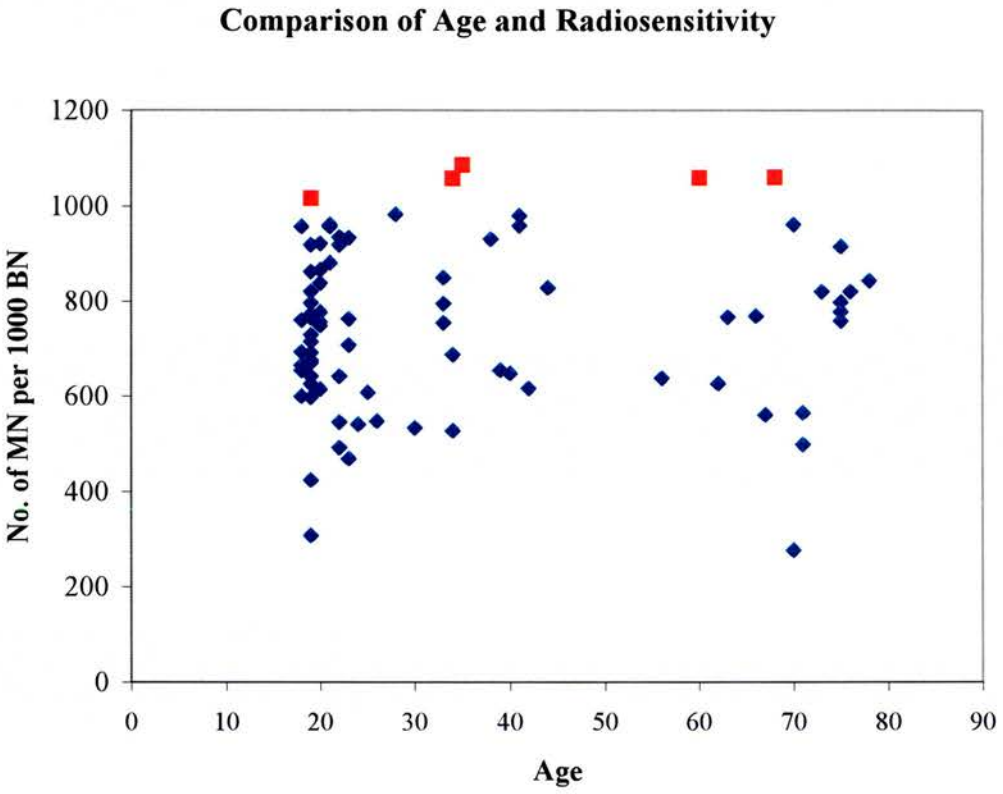

Figure 3.13. Comparison of age and radiosensitivity as measured by the CBMN assay.

Scatter plot showing the normal control population and the BPH populations MN scores in relation to the age of each individual. Each marker represents one person; the red square markers represent the sensitive individuals and the blue rhomboid markers the non-sensitive individuals.

3.3. Comparison of G2 and G0 radiosensitivity.

In this study it has been shown that sensitivity to the induction of chromosome damage by ionising radiation is on average higher in G2 and G0 peripheral blood lymphocytes from prostate cancer patients than of healthy controls. The G2 and CBMN assays have both been performed on the same blood samples from 28 people and a comparison made between the results.

There was no correlation between G2 and G0 sensitivities for the 28 individuals drawn from both the control and patient population using the same blood sample for both assays. A total of 17.86% of the men were G2 sensitive and 35.71% G0 sensitive, giving a total of 53.57% sensitive in either assay, but only 3.57% (1 individual) was sensitive in both. A clear example of the lack of correlation between the two results of the two assays is that the patient with the highest G2 score (indicated by the arrow in Figure 3.14.) had the second lowest G0 score. This is illustrated in Figure 3.14, which is a scatter plot of MN score in 50 binucleate cells versus the number of breaks in 50 metaphase spreads. The 90^{th} percentile cut off points are 81 breaks per 50 metaphases and 53 micronuclei per 50 binucleate lymphocytes (indicated by the lines on the graph).

Figure 3.14.

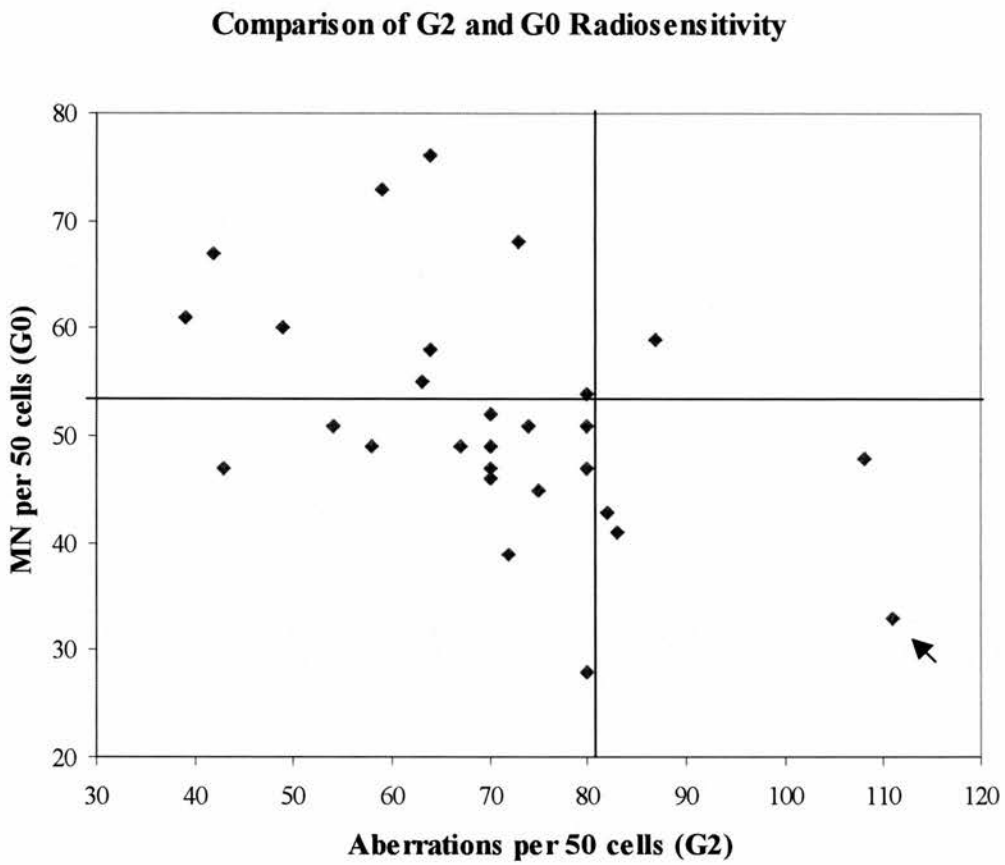

Figure 3.14. Comparison of G2 and G0 radiosensitivity.

Radiation induced micronucleus yields in G0 cells (y-axis) and metaphase aberrations in G2 cells (x-axis) for the same 28 men using the same blood samples for both assays. Each marker represents one patient. The lines represent the 90th percentile cut off points for both G2 (the vertical line) and G0 (the horizontal line). Arrow indicates the highest G2 score.

3.4. Discussion.

A significant proportion of prostate cancer patients in this study exhibit an elevated MN score indicating there is an identifiable group of men with increased G0 radiosensitivity within the cancer population. The proportion of radiosensitive prostate cancer patients in this cohort is similar to that reported for breast cancer patients. Using the CBMN assay and the 90th percentile of a normal control population as the cut off point for sensitivity, 33% (17/52) of the prostatic carcinoma cases were sensitive (see Figure 3.12. and Table 3.4.). A similar assay giving rise to the same endpoint was employed in a breast cancer study by Scott *et al* (1999) which reports that 27% (35/130) of the breast cancer patients were G0 sensitive.

However, using the G2 assay to investigate the chromosomal radiosensitivity of prostate cancer patients and again the 90th percentile of normal controls as a cut off, no significant difference was found (see Table 3.1). This could be due to the small number of patients for which the G2 assay was successfully completed. It was only possible to obtain good metaphase spreads for 22 out of the 52 prostate cancer patients recruited for this study. The reason for this is unknown as the same protocol was applied to all samples. To improve the numbers of scoreable metaphase spreads different spreading techniques and fixation procedures were tried but there were still insufficient well spread metaphases to allow analysis.

There is an indication that with a larger number of patients a discernible difference similar to that seen in breast cancer patients, head and neck cancer

patients and colon cancer patients may become apparent, because a significant difference between the medians of the controls and prostate cancer patients existed when the non-parametric Mann Whitney U test was applied ($p = 0.0001$) (see Table 3.1.). The G2 assay identified 27.27% (6/22) of the prostate cancer patients as being sensitive, if this percentage is also compared with the breast cancer studies (those using the G2 assay are numerous) it can be seen that it is considerably lower than could be expected. The majority of studies report that approximately 40% of their respective populations are demonstrably G2 radiosensitive (Riches *et al* 2001, Terzoudi *et al* 2000, Scott *et al* 1999 to mention a few of the most recent).

If it had been possible to recruit a larger prostate cancer patient population, this study might have been able to confirm whether or not the prostate cancer population on average exhibits a greater radiosensitivity than the normal population as has been shown for breast cancer patients (Scott *et al* 1999). At the moment it can only be stated that the prostate cancer patients tested in this cohort exhibit sensitivity to ionising radiation damage at G0.

3.4.1. Comparison of G2 and G0 sensitivity in prostate cancer patients.

There is no correlation between the G2 and G0 radiosensitivity of prostate cancer patients. The same lack of correlation has been shown in breast cancer patients (Scott *et al* 1999), so it is perhaps not surprising. Many different mechanisms of chromosomal radiosensitivity have been identified to date. These include defects in DNA repair (Parshad *et al* 1983), cell cycle checkpoint control (Wang *et al* 1996), the influence of differences in chromatin structure on

the conversion of DNA damage into chromosome breaks (Mozdarani and Bryant 1989, Hittelman and Pandita 1994) and the elimination of damage by apoptosis (Schwartz *et al* 1995). All of these mechanisms mentioned are likely to be cell cycle stage specific. It appears from this study and Scott *et al* (1999) that any chromosomally radiosensitive individual is defective in only one such mechanism.

However, in some inherited cancer prone conditions, cells display 40% sensitivity to both G2 and G0 radiation damage (Scott *et al* 1999). This is perhaps explainable by the fact that most of these are multi-systems disorders with the characteristic germline mutations in regulatory genes that influence the activity of numerous genes. The affected genes probably include those involved in the detection and processing of DNA damage such as that inflicted by ionising radiation, leading to the chromosome damage displayed in the G2 or G0 phases of the cell cycle. In the cases of breast and prostate cancer, it might be these downstream genes that are mutated, with only one mutation per patient, conferring either G2 or G0 radiosensitivity (Scott *et al* 1999).

The results of this study into prostate cancer patient radiosensitivity confirm the general trend that individuals exhibiting chromosomal radiosensitivity are defective in only one mechanism (other than those with multi-systems disorders mentioned above) implying that G2 and G0 sensitivities are largely independent.

3.4.2. Predisposition to prostate cancer.

Chromosomal aberrations in peripheral blood lymphocytes predict human cancer. The frequency of these aberrations is a relevant biomarker for cancer risk in humans, reflecting either early biological effects of genotoxic carcinogens or individual cancer susceptibility. Such aberrations could be useful as early diagnostic and predictive markers for prostate cancer (Ozen *et al* 2000).

There is already evidence for a link between G2 chromosomal radiosensitivity and breast cancer predisposition. Healthy women with a family history of breast cancer (on affected first degree relative or two affected second degree relatives) have a greater mean G2 score than healthy women without such familial history (Helzslouer *et al* 1996, Parshad *et al* 1996, Patel *et al* 1997). It has also been shown that the proportion of G2 sensitive individuals is greater in breast cancer patient populations with a family history than for those cases without (Parshad *et al* 1996).

The implications of this increased radiosensitivity for prostate cancer patients are as yet unclear, but it is indicative of increased chromosomal fragility and possibly associated with malignant transformation. For a similar link to be made with prostate cancer and predisposition, family studies need to be carried out. There is a distinct possibility that the same is true for prostate cancer as 10-15% of all prostate cancer cases are familial (Pentyala *et al* 2000) and having a family history is a definitive risk factor.

The increased radiosensitivity shown in the prostate cancer population (32.68% compared with 6.25% of controls for G0 radiosensitivity (Figure 3.12. and Table 3.4.) and 27.27% compared with 13.33% of controls for G2 (Figure 3.1.and Table 3.1.)) is perhaps not surprising, as it has been shown in both heritable and sporadic cases of other carcinomas. Elevated sensitivity is indicative of defects in the repair and processing of DNA damage (Rothfuβ *et al* 2000).

Various groups have demonstrated that sensitivity to the induction of chromosome damage by ionising radiation is on average higher in PBLs of breast cancer patients than those of normal controls (Scott *et al* 1999). There is now clear evidence that radiosensitivity as measured by the G2 assay in breast cancer patients is inherited and likely to be a marker for a small number of low penetrance predisposing genes (Roberts *et al* 1999). This may be the case with prostate cancer as well. There have been tentative links made between prostate cancer and breast cancer. Commonalities between breast and prostate cancer suggest similar aetiological risk factors and the involvement of the same causative agents (Grover and Martin 2002). This is backed up by evidence gathered from migrant populations who move from low risk to higher risk countries. Increases in incidences of both prostate and breast cancer in migrants cannot be attributed to ethnicity, but to changes in other factors, such as lifestyle, environment, diet and cooking practices (Grover and Martin 2002). The breast cancer predisposition genes BRCA1 and BRCA2 have also been implicated in prostate carcinogenesis and the ATM gene is also thought to play a role in both prostate and breast cancer (from Part 4 mechanisms of

carcinogenesis). Human mammary and prostatic epithelial cells are capable of metabolically activating members of different classes of chemical carcinogens to DNA reactive species and in rodents, five out of six mammary carcinogens can also induce prostatic neoplasms (Grover and Martin 2002).

High lymphocyte G2 radiosensitivity appears to be unrelated to sex, age or to environmental variables (Roberts *et al* 1999, Riches *et al* 2001). The possible mechanisms underlying this radiosensitivity and the identity of the low penetrance genes involved in cancer predisposition have not yet been established but it seems likely that chromatid breaks arise by a process involving genomic rearrangements (Bryant *et al* 1998, Rogers-Bald *et al* 2000, Riches *et al* 2001). A network of proteins regulates the cellular response to DNA damage. Following the induction of double strand breaks in chromosomal DNA, a complex network is activated that regulates DNA repair and progression through the cell cycle (Wang 2000, Riches *et al* 2001). Genes in this network protect the integrity of the genome; hence mutational events in these genes can predispose individuals to cancer. According to one scheme, cancer susceptibility genes can be conveniently classified into two groups: gatekeepers and caretakers (Kinzler and Vogelstein 1997, Riches *et al* 2001). Gatekeeper genes are important in cell cycle control and apoptosis. Caretaker genes are involved in DNA repair and inactivation can result in genetic instability. The hypothesis is that inactivation of caretaker genes influences the processing of DNA damage and thus leads to increased chromatid damage (high G2 radiosensitivity). The low penetrance gene/s involved in predisposition to breast cancer might thus be members of the caretaker gene family (Riches *et al*

2001). Members of the caretaker family may also be involved in the G2 and G0 radiosensitivity seen in the prostate cancer patients.

Observations may facilitate the identification of low penetrance genes involved in prostate cancer. It would not be possible to use the G2 assay as a population screening method, as it is technically demanding and time consuming. However, if these low penterance genes can be identified then it will be possible to determine whether specific polymorphisms serve as markers for increased risk of breast cancer (Riches *et al* 2001).

If it were possible to identify the genes involved in G2 and G0 radiosensitivity of prostate cancer patients they might serve as biomarkers for increased risk. The increase in radiosensitivity demonstrated might provide a means of identifying candidate genes, which for prostate cancer have proved elusive. The chromosomes most commonly seen in the micronuclei and those most commonly damaged in the G2 assay could be identified by FISH probes and the specific gene locations identified by cytogenetics.

3.4.3. DNA repair ability and cancer risk.

The importance of DNA repair in maintaining genomic integrity and protecting against development of cancers has been shown in studies involving cancer patients and cancer prone individuals, as well as in studies involving genetically altered mice exhibiting DNA repair deficiencies. The connection between DNA repair defects and cancer predisposition was first recognised by Cleaver (1968 and 1969) in studying cells from individuals with Xeroderma-pigmentosum.

Studies of PHA stimulated lymphocytes from individuals with breast cancer and from breast cancer families showed that these cells were deficient in their DNA repair capacity compared with lymphocytes from control individuals, as measured by directly quantifying the generation of chromatid abnormalities following DNA damage (Parshad *et al* 1996, Rao *et al* 1998, Shackelford *et al* 1999). Defects in DNA repair, specifically mismatch repair pathways are important in the development of a variety of human cancers. The post-replication DNA mismatch repair system recognises and removes inappropriately paired nucleotides that may have been generated by DNA replication errors, errors generated in DNA recombination events, or base damage following exposure to genotoxic agents (Shackelford *et al* 1999).

3.4.4. Ionising radiation as a therapy and the implications and relevance of increased radisoensitivity.

The objective of ionising radiation (IR) therapy is to deliver a lethal dose to cancer cells but attenuate the toxic effects on adjacent normal tissue. Undesirable sequels of radiotherapy are the development of tumour resistance and damage to normal tissue. Various types of DNA damage including that induced by IR are recognised and repaired by specialised pathways. Many of the genes involved are conserved. Transcriptional induction of DNA repair genes, immediate early genes and a variety of cytokine and growth factor genes have been proposed as the mechanisms by which cells survive after IR in mammalian cells, tissues and tumours. Gene induction has been reported after exposure of mammalian cells to IR. Few studies of gene induction profiled RNA changes. A

central unresolved issue is whether, within a range of cytoreductive doses administered in clinical practice, the gene response to IR is dose dependent. Daily doses from 1Gy to 3Gy are commonly used in RT, whereas 10Gy is frequently used to study biochemical responses of mammalian cells to IR and in radiosurgery of some brain tumours. It is reported that cell-common and cell-type specific, dose-dependent and time-restricted patterns of gene up-regulation are seen after IR. Analysis of this type of data provides valuable information for the design of effective therapy and could also point to adjunct therapies that specifically target undesirable activation of specific genes by IR. Analyses of the products of the genes activated by irradiation will take considerable effort (Khoderav *et al* 2001). IR is administered to specifically destroy cancer cells. Detailed analyses of each tumour type will be necessary to determine idealised treatment. Knowledge of the patterns of response of the tumour cells would be required. Contrary to expectation, IR administration within the lethal range produced a dose dependent response with a significant number of genes. Some genes were induced at low IR doses and some induced only at high IR doses. This finding is in conflict with the prevailing notion that within certain parameters the sum total rather than the individual doses predicts success of IR treatment. Also of considerable interest is the finding that the temporal pattern of gene expression was also dose dependent. It is conceivable that the brief expression of certain genes may play a significant role in determining whether the cell survives or dies after irradiation.

A fraction of genes were activated at a relatively high level and it would be interesting to discover whether the promoters of these genes are particularly

sensitive to IR or whether they are induced by products of IR-inducible genes. Data suggests that pathways of nuclear pre-mRNA processing and nuclear/cytoplasmic transport of RNA may be activated by IR and may provide potential therapeutic targets. To properly assess the function of responders in the environment of the irradiated cell, it will be necessary to modify or suppress the expression of up-regulated genes. This approach may ultimately shed light on the development of resistance to IR (Khodarev *et al* 2001).

Patients receiving similar RT may experience widely differing levels of normal tissue injury ranging from undetectable or minimal to unacceptably severe. Possible factors contributing to this can be grouped into two general categories: those related to the treatment such as fractionation schedule, volume treatment field or radiation quality and those inherent to the patient such as intrinsic cellular radiosensitivity, age and physiological characteristics (Nunez *et al* 1998). Clinical observations of normal tissue damage following RT support the notion that intrinsic differences in individual radiosensitivity account for much of the variation in the severity of acute and chronic radiation injury to normal tissues (Tucker *et al* 1996, Nunez *et al* 1998).

A variety of heritable syndromes indicate that specific genes influence the response of normal tissues and cells to radiation, but even cells cultured from apparently normal populations exhibit a wide range of cellular radiosensitivity. If tissue radiosensitivity has a significant genetic component, then it is reasonable to suggest that the radiosensitivity of cells in culture reflect the genetic constitution of the individuals from whom they were derived. At present

selection of dose for all patients is based on a balance between minimising the incidence of severe normal tissue complications and maximising the probability of local control (Burnet *et al* 1996, Nunez *et al* 1998). The existence of inter-patient variability in radiation sensitivity implies that the dose used in conventional treatment is determined primarily by the most sensitive patients. This recognition has raised hopes that predictive assays could be developed to identify the most sensitive and most resistant patients prior to treatment. Studies have demonstrated the theoretical feasibility of a predictive assay of radiosensitivity. However, such an assay is a long way from a clinical application (Nunez *et al* 1998).

It is generally accepted that IR kills eukaryotic cells by damaging the structure and function of genomic DNA. Much effort has consequently been focussed on understanding how cells respond to radiation-induced damage. However, the exact nature of the link between DNA damage and cell lethality in mammalian cells still has to be clearly established. Two main hypotheses, not mutually exclusive, have been proposed to explain the differences in sensitivity among cells: (i) cells may vary in the amount of damage induced by a given dose of radiation and (ii) cells may differ in their capacity to repair radiation damage to DNA. Radiation damage in mammalian cells can be divided into two categories: (i) lethal damage which is irreparable and leads irrevocably to cell death and (ii) sub-lesions resulting from potentially lethal damage, which under normal circumstances can be repaired in hours unless additional damage is added or interaction of sub-lesions take place. Briefly, sensitive cells suffer more DNA double strand breaks per unit dose (Gy) and the process of DNA double strand

break rejoining is slower than resistant cells (Nunez *et al* 1996, Nunez *et al* 1998). Highlighting the need to elucidate the molecular basis for radiosensitivity. Knowledge of the reasons for radiosensitivity would have important implications with regard to RT, since a strategy based on testing normal tissue sensitivity might permit individualisation of treatment. Results from overreacting patients suggest that a relationship may exist between DNA damage in lymphocytes and clinical response (Nunez *et al* 1998). Radiosensitivity as measured by the two assays (CBMN and G2) was not a strong prognostic factor following standard RT (Roberts *et al* 2003).

It is now well established that the sensitivity of lymphocytes to the induction of chromosomal damage to ionising radiation is greater in breast cancer patients than in normal controls and this is true if the lymphocytes are irradiated in either the G2 (Scott *et al* 1994 and 1999, Terzoudi *et al* 2000, Baria *et al* 2001, Riches *et al* 2001, Baeyens *et al* 2002) or G0-phase of the cell cycle (Scott *et al* 1998 and 1999, Baeyens *et al* 2002). There is also evidence, at least as far as the G2 assay is concerned, that it has a heritable genetic basis and is therefore related to cancer predisposition (Roberts *et al* 1999, Burrill *et al* 2000). Genetic analysis by Roberts *et al* (1999) implies that around 40% of breast cancer patients express a radiosensitive genotype in their lymphocytes. In testing for chromosomal radiosensitivity there was no significant correlation between the results of the G0 and G2 assays, suggesting different mechanisms of radiosensitisation are involved at these two stages of the cell cycle (Scott *et al* 1999, Baeyens *et al* 2002, Roberts *et al* 2003). This lack of correlation between the Go and G2 assays was also found during the course of this investigation (see

Figure 3.14.). It is plausible that the tumours generated in these radiosensitive individuals might have a different phenotype from those occurring in individuals whose radiosensitivity falls in the normal range and this might be reflected in a different pattern of tumour progression (Roberts *et al* 2003).

In view of the close relationship between radiation induced chromosome damage and cell death, it is also plausible that tumours occurring in the radiosensitive patients might themselves be more radiosensitive and show a more improved control rate following RT again leading to an improved prognosis in the radiosensitive patients (Roberts *et al* 2003).

The concept that inherent radiosensitivity of both normal cells and tumour cells varies from one individual to the next is well established. This is clinically relevant because large patient to patient variation in radiation morbidity is documented after a fixed dose fractionated RT schedule. Patients with the same type of tumour at the same anatomic site are treated with standardised RT, the difference in individual radiosensitivity could mean that the dose is sub-optimal for radioresistant patients and could induce tissue morbidity in radiosensitive patients. The identification of individual intrinsic cellular radiosensitivity would allow prescribed radiation doses to be individualised, thereby optimising treatment. Lee *et al* (2003) tried to address directly the potential relationship between G0 radiosensitivity of prostate cancer patients established by the CBMN assay before RT treatment and RT-related morbidity after treatment and

found that MN yields in peripheral blood lymphocytes induced by the CBMN assay before inititiation of RT correlates with RT-related morbidity. This means that this could form the basis for individualising treatment.

3.4.5. Clinical parameters and radiosensitivity.

Unfortunately in the present study, it was not possible to obtain sufficient clinical information for the patients tested with the G2 and CBMN assays to draw any conclusions about the correlation of clinical parameters and the elevated radiosensitivities observed. However, other studies have shown that chromosomal abnormalities can be used as diagnostic and predictive markers for metastasis and the development of androgen independent disease in prostate cancer (Ozen *et al* 1999 and 2000).

Ozen *et al* showed that chromosomal abnormalities can be detected in the PHA stimulated PBL cultures of prostate cancer patients and suggested that the aneuploidy index in blood can be used as an early diagnostic and predictive marker for prostate cancer metastasis and androgen independent disease (Ozen *et al* 1999). Non-random chromosomal alterations can be detected in PBLs of prostate cancer patients and these can be used as an early diagnostic and predictive marker for prostate cancer metastasis and androgen independent disease. In addition specific chromosomal alterations can be correlated with specific clinical parameters (Ozen *et al* 2000). The damage reported could help pinpoint primary genetic aberrations in prostate cancer.

Are the genetically abnormal PBLs circulating prostate cancer cells or bone marrow derived lymphocytes showing genetic instability? If the latter, why do these patients not have leukaemia or lymphoma? The presence of abnormal metaphases in asymptomatic family members would indicate that these are not tumour cells (Ozen *et al* 2000). Another possibility is that these lymphocytes are derived from stem cells of specific adult organs (Bjornsen *et al* 1999, Ozen *et al* 2000). Organ specific stem cells were investigated to see if they were restricted to producing specific cell types from the tissue in which they reside. It was found that genetically labelled neural stem cells produce a variety of blood cell types (Bjornsen *et al* 1999, Ozen *et al* 2000). Bone marrow stem cells are also capable of producing epithelial like cell types as well as lymphocytes, this means that they may be the stem cells for different organs (Petersen *et al* 1999 Ozen *et al* 2000). This work was very labour intensive so it would probably not be feasible to test every patient using these methods, but if the CBMN assay could be shown to detect similar aberrations that correlate with specific clinical parameters it has great potential

Roberts *et al* (2003) did not show any significant association between radiosensitivity in either of the assays and breast cancer patient survival. Also there was no association between radiosensitivity and the clinical characteristics of the tumours except for G0 sensitivity and tumour grade. However, Riches *et al* (2001) showed an association between the score in the G2 assay with conventional prognostic indicators as expressed in the Nottingham prognostic index, an indicator of survival calculated using data on the size of the primary tumour, axilliary lymph node status and histological grade (Riches *et al* 2001).

There are two potential mechanisms that the intrinsic radiosensitivity of patients might be expected to affect their prognosis. Originally, it was hypothesised that the mechanism of tumourigenesis might be different if the radiosensitivity had a genetic basis and that the clinical characteristics of the resulting tumours might be different. Secondly, one might expect that any intrinsic difference in radiosensitivity could translate into differences in radio-curability of the tumours arising in the patients. One possibility is that these two mechanisms are operating in opposite directions and the real biological effects related to radiosensitivity are being masked in the use of a simple survival endpoint (Roberts *et al* 2003). Given the likely interaction between radiosensitivity and the RT component of treatment, studies need to be undertaken to investigate the alternative radiation fractionation regimes.

If the genome's susceptibility to damage can be ascertained before the onset of any disease and then the specific genes involved could be identified, it could be possible to pinpoint the most vulnerable organs/ areas and monitor them and catch the disease in its early stages. It has to date been impossible to ascertain whether variation in the intrinsic radiosensitivity of human tumour and normal tissue is recognisable and whether these inherent difference can be related to clinical curability and tolerance to treatment. The success of RT depends on total radiation dose, which is limited by the tolerance of surrounding normal tissues (Nunez *et al* 1998).

3.5. Conclusions.

The differences in G2 and G0 radiosensitivity reported in this preliminary study require a more detailed study on a larger cohort of prostate cancer patients and their families. There is an identifiable G0 radiosensitive group of men within the prostate cancer population and potentially a G2 radiosensitive group. For this population of prostate cancer patients, the combined percentages of identifiably sensitive patients account for approximately 60%. Which in turn suggests that large proportions of prostate cancers are associated with low penetrance genes as G2 and G0 sensitivities are largely independent.

Correlations between chromosomal damage highlighted by the radiosensitivity assays and specific clinical parameters could be used to identify a patient's risk and response to therapy but unfortunately in this study it was not possible to obtain sufficient data to make any meaningful conclusion.

3.6. Future directions.

Further investigation is needed to evaluate the combination of markers required to form an ideal criterion for determining early onset potential, androgen independence and metastasis in prostate cancer and to understand the origin of genetically abnormal PBLs.

Recruiting more prostate cancer patients and obtaining complete data sets for each patient would expand on the already interesting information emerging from this study and help answer the questions mentioned above. It would also be interesting to recruit a larger cohort of BPH patients and see if the radiosensitivity assays could distinguish between benign and malignant prostatic disease.

Although there are no obvious correlations between G2 and G0 sensitivities it would be interesting to investigate the chromosome angle (are the same chromosomes involved in both sensitivities?). It would be interesting to find out which chromosomes most commonly exhibit damage in both the assays. This could be done by using fluorescence *in situ* hybridisation (FISH) probes to identify which chromosomes were most commonly included in the micronuclei and those which incurred breaks most often with the G2 assay and allowing checks for commonality.

CHAPTER 4

THE EFFECTS OF DIHYDROTESTOSTERONE ON THE RADIOSENSITIVITY OF PROSTATE CELL LINES.

CHAPTER 4

THE EFFECTS OF DIHYDROTESTOSTERONE ON THE RADIOSENSITIVITY OF PROSTATE CELL LINES.

4.1. Introduction.

4.1.1. Chapter rationale.

Previous studies have indicated that radiosensitivity may vary with physiological status. To date, these studies have concentrated on the female population and the hormonal changes involved in the menstrual cycle and the progression of pregnancy as most intra individual variation has been observed in women when using the peripheral blood lymphocyte based radiosensitivity assays. There is some evidence that increasing levels of the hormones, oestrogen, progesterone (Ricoul *et al* 1997 and Roberts *et al* 1997) and oestradiol (Kanda and Hayata 1999) can cause an increase in lymphocyte radiosensitivity.

As it has been established that the hormones mentioned above affect radiosensitivity, it was important for this study to find out whether or not dihydrotestosterone (DHT), a hormone intimately concerned in the control of growth factors involved in the growth processes of the prostate and hence, in the proliferative disorders such as prostate cancer, influences radiosensitivity.

In order to ascertain the effects, if any, of DHT on radiosensitivity a number of approaches were employed. Two established prostate cell lines were used, LNCaP, which was immortalised from a lymph node metastasis and is known to

be androgen sensitive and PC3, derived from a bone marrow metastasis and androgen insensitive. The radiosensitivity of these cell lines was assessed in two ways, one, using the MTT assay (materials and methods sections 2.10. and 2.10.1.) to establish the effect of different radiation doses on cell survival/proliferation and the cytokinesis-block micronucleus assay (CBMN) assay (materials and methods section 2.3.2.) was used to establish the amount of chromosomal damage incurred by the cells when exposed to increasing doses of ionising radiation. The effect of DHT on the radiosensitivity of peripheral blood lymphocytes (the cells obtained from patients) was also assessed by using the CBMN assay (described in materials and method section 2.3.).

4.2. Experimental procedures and results.

4.2.1. The MTT assay.

The MTT assay is a commonly utilised colourimetric assay that estimates cell viability. It does this by measuring the activity of dehydrogenase enzymes present in the cell. When viable cells take up MTT, (a yellow tetrazolium salt solution), it is reduced by the dehydrogenase enzymes to blue-purple formazan crystals. This process requires active mitochondrial function; hence, it occurs with proportionality to cell activity. Thus, giving a measurement of cellular survival and proliferation. The crystals precipitated can be solubilised with DMSO and Sörensens buffer (organic solvents) and the optical density of the purple colour in the solution can then be determined spectrophotometrically at 570nm. (Slavotinek *et al* 1994 and Banasiak *et al* 1999). This makes the assay a powerful tool for quickly determining cell growth or substance cytotoxicity.

4.2.2. Optimisation of MTT assay conditions.

4.2.2.1. Optimum cell number and time.

Growth characteristics of the two prostate cell lines, LNCaP and PC3 were established by measuring cellular survival and proliferation using the MTT assay (described in materials and methods section 2.10. and 2.10.1.). A series of cell doses, (200 cells, 1000 cells, 2000 cells and 4000 cells per well) were grown in 96 well plates and monitored over a period of 6 days. After incubation, the cell cultures were assessed for viability, 50µl of 5mg/ml of tetrazolium salt solution (MTT) in PBS was added to each well and incubated in the dark. The medium and MTT was removed by snap inversion of the plate and the crystals resuspended in 200µl DMSO and 50µl Sörensens buffer. The optical density resulting from the solubilisation of the crystals was read immediately using an MRX-ELISA multiwell scanning spectrophotometer at 570nm, values were corrected by the subtraction of the optical density of blanks (this was done automatically by the machine). The mean absorbance of each column of 8 replicate wells was calculated and an optical density obtained for each data point, allowing quantitative expression of cell viability. Results for the prostate cell lines are illustrated below in figure 4.1 for LNCaP and figure 4.2 for PC3 (see Appendix VII, Table VII.1.).

A cell dose of 2000 cells per well was found to produce a slow enough growth rate to allow meaningful comparisons between the effects of different radiation doses and DHT treatment. In light of this future MTT assays were set up using this cell dose.

The growth rates of the cell lines were measured over 6 days (illustrated in figures 4.1 for LNCaP and 4.2 for PC3). The graphs for both cell lines show an initial period of slow growth for each cell dose, followed by a period of rapid growth which reached a plateau and then there was a subsequent decline in cellular survival for the two highest cell doses investigated, 2000 and 4000 cells per well. The cell dose decided upon (2000 cells per well) showed most rapid growth on day five with a slight decline by day six. In subsequent MTT assays, growth was recorded at day five in order to measure the maximum cellularity attained before plateau.

Figure 4.1.

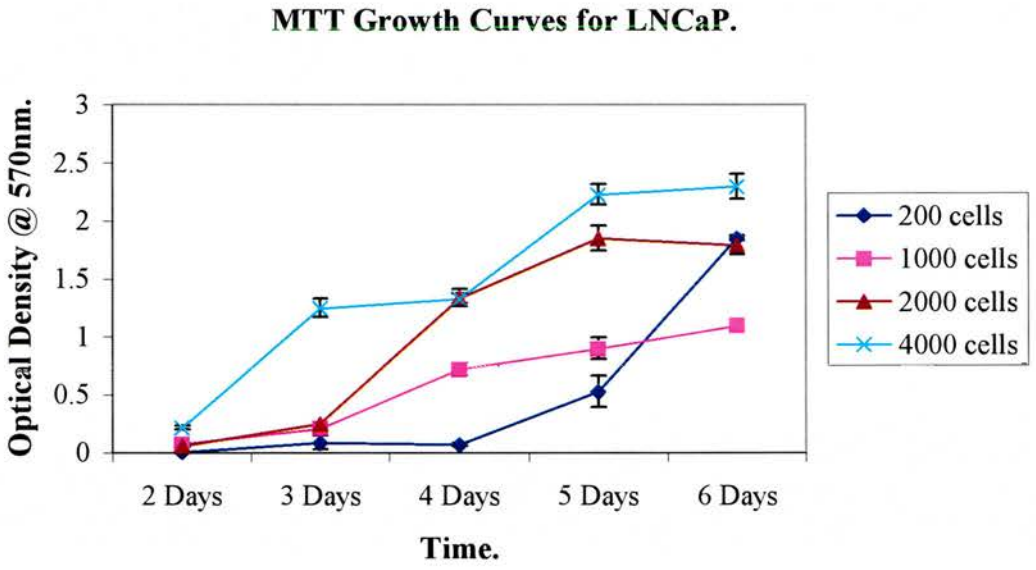

Figure 4.1. MTT growth curves for LNCaP.

Graph shows growth curves obtained by the MTT assay for LNCaP. Different cell doses were plated and monitored over a period of 6 days with readings being taken every day. Data points are mean optical densities of recordings taken from 8 identical wells with standard error bars.

Figure 4.2.

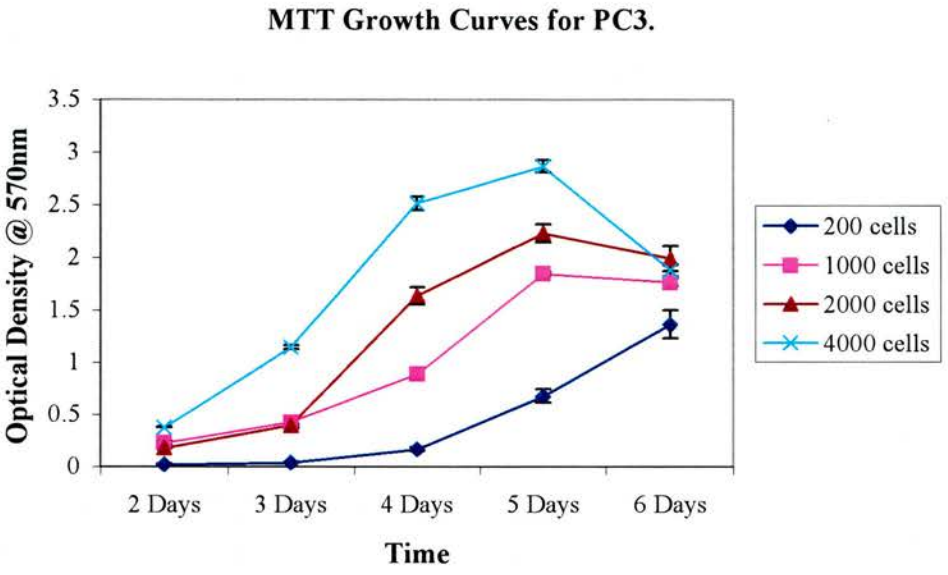

Figure 4.2. MTT growth curves for PC3.

Graph shows growth curves obtained by the MTT assay for PC3. Different cell doses were plated and monitored over six days with readings being taken every day. Data points are mean optical densities of recordings taken from 8 identical wells, showing standard errors.

4.2.2.2. Optimum concentration of DHT.

After the optimisation of MTT assay conditions, the prostate cell lines were acutely exposed to a range of DHT concentrations so that an optimum dose for use in further experiments (into its effect on radiosensitivity) could be ascertained. Cells were plated for the MTT assay and DHT (rehydrated in absolute alcohol, from which serial dilutions were made using the appropriate culture medium) was added at concentrations ranging from 10^{-6}M (1μM) to 10^{-10}M (0.1nM) and a negative control included, results are illustrated in figure 4.3 (see Appendix VII, Table VII.2.). The higher concentrations of DHT appear to

inhibit the growth of LNCaP cells, as the readings observed are lower than that of the negative control grown without DHT. Both 10^{-9} M (1nM) and 10^{-10}M (0.1nM) concentrations of DHT increased the optical density observed implying that there had been an increase in the proliferation of LNCaP cells. However, as expected, PC3 showed no response to any of the DHT concentrations used, as all optical densities recorded were almost identical to those observed in the negative control. From these results, it was decided that 10^{-9} M should be used in future experiments, as it elicited a growth response and is within the normal physiological range found in men.

Figure 4.3.

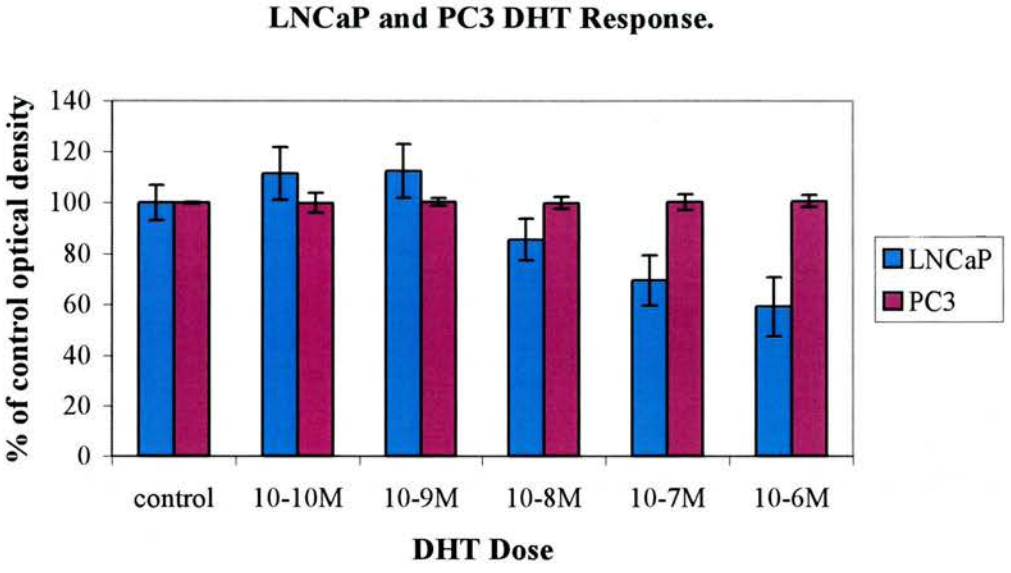

Figure 4.3. LNCaP and PC3 DHT response.

Histogram showing the prostate cell lines (LNCaP and PC3) response to a range of DHT doses expressed as a percentage of the optical density of the negative control. An increase in optical density can be seen with LNCaP in the presence of 0.1nM and 1nM DHT and a decrease in the optical density in the presence of 10nM, 100nM and 1μM DHT. Whereas, PC3 shows no change in optical density with any of the DHT doses, implying that this cell line does not respond to DHT. Each bar is the mean optical density of 8 identical wells with standard error bars.

4.2.3. Radiosensitivity of the prostate cell lines and the effects of DHT as measured by the MTT assay.

To establish radiation dose response curves for PC3 and LNCaP, cell suspensions were set up and irradiated using the caesium gamma source (IBL473C) at doses ranging from 1-10 Gy. These suspensions were then plated

out in 96 well flat plates (2000 cells per well) in the presence and in the absence of 1nM (10^{-9}M) DHT and grown in a gassed humidified incubator for 5 days after which the cultures were assessed for cell viability using MTT.

As expected, the survival of both cell lines decreased with an increasing radiation dose (see figures 4.4. and 4.5.). However, the cell lines show different growth rates under the experimental conditions i.e. different radiosensitivities. In the case of LNCaP, (see figure 4.4.) there was not as much change in the optical density between 5 and 10Gy as there was between 0 and 4Gy suggesting that the larger doses of radiation may have stopped the cells cycling, possibly so that repair can be initiated and that no severely detrimental mutations are passed onto future generations, or the number of viable cells has been reduced sufficiently for the lag phase of the cells to have been increased beyond the 5 day limit of these experiments. An illustration of this can be seen in figure 4.1. where significantly lower numbers of cells were plated and the exponential growth phase of the cells is not reached before the end of the experimental time course. PC3 cells behaved in a different way showing a steady decrease in optical density with each incremental increase in radiation dose. The growth curves for the cell lines treated with DHT were very similar to those of the untreated cultures; this is illustrated in figure 4.4. for LNCaP and figure 4.5. for PC3 (see Appendix VII, Table VII.3 and Table VII.4). Therefore, DHT had no significant effects on the dose response curves of either cell line. These results were confirmed statistically by analysing the variance using the General Linear Model (this was used as the numbers were unequal). As mentioned above the radiosensitivity of the cells did not change when they were treated with DHT

this is reflected in the p values for PC3, p = 0.816, and for LNCaP, p = 0.997. Both of these p values are greater than 0.05, therefore, this supports the conclusion that the radiosensitivity when measured by the MTT assay is not affected by the presence or absence of DHT.

Figure 4.4.

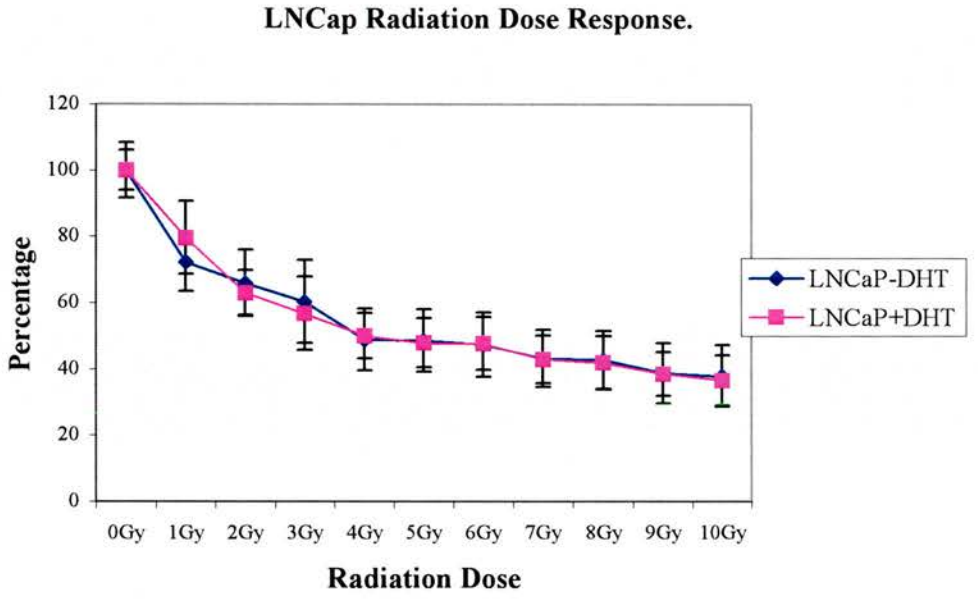

Figure 4.4. Radiation dose response of DHT treated and untreated LNCaP cells.

Line graph showing the radiation dose response curves for LNCaP cultured in the presence of DHT and LNCaP grown under normal culture conditions. The y-axis is the optical density expressed as a percentage of the mock-irradiated (0Gy) sample. It can be seen that the dose response curves of both the treated and untreated cells are very similar. Each point is derived from the mean optical density of 8 identical wells and standard errors are displayed.

Figure 4.5.

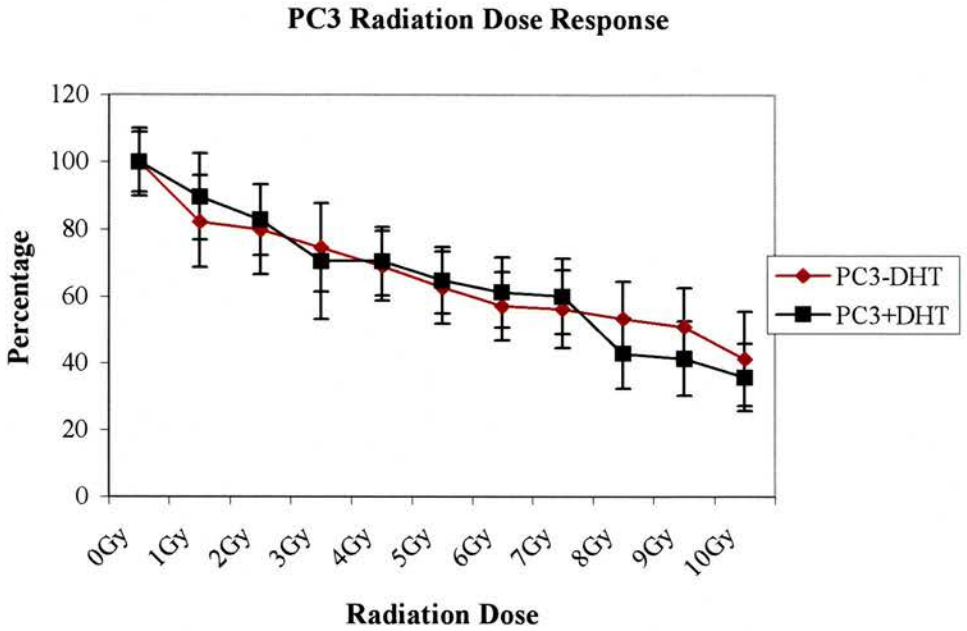

Figure 4.5. Radiation dose response of DHT treated and untreated PC3 cells.

Line graph showing the dose response curves of untreated PC3 cells and PC3 cells treated with DHT. The y-axis is optical densities expressed as a percentage of the unirradiated cells (0Gy). It can be seen that there is not much visible difference between the dose response curves of the cells grown under normal culture conditions and those treated with DHT. Each data point mean optical density of 8 identical wells with standard errors.

4.3. The cytokinesis block micronucleus (CBMN) assay.

The CBMN assay is reliable and simple way to visualise DNA lesions. It enables the measurement of chromosomal damage incurred by cells in the G0 phase of the cell cycle. The CBMN assay quantifies the post mitotic micronuclei, which arise from chromatid, chromosomal fragments or whole chromosomes not incorporated in the daughter nuclei, hence providing a

measurement of chromosome loss and chromosome breakage. It is now one of the preferred methods for assessing chromosomal damage.

4.3.1. Radiosensitivity of the prostate cell lines and the effects of DHT as measured by the CBMN assay.

Cell suspensions of both the prostate lines were irradiated with doses ranging from 0-10Gy. The cells were set up for the micronucleus assay as described in materials and methods section 2.3.2. and cultured either in the presence or absence of 1nM DHT for 24 hrs. 2μg/ml of cytochalasin B was added to block cytokinesis and the cells were grown for a further 48hrs. After a total of 72hrs, the cells were trypsinised and cytospun onto slides. The resulting spreads were scored for micronuclei in accordance with the criteria in materials and methods section 2.3.1.

Both cell lines showed an increase in the number of micronuclei with increasing radiation dose, this can be seen in figure 4.6. for LNCaP and figure 4.7. for PC3 (see Appendix VII, Tables VII.5 and VII.6). Again there is a difference between in the responses of the cell lines to radiation damage. The increase in the number of micronuclei shown by LNCaP is much greater from 5Gy to 10Gy than it is from 0Gy to 4Gy, this can be seen in figure 4.6. For PC3, there is a steady increase in the number of micronuclei with the increase in dose from 0Gy to 6Gy and between 8Gy and 10Gy, but at 7Gy there is a large decrease in the number of micronuclei expressed (see figure 4.7.).

LNCaP cells grown in the presence of DHT showed a significant decrease in the amount of chromosomal damage expressed especially at radiation doses higher than 4Gy, (see figure 4.6.). PC3 cells, although a little less prominent on the graph, also shows a decrease in the number of micronuclei in the presence of DHT (see figure 4.7.). This suggests that DHT does affect radiosensitivity to some degree. The differences in chromosomal damage illustrated in the graphs in treated and untreated cells of both cultures are significant. These changes in the number of micronuclei were confirmed as statistically significant by two way analysis of variance (ANOVA), with both cell lines having a p value of less than 0.05, LNCaP with $p = 0.037$ and PC3 $p = 0.040$.

Figure 4.6.

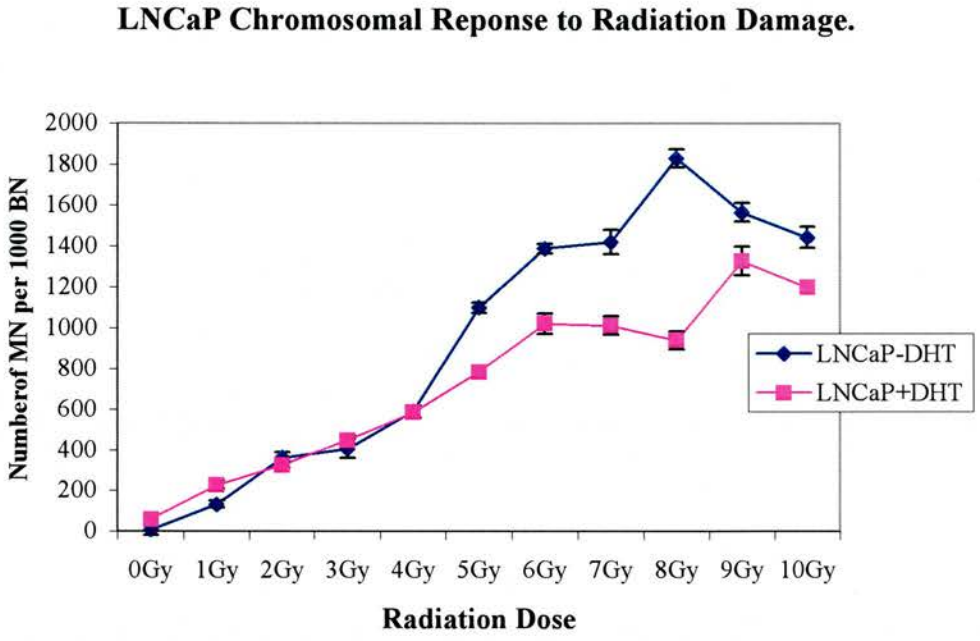

Figure 4.6. Chromosomal response of DHT treated and untreated LNCaP cells to radiation damage.

Line graph showing LNCaP chromosomal response to radiation damage. This graph shows that the cells grown in the presence of DHT express a smaller number of micronuclei than the untreated cells. Each point is the number of micronuclei (MN) per 1000 binucleated cells (BN).

Figure 4.7.

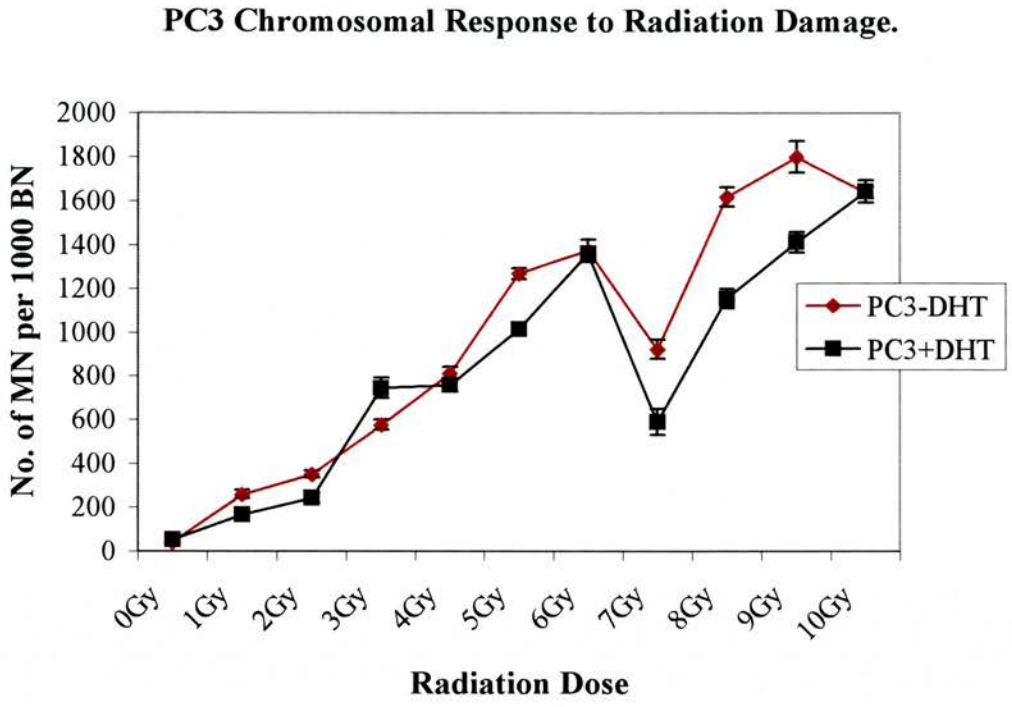

Figure 4.7. Chromosomal response of DHT treated and untreated PC3 cells to radiation damage.

Line graph showing PC3 chromosomal response to radiation damage. This graph shows that the cells grown in the presence of DHT show a reduced amount of chromosomal damage. Each point on the graph represents the number of micronuclei (MN) per 1000 binucleated (BN) cells.

4.3.2. Radiosensitivity of peripheral blood lymphocytes and the effects of DHT as measured by the CBMN assay.

Blood cultures were set up for the CBMN assay as described in materials and methods section 2.3. 1ml of whole blood was added to 9mls of pre-warmed medium, this was allowed to equilibrate for 1hr in the incubator. If the cultures

were treated with 1nM DHT, it was added at this point and equilibrated with the medium. Two flasks were set up for each experiment, one was mock irradiated (0Gy) and the other exposed to 3.5Gy gamma radiation from a caesium gamma source (IBL473C). The lymphocytes were then stimulated to divide by the addition of PHA and the cultures left for 42 hrs. At this point 6μg/ml cytochalasin B is added to block cytokinesis and accumulate binucleate cells and the cultures are returned to the incubators for a further 30hrs. After a total incubation of 72hrs cells are a harvested and dropped on slides for scored using the criteria described in materials and methods section 2.3.2.

A small number of samples obtained from patients were used for this study. For each sample, 4 flasks were set up as described above and two were treated with DHT and two left untreated. One flask from the treated group and one flask from the untreated group were exposed to radiation (3.5Gy) and the others mock irradiated (0Gy). Each patient had a different response to radiation damage, shown by the number of micronuclei expressed (see the histogram in figure 4.8. and Appendix VII, Table VII.7.) The samples treated with DHT all show a marked reduction in the number of micronuclei expressed at 3.5Gy (see figure 4.8. and Appendix VII, Table VII.7.) when the student T-test was applied the p value was less than 0.05, $p=0.001$, confirming that the presence of physiological concentrations of DHT does affect lymphocyte radiosensitivity.

Figure 4.8.

Lymphocyte Chromosomal Response to Radiation Damage.

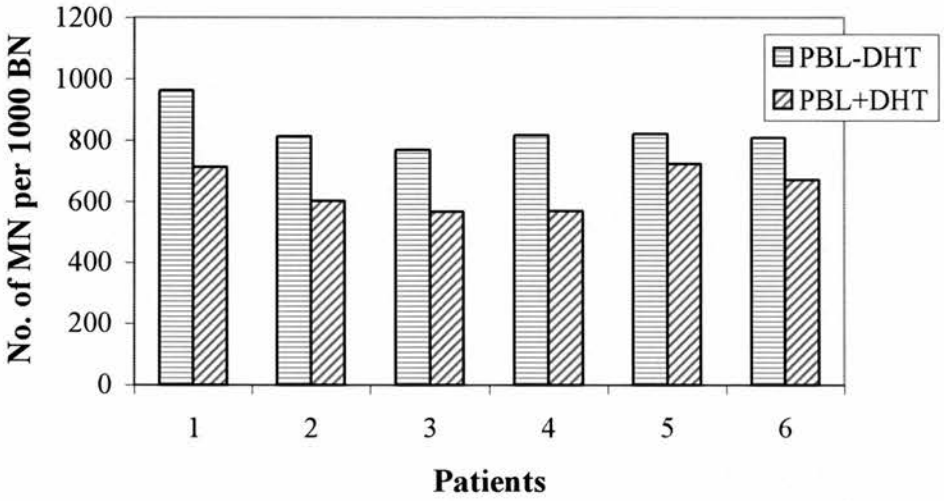

Figure 4.8. Chromosomal response of DHT treated and untreated peripheral blood lymphocytes (PBL) to radiation damage.

Histogram showing the chromosomal responses of lymphocytes, obtained from six different patients, to radiation damage. This graph shows that lymphocytes cultured in the presence of DHT show a reduction in the amount of chromosomal damage expressed. Each bar represents the number of micronuclei (MN) per 1000 binucleated cells (BN) irradiated at 3.5Gy with the number of spontaneous micronuclei deducted.

4.4. Discussion.

4.4.1. Summary.

This chapter aimed to establish if the androgenic hormone DHT affected the radiosensitivity of prostate cells and PBLs taken from male patients, as other hormones have been shown to alter radiosensitivity. In order to investigate this,

the effect of DHT on radiation induced damage was evaluated in two prostate cell lines LNCaP which is hormone sensitive and PC3 which is hormone insensitive, by using the MTT assay and the CBMN assay. The effects of DHT on PBL radiosensitivity were also assessed by the CBMN assay using lymphocytes obtained from male patients.

Results obtained using the MTT assay, showed that DHT had little or no affect on the radiation dose response curves of either cell line and that it didn't really stimulate growth of LNCaP (the hormone responsive cell line). However, using the CBMN assay, it was found that DHT had a radioprotective effect, decreasing the radiosensitivity of LNCaP cells (which showed the greatest difference between treated and untreated), PC3 cells and PBLs. All of the cell cultures, LNCaP, PC3 and lymphocyte, treated with 1nM DHT showed significantly less chromosomal damage by a reduction in micronucleus expression. The cell lines showed lower levels of chromosomal damage especially after exposure to the higher radiation doses.

4.4.2. Physiological status and radiosensitivity.

Previous studies have indicated that radiosensitivity might vary with physiological status. It has been noted that females display a greater variability in radiosensitivity, with a wider range of sensitivities being recorded in the female population, than in the male population. There is also a greater degree of intra-individual variation in the female population; radiosensitivity differs among blood samples taken from the same person on separate occasions (Roberts et al 1997). This effect must be mitigated by something transitory,

hence, the suggestion that the female hormonal cycle could be contributing to the variation observed. A number of studies have been conducted into this, the variation of radiosensitivity seen in mothers, paralleled that of the pregnancy hormones, suggesting that progesterone and oestrogen, or both might increase radiosensitivity. The increase in radiosensitivity observed was proportional to the amount of progesterone found in the serum (Ricoul et al 1997). A possible explanation for this is that the progesterone decreases the DNA repair synthesis. Various oestrogens have also been shown to generate increases in radiosensitivity. Oestrogens are epigenotoxic, not directly mutagenic or DNA damaging, but they cause heritable changes by an as yet unknown mechanism (Roberts et al 1997). More recently it has been shown that oestradiol also increases radiosensitivity (Kanda and Hayata 1999).

Although there is no obvious male hormonal cycle, the prostate does appear to go through growth phases, initially at birth, at puberty and again after the age of 50. The current understanding is that physiological levels of DHT are required for the growth and development of the prostate. Hence, the use of androgen ablation for prostate cancer.

The levels of circulating testosterone fluctuate and change especially with increasing age. These fluctuations have been implicated in glandular enlargement and hence the progression to prostate cancer and it was important under the contexts of this study to discover whether radiosensitivity was affected.

It has been previously stated that the in vitro radiosensitivity of androgen receptor positive prostate cancer cell lines is not necessarily altered by the presence of androgen before or after irradiation (Wollin et al 1989). This study looked at cell survival of the prostate carcinoma cell lines PC3 and DU145 in the presence of testosterone and beta-oestradiol after X-irradiation and found that these hormones did not alter radiosensitivity (Wollin et al 1989). This is similar to the findings of this research; the radiation dose response survival curves of LNCaP and PC3 measured by the MTT assay were not affected by the presence or absence of DHT.

However, a decrease in radiosensitivity of LNCaP and PC3 in the presence of DHT was detected via the CBMN assay. Both cell lines displayed a significant decrease in the number of micronuclei expressed when grown in the presence of physiological concentrations of DHT. This decrease could give clues to and be indicative of other functions of DHT within the prostatic environment. This decrease in radiosensitivity has several possible explanations. One possibility is that DHT in some way enhances radiation-induced apoptosis of damaged cells, resulting in the lower frequency of chromosome aberrations observed. The MTT assay results presented argue against this hypothesis, as survival was not affected. However, TGF-β mediated apoptosis in prostate cancer cells has been shown to be enhanced by androgens (Bruckheimer and Kyprianou 2001). This is thought to act through specific mechanisms involving cell cycle and apoptosis regulators. This provides initial evidence that physiological levels of androgens (namely DHT) have the ability to stimulate the intrinsic apoptotic potential of prostate cancer cells (Bruckheimer and Kyprianou 2001). The opposite of this

has been suggested for the hormones, which increase radiosensitivity, these hormones could decrease radiation induced apoptosis (Roberts et al 1997).

Another related explanation could be that DHT might enhance the repair of radiation-induced damage. This is also the reverse of what has been suggested as the mechanism of action of the other radiosensitivity increasing hormones (that they reduce repair through unknown routes). However, causative mechanisms of hormonal influence on radiosensitivity remain speculative and need further investigation.

It is fundamental to the appropriate development of clinical strategies involving androgen deprivation and radiation therapy to understand the basic principles governing the possible interaction of androgens and radiosensitivity. It has been shown that androgen blockade does not result in a reduction of prostate volume, but it changes the histology; glands atrophy and stromal tissue increases (De Voogt et al 1987). This in itself could affect the radiosensitivity as cell types with different receptors show different radiation responses as illustrated by PC3 and LNCaP in this study. If as suggested, by the results of this study DHT is radioprotective, does androgen deprivation cause radiosensitisation? Pollack et al (2001) suggests that this is not the case, as apoptosis does not appear to be the major mechanism responsible for the combined effects of androgen deprivation and radiotherapy. This combination of therapies alters the kinetics of prostate cancer growth. Therefore, additive cell killing and reduced cell proliferation account for the effects of combined therapy (Pollack et al 2001).

If DHT sensitises prostate cancer cells to undergo apoptosis, this could be used as an addition to the combined androgen deprivation and radiotherapy as a priming mechanism. A molecular interaction between TGF-β and androgen signalling pathways has been suggested. A further understanding of this cross talk will facilitate the design of new mechanistic apoptosis driven therapeutic modalities to effectively treat androgen dependent and independent prostatic tumours (Bruckheimer and Kyprianou 2001).

As well as in the cell lines, a decrease in radiosensitivity has also been shown in PBLs (from male patients) cultured in the presence of DHT. All of the samples tested using the CBMN assay showed a significant decrease in the number of micronuclei expressed. One possible explanation of this could be that DHT promotes the growth of a particular subset of lymphocyte and that possibly this type of lymphocyte is less susceptible to the induction of chromosomal damage by irradiation, or it has more effective DNA damage detection and repair synthesis.

4.5. Conclusions.

Hormones do appear to affect radiosensitivity, although their mode of action is not understood. DHT, according to the results of this study reduces the amount of chromosomal damage whereas, other studies show that progesterone, oestrogen and oestradiol increase radiosensitivity. These hormones could exert their opposite effects on the same processes of apoptosis and repair. Their effects could be seen in patients when the hormonal balance is altered.

4.6. Future work.

For the continuation of this study, it would be important to repeat all the experiments with charcoal stripped serum so that no other DHT is present apart from that added this should cut down variability. Also, pre-treatment of the cells with DHT instead of an acute exposure might lead to a greater response from LNCaP. In addition to using the MTT assay, colony assays should be performed as an additional means of assessing proliferation and survival. If possible, it would be interesting and potentially important for the treatment of prostate cancer to look at the radiosensitivity of and hormonal effects in the target tissue, primary prostate epithelial cells and to make comparisons between the different epithelial and stromal compartments and their influences on each other and the different cell populations inherent radiosensitivity.

The radiosensitivity of prostate cells is altered by DHT. Do other hormones not yet investigated have any affect on prostate cells or PBLs? These investigations may give clues for the improvement of currently available treatment and aid in making it more effective. Also, the effects of hormone cocktails with different proportions should be investigated, as that is what is normally found in circulation and different proportions and combinations could elicit different responses.

The effects of DHT on G2 radiosensitivity should be explored as the damage incurred is thought to occur through different mechanisms and DHT might have an affect on these as well.

CHAPTER 5

PROSTATE CELL CULTURE.

CHAPTER 5
PROSTATE CELL CULTURE.

5.1. Introduction.

5.1.1. Chapter Rationale.

The transformation of normal human epithelial cells into cancer cells is associated with major phenotypic changes; these include immortalisation, aberrant growth control and malignancy (Riches *et al* 2001). Human prostate cancer is a disease of increasing frequency and one for which the genetic and environmental contributory factors are largely unknown (Maitland *et al* 2001). Although mutations have been observed in a wide variety of oncogenes and tumour suppressor genes, little is known regarding the genes that control prostate cancer susceptibility (Yasunaga *et al* 2001). Unlike other major cancers, such as breast, lung and colon cancer, little is known about prostate cancer at the molecular level and there is no long-term treatment available and a site-specific model for prostate cancer has not emerged.

The major prostate cell lines currently readily available are all approximately 20 years old having been established at a time when in vitro cell culture was in its infancy (Maitland *et al* 2001). To date, only three readily available and well studied long-term human prostate carcinoma cell lines exist: DU145, PC3, LNCaP. All three were isolated from extraprostatic metastatic lesions, thus there is a lack of long-term human cell lines derived from and representing primary localised adenocarcinoma of the prostate (Ko *et al* 2003).

The use of normal human cells, including prostate epithelial cells, for research, biotechnology and therapeutic purposes has been restricted in part by their

limited proliferative potential. Normal somatic cells divide a finite number of times before permanently exiting the cell cycle and becoming senescent. For many applications it would be ideal to have essentially unlimited numbers of well characterised cells with the desired phenotype especially for the purposes of investigating carcinogenesis.

Ionising radiation has been successfully used as a tool for neoplastic transformation in rodent cells. These cells have been successfully exploited, dose response relationships and dependence of transformation after ionising radiation exposure established (Hall and Hei 1986 and Hei *et al* 1988). However, in contrast, it has been difficult to establish models of human carcinogenesis in vitro.

Previously, immortalised human cell lines have been used as models for the induction of transformation with ionising radiation. These lines have been immortalised from primary cultures using viral vectors from SV40, Epstein Barr, adenovirus and HPV. Studies of radiation carcinogenesis using this approach have investigated human keratinocytes (Thraves *et al* 1990), human fibroblasts (Morgan *et al* 1991), human bronchial epithelium (Hei *et al* 1994), human thyroid cells (Riches *et al* 1994 and 1997), and human prostatic epithelium (Kuettel *et al* 1996). Virally immortalised cell lines are often genetically unstable with variable chromosome numbers and polyploid karyotypes (Riches *et al* 2001). These factors complicate and limit the use of these models, as genomic instability confounds studies of genetic change in carcinogenesis. Consequently, investigators studying radiation-induced cancers

have been searching for improved in vitro models of human neoplastic transformation (Riches *et al* 2001).

Immortalisation of primary cells using retroviral technology has been widely reported in many different tissue types. The retroviruses used for this work were modified from MoMLV where the key elements required for infection were separated over three vectors. Viral coding regions of retroviral vectors have been removed and viral elements have been reduced as a result these retroviral vectors can be used to transfer only the gene of interest. This also means that viral replication is limited to a single round, which reduces the mutation and recombination rates, hence, limiting the genomic instability.

Recent advances in telomerase research and stem cell technology are opening up the possibility of large-scale culture of human cells capable of attaining a variety of differentiated phenotypes. This should be an enabling research tool for renewed efforts to optimise cell culture conditions for human cells of various lineages (Yeager and Reddel 1999).

Primary cultures of mammalian cells undergo senescence *in vitro*. This seems to be associated with gradual shortening of the telomeres until a critical length is reached after which the result is cell death. Telomerase activity is repressed in most normal human somatic cells, limiting the potential number of cell divisions available, but is almost universally expressed in cancer cells and in stem cells (Kim *et al* 1994, Shay and Wright 1996, Riches *et al* 2001). The mechanism by which telomerase is normally repressed appears to operate at the

level of transcriptional regulation of the catalytic subunit (the protein component) of the telomerase ribonucleoprotein. The life span of human primary cell cultures has been extended substantially by transfection with a vector expressing TERT, the catalytic subunit of telomerase (Bodnar *et al* 1998, Riches *et al* 2001). These cells express telomerase and maintain their telomere length during culture; this does not seem to induce a malignant phenotype on its own. Thus, it has been concluded that induction of an immortal phenotype is a necessary prerequisite and probably an early event in malignant transformation, but insufficient on its own (Morales *et al* 1999, Riches *et al* 2001).
Indeed transfection of hTERT into selective human cell types for example fibroblasts, retinal pigment epithelial cells, mammary epithelial and endothelial cells, can itself induce immortalisation. Interestingly, telomerase expression in human somatic cells does not induce any change associated with a transformed phenotype. Primary cells transduced with the gene that delays replicative senescence will show increased growth potential without converting transformation or showing any karyological artefacts, thus making them ideal *in vitro* models for the study of prostate carcinogenesis (Yasunaga *et al* 2001).

The goal of this research was to generate new, continuously proliferating human prostate epithelial cell lines from primary prostate tissue obtained from BPH patients using telomerase and to use ionising radiation as a tool to neoplastically transform the cells and study the changes induced at the molecular and genetic level.

5.2. Experimental procedures and results.

5.2.1. Primary prostate epithelial isolation and culture.

Primary prostate cells were extracted from tissue removed from BPH patients undergoing transurethral resection (TUR). The tissue was processed as described in materials and methods section 2.6. The TUR chips were chopped into small fragments with a sterile scalpel, suspended in a collagenase solution and gently agitated at 37°C for 20hrs. The remaining small clumps of tissue were then rinsed thoroughly and placed in a $75cm^3$ flask(s), incubated at 37°C 5% CO_2 in a humidified incubator, until subconfluency was reached. Aliquots of the primary cultures were frozen down (as described in materials and methods section 2.6.4.) and stored in liquid nitrogen. The cells were then re-established for secondary culture, further serial passages and future experiments. Tissue was received from 16 patients in total all of these samples were treated in the manner described in materials and methods section 2.6. and above. Each sample responded slightly differently, mainly in the time taken for epithelial outgrowths to appear. It was found that the primary prostate epithelial cells have a doubling time of approximately 52hrs (this was established by seeding cells at a known density and passaging after a known period of time and performing counts of the cell suspension after trypsinisation) and will grow in serum free medium (PrEGM) for approximately 5 passages until senescence ensues see table 5.1 for details of each primary tissue received.

Table 5.1.

Prostate tissue	Number of passages.	Comments.
PT1	5	
PT2	6	
PT3	5	
PT4	5	
PT5	4	
PT6	4	
PT7	1	Did not grow after passaging from original flask.
PT8	4	
PT9	4	
PT10	1	Flask became contaminated
PT11	6	
PT12	0	Dropped in formalin at hospital, rinsed immediately, but did not attach to flask after collagenase treatment.
PT13	5	
PT14	5	
PT15	5	
PT16	5	

Table 5.1. Primary growth of tissues.

Table shows the number of passages cells undertook before senescence.

Initial growth of prostate tissue occurs from the small lumps of tissue which are left undisturbed in the incubator attach to the flask after collagenase treatment. Figure 5.1. panel a) shows this initial outgrowth of epithelial cells, after the first change of medium at approximately day 5 of culture. These initial outgrowths were then left until they had formed colonies, spread across the flask in a monolayer grown into a subconfluent culture; this culture was then passaged (see sections 2.6.1 and 2.6.3). Healthy epithelial cells from a secondary culture flask can be seen in Figure 5.1 panel b).

Figure 5.1.

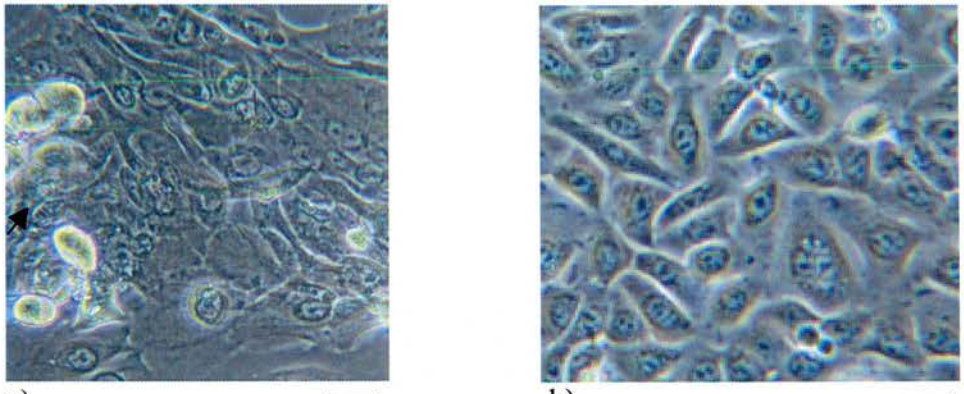

Figure 5.1. Primary prostate epithelial culture.

Photographs show appearance of primary prostate epithelial culture. Panel a) shows initial epithelial outgrowths from a clump of tissue (indicated by the arrow) after about a week in culture. Scale bar ~20μm. Panel b) shows sub-confluent secondary culture of epithelial cells. Scale bar ~10μm.

5.2.1.1. Growth of primary prostate epithelial cells on feeder layers.

It has been thought that the use of feeder layers provides various epithelial cell types with a supply of growth factors and extracellular matrix support allowing growth from lower seeding densities, this has also been advocated for prostate epithelial cells (Hudson *et al* 2002). For comparison epithelial cells were grown with and without feeder layers. In this investigation, primary prostate epithelial cells were seeded straight onto the flasks and onto layers of mitomycin C treated J2 3T3s (prepared as described in materials and methods section 2.6.5.). Mitomycin C treatment inhibits cell division so that only the epithelial cells are proliferating. Both of these cultures were monitored by observing the cells using an inverted phase contrast microscope.

There was little or no difference between the growth and survival of prostate epithelial cells with or without feeder layers. However, after passaging the cells it was difficult to separate the epithelial cells from the feeder layer. As the main of objective was to produce pure epithelial culture it was decided to grow cells without feeder layers.

5.2.2. Immunocytochemical determination of epithelial cell type.

In order to determine the epithelial origin (luminal, basal or intermediate) of the cells isolated from the prostate tissue, the expression of cell type markers was investigated by immunocytochemistry. Cytokeratins, intermediate filament proteins, are an integral part of the cell cytoskeleton and are often used for the identification of tissue components. Subgroups of heterodimerised cytokeratins (CK) are expressed by epithelial cells, thus enabling the discrimination between

basal and luminal localisation. As in all simple epithelia, luminal cells in the human prostate express CK8 and CK18. Basal cells were originally identified by their expression of CK5 and CK14, but CK8 and CK18 have since been shown to be present in at least a subset of them (Nagle *et al* 1991, Yang *et al* 1997, Van Leenders 2000). In order to confirm the presence or absence of basal cells in the isolated culture, the expression of p63, a selective marker of basal cells within the prostatic epithelium (Signoretti *et al* 2000) was also studied.

Cells were trypsinised from flasks (materials and methods section 2.6.3.) and cell suspensions made. The resulting cell suspensions were cytospun onto glass slides then fixed with acetone. Immunocytochemistry was performed using primary antibodies against CK5, CK14, CK18 and p63 and the Vectastain Elite ABC kit (materials and methods section 2.8.). After application of the primary antibody the slides were blocked with serum and then incubated in biotinylated secondary antibody. Following this, slides were incubated with Vectastain ABC solution (with avidin and biotinylated horse radish peroxidase complex), developed with DAB and counterstained with haemotoxylin. The slides were then mounted and scored.

All of the primary antibodies used showed positive staining of some degree in each of the epithelial cultures tested. Representative examples of staining with each primary antibody used can be found in Figure 5.2. The epithelial cells isolated and cultured from the tissues seem to be of both luminal and basal origin or possibly an intermediate cell population expressing both luminal and

basal markers. The other tissue samples had a fairly similar cytokeratin and p63 profile although the proportions of each marker differed.

Figure 5.2.

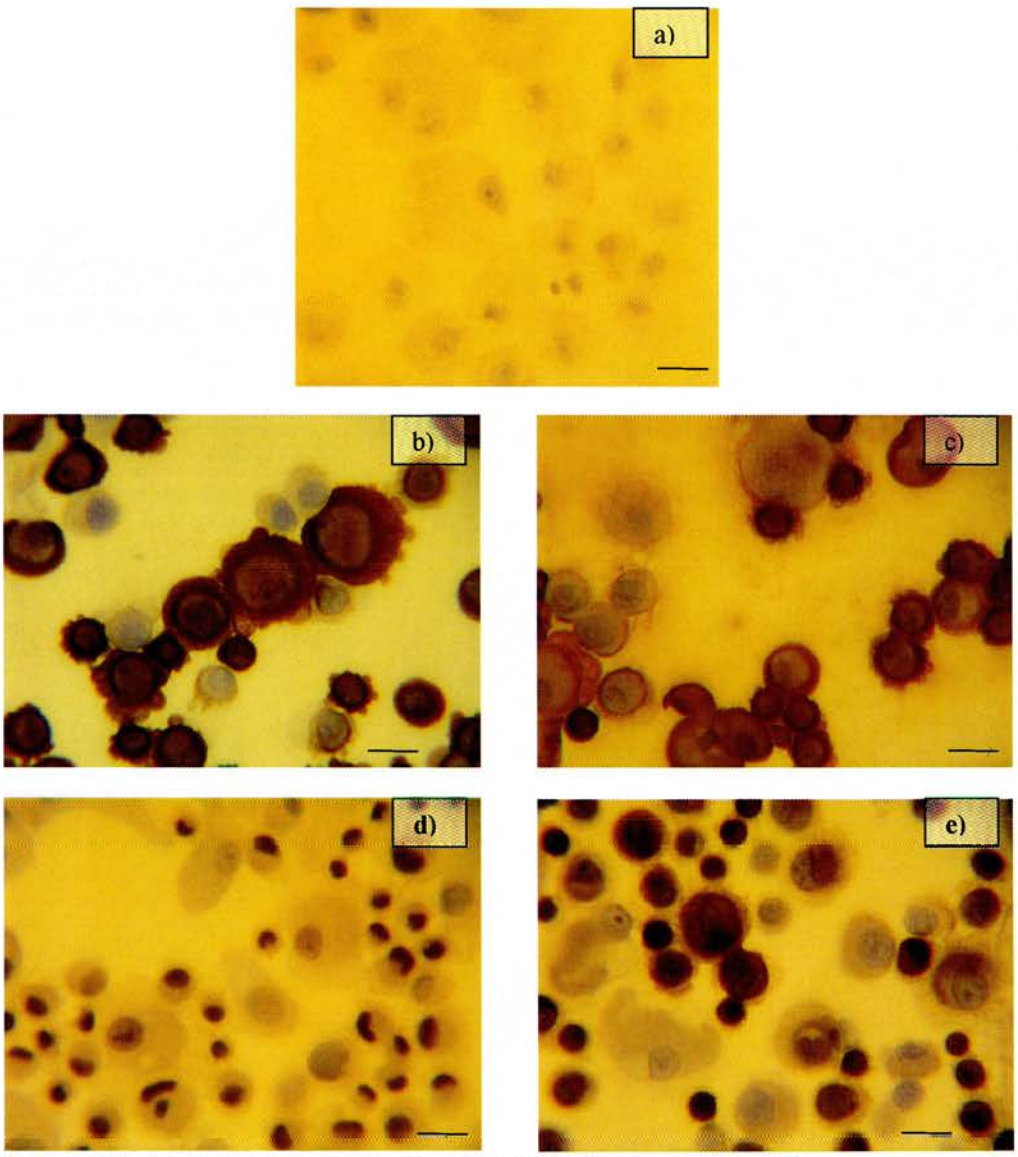

Figure 5.2. Immunocytochemistry for epithelial type markers.

Prostate epithelial cells were cytospun onto slides and immunocytochemistry was performed as described above. These pictures are representative examples of the staining observed. a) Negative control, no primary antibody used. b) Cells stained following exposure to CK5 antibodies. c) Cells stained following exposure to CK14 antibodies. d) Cells stained following exposure to p63 antibodies. e) Cells stained following exposure to CK18 antibodies. Scale bars ~10μm

A representative count of the proportion of cells positive for each marker can be seen in Table 5.2. The slides were scored by counting the positively stained cells in eight fields of view, the percentages and standard errors for the isolated epithelial can be seen below. In this culture, the larger proportion of cells were of basal origin with 73% staining positive for p63.

Table 5.2.

Cell type marker	**Percentage of Positive Staining ± Standard Error**
CK5	66.11 ± 4.93
CK14	67.97 ± 5.79
p63	73.53 ± 27.91
CK18	49.74 ± 7.86

Table 5.2. The expression of cell markers by primary prostate epithelial cells cultured.

Data values are the mean percentages of positively stained cells recorded in 8 samples from the same slide with the standard error quoted.

5.2.3. Immortalisation of prostate cells.

5.2.3.1. Prostate fibroblasts.

Fibroblasts were isolated from the primary prostate tissue alongside the epithelial cells (see materials and methods section 2.6.2.) from the collagenase digested stroma. The fibroblasts grew well in culture so it was decided to test the retroviral infection protocol on these prostate derived cells. The protocol is

described in materials and methods section 2.7.3. Actively proliferating cells were exposed to a retroviral supernatant containing a construct with hTERT and puromycin (see Appendix IV for a vector map). Cells were transduced using the retrovirus and selected for using puromycin. Non-infected cells did not survive the antibiotic selection, however, after the introduction of the retroviral construct, the fibroblasts continued to proliferate in the presence of 1μg/ml puromycin. This suggested that at least part of the cassette had been inserted as they survived puromycin pressure and they continued dividing. The antibiotic pressure was removed and the resulting clones were successfully passaged. The introduction of hTERT into these cells was confirmed by immunocytochemistry performed using a primary antibody against hTERT and the Vectastain Elite ABC kit and protocol (described in materials and methods section 2.8.).

Figure 5.3. shows the staining of prostate fibroblasts after incubation with an hTERT antibody. All the surviving cells seem to have incorporated the hTERT construct, indicated by the brown colour within the nuclei. The cells were not counterstained with haematoxylin so it is difficult to distinguish any other features.

Figure 5.3.

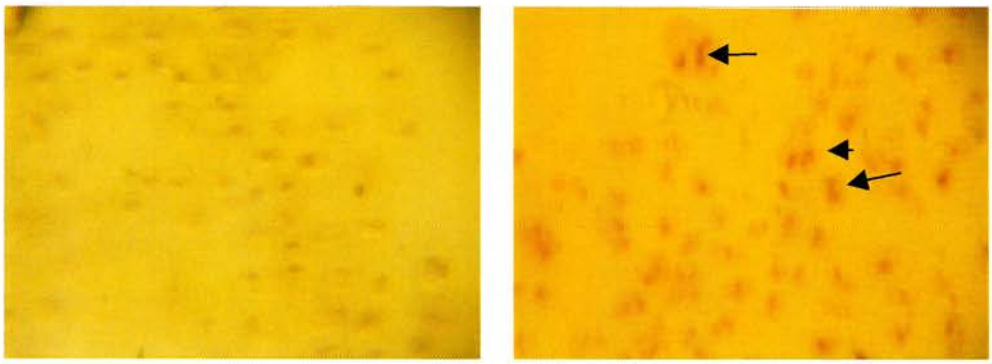

Figure 5.3. Immunocytochemical detection of hTERT.

Prostate fibroblasts were cytospun onto slides and immunocytochemistry was performed (as described above and in materials and methods section 2.8.). a) is the negative control, no primary antibody used. b) Cells stained following exposure to hTERT antibody. It can be seen that there is nuclear staining present in the fibroblasts in panel b) other cell features are not visible as haematoxylin counterstain was not used. Arrows indicate some of the positive staining. Scale bars ~20μm.

5.2.3.2. Prostate epithelial cells.

In an attempt to establish immortal human prostate epithelial cell lines, the human catalytic subunit of telomerase (hTERT) was introduced into the actively proliferating isolated epithelial cell cultures (as described above in section 5.2.3.1.). After the first passage, the actively proliferating prostate epithelial cells were infected with a retroviral construct containing hTERT and a puromycin resistant gene. Cells were transduced with the retrovirus using

polybrene to allow better cell surface attachment and selected for using puromycin pressure (as described in materials and methods section 2.7.3.). Non-infected cells did not survive and could not be propagated in the presence of the antibiotic selection agent (puromycin). In contrast some of the epithelial cells continued to proliferate in the presence of puromycin after the introduction of hTERT, which suggested that at least part of the cassette had been inserted into the cells. The puromycin pressure was removed from these cells and some of the resulting surviving colonies were successfully subcultured. The cells survived for a further 10 passages before senescing. In prostate epithelial cells it does not look like the addition of hTERT alone is sufficient to give these epithelial cells an unlimited life span, but it does extend their proliferative life. Unfortunately the cells senesced before the insertion of hTERT could be confirmed by immunocytochemistry or another means of telomerase detection.

Figure 5.4. shows a subculture of retrovirally infected cells which have survived and proliferated after puromycin pressure, also some of the morphological changes that occur as senescence ensues can be seen. As the epithelial cells senesce, indicated by morphological changes such as the flattening of the cells profile and increasing vacuolation. The cells are still metabolically active in this state, but do not divide.

Figure 5.4.

a) **b)**

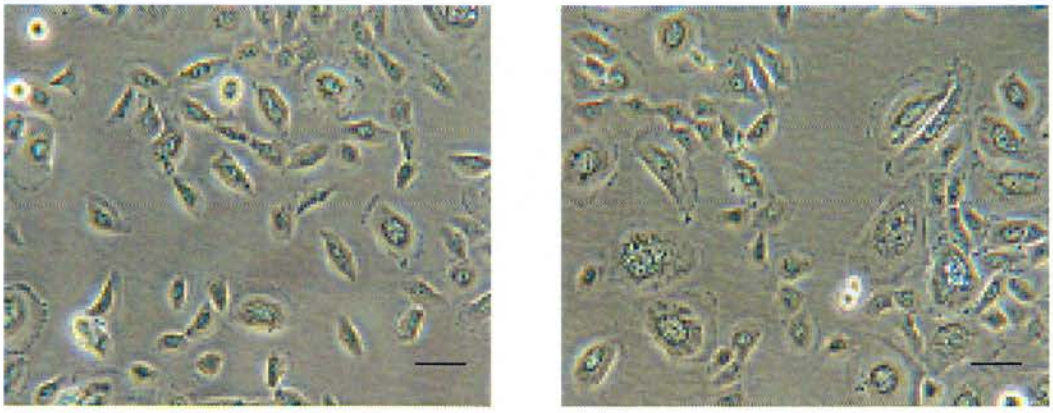

Figure 5.4. Prostate epithelial cells after the retroviral introduction of hTERT and puromycin selection.

Photographs show the appearance of human prostate epithelial cells after the introduction of hTERT and selection by puromycin. Panel a) shows a profile with a mixture of stressed, flattening cells and healthy cells with an intact refractile membrane, whereas panel b) shows senesced cells which are vacuolated and flattened. Scale bars ~20μm.

5.3. Discussion.

5.3.1. Summary.

The aim of the work described in this chapter was to isolate and culture primary prostate cells, especially those of epithelial origin and produce immortalised lines via the introduction of hTERT by retroviral transfer. Then once having established the cell lines to use them in conjunction with gamma radiation to induce neoplastic transformation in the attempt to unravel the cytogenetic changes involved.

Fibroblasts were successfully isolated from the stromal component of the tissue and epithelial cells from the glandular region. It was important to establish whether the epithelial cells were of basal or luminal origin (example of results shown in Figure 5.2.), as the cells have different properties that could be of interest in future experiments.

Cytokeratins and p63 were used to determine epithelial origin. p63 is a recently cloned homologue of the p53 tumour suppressor gene and is selectively expressed in the basal compartments of a variety of epithelial tissue and has been confirmed as a basal marker in prostate epithelium. It may be essential for the maintenance of stem cell populations in various epithelial tissues (Signoretti *et al* 2000).

The cultures isolated from the tissue obtained from BPH patients were a mixture of luminal and basal cells. Proportionally in most of the primary epithelial cultures, the majority of cells were of basal origin, shown by p63 (see Figure 5.2. and Table 5.1.). However, no dual staining immunocytochemistry was performed, as the cytokeratin profiles were fairly mixed, therefore making it difficult to determine with any certainty whether the other cells were definitely luminal, or of an intermediate phenotype.

Numerous immortalisation attempts were made. The catalytic subunit of human telomerase (hTERT) was retrovirally introduced into the epithelial cell cultures. Immortalisation of a number of different types of epithelial cells including those

derived from primary prostate tumours (Yasunaga *et al* 2001) have already been successfully achieved using a similar method.

Primary prostate cell cultures isolated from different patients were exposed to the retroviral supernatant containing hTERT, puromycin selection cassette. Unfortunately to date it has not been possible to immortalise the primary epithelial cells from any of the BPH patients. It seems that hTERT alone is insufficient for immortalisation, but an extension of proliferative life span has been observed in some cultures.

5.3.2. Prostate epithelium and markers of epithelial cell type.

Prostate epithelium contains morphologically basal cells, which make up the proliferative layer and luminal cells, which comprise the secretory layer (Bonkhoff and Remberger 1996, Hudson *et al* 2001). These layers can be discriminated by differentiation specific markers. The luminal cells express PSA, prostatic acid phosphatase (PAP), androgen receptor and CK8 and CK18. CK5, CK14, CD44 (Liu *et al* 1997), bcl-2 (McDonnell *et al* 1992) and p63 (Signoretti *et al* 2000).

Cells with intermediate phenotypes have also been described, these express a mixture of basal and luminal markers, CK5 and CK18 in the absence of CK14, or CK5 with PSA phenotypes have been described (Verhagen *et al* 1992, Bonkhoff *et al* 1994, Xue *et al* 1998). Figure 5.5. shows a possible pathway for prostate epithelial differentiation based on cytokeratin expression. However, the

lineage relationships between secretory, basal and neuroendocrine cells and the existence of a common precursor are still being debated.

Figure 5.5.

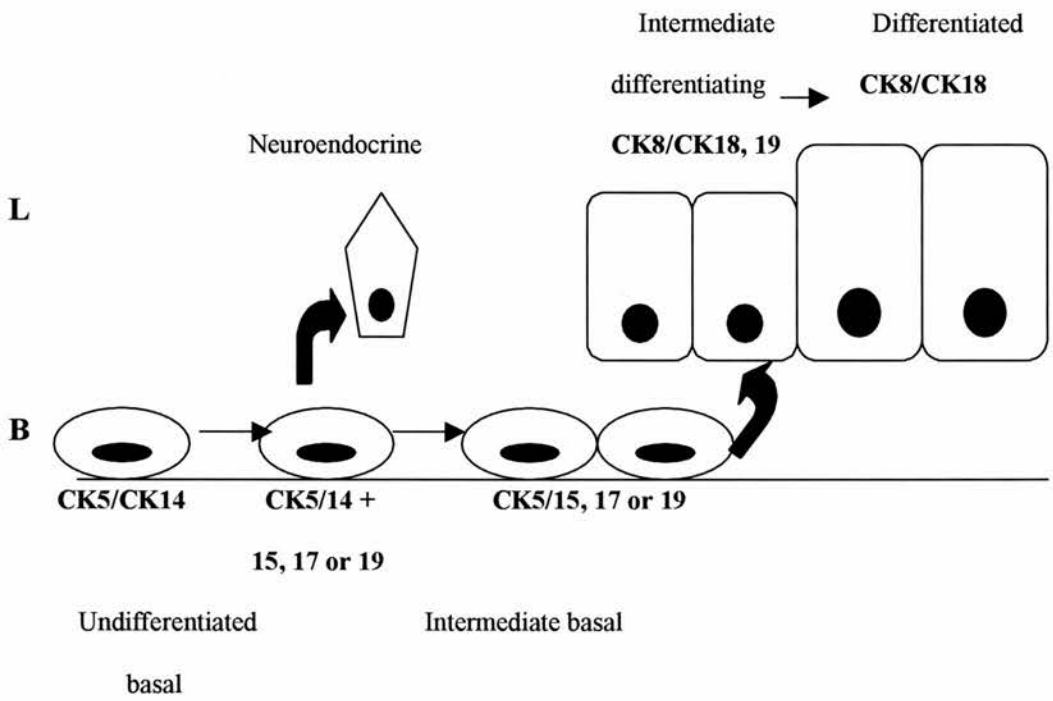

Figure 5.5. Hypothetical differentiation for human prostate epithelial cells. *Possible differentiation pathway based on patterns of cytokeratin staining. Basal cells* **B** *(expressing CK5 and CK14) give rise to a transit amplifying population expressing CK19. These cells differentiate into luminal cells* **L** *with transient coexpression of CK19, CK8 and CK18 before complete differentiation into cells expressing CK8/18 only. Picture adapted from Hudson et al 2001.*

CK 5, CK14, for identification of cells of luminal origin, and CK18, p63 for cells from the basal compartment were used during the course of this investigation. In future investigations, other markers should be added and the

same cells should be stained for the different cytokeratins and other markers. Then that it can be clearly determined whether the cells are secretory luminal, neuroendocrine or of an intermediate phenotype. Putative prostate epithelial stem cells have also been distinguished from other basal cells by their expression of integrins. $\alpha_2\beta_1$-integrin is expressed at higher levels in 1% of cells with basal phenotype (CK5 and CK14), these cells are distinguished from other basal cells by their ability to generate prostate like glands *in vivo* and immunohistochemical evidence of prostate specific differentiation (Collins *et al* 2001). If this particular population of cells could be isolated and cultured, a major impediment to the understanding of prostatic disease could be removed.

The stem cell population is believed to be primarily basally located in the prostate. This small population has the capacity for extended or unlimited growth and its progeny are either stem cells or cells with a more limited proliferative capacity, transiently proliferating/amplifying cell population. These daughter cells proliferate rapidly to maintain tissue balance, but have a shorter life span and a limited cyclic activity before terminal differentiation (Isaacs and Coffey 1989, Bonkhoff and Remberger 1996,Van Leenders 2000). This cell population is situated in the basal as well as the luminal cell layer and finally differentiates into exocrine or neuroendocrine cells (luminal secretory cells) (Xue et al 1997 and 1998,Van Leenders 2000). Exocrine differentiation occurs in the majority of the luminal cells and is identified by expression of PSA. Neuroendocrine cells are dispersed in the prostate epithelium and secrete neuropeptides like chromogranin A and serotonin. Recently, this population of cells has gained attention because they indicate the existence of a hierarchical

relationship in the prostate epithelium and putatively play a role in neoplastic diseases (Van Leenders *et al* 2000).

Imbalances in the stromal-epithelial interactions are implicated in the pathogenesis of prostate cancer and BPH. Numerous mitogenic and inhibitory substances are produced by stromal and epithelial cells, which together react in an autocrine, paracrine and/or intracrine manner resulting in growth stimulation, inhibition or apoptosis. Stromal cells structurally support epithelial cell growth and in addition seem to play an active role in carcinogenesis. For example in the breast it has been suggested that without the stromal cells there would be no tumour (Planz *et al* 1999). The stromal cells in the breast have roles similar to those exhibited in the prostate.

The vast majority of prostate cancers express markers of secretory cells such as androgen receptor and PSA and are negative for basal cell markers (Liu *et al* 1997, Signoretti *et al* 2000). Consequently, it has been generally accepted that prostate cancer arises from malignant transformation of secretory cells. However, prostate carcinomas may also express gene characteristics of basal cells (Liu *et al* 1997, McDonnell *et al* 1992, Signoretti *et al* 2000). Thus, the cell from which prostate cancer arises remains unknown so the isolation and immortalisation of epithelial cells from different discrete compartmental origins would be interesting and beneficial to furthering understanding.

5.3.3. Immortalisation of primary prostate epithelial cells.

The development of normal human prostate cell lines would be of considerable value for the *in vitro* study of molecular mechanisms involved in neoplastic transformation. Recent advances in understanding the role of telomeres in the regulation of cellular proliferative capacity have provided novel approaches for the immortalisation of normal human cells.

The proliferative capacity of most normal human somatic cells in culture is a function of telomere length. Telomeric repeats are lost with each round of cell division and eventually, this shortening triggers entry into a permanent state of growth arrest known as cellular senescence. Telomerase is a ribonucleoprotein that utilises its RNA as a template for the *de novo* addition of telomeric DNA repeats to chromosome ends. Most normal somatic cells lack telomerase activity, 85-90% of human epithelial cancers express high levels of the enzyme (Shay 1996), suggesting that telomerase reactivation might be a major mechanism by which most human cancers become malignant.

The introduction and forced expression of the human catalytic subunit of telomerase (hTERT) gene can extend the life span of human cells without any change in the fundamental cellular characteristics. Cells with introduced hTERT still maintained normal cell cycle controls, exhibited functional p53 and RB checkpoints and growth factor dependent proliferation. In addition these cells possess a normal karyotype (Jiang *et al* 1999, Morales *et al* 1999, Oullette *et al* 2000, Wood *et al* 2001, Mackenzie *et al* 2000). Several groups have introduced hTERT into cultures of human fibroblasts, retinal pigment epithelial cells,

myometrial cells, mammary epithelial cells, endothelial cells and skin keratinocytes and shown stabilisation of telomere length and immortalisation. This method of life span extension/immortalisation seems to give rise to cell lines that could prove to valuable models for many tissue types and disease processes. They may provide suitable models to analyse the effects of various substances on human cells and also for large-scale biochemical and molecular experiments such as gene transfer or gene inactivation (Nakamura *et al* 2002) and for examining the neoplastic transformation and cytogenetic changes after gamma irradiation (Riches *et al* 2001). Telomere maintenance seems to be an excellent strategy for immortalising normal human epithelial cells without affecting the integrity of their important phenotypic characteristics (Morales *et al* 2003).

There has only been one publication to date using telomerase immortalisation and human prostate epithelium. However, the prostate epithelial cells were derived from a prostate tumour in a patient with a family history of this disease (Yasunaga *et al* 2001). These cells have probably already accumulated abnormalities, hence, are of little value when investigating predisposition and the early events of prostate carcinogenesis.

This study demonstrates that the expression of hTERT can extend the life-span of primary prostate epithelial cells, but not to the extent of the immortalisation displayed by fibroblasts and other normal epithelial cell types, for example the introduction of telomerase into normal human oesophageal squamous cells established a cell line retaining normal cell cycle checkpoints and differentiation

markers which has reached 100 population doublings with no decrease in the growth rate whereas uninfected cultures normally senesce after 40-45 population doublings (Morales *et al* 2003).

In this study of the immortalisation of primary prostate epithelium from BPH patients, the ectopic expression of telomerase, although a conceptually a sound strategy, was insufficient for generating immortal prostate epithelial cells. This suggests that there are other mechanisms involved in determining the finite proliferative potential of the cells and stabilisation of the telomeres is not enough. An understanding of the genes controlling normal prostate epithelial mortality may provide relevant information on the mechanisms underlying abnormal cell growth and tumourigenesis. Prostate epithelial cells have been immortalised by expression of DNA tumour virus oncoproteins, including SV40 (Kuettel *et al* 1996), the Human Papilloma Virus (HPV) 16 E6 and E7 (Jarrard *et al* 1999, Webber *et al* 2001, Ko *et al* 2003). These tumour viruses are known to inactivate p53 and Rb thus implicating these antiproliferative proteins in the process of overcoming senescence. Maybe in this case, the hTERT has allowed the cells to overcome the initial block to proliferation, but further additional genetic events are needed. Immortalisation occurs infrequently suggesting that the inactivation of several pathways is required, perhaps the use of hTERT in combination with HPV could provide the prostate epithelial cells with the tools to grow ad infinitum, Jarrard *et al* (1999) suggests that alterations in the p16/pRB pathway are a requirement for bypassing senescence in human prostate epithelial cells. Alterations in this cell cycle checkpoint occur with some frequency in primary and metastatic human prostate cancer (Jarrard *et al* 2002).

Development of a functional and morphologically correct prostate *in vitro* is dependent on extracellular matrix, steroid hormones and factors from stromal cells and serum (Lang *et al* 2001). It is a possibility that using prostate stromal cells as a feeder layer and stabilisation of the epithelial cells telomeres by ectopic expression hTERT may provide an alternative solution to those suggested above and still provide a cell line with the desired phenotype and unaltered karyotype.

5.3.4. Prostate cancer and in vitro cell models.

Prostate cancer cell lines derived from primary tumours and immortalised using HPV 16 E6 and E7 genes are not ideal models, because the immortalisation process frequently leaves viral oncogenic DNA in the cells which accompanies major cytogenetic alterations and growth dysregulations. The immortalising agent may introduce many genetic and epigenetic artefacts into these cells, making it difficult to investigate specific alterations unique to tumours.

The development of *in vitro* human cell culture models that mimic human prostate cancer progression would be ideal. HPV infection does not appear to be related to prostate cancer development although it is present in many prostate specimens. The question of the involvement of HPV in the pathogenesis of prostate cancer has caused great controversy.

As already mentioned, Yasunaga *et al* (2001) have succeeded in immortalising primary human prostate epithelial cells derived from a familial prostate cancer patient by the introduction of telomerase. The cells show a transformed morphology and express CK8, prostatic stem cell antigen and p16, but did not

express AR and PSA. The cell line is near diploid with loss of chromosomes 8, 13, X and Y and an alteration of chromosome 4 (Yasunaga *et al* 2001).

Ko *et al* (2003) have demonstrated that their immortalised primary tumour derived human prostate epithelial cell line RC-9/E6E7, retained its original malignant phenotypes and expresses prostate specific markers. The androgen responsive properties of this cell line should help answer questions related to androgen regulation of prostate cells. The novel *in vitro* model will become a powerful tool for elucidation of prostatic carcinogenesis and also provide the means for testing new modalities for both prevention and progression of prostate cancer as well as provide methods for testing both chemopreventative and chemotherapeutic agents (Ko *et al* 2003).

Webber *et al* (2001) have developed a family of human prostate epithelial lines (four tumourigenic cell lines with progressive malignant characteristics) by exposure of non-tumourigenic RWPE-1 cells (immortalised from non-tumourigenic tissue) to the direct acting complete carcinogen N-methyl-N-nitrosourea (MNU). MNU has been used for prostate cancer induction in rats (McCormick *et al* 1999, Pollard 1998, Okamoto *et al* 1998) and transformation of prostatic epithelial cells in vitro (Rhim *et al* 1997). The RWPE-1 derived, MNU-transformed cell lines show progressive changes from pre-neoplastic cells to non-invasive and then progressively increasing invasive cancer cells. Webber *et al* (2001) demonstrated that the MNU cell lines exhibit characteristics of malignant epithelial cells and yet retained the differentiated functions of prostatic epithelial cells. All the cell lines express CK8 and CK18 confirming

epithelial origin, respond to androgen by upregulation of nuclear AR and expression of PSA. Furthermore, it is proposed that these cell lines mimic early stages of cancer progression from non-invasive latent carcinoma as seen in PIN to invasive cancer (Webber *et al* 2001).

The multi-step processes of carcinogenesis and tumour progression, which includes progressive changes from early to late PIN and then to invasive carcinoma, are not well understood. Many cellular and molecular changes including cellular heterogeneity, loss of differentiation, expression of early growth response genes as well as abnormal growth control have been proposed as being associated with PIN. A wealth of information has been gained from employing the commonly used; metastasis derived prostate carcinoma cell lines such as DU145, PC3 and LNCaP. These cell line however, represent advanced cancer and are not suitable for elucidating the multistep process of carcinogenesis and tumour progression in the prostate (Webber *et al* 2001)

The tumour cell lines are mostly derived from late-stage malignant metastatic tumours that have undergone radical treatment with either x-rays or chemotherapy or a combination of both. These limitations are imposed by the lack of consensus culture conditions and factors produced by extracellular matrix or secreted factors from stromal components (Chung, 1993, Maitland *et al* 2001) that are essential for the induction of genes responsible for the correct epithelial phenotype (Lee, 1997, Maitland *et al* 2001).

Research into molecular and genetic mechanisms underlying familial prostate cancer would be greatly advanced by *in vitro* models of primary tumours. The recent progress made in identifying cancer genes and understanding cancer genetics has been impressive. However, our understanding of the molecular and genetic mechanisms underlying prostate carcinogenesis remains limited. Hereditary factors are estimated to be responsible for about 9% of all cases of prostate cancer in the USA. Analysis of familial prostate cancer has supported an autosomal dominant mode of inheritance for prostate cancer susceptibility alleles with some evidence of heterogeneity (Carter *et al* 1992, Yasunaga *et al* 2001). Genome wide scan analysis implicated a region of chromosome 1 (1q24-25) as being the most likely region of the genome to contain a major prostate cancer susceptibility gene (Smith *et al* 1996). Subsequent analysis of the linkage data indicates that evidence for linkage to a hereditary prostate cancer locus, termed HPC1, is primarily restricted to families containing five or more men affected with prostate cancer, in which the average age of diagnosis is under 65 (Grönberg *et al* 1997, Yasunaga *et al* 2001). In addition to HPC1, at least four other candidate prostate cancer susceptibility loci have been reported, although the genes on these loci have not been identified (Ostrander and Stanford 2000, Yasunaga *et al* 2001). More studies are needed to identify these prostate cancer genes.

Elucidation of molecular and genetic events involved in familial prostate cancer progression remains poorly understood. This is in part due to the lack of suitable *in vitro* models for the study of familial prostate cancer. To study early genetic and molecular lesions of familial prostate cancer, cell lines derived from

primary tumours are urgently needed, as they are not presently available. *In vitro* human cell culture models are critical for defining the mechanism of prostate cancer progression and for testing preventative and therapeutic regimens. The generation of immortalised human prostate epithelial cell culture that will accurately reflect the *in situ* characteristics of malignant prostate epithelium is imperative. *In vitro* cell culture models of human prostate carcinogenesis have not been widely available or well characterised until recently. To study early genetic and molecular lesions of prostate cancer, cell lines derived from primary tumours are urgently needed (Ko *et al* 2003).

Androgen regulation of prostate growth as well as the widely used androgen deprivation therapy for prostate cancer treatment necessitates a better understanding of the role of androgen in prostate cancer biology. The well characterised prostate cancer cell line LNCaP is known to express AR, but it is a mutant receptor. Therefore the generation of primary prostate tumour derived cell lines expressing AR will have significant impact in evaluating the role of androgen signalling pathway. However, such lines are presently not available. The establishment and maintenance of long-term human prostate epithelial cell lines that retain their original malignant phenotypes of primary prostate tumours have been unsuccessful in the absence of *in vitro* immortalisation. Successful generation of immortal human prostate epithelial cell lines from primary prostate cancer specimens by HPV has been described (Weijerman *et al* 1994, Bright *et al* 1997, Ko *et al* 2003). However, these immortalised cells are not usually tumourigenic in nude mice and do not express AR as well as PSA. To our knowledge, no successful establishment of AR and PSA positive primary

human prostate cancer cell lines with neoplastic phenotypes has been reported (Ko *et al* 2003).

5.3.5. Neoplastic transformation after gamma irradition.

Marked changes in the properties of parental human epithelial cell lines have been induced after exposure to fractionated doses of radiation. Repeated doses of radiation seem necessary to induce changes, this has been shown using human retinal pigment epithelial cells (Riches *et al* 2001), human prostate epithelial cells (Kuettel *et al* 1996) and human keratinocytes (Thraves *et al* 1990).

Immortalisation with hTERT helps the cells to retain a stable diploid karyotypes, this is a major advantage over cell lines immortalised by other agents as they usually exhibit an unstable polyploid karyotype (Reddel *et al* 1993). This makes the analysis of radiation induced changes more complex. In the RPE cells it has been shown that there is a dose dependent increase in ploidy and structural aberrations. Recurrent chromosome changes could also be detected in different clones, this may point to early changes being induced reproducibly after irradiation and may also provide suitable starting points for positional cloning of genes involved in radiation carcinogenesis (Riches *et al* 2001).

Malignant transformation of human prostate epithelial cells has been achieved by the cumulative action of the DNA tumour virus SV40 and x-ray irradiation (Kuettel *et al* 1996). Alterations in the growth properties of cells are required;

the addition of SV40 allows immortalisation and the development of an apparently unlimited growth potential. Treatment of early non-tumourigenic passages with IR resulted in additional changes in the cells growth properties. Concomitantly acquired properties of the radiation transformed cells included morphological alterations, the ability to grow in soft agar and the formation of rapidly growing adenocarcinomas in athymic mice. The alterations in the growth patterns made by irradiation required several subcultures for their visualisation. This suggests that multiple cell divisions are required for fixation and expression of the transformed phenotype in response to radiation. It is also possible that more than one genetic lesion is required (Kuettel *et al* 1996). The carcinogenic action of ionising radiation in humans has been well recognised from epidemiological data.

If a line can be generated using hTERT and prostate epithelial cells it may prove a useful in vitro tool for dissecting the process of radiation induced malignant transformation in human prostate epithelial cells. Additionally, molecular analysis of these cells would be needed to determine the specific events that are responsible for neoplastic transformation induced by IR.

5.4. Conclusions.

The hTERT-immortalised lines provide an exciting model with which to investigate and link cytogenetic changes to molecular events induced by ionising radiation. However, in this study it has not been possible to produce an hTERT immortalised cell line retaining normal characteristics of prostate epithelium although extended life span has been observed. The cells isolated

from prostate tissue appeared to come from both the luminal and basal compartments, whether the intermediate cells were present is unclear as dual staining of the same cells was not undertaken in this study.

5.5. Future directions.

Growth and immortalisation of the prostate epithelial cells from different regions of the prostate and from different epithelial compartments, is of interest. Prostate cancer arises mainly from the peripheral zone, whereas BPH arises from the transitional zone. What properties make these cells more likely to escape the normal growth controls and why are the cells of the peripheral zone more likely to acquire malignancies? Also to be able to identify whether the prostate cancers arise from the basal or secretory epithelial compartment would be of great advantage.

If prostate epithelial cells can be successfully and stably immortalised with hTERT, if a diploid karyotype is exhibited, and it responds to cytotoxic agents by cell cycle arrest, the resulting cell line could be transformed using fractionated doses of gamma radiation and the properties of the cell lines derived by exploring cell proliferation, anchorage independent growth, chromosome analysis, comparative genomic hybridisation and spectral karyotyping.

Different retroviral vectors with other hTERT cassettes should be investigated and possibly hTERT in combination with other agents such as HPV E6 and E7 genes might help the cells overcome the senescence signals.

In future other epithelial cell markers should be used in addition to the CKs to identify which population of epithelial cells is being cultured and methods where different markers are probed for in the same cell population should be employed. It might also be useful to investigate the coculture of stromal and epithelial cells from the same individual and stromally derived feeder layers to determine whether this provides a more suitable microenvironment for prostate epithelial cell growth.

CHAPTER 6

GENERAL DISCUSSION.

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6.1. Summary and Discussion.

6.1.1. Radiosensitivity of peripheral blood lymphocytes from prostate cancer patients.

Research in radiobiology is currently directed towards developing an assay that can predict cancer predisposition and adverse responses to radiotherapy prior to the commencement of treatment. The concept that the inherent radiosensitivity varies from one individual to another is well established.

Every cell within the adult human is a progeny of the single diploid cell formed at fertilisation. As this single cell multiplied, its progeny differentiated from it producing functionally specialised cells designed to perform physiological functions. A tumour represents the breakdown in this regulation and growth differentiation.

Prostate cancer is the second leading cause of cancer deaths in the male population of the western world, but unlike some other types of cancer, it lacks any overt warning signs. Although approximately 10-15% of prostate cancer cases have a familial component (Pentyala *et al* 2000), predisposition to prostate cancer seems to be present in a much larger proportion of the population, possibly attributable to the carriage of low penetrance genes involved in the processing of DNA damage (Pentyala *et al* 2000). It would be highly beneficial if an effective screening test existed and a means of determining which prostate cancers would progress to clinical relevancy. Excessive chromatid aberrations

(chromatid gaps and breaks or micronucleus yields) constitute a potential warning phenotype of genetic cancer predisposition (Scott *et al* 1994).

An implication of the findings of studies into the consequences of DNA damage in patients who suffer from heritable conditions, such as ataxia telangiectasia and Xeroderma pigmentosa, which genetically predispose the sufferers to a high risk of developing cancer, is that the cell lines derived have a defective DNA repair capacity and thus an increased potential for neoplastic transformation. A defective repair capacity could play a role in the predisposition to prostate cancer.

Further evidence that neoplastic potential is linked to inefficient DNA repair comes from successive studies using the G2 assay into diseases such as breast cancer. As already described the G2 assay involves exposing the cells to irradiation in the G2 phase of the cell cycle. As the cells enter mitosis, the chromosomes condense and aberrations in their structure can be observed. A continuous double strand of DNA forms each chromatid, the gaps and breaks represent unrepaired damage. The extent of the damage presented shortly after irradiation are a measure of the cells' sensitivity, while the decline, or persistence of chromatid gaps and breaks in cells arrested later after irradiation is a measure of their repair capacity.

The CBMN assay also measures chromosomal damage; it provides a reliable measurement of both chromosome loss and chromosome breakage. The micronuclei are expressed after one round of cell division and consist of small

amounts of DNA left in the cell cytoplasm when chromatid and chromosomal fragments or whole chromosomes are not incorporated in the daughter nuclei. The CBMN assay is becoming the more favoured of the two assays as it is easier to interpret the results. The assay is simpler to conduct and the scoring less arduous and specialised.

The work presented in chapter 3 of this thesis established that there is an identifiable group of men within the prostate cancer population that exhibit an increased radiosensitivity, indicating that these patients have either a reduced damage repair capacity or that they have fragile sites within their genome. The identification of fragile sites or repair defects could help in the understanding of prostate cancer and its progression.

The CBMN assay and the G2 assay were both used in this study as they assess chromosomal sensitivity at different phases of the cell cycle. There is no correlation between G2 and G0 sensitivity (shown in Figure 3.14.), within this population, confirming the findings of other groups studies in relation to breast cancer (Scott *et al* 1999). The lack of correlation between G2 and G0 radiosensitivity could be explained by the fact that there are many known mechanisms of chromosomal radiosensitivity and these are cell cycle stage specific. This implies that G2 and G0 sensitivities are largely independent, because if the same mechanisms were involved, then the defect giving rise to the increased chromosomal damage would be apparent in both assays. However, if the results of these assays are combined, 60% of the prostate cancer patients were identifiable by these methods in comparison to the normal population. The

implications of this increased radiosensitivity for prostate cancer are unclear. However, these changes in radiosensitivity are indicative of increased chromosomal fragility and might be associated with a predisposition and malignant transformation. Additionally in the case of prostate cancer may provide a tool with which to predict disease progression. Unfortunately in this study it proved very difficult to obtain patient information so only limited comparisons could be made. For the comparison of G2 assay results and PSA levels and Gleason score see Figures 3.3 and 3.4 respectively and for the CBMN assay results and the clinical parameters mentioned see Figures 3.10 and 3.11.

Apart from the application of the CBMN assay and G2 assay in the prediction of genetic predisposition to cancer, the excessive chromatid aberrations detected by these methods have further clinical significance. Each individual's intrinsic radiosensitivity means that there is considerable variation in the effects of ionising radiation and this presents a constant clinical problem. In combination and with further refinements, the G2 and CBMN assays may provide the basis for a test allowing the early identification of those patients susceptible to life threatening complications. Therefore permitting a more appropriate treatment programme for the individual to be compiled before the commencement of radiotherapy. This kind of assay would enable early detection, the individualisation of treatment and the prediction of the early and late effects of radiotherapy.

Lee *et al* (2003) conducted prospective CBMN assays on the PBLs of prostate cancer patients before RT and found that the development of RT related

morbidity correlated with the radiation dose response relationship of micronucleus yield of the lymphocytes before treatment. This suggests that the relationship of micronucleus yield in PBLs might be a predictive factor for morbidity in prostate cancer patients after pelvic RT. Perhaps the information gathered in this study could be used in a similar context for the patients involved.

This study has shown that there is a group of prostate cancer patients who can be identified because of their increased radiosensitivity. This group accounts for 60% of the prostate cancer population tested when the G2 and CBMN assay results are added together. Also there is a lack of correlation between G2 and G0 sensitivity implying that they mechanisms involved are entirely independent.

6.1.2. The prostate gland and prostate cancer.

The prostate gland and the process of tumour development within it still remain a bit of an enigma. It is still unknown exactly, which cells give rise to prostate cancer, although it has been found that it is most likely to arise in the histological defined peripheral zone. Nonetheless, advances have been made in the understanding of the normal gland and into the disease processes that affect it. The structure of the gland initially seems to possess a fairly straightforward histological structure, with a glandular epithelial component and a stromal supporting part. However, upon closer examination more complex relationships exist between the stroma and the epithelial layers and also between the various

cells found in each compartment (see figures 1.1 and 1.2 for pictures of the prostate gland).

Stromal cells obtained from BPH consist predominantly of smooth muscle cells. Location within the prostate may influence cellular differentiation this has been demonstrated in the rat prostate. Regional variations of various types of stromal cells, their distribution and their corresponding secretory products may be responsible for the different responses to androgen in the rat prostate (Planz *et al* 1999). If the same is true in humans this might be a possible explanation for the zonal nature of disease in the prostate and may play a role in the determination of the type of prostate cancer, as some cancer remain latent, within the confines of the gland and others are aggressive and metastasise progressing to androgen independent disease.

Phenotypic heterogeneity in prostatic fibroblasts showing muscle differentiation features occur under physiological and pathological conditions and may behave differently (Planz *et al* 1999). Cell behaviours need to be investigated, as they are also contributory factors. Hence the attempts made to characterise the cells isolated from primary tissue during the course of the study, the results showed that the epithelial cells were mainly of basal origin, indicated by the presence of p63 (see Figure 5.2.) and that the stromal cells were fibroblastic in origin (results not shown).

Further characterisation of stromal cells involved investigation of their functional properties. Primary cultures of stromal cells very often lose or lack

androgen receptor (AR) gene expression. Evidence suggests that there is a specific androgen receptor mediated pathway of DHT in the prostatic stromal cells. There is still controversy about the androgen action on epithelial and stromal cells. It is believed that the proliferative effect of androgen on the epithelium is mainly mediated by the stroma and the release of mitogenic substances like growth factors (Planz *et al* 1999).

The proliferative capacity of prostatic carcinoma appears to be under the control of factors produced by the local microenvironment. In the primary site, prostatic carcinomas expand slowly, often taking years or decades to reach a size and stage compatible with metastatic spread. In contrast, metastatic colonies especially those in the vertebral cavity, expand rapidly often leading to death within a few years. Human bone marrow extracts contain agents that stimulate prostatic carcinoma growth and β1 integrin plays a role in attachment of prostate cells to bone marrow (Scott *et al* 2001) and its increased expression has been associated with higher grades of prostate cancer (Murant *et al* 1997). Normal human prostate tissue contains an inhibitor of prostatic cell growth called spermine (Smith *et al* 1995).

Spermine is a polyamine that is locally abundant in the prostate, it is speculated that the growth of early stage prostatic carcinoma cells may be retarded by the locally high concentrations. Hence, the levels of spermine within the prostatic microenvironment might dictate the slow growth of prostatic carcinoma at its site of origin. Escape from this regulation could reflect changes in the amount of spermine produced by the tumour cells as well as the extent to which

surrounding secretory ducts have been compromised by tumour invasion (Smith *et al* 1995).

Although biology of the human prostate is complex, in clinical practice the differentiated state of tumours provides a good indication of the prognosis for the patient (Maitland *et al* 2001). In colon cancer biology, the stepwise development of cancer from a well-differentiated lesion to a more malignant phenotype is well established (Vogelstein and Kinzler, 1993, Maitland *et al* 2001) although there is some controversy at present. This multistage carcinogenesis has also been related to both genome instability and an increasing mutagenic burden in the tumour cells. Despite searches for such instabilities in prostate cancer, the evidence remains equivocal (Maitland *et al* 2001).

Prostate cancer is a complex, mulitfactorial disease with genetic and environmental factors involved in its aetiology. The search for genetic determinants involved in the disease has proven to be challenging, partly because such complex diseases are not amenable to characterisation by linkage analysis and positional cloning as is the case for diseases with simple Mendelian genetic inheritance.

The molecular mechanisms underlying the development and progression of prostate cancer are poorly understood. Epidemilogical studies have suggested that 9% of all prostate cancers are familial and numerous chromosomal loci have been associated with prostate cancer in multicentre linkage studies.

However, no putative susceptibility genes located in these chromosomal regions have been identified. Several recurrent chromosomal alterations already reported in prostate cancer have been detected by comparative genetic hybridisation (CGH) and loss of heterogeneity (LOH) analysis. The target genes for many of these aberrations are still unknown.

Previously AR expression in human primary prostate has been observed in both epithelia and stroma when co-cultured but not in isolation emphasising the importance of both cell types for terminal epithelial differentiation. Stroma is required to produce the correct architectural organisation. Studies have shown that different stroma from different reproductive states of prostate tumour can modulate invasion and motility of the epithelium (Lang *et al* 2001).

6.1.3. Prostate cell culture.

A limited proliferative lifespan is a major problem encountered in culturing human cells. It is very rare for primary human cell strains to spontaneously become immortal therefore techniques have been developed to induce immortality. Developing prostate epithelial and stromal cell lines has proved challenging, and to date there are really only three well characterised lines LNCaP, PC3 and DU145 but, these have been developed from prostate cancer metastases.

Recently, some other cell lines have started to emerge, Webber *et al* (2001) have developed a series of cell lines showing progressive characteristics from

"normal" to invasive phenotypes and Yasanuga *et al* (2001) used hTERT to immortalise cells from a prostate cancer patient with a family history.

A number of primary human cell lines transfected with TERT have been shown to retain normal growth control (Morales *et al* 1999, Jiang *et al* 1999, Riches *et al* 2001). Their growth rate decreases markedly in serum free medium and they also respond to cell cycle checkpoints after exposure to cytotoxic agents including radiation (Morales *et al* 1999, Jiang *et al* 1999, Riches *et al* 2001).

Interestingly, it has recently been reported that human endothelial cells also bypassed replicative senescence after introduction of hTERT, that hTERT expression in these life extended endothelial cells did not affect their differential and functional phenotype and that they maintained their angiogenic potential *in vitro*. Furthermore hTERT expressed endothelial cells had a normal karyotype and did not exhibit a transformed phenotype (Yang *et al* 1999, Yasunaga *et al* 2001).

Conceptually, the successful establishment of spontaneous immortalised human prostate cell lines derived from familial prostate cancer patients would be ideal and a major breakthrough in prostate cancer research. However the generation of such cell lines is an extremely rare event and to date has not been reported (Yasunaga *et al* 2001).

The prostate derived cell lines currently available all have drawbacks, they have either originated from tumour tissue or they have been immortalised by use of

viruses that interfere with the cell cycle machinery. Viral oncogenes such as E6 and E7 of the human papillomavirus type 16 or the large T-antigen of SV40 virus allow cells to escape the short telomere associated checkpoint and proliferate for a further 20 to 40 population doublings until crisis. Rare immortal clones then recover from after crisis reactivating telomerase or alternative telomere lengthening pathways (Milyavsky *et al* 2003).

The ideal prostate cell lines would be those that displayed the characteristics of cells *in vivo* but had the ability to divide indefinitely. During the course of this investigation, attempts were made to immortalise primary prostate cells obtained from BPH patients by retroviral introduction of hTERT into the genome (see section 5.2.3.). hTERT over expression in primary cells has not been found to be associated with a malignant phenotype, its ability to immortalise the cells allows the expansion of a cell population beyond the limits imposed by replicative senescence. Unfortunately, immortalisation of prostate epithelial cells has not been achieved to date in this study, but extension of proliferative lifespan was seen after the introduction of hTERT. This suggests that there is another complimentary or supplementary mechanism for senescence.

The introduction of hTERT has successfully immortalised several cell types giving rise to useful cell lines. However, immortalisation can be hallmark for malignant transformation. Changes in genome integrity and growth control accompany the immortalisation process. hTERT mediated immortalisation escapes the genome instability step but retains the intact checkpoints that limit

infinite proliferation (Varizi and Benchimol 1998, Drayton and Peters 2002, Yaswen and Stampfer 2002). However, studies of hTERT immortalised embryonic lung fibroblasts have provided evidence that potentially malignant genetic alterations are selected for during prolonged culture *in vitro* (Milyavsky *et al* 2003). This phenomenon could also occur naturally, as with each cell division cell cycle, the genetic damage increases and is amplified.

6.2. Future perspectives for the field of radiosensitivity of prostate cancer patients.

Predictive tests developed from the radiosensitivity angle need to be tailored toward the tissue at risk. The CBMN assay needs to be carried out on the cells from which the tumour arises, in the case of prostate cancer, it is known that it is epithelial cells, but not which compartment, as the cells from the basal and luminal areas have different phenotypes, they may have different radioresponses. The currently available immortalised prostate cells are probably unsuitable for this purpose as the method of immortalisation affects the cells kinetics and hence ability to deal with damage so the results obtained would not be a true reflection of the individual radiosensitivity.

CBMN and perhaps G2 assays need to be carried out on the primary fibroblasts and epithelial cells isolated from "normal" tissue obtained from BPH patients and prostatic carcinoma patients so that scores can be compared for the tissue of interest and it can be established whether other cells show the same radiosensitive patterns as lymphocytes.

6.3. Future perspectives for the field of human prostate cell immortalisation.

A differentiated in vitro prostate model would be invaluable to the study of prostate cancer. The impact of such a cell line would be immense with applications ranging from susceptibility and prediction of disease development and progression to therapeutic treatment regimes. Recent work in the field of prostate cell immortalisation has utilised viral technology to immortalise prostate epithelial and stromal cells. The SV40 large T antigen, HPV E6 and E7 genes and hTERT have been transduced into human prostate cells. Unfortunately, the value of all constituitively immortalised cells is limited by the long term effects of oncogenic expression. The SV40 large T antigen has been shown to interfere with DNA damage repair mechanisms and generate chromatin instability through the interactions with histone acetylases with time, this has inevitable repercussions for differentiated gene expression. (Digweed *et al* 2002, Caron *et al* 2002).

A promoter based system suitable for the immortalisation of prostate epithelial cells might have the ability to produce an expandable cell line with a better differentiated phenotype. The regulatable element would have to be something that was tightly regulated *in vivo* and it would have to be shown that promoter integration does not interfere with the transcription. Promoter regulated expression of an immortalising gene is interesting. The ability to switch off proliferation would allow the potential for further study of impact of division upon differentiation, it is a possibility that this could result in an immortalised cell line with a differentiated phenotype more closely related to that seen in

vivo. It is also a possibility that some promoters could select for certain types of cells.

Promoter regulatable systems reduce destabilisation of the genome because the large T antigen expression can be switched off limiting the damage to the chromatin structure. However, the use of alternative immortalising pressure also may provide a solution to the problem of chromosome rearrangement. The SV40 large T antigen releases the cells from normal cell cycle checkpoints, which ultimately leads to the accumulation of DNA mutations. In the short term, prostate cell immortalisation can make a contribution to the *in vitro* study of the prostate cell phenotypes and perhaps contribute to the understanding of the diseases which affect this gland, but in the long term another renewable source of differentiated prostate cells must be explored.

The approach of isolation of undifferentiated foetal cells which are culturable and then induction of the prostatic phenotypes may offer an opportunity for the isolation of a sustainable prostate like cell line. The development of cell lines from embryonic cells does not require the presence of an immortalising pressure, which therefore provides a better chance of maintaining a stable differentiated phenotype.

More work is needed to explore the actual conditions in the prostate so that isolated cells can be grown in the same environment. Medium conditions that are suitable for maintaining high levels of prostate epithelial proliferation need to be identified and chemically defined. The commercially available medium

PrEBM and its additives is successful in establishing primary cultures, but the company will not release the recipe so the exact contents are unknown, bovine pituitary extract is included and this could contain anything, this is prohibitive for many studies, especially those concerning growth factors and hormones.

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APPENDICES.

APPENDIX I.

STAGING OF PROSTATE CANCER.

1.1 The Gleason grading system. (Images below are adapted from http://www.phoenix5.org/Infolink/GleasonGrading.html)

Figure I.1.

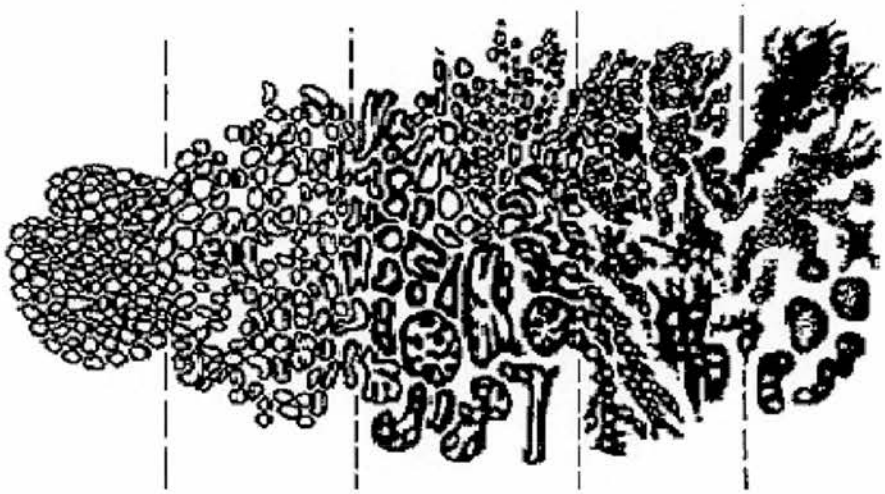


Figure I.1. Drawing of the Gleason grades.

Conceptual diagram showing the deteriorating cell architectural pattern of the gland with increasing aggressive malignancy. The nearer the tumour structure to normal, the closer the biological behaviour is to the normal gland. The dividing lines on the drawing indicate the increasing grade of the tumour with grade 1 appearing on the left and moving right up to grade 5. The patients' prognosis differs significantly depending on the grade at diagnosis.

Figure I.2.

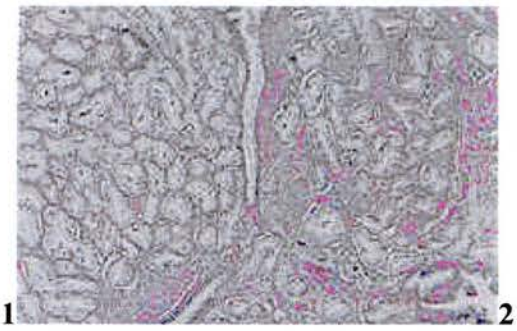


Figure I.2. Gleason grades 1 and 2.

The left hand side of this image shows grade 1 prostate adenocarcinoma and the right hand side grade 2. In both grades the cells are pale staining and form separate glands with easily identifiable lumens. These two grades closely resemble the normal prostate. Grade 1 the tumour is very well differentiated. Grade 2 the glands are more loosely aggregated with some invasion into the surrounding stroma.

Figure I.3.

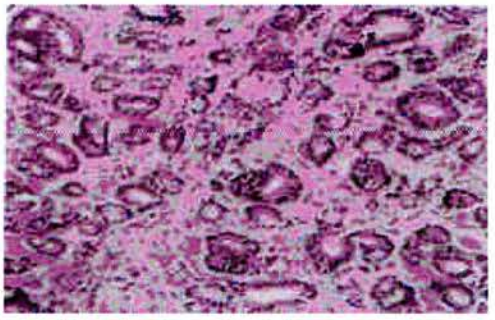


Figure I.3. Gleason grade 3.

Grade 3 prostate carcinoma. Individual glands can be seen invading the surrounding stroma. grade 3 is the most common grade and is described as well differentiated as the 'gland unit' is like that seen in the normal prostate.

Figure I.4.

a) *b)*

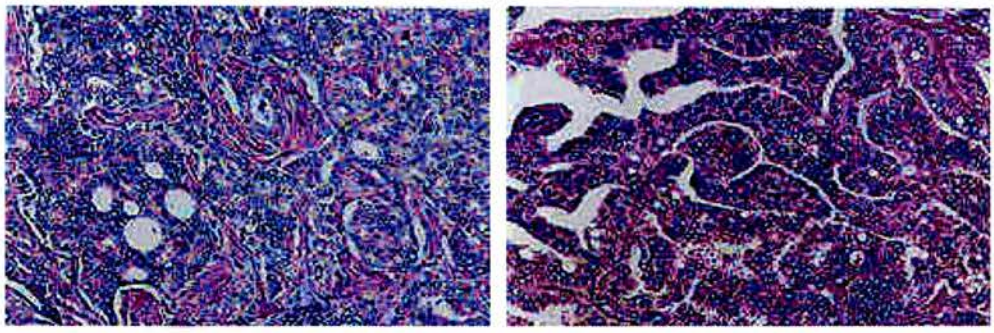


Figure I.4. Gleason grade 4.

Panels a) and b) both show grade 4 carcinomas, however, they have different architectural patterns. It can be seen that there is a great disruption to the gland. There are no distinct units with lumens, but sheets of cells with scattered spaces.

Figure I.5.

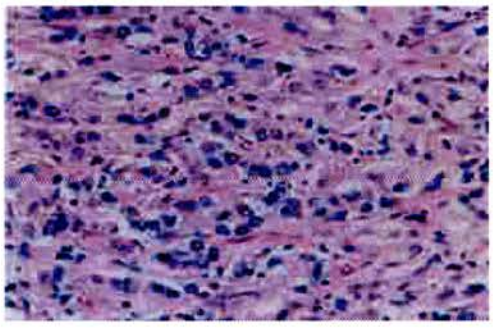


Figure I.5. Gleason grade 5.

Grade 5 adenocarcinoma consisting of sheets of cells with no distinguishable pattern in nuclear arrangement or architecture. There are a variety of patterns within this grade all of which are undifferentiated, giving no clue as to the tissue origin of the tumour.

I.1.2. The Gleason score.

The Gleason grading system is dependent on the histological appearance of the architecture and glandular pattern of the malignancy, it assigns primary and secondary grades ranging from 1 to 5 as seen in the images above. Gleason score is obtained by adding the primary and secondary grades and ranges from 2 ((1+1), where both the primary and secondary patterns have a Gleason grade of 1) to 10 ((5+5) when the primary and secondary patterns have the most disordered Gleason grade 5).

Table I. (Cussenot 2003).

Tumour Differentiation	Gleason score
Well- differentiated tumours	2-4
Moderately differentiated tumours	5-6
Moderately to poorly differentiated tumours	7
Poorly differentiated tumours	8-10

Table I. Gleason score and tumour differentiation.

Table shows the range of Gleason scores and how they equate to tumour differentiation.

I.2. The Whitmore Jewitt staging system. (Cussenot et al 2003)

Stage A	Clinically undetectable tumour confined to the prostate gland and found incidentally at prostatic surgery.
A1	Well-differentiated with focal involvement usually left untreated.
A2	Moderately or poorly differentiated or involves multiple foci in the gland
Stage B	Tumour confined to the prostate gland.
B0	Non-palpable, PSA detected.
B1	Single nodule in 1 lobe of the prostate.
B2	More extensive involvement of 1 lobe or involvement of both lobes.
Stage C	Tumour clinically localised to the periprostatic area, but extending through the prostatic capsule; seminal vesicles may be involved.
C1	Clinical extracapsular extension.
C2	Extracapsular tumour producing bladder outlet or ureteral obstruction.
Stage D	Metastatic disease.
D0	Clinically localised disease (prostate only) but persistently elevated enzymatic serum acid phosphatase titres.
D1	Regional lymph nodes only.
D2	Distant lymph nodes, mestastases to bone or visceral organs.
D3	Patients who relapsed after adequate endocrine therapy.

I.3. The TNM staging system. (Cussenot et al 2003)

I.3.1. Primary tumour (T).

TX	Primary tumour cannot be assessed.
T0	No evidence of primary tumour
T1	Clinically inapparent tumour not palpable nor visible by imaging.
T1a	Tumour incidental histological finding in 5% or less of tissue resected.
T1b	Tumour incidental histological finding in more than 5% of tissue resected.
T1c	Tumour identified by needlie biopsy (e.g. because of elevated PSA).
T2	Tumour, palpable or reliably visible by imaging, confined within prostate.
	Tumour found in 1 or both lobes by needle biopsy.
T2a	Tumour involves 1 lobe.
T2b	Tumour involves both lobes.
T3	Tumour extends through the prostatic capsule. Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3, but T2.
T3a	Extracapsular extension (unilateral or bilateral).
T3b	Tumour invades seminal vesicle(s).
T4	Tumour is fixed or invades adjacent structures other that the seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, and.or pelvic wall.

I.3.2. Regional lymph nodes (N).

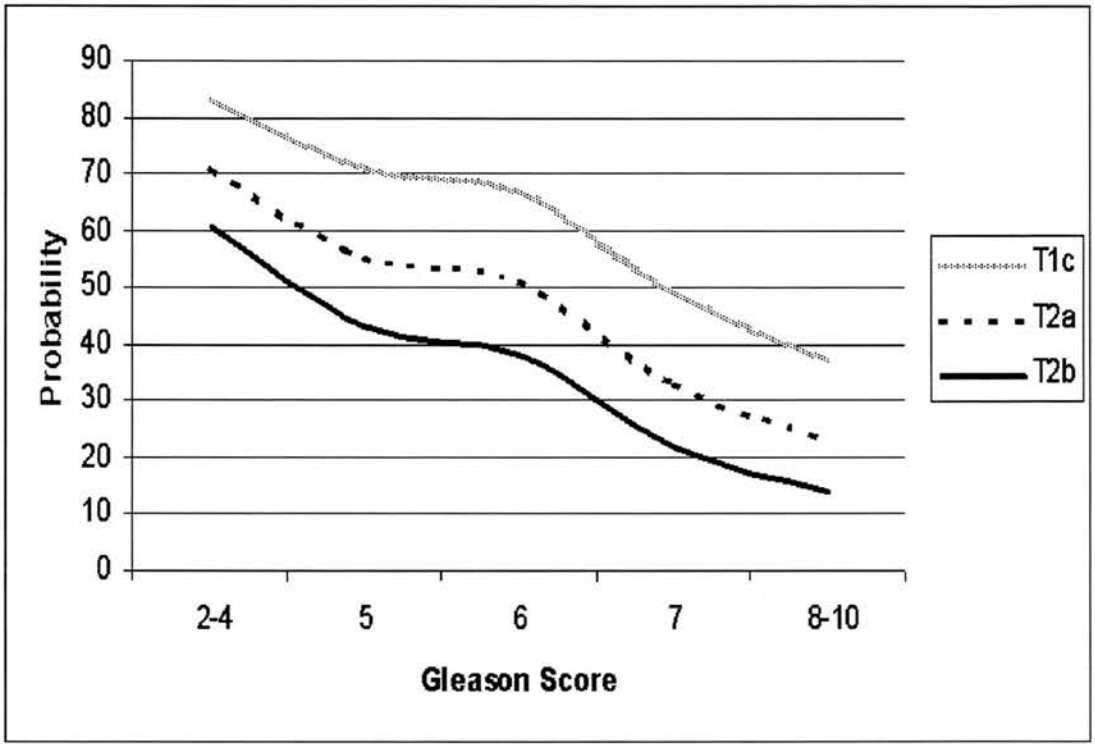
Regional lymph nodes are the nodes of the pelvis located below the bifurcation of the common iliac arteries.

NX	Regional lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Metastasis in the regional lymph node or nodes.

I.3.3. Distant metastasis (M).

MX	Distant metastasis cannot be assessed.
M0	No distant metastasis.
M1	Distant metastasis.
M1a	Nonregional lymph node(s).
M1b	Bone(s).
M1c	Other site(s).

I.4. Probability of extraprostatic extension of prostate cancer in the range of PSA 4-10 ng/ml (using Partins table).



Graph from Cussenot et al 2003.

APPENDIX II

EXAMPLES OF G2 ASSAY AND CBMN ASSAY SCORE SHEETS.

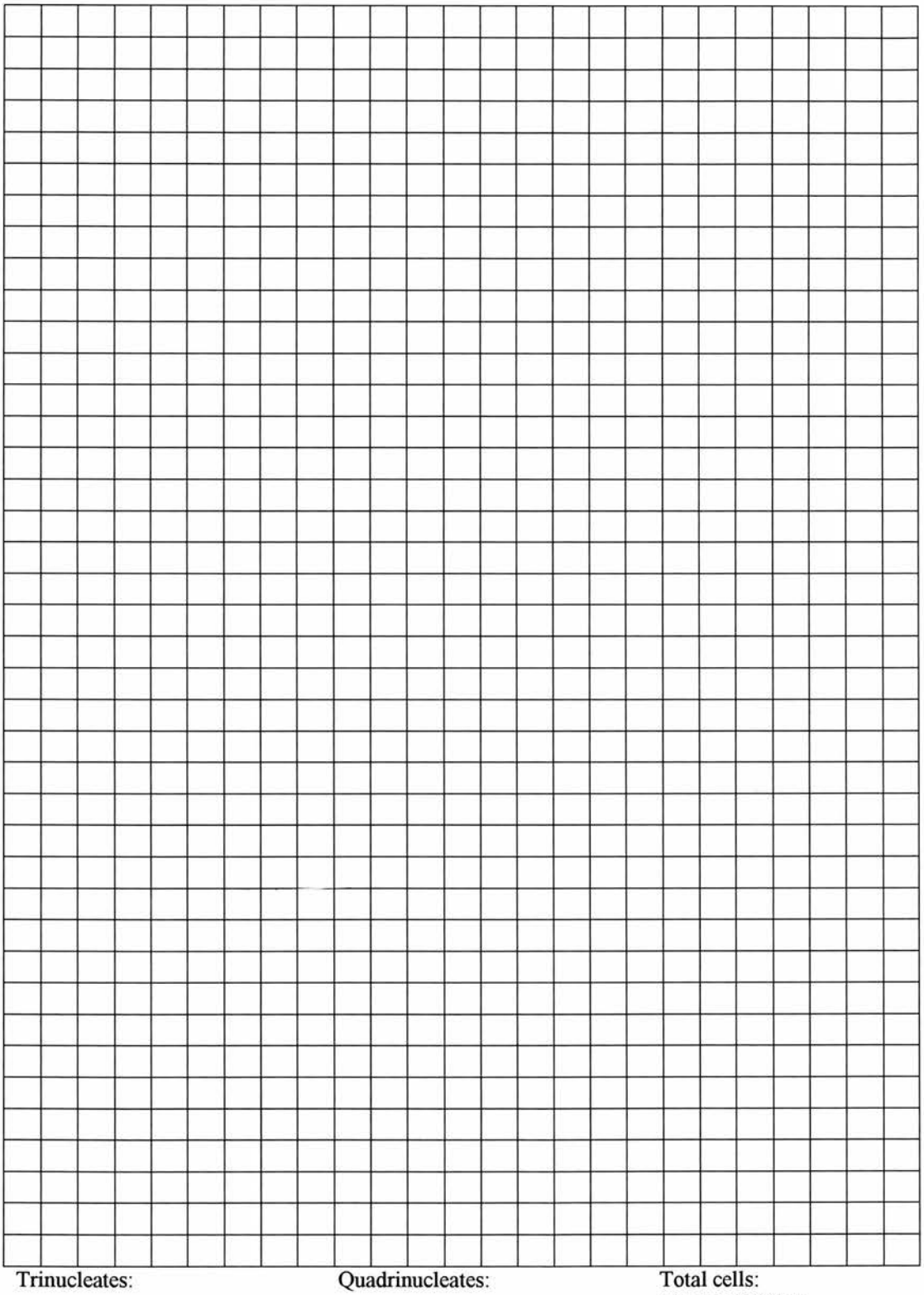
II. G2 Assay score sheet.

Experiment:	Date:

Co-ordinates	Breaks or gaps	Co-ordinates	Breaks or gaps
Totals:			

II.1. CBMN assay score sheet.

Experiment: Date:



Trinucleates: Quadrinucleates: Total cells:
Mn per 1000 bn:
Binucleate Index:

APPENDIX III.

CBMN ASSAY SCORING CRITERIA.

Figure III.1.

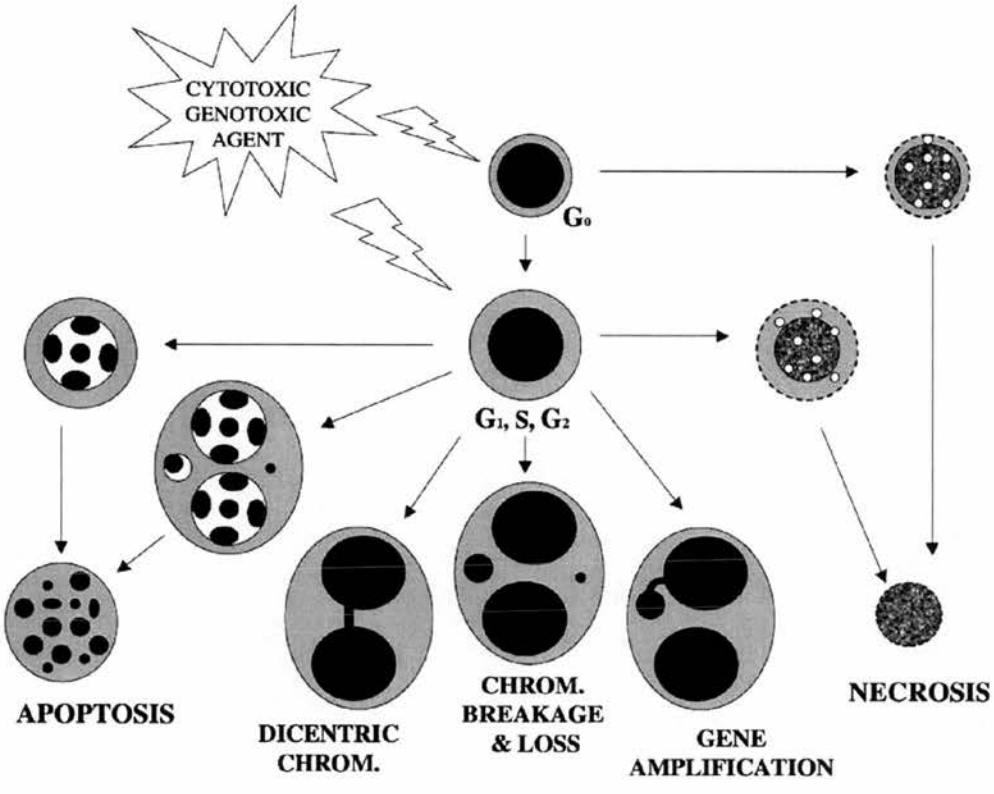


Figure III.1. The various possible fates of cultured cytokinesis blocked cells following exposure to toxic agents.

Using these biomarkers within the CBMN assay, it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, gene amplification necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mononucleated, binucleated and mutinucleated cells. Chromosome loss can be distinguished from chromosome breakage using pancentromeric probes or kinetochore antibodies (Image from Fenech et al 2003).

Figure III.2.

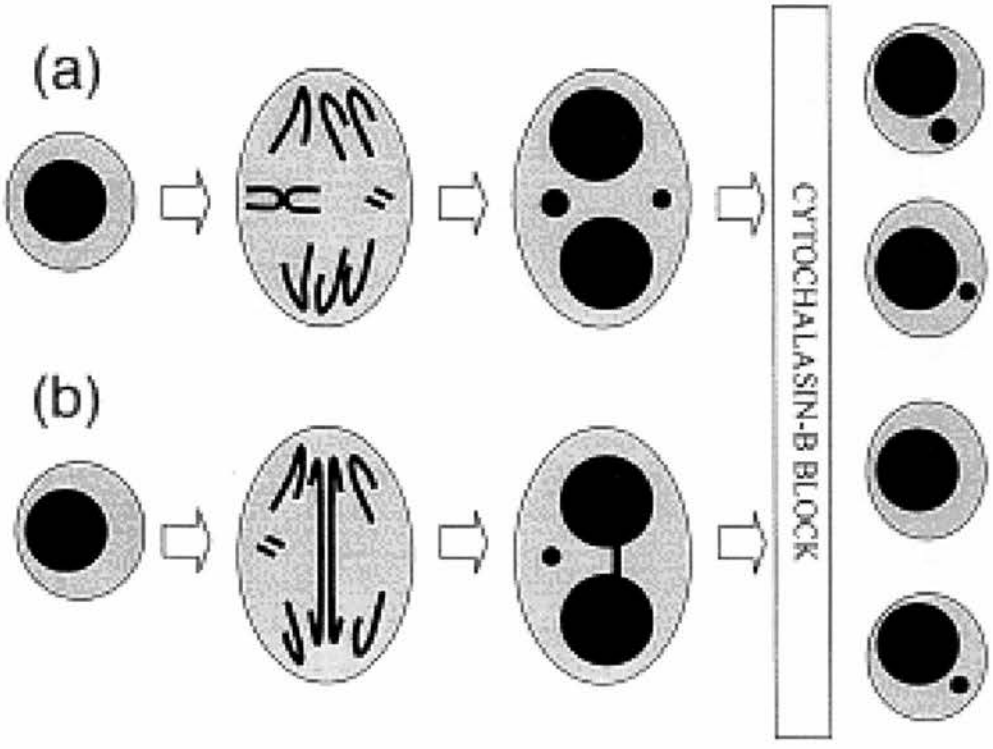


Figure III.2. The origin of micronuclei.

Panel a) shows the origin of micronuclei from lagging whole chromosomes and acentric chromosome fragments at anaphase. Panel b) shows the formationof a nuceloplasmic bridge from a dicentric chromosome in which the centromeres are pulled to opposite poles of the cell; the formation of a micronucleus from the accompanying acentric fragment is also illustrated. This image shows a hypothetical cell with only two chromosomes (Image from Fenech 2000).

Figure III.3.

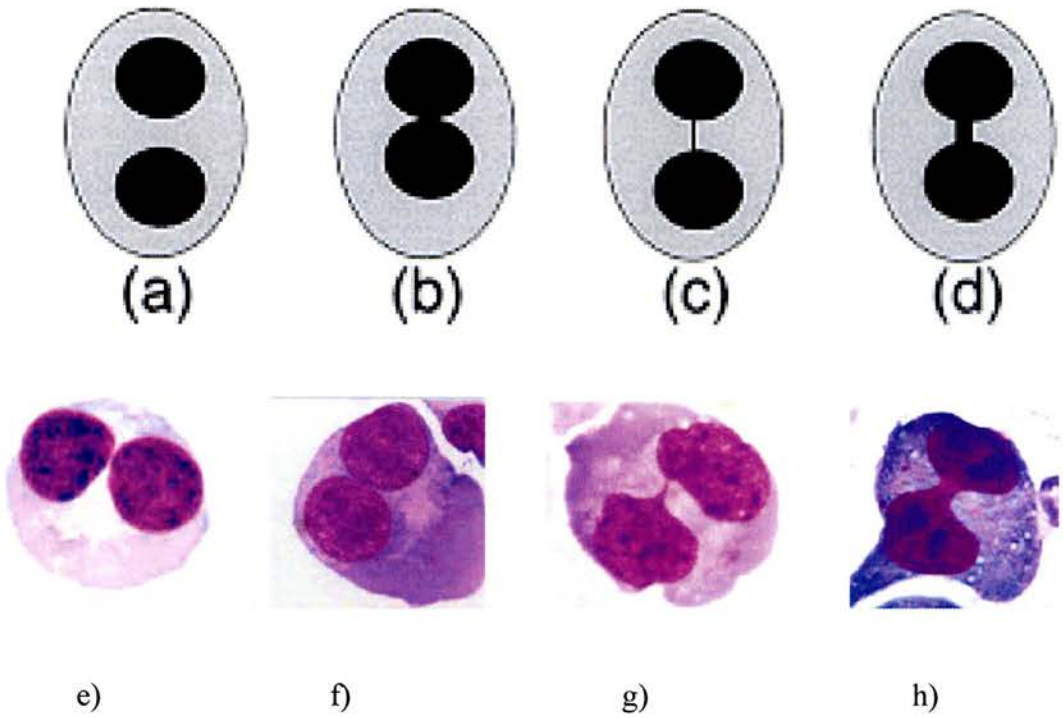


Figure III.3. Criteria for choosing binucleate cells in the CBMN assay.

Panels a) and e) show an ideal binucleate cell; b)and f) binucleate cell with touching nuclei; c) and g) binucleate cell with narrow nucleoplasmic bridge; d) and h) binucleate with wide nucleoplasmic bridge. Drawings taken from Fenech 2000 and images adapted from Fenech et al 2003.

Figure III. 4.

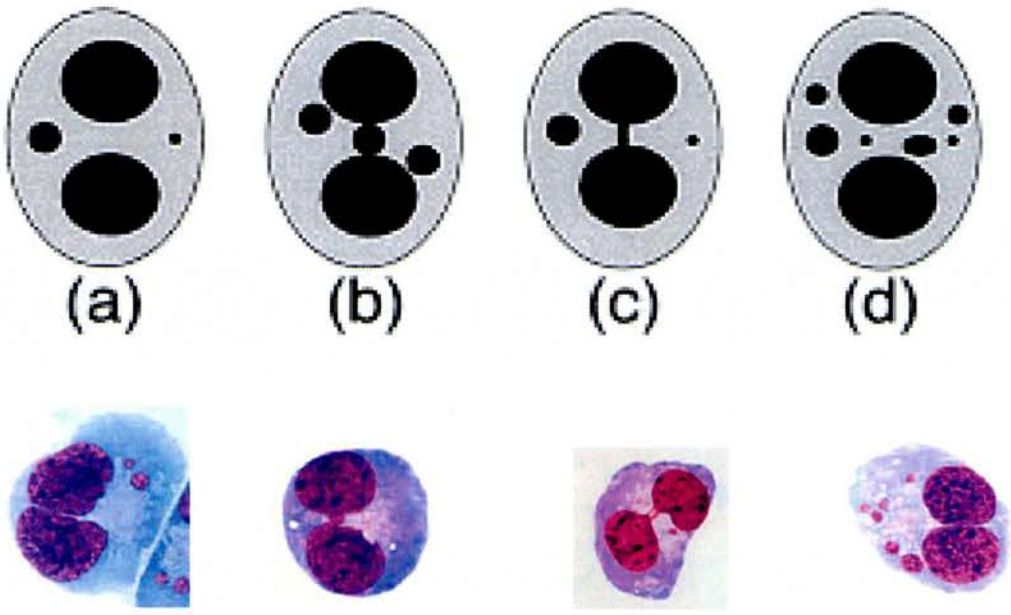


Figure III.4. Typical appearance and relative size of micronuclei in binucleates.

Panels a) and d) cells show a binucleate cell with two micronuclei approximately 1/3rd and 1/9th of the diameter of the main nuclei. b) and e) micronuclei touching but not overlapping the main nuclei. c) and f) binucleated cell with nucleoplasmic bridge and micronuclei. d) and g) binucleated cells with micronuclei of various sizes, these are rarely seen. (Figure adapted from Fenech et al 2003).

APPENDIX IV

RETROVIRAL VECTOR.

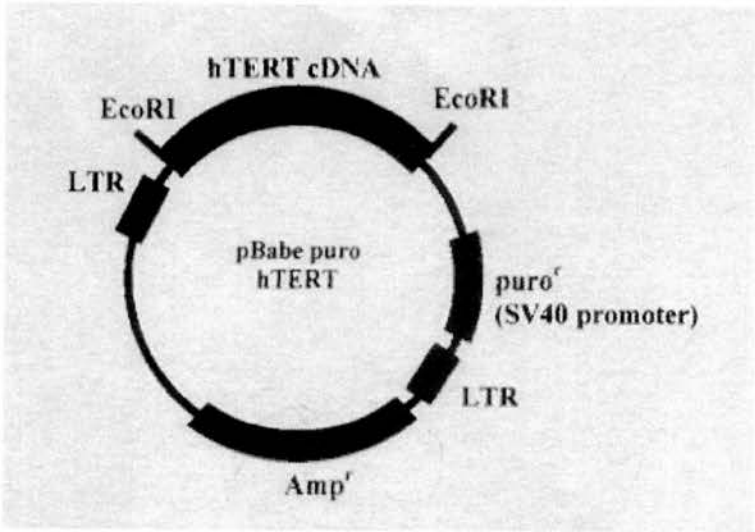


Diagram of the construction of pBabe puro hTERT.

APPENDIX V

G2 ASSAY DATA

Code	Age	No of gaps or breaks per 50 metaphases	Diagnosis
P16	72	58	C
PM	72	74	C
P17	84	80	C
P18	23	70	N
P19	39	111	N
P20	69	83	C
P21	74	80	C
P22	41	108	N
P23	33	82	N
P24	91	80	C
P25	78	39	C
P26	88	42	C

P27	75	70	B
P28	71	110	C
P31	72	70	C
P32	41	70	N
P33	38	80	N
P34	68	63	C
P35	75	87	C
P36	82	64	C
P37	90	59	C
P38	75	73	C
P39	73	49	C
P40	73	56	C
P41	79	67	C
P42	90	64	C
P43	85	72	C
P44	74	75	C
P67	66	43	C

M1	66	25	N
M2	25	34	N
M3	61	28	N
M4	25	21	N
M5	53	39	N
M6	55	33	N
M7	50	33	N
M8	73	39	N
M9	45	30	N
M10	26	28	N
M11	48	18	N
M12	34	29	N
M13	75	20	N
M14	27	20	N
M15	31	17	N
M16	47	19	N
M17	53	44	N

M18	52	39	N
M19	71	39	N
M20	36	52	N
M21	26	36	N
M22	57	44	N
M23	53	40	N
M24	31	37	N

APPENDIX VI

MICRONUCLEUS ASSAY DATA.

Code	Age	No. of MN per 1000 BN	Total no. of cells	No. of tri-nucleates	No. of quadra-nucleates	BN Index (%)	Diagnosis
P1	71	567	6249	80	48	16	B
P2	75	800	4216	57	55	23.72	B
P3	66	593	4166	73	35	24	C
P4	72	778	3645	78	50	27.43	C
P5	70	438	3552	97	53	28.15	C
P6	76	614	2707	137	72	36.94	C
P7	62	629	3119	97	54	32.06	B
P8	61	559	2791	93	82	35.83	C
P9	71	501	3072	89	58	32.55	B
P10	80	441	4412	91	62	22.67	C
P11	64	607	1764	107	46	56.69	C
P12	65	780	1857	137	118	53.85	C

P13	70	708	2276	100	79	43.94	C
P14	76	823	2264	188	120	44.17	C
P15	69	974	2163	171	126	23	C
P16	72	1029	3866	66	64	25.87	C
P17	84	1021	3745	71	59	26.7	C
P18	23	935	2727	238	206	36.67	N
P19	39	657	2704	220	145	36.98	N
P20	69	816	2652	84	37	37.71	C
P21	74	564	4819	123	33	20.75	C
P22	41	960	3211	76	86	31.14	N
P23	33	851	3183	221	212	31.42	N
P24	91	1079	3256	131	102	30.71	C
P25	78	1222	3243	158	99	30.84	C
P26	88	1338	2608	164	107	38.34	C
P27	75	917	3363	153	90	29.74	B
P29	28	984	2719	135	125	36.78	N
P30	35	1087	2793	234	162	35.8	N

P31	72	1044	4483	89	62	22.31	N
P32	41	981	1946	76	79	51.39	N
P33	38	932	2429	150	110	41.21	N
P34	68	1099	2846	110	60	35.14	C
P35	75	1170	3559	160	76	28.1	C
P36	82	1512	4856	50	36	20.51	C
P37	90	1468	3822	33	17	26.16	C
P38	75	1371	1991	77	78	50.23	C
P39	73	1193	2070	84	70	48.31	C
P40	73	1029	2788	115	92	35.87	C
P41	79	977	2943	78	54	33.98	C
P42	90	1160	2350	105	70	42.55	C
P43	85	778	2122	110	82	47.13	C
P44	74	893	2512	89	62	39.8	C
P45	34	1059	2817	277	94	35.49	N
P46	26	550	2635	182	168	37.69	N
P47	23	471	2881	320	180	34.71	N

P48	34	530	2330	193	149	42.92	N
P49	24	543	2370	215	90	42.19	N
P50	30	536	2260	218	83	44.25	N
P51	62	1049	2038	149	101	49.07	C
P52	77	748	1878	107	59	53.25	C
P53	75	780	4055	160	102	24.66	B
P54	79	850	2076	112	43	48.17	C
P55	67	563	2537	71	18	39.42	B
P56	19	600	1674	135	41	59.74	N
P57	19	672	2659	33	7	37.61	N
P58	19	644	2185	94	37	45.77	N
P59	19	628	2084	135	37	47.98	N
P60	18	667	1865	190	40	53.62	N
P61	18	602	1719	157	57	58.17	N
P62	65	869	1858	95	115	53.82	C
P63	20	751	2211	179	97	45.23	N
P64	20	867	2198	184	104	45.49	N

P65	20	617	1863	106	54	53.68	N
P66	20	758	2025	151	92	49.38	N
P67	66	940	1899	56	44	52.66	C
P68	19	731	2124	226	108	47.08	N
P69	19	798	2207	202	114	45.31	N
P70	18	695	2174	116	37	45.99	N
P71	18	656	1951	133	44	51.26	N
P72	19	717	2258	104	101	44.29	N
P73	19	775	2231	229	92	44.82	N
P74	19	425	2826	186	60	35.38	N
P75	19	694	2190	174	71	45.66	N
P76	22	492	2570	319	110	38.91	N
P77	22	644	2330	271	91	42.92	N
P78	19	399	2414	213	66	41.43	N
P79	19	822	2568	253	93	38.94	N
P80	75	761	1570	57	59	63.69	B
P81	72	1012	1954	73	29	51.18	C

P82	81	915	2007	178	83	49.83	B
P83	76	751	2215	51	25	45.15	C
P84	70	793	2006	124	116	49.85	C
P85	78	845	1645	58	37	60.79	B
P86	68	705	2405	109	49	41.58	C
P87	78	987	2249	134	76	44.46	C
P88	70	278	1719	122	57	58.17	B
P89	68	1062	2157	208	211	46.36	B
P90	62	792	2331	102	38	42.90	C
P92	72	682	1977	175	76	50.58	C
P93	78	786	1995	225	130	50.13	C
P94	21	962	2475	294	191	40.40	N
P95	18	958	2719	402	281	36.78	N
P96	19	1018	2453	289	194	40.77	N
P97	19	920	2632	463	263	37.99	N
P98	20	840	2431	357	195	41.14	N
P99	19	678	2620	458	221	38.17	N

P100	21	958	2242	306	200	44.60	N
P101	76	657	2319	67	35	43.12	C
P102	18	762	1823	177	102	54.85	N
P103	21	882	2304	251	178	43.40	N
P104	23	710	2488	317	223	40.19	N
P105	20	779	2141	186	117	46.70	N
P106	23	765	2042	237	133	48.97	N
P107	20	923	2147	356	230	46.58	N
P108	19	767	2906	427	249	34.41	N
P109	19	864	2320	414	208	43.10	N
P110	82	849	1836	122	69	54.47	C
P111	70	720	2052	166	159	48.73	C
P112	73	822	2783	134	111	35.93	B
P113	66	771	1708	125	81	58.55	B
P114	61	1155	2676	132	86	37.38	C
P115	70	963	2121	87	51	47.15	B
P116	85	812	2530	94	65	39.53	C

P117	77	769	1790	177	92	55.86	C
P118	66	816	1834	204	163	54.53	C
P119	76	822	2139	157	80	46.75	B
P120	66	808	2131	149	150	46.93	C
PT10	75	933	1817	117	56	55.04	C
MM	22	548	6800	154	80	14.70	N
AR	56	640	4740	50	90	21.10	N
PB	60	1060	2610	130	150	38.31	N
TB	63	769	3770	60	40	26.53	N
CP	44	830	3911	130	160	25.64	N
NF1	34	690	3660	140	160	27.32	N
NF2	25	610	3970	160	90	25.19	N
NF3	42	619	5300	100	70	18.87	N
NM1	40	650	5870	100	90	17.04	N
NM2	33	797	5130	180	40	19.49	N
NM3	33	757	3110	254	200	32.15	N
IB	22	936	3246	209	208	30.80	N

JM	22	920	3240	400	151	30.86	N

APPENDIX VII

CELL LINE DATA.

Table VII.1

Cell Line	Cell Dose	Day 2	Day 3	Day 4	Day 5	Day 6
LNCaP	200	0.0114±0.00117	0.09±0.06006	0.0748±0.01378	0.5306±0.13306	1.854±0.2024
LNCaP	1000	0.0792±0.00684	0.215±0.01563	0.7252±0.05631	0.9002±0.09304	1.1±0.03964
LNCaP	2000	0.062±0.00787	0.2558±0.01679	1.3386±0.07557	1.854±0.1078	1.7974±0.08159
LNCaP	4000	0.2204±0.01544	1.2506±0.08028	1.332±0.04115	2.2302±0.08792	2.3±0.10876
PC3	200	0.0234±0.00286	0.043±0.00277	0.1704±0.01786	0.6772±0.06421	1.3638±0.13174
PC3	1000	0.2318±0.0184	0.4286±0.05728	0.8896±0.01741	1.8502±0.01657	1.768±0.03774
PC3	2000	0.1816±0.00781	0.4008±0.01422	1.6392±0.08418	2.2342±0.08339	1.9946±0.11923
PC3	4000	0.3788±0.0296	1.147±0.01891	2.5172±0.06579	2.868±0.05809	1.8856±0.05699

TableVII.1. Growth of different cell doses for both cell lines.

Data points are mean optical densities at 570nm recorded in 8 identical wells of one plate with standard error (see figures 4.2 and 4.3 for graphical representation).

Table VII.2.

DHT Dose	LNCaP (OD)	LNCaP (%)	PC3 (OD)	PC3 (%)
0M (control)	1.6205±0.109755	100±6.83	1.33925±0.004191	100±0.31
10^{-10}M	1.806±0.13764	111.44±10.24	1.338875±0.050181	99.97±3.82
10^{-9}M	1.80775±0.147243	112.54±10.63	1.3445±0.018784	100.39±1.58
10^{-8}M	1.3875±0.063308	85.56±8.22	1.337875±0.02995	99.9±2.36
10^{-7}M	1.12665±0.081726	69.52±9.97	1.34275±0.04083	100.26±3.13
10^{-6}M	0.959125±0.090414	59.18±11.64	1.349125±0.031462	100.73±2.45

Table VII.2. LNCaP and PC3 DHT response.

Data expressed mean optical density (OD) at 570nm and as a percentage of the optical density of the negative control. Each point is the mean optical density or percentage of the mean optical density of 8 identical wells with standard errors. (For graphical representation see figure 4.3.).

Table VII.3.

Radiation Dose	LNCaP-DHT (OD)	LNCaP-DHT (%)	LNCaP+DHT (OD)	LNCaP+DHT (%)
0Gy	1.15075±0.09565	100±8.31	1.2615±0.06965	100±6.05
1Gy	0.833±0.02364	72.39±8.78	1.00463±0.0921	79.64±10.99
2Gy	0.75675±0.04347	66.02±10.09	0.79613±0.02484	63.11±6.81
3Gy	0.69525±0.06493	60.42±12.5	0.71725±0.06693	56.86±11.12
4Gy	0.56458±0.00436	49.04±9.37	0.6325±0.01904	50.14±6.76
5Gy	0.56038±0.02485	48.69±9.42	0.60538±0.02672	47.99±7.49
6Gy	0.54588±0.02701	47.44±9.67	0.60313±0.03095	47.81±7.94
7Gy	0.497±0.01109	43.26±8.61	0.54313±0.02143	43.05±7.23
8Gy	0.493±0.01447	42.84±8.81	0.52938±0.02901	41.96±8.16
9Gy	0.44638±0.01632	38.79±9.08	0.48675±0.01243	38.59±6.57
10Gy	0.43713±0.01845	37.99±9.32	0.46275±0.0216	36.68±7.59

Table VII.3. Radiation dose response of DHT treated and untreated LNCaP cells.

Data shown as mean optical densities at 570nm and percentage of control optical density. Each number represents the mean of 8 identical wells with standard errors.

Table VII.4.

Radiation Dose	PC3-DHT (OD)	PC3-DHT (%)	PC3+DHT (OD)	PC3+DHT (%)
0Gy	0.703±0.0787	100±10.06	0.714±0.063	100±8.82
1Gy	0.579±0.0535	82.36±13.63	0.641±0.0602	89.76±12.59
2Gy	0.563±0.0499	79.97±13.15	0.592±0.034	82.91±10.53
3Gy	0.525±0.0445	74.68±13.41	0.504±0.0748	70.59±17.26
4Gy	0.487±0.0124	69.18±10.37	0.504±0.0266	70.59±10.28
5Gy	0.442±0.0171	62.78±10.78	0.463±0.0213	64.85±9.95
6Gy	0.403±0.0068	57.24±10.19	0.438±0.0246	61.34±10.47
7Gy	0.397±0.0234	56.39±11.66	0.429±0.0302	60.08±11.28
8Gy	0.377±0.0173	53.55±11.06	0.307±0.018	42.99±10.59
9Gy	0.359±0.0213	50.99±11.68	0.296±0.0203	41.46±11.17
10Gy	0.292±0.0291	41.48±14.16	0.256±0.013	35.85±10.18

Table VII.4. Radiation dose response of DHT treated and untreated PC3 cells.

Table showing mean optical densities and percentages of mean optical density using the control. Each number is the mean of 8 identical wells and standard errors are shown.

Table VII.5.

Radiation Dose	LNCaP-DHT No. of MN per 1000 BN	LNCaP +DHT No. of MN per 1000 BN
0Gy	11±8.06	63±29.83
1Gy	134±20.02	230±16.97
2Gy	365±16.30	329±25.13
3Gy	409±23.98	451±48.81
4Gy	592±30.65	588±29.19
5Gy	1101±24.94	787±25.18
6Gy	1390±50.99	1022±22.68
7Gy	1422±44.25	1014±58.98
8Gy	1830±44.08	941±44.29
9Gy	1568±71.51	1330±46.25
10Gy	1446±27.47	1202±51.67

Table VII.5. Chromosomal response of DHT treated and untreated LNCaP cells to radiation damage.

Raw data for LNCaP cells cultured in the presence of DHT and without the hormone. Each point is the number of micronuclei per 1000 binucleated cells and standard error.

Table VII.6.

Radiation Dose	PC3-DHT No. of MN per 1000 BN	PC3+DHT No. of MN per 1000 BN
0Gy	43±11.83	52±5.58
1Gy	260±32.76	166±12.09
2Gy	351±22.76	242±20.76
3Gy	577±37.61	745±37.15
4Gy	812±30.77	758±39.36
5Gy	1269±42.41	1014±41.52
6Gy	1374±64.15	1361±45.49
7Gy	923±97.42	589±53.48
8Gy	1620±47.41	1156±47.74
9Gy	1802±77.52	1414±59.44
10Gy	1642±102.62	1645±73.96

Table VII.6. Chromosomal response of DHT treated and untreated PC3 cells to radiation damage.

Table shows raw data from cells grown in the presence of DHT and without. The number of micronuclei expressed per 1000 binucleate cells are quoted along with standard error.

Table VII.7.

Patient	PBL-DHT No of MN per 1000 BN	PBL+DHT No of MN per 1000 BN
1	963	713
2	812	602
3	769	567
4	816	568
5	822	723
6	808	670

Table VII.7. Chromosomal response of DHT treated and untreated peripheral blood lymphocytes (PBL) to radiation damage.

Micronucleus scores for six patients, the number of micronuclei per 1000 binucleates. Lymphocytes cultured with and without DHT and irradiated at 3.5Gy.