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An Examination of the Bystander Effect in Multiple Models: from Cell Line to In-vivo Exposure

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An examination of the bystander effect in multiple models; from cell line to *in-vivo* exposure.

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Abstract

In recent years there has been a paradigm shift in radiobiology, to one which includes non-targeted effects, such as the bystander effect and genomic instability. The bystander effect has been defined as effects seen in cells that although never exposed to radiation, display similar effects to those that have, due to some form of communication with directly hit cells. This investigation aims to further the current understanding of this effect using three different models, cell lines, primary explant cultures and whole animals. Multiple cell lines were used to determine the relative importance of bystander signal production versus the response in the exposed cell line. In the case of responding cell lines, the signal generated from the cells exposed to direct radiation determined the magnitude of the response in the bystander cell. A primary tissue culture model, in conjunction with a cell line reporter system was used to investigate the bystander effect generated by epithelial tissue *in-vitro*. Apoptosis related protein expression was determined over a range of doses for both direct radiation and bystander signal exposure. The signal generated from tissue cultured *in-vitro* induced protein expression changes in the exposed tissue and a reduction in cell survival. To further examine signal production in tissue, mice on an antioxidant diet were used for both *in-vitro* and *in-vivo* exposures using the tissue culture / reporter cell line system to measure effects. While there were significant differences between normal and anti-oxidant mice, the most interesting result of this study was in the differences between *in-vitro* and *in-vivo* exposure, and the male and female mice. These results indicate that while much valuable information about the bystander effect may be gained from various types of *in-vitro* models, it is important that more *in-vivo* models are developed, while they are ultimately more complex, they are also a more accurate reflection of the true nature of this phenomenon.

Declaration

I certify that this thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature Date

Candidate

Dedication

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Abbreviations

PMA	20-pentahydro-xytiglia-1, 6-dien-3-one 12β -myristate 13-acetate
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AIF	Apoptosis Inducing factor
Apaf-1	Apoptosis Protease Activating Factor - 1
Bq	Becquerel
BaD	Bystander and Direct model
BSDM	Bystander Diffusion Model
c–PTIO	carboxy-2-phenyl-4, 4, 5, 5-tetramethyl-imidazoline-1-oxyl-3-oxide
CHO	Chinese Hamster Ovary
c–JNK	c–Jun N-terminal kinase
COX–2	Cyclooxygenase–2
DIE	Death Inducing Effect
DAB	Diaminobenzidine
DCF	Dichloroflourescein Diacetate
DMSO	Dimethyl sulfoxide
DPI	Diphenylene iodonim
DNA	Deoxyribonucleic Acid
dsb	Double strand breaks
DMEM	Dulbecco Minimal Essential Medium
ERK	Extracellular Related Kinase
FBS	Fetal Bovine Serum
GJIC	Gap Junction Intercellular Communication
G6DP	Glucose–6–Phosphate Dehydrogenase

Gy	Gray
HSP	Heat Shock Protein
HMP	Hexose Monophosphate Pathway
HPV-G	Human Papillomavirus
HEPES	Hydroxyethyl Piperazineethanesulfonic Acid
HRS/IRR	Hypersensativity/Increased Radioresistance
HPRT	Hypoxanthine Guanine Phosphoribosyl Transferase
IL-3	Interleukin-3
ICCM	Irradiated Cell Condition Medium
ITCM	Irradiated Tissue Condition Medium
LET	Linear Energy Transfer
LNT	Linear No Threshold Model
MeV	Mega Electron Volts
MAPK	Mitogen–Activated Protein Kinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NFκB	Nuclear Factor κB
PBS	Phosphate buffer saline
PET	Positron Emission Tomography
PCNA	Proliferating Cell Nuclear Antigen
PKC	Protein kinase C
rad	Radiation Absorbed Dose
ROS	Reactive Oxygen Species
REDOX	Reduction and Oxidation
RBE	Relative Biological Effectiveness
RNA	Ribonucleic Acid

R	Roentgen
Sv	Sievert
ssb	Single Strand Breaks
SCE	Sister Chromatid Exchanges
SOD	Superoxide Dismutase
TCF β –1	Transforming Growth Factor β 1
^{3}H dTTP	Tritiated Thymidine
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 History of Radiation

Wilhelm Roentgen discovered the first type of radiation, X-rays, in 1895. Soon after in 1896 and 1898, Antoine Becquerel and Marie Curie discovered the radioactive properties of uranium and radium which emit γ rays. Use of radiation in diagnostics was quickly developed, and within two years was being used to treat abnormal tissue. However, it became apparent to those working in the field that radiation was not only beneficial, but caused damage to normal tissue that had been exposed. In 1931, Irene Curie and Francois Joliot found that radioactivity could be artificially induced, and in 1932 James Chadwick discovered the neutron.

In the early days of radiotherapy, which began with the treatment of a benign hairy tumor in 1897, measurement of radiation was very crude. 'Skin erythema dose' was used, defined as the amount of radiation exposure that caused the skin to redden after 1-2 weeks. In 1928, this was replaced by the Roentgen (R), which was based on radiation induced ionisation in air. At this time, an important discovery was made by Muller [1927] who studied mutations in the *Drosophila* post exposure to x-rays, and found a dose-response relationship. Gray et al. [1940, 1953] showed some of the biological effects of fast neutrons and showed that oxygen concentration influences the radiosensitivity of the tissue exposed

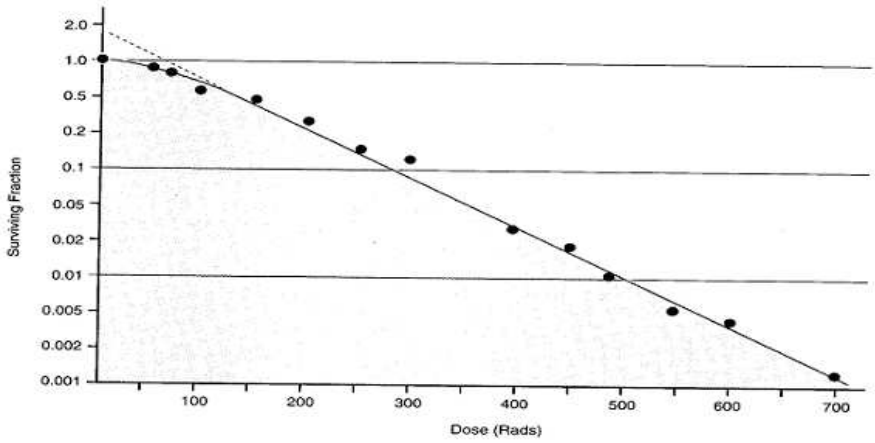

Figure 1.1: A typical survival curve displaying the dose-response relationship. *(From Hall [2005], originally from Puck and Marcus, 1956)*

[Nias, 1998].

One of the most important steps in the investigation of radiation and radiobiology was the development of the clonogenic assay by Puck and Marcus [1956]. This is a cell culture technique that allowed the quantitative measurement of cell death and demonstrated an exponential radiation dose response relationship *in-vitro*. This assay has since been used in a number of fields, as it is an extremely useful tool for assessing damage to a cell population post exposure to toxins. It became one of the main tools for investigating the dose–response relationship, and is still in use today. A typical survival curve is shown in figure 1.1.

1.1.1 Units

As the field of radiation physics and radiobiology progressed, so to did the units of measurement. The original unit known as the Roentgen, was a measure of ionisation in air, while the radiation absorbed dose (rad) was used for the amount of energy actually absorbed. Currently the SI unit of exposure is the coulumb/kg, and so the Roentgen is no longer in use. The rad has been replaced by the Gray (Gy), with one gray equivalent to

one joule of radiation energy absorbed per kilogram of tissue. To account for the different energies of various types of radiation the Sievert (Sv) was used, which was defined as the amount of radiation absorbed multiplied by the weighting factor of the type of radiation in question. The weighting factor for γ radiation is 1, while for higher energy radiation such as α particles, the weighting factor is 20. For measuring the dose from radioactive isotopes such as radium, the becquerel (Bq) is used. One becquerel is defined as one disintegration per second.

1.2 Interaction with Matter

1.2.1 Linear Energy Transfer

With regard to the different types of radiation, much was learned early in the last century. Radiation could be described depending on the source and the amount of energy it carried. Linear energy transfer (LET) was defined as the amount of energy that is transferred per unit track; charged particles with high LET such as neutrons or α particles, deposit large amounts of energy into their target but do not penetrate very far. These types of radiation are directly ionising, i.e. the probability that they will cause direct damage to the tissue they pass through is very high. For high LET radiation, 1 α particle track through a cell will result in a dose of 500mGy. In contrast, low LET radiation, such as γ rays carry low amounts of energy, but can penetrate much further into the target. These types of radiation, known as electromagnetic radiation are indirectly ionising, i.e. they do not themselves cause the majority of damage to the tissue they pass through, but instead their absorbtion into the tissue causes damage. There is some direct damage where the energy is deposited. For low LET radiation, 1 electron track through a cell will result in a dose of 1mGy. Thus, with increasing LET there is increasing relative biological effectiveness, which is described below in section 1.2.2, [Hall, 2005].

1.2.2 Relative Biological Effectiveness

Relative biological effectiveness (RBE) is used as a measure of the ability of radiation to cause a significant reduction in the survival fraction of cells. As the LET increases, so to does the RBE, with a maximum of 100 keV/μm. At energies over this, there is no increase in the RBE due to the 'overkill' effect, i.e. once a cell has received 100keV/μm it has already received a lethal dose, so increasing the energy deposited into the cell can do no further damage. Due to the amounts of energy deposited by various different types of radiation, there are different levels of response within a cell that a particle has traversed. As mentioned above, when an electron traverses a cell it deposits 1mGy to the

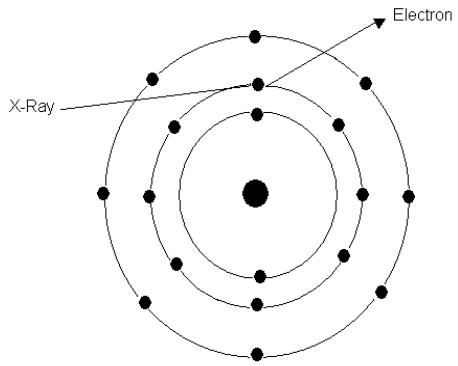

Figure 1.2: Photoelectric effect. The X-ray transfers all energy to an orbital electron and the electron is ejected, resulting in the ionisation of the atom. *Adapted from Hall [2005]*

cell, whereas an α particle deposits 500 times that amount. Therefore, low LET radiation such as γ rays have relatively low RBE, whereas high LET radiation such as α particles have high RBE [Nias, 1998].

1.2.3 Absorption

As mentioned above, (section 1.2.1) electromagnetic radiation such as γ rays do not cause direct damage to the tissue they pass through, but cause damage indirectly via interaction with water molecules in the tissue. Electromagnetic radiation is absorbed into matter in one of a number of ways. The first is the photoelectric effect and occurs when the x-ray (or photon) is at low energy, < 0.05MeV. In this case the photon will interact with a tightly bound electron and give all of its energy to that electron, see figure 1.2. The atom that loses this electron then becomes ionised. If the electron is knocked out of an inner shell, another electron either from the same atom or a free electron, fills its space. This causes the electron to lose some kinetic energy, which is given off as characteristic x-rays. The electron that had been knocked out of its shell becomes a fast electron, and has the potential to cause damage in the tissue. The probability of an x-ray interacting with an atom via the photoelectric effect decreases with increasing energy of the photon.

The second type of absorption is known as the Compton process, which dominates at

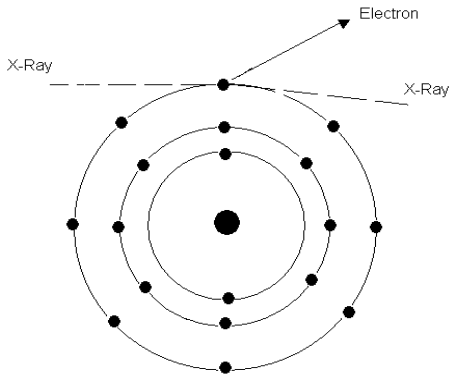

Figure 1.3: Compton scattering. The X-ray transfers some of its energy to an orbital electron and the electron is ejected. The x-ray continues through the material, interacting with more electrons. *Adapted from Hall [2005]*

higher energies, 0.1 - 10MeV. In this case the photon interacts with an electron which is loosely bound to an atom, and knocks it out of its shell, see figure 1.3. The electron now becomes a fast electron, as it has taken on some of the energy of the photon. The photon continues through the tissue with a reduced energy, interacting with more electrons. It is these fast electrons that cause chemical and biological changes in the tissue. The probability of a photon interacting with an atom via Compton scattering is dependent on the energy of the photon.

A third type of interaction occurs when the x-ray is 1.02MeV or higher. This is known as pair production and it involves an x-ray passing through the electric field of a nucleus, where it creates an electron and a positron due to conversion of energy to matter. The energy of the incident ray is shared between the electron and the positron, see figure 1.4. The electron and positron interact with and can ionise other molecules. The positron eventually interacts with another electron resulting in annihilation and the emission of two 0.51MeV photons. These photons can produce further ionisations via photoelectric effect or Compton scattering. At energies below 1.02MeV, there is no possibility of pair production, however the probability increases with increased energy and is dominant at energies above 10MeV [Chandra, 1992, Hall, 2005].

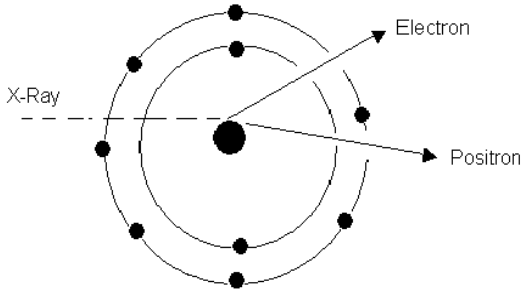

Figure 1.4: Pair production. An electron with an energy of more than 1.02 MeV interacts with a positively charged nucleus and produces an electron and a positron. *Adapted from Chandra [1992]*

When radiobiologists began to describe how radiation of various energies interacted with matter such as tissue they developed the target theory, which is described below in section 1.3.1.

1.3 Models of Radiation Damage

1.3.1 The Target Theory

When radiation was first used in the treatment of cancer early in the 20th century, nothing was known about the way radiation induced cell death. Blau and Attenburger [1922] proposed a critical target in the cell, that if damaged would cause cell death. Once DNA was established as the control center for the cell, it was suggested as the target for radiation and so target theory was born. The target theory of radiation states that there is a critical point in the cell that must be hit in order to induce cell death [Lea, 1946, Marshell et al., 1970]. As the field of biology advanced, there were numerous discoveries that confirmed this theory and DNA as the target. These included the clonogenic assay, developed by Puck and Marcus [1956] to determine cell survival and the ability of radiation to induce strand breaks into the DNA double helix by Lett et al. [1961]. Thus for many years it has been thought that DNA is the critical target in a cell exposed to radiation, and although much evidence has accumulated to suggest that there are other targets within the cell that can lead to significant damage, DNA is still of critical importance. The damage and repair of DNA is discussed below in section 1.4

Within the target theory two types of interaction are described, direct and indirect, illustrated in figure 1.5. Direct action occurs as a result of absorption of high LET causing single strand breaks, ssb, double strand breaks, dsb and base damage within the DNA helix. Depending on how the cell handles the repair of the damage, these can be non-lethal or lethal mutations. In the case of low LET radiation, such as γ rays, interaction is more likely to occur with water via one of the mechanisms described above, 1.2.3. The water molecule becomes ionised, as the photon knocks one of its electrons out of its orbit. This highly reactive water molecule will then react with another water molecule, and form the hydroxyl radical (see equation 1.1), which is capable of traveling the short distance to the nucleus. This damages the DNA strand in much the same way as the charged particle.

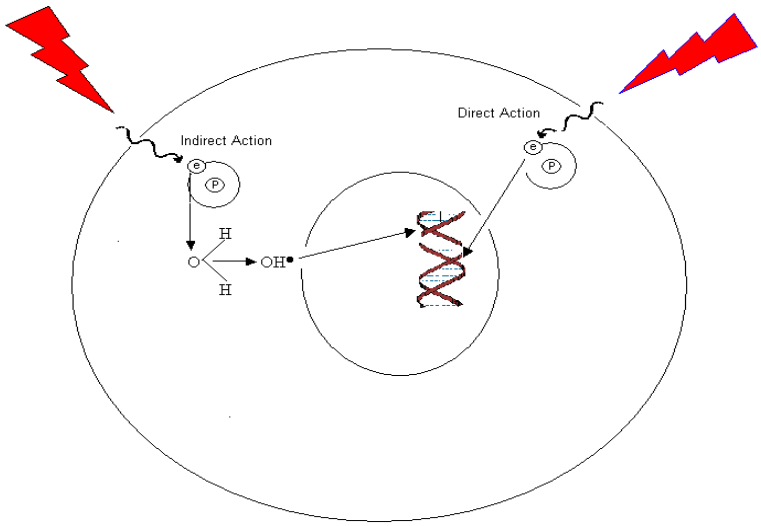

Figure 1.5: Direct and indirect action of radiation. *Adapted from Hall [2005]*

$$\boxed{\begin{array}{c} H_2O \rightarrow H_2O^+ + e^-. \\ \Downarrow \\ H_2O^+ + H_2O \rightarrow H_3O + OH\cdot \end{array}} \tag{1.1}$$

As a result of advances in radiobiology and the need to quantify the damage caused by radiation, Kellerer and Rossi [1972] put forward a linear quadratic equation, which was modified by Chadwick and Leenhouts [1973], to accommodate the survival curve data at the time, (see equation 1.2). This equation states that at low doses of radiation, the majority of the damage to DNA is ssb, however, as the dose increases, dsb tend to dominate and so the level of cell death is greater. This equation, based on the target theory, is still used as a measure of radiation damage in risk estimation today, and is the basis of the linear no threshold model, discussed in section 1.3.2.

$$\boxed{\begin{array}{c} \text{Ln F} = -(\alpha\ \text{D} + \beta\ \text{D}^2) \\ \text{F} = \text{Survival Fraction} \quad \alpha = \text{initial slope survival curve} \\ \beta = \text{terminal slope survival curve} \quad \text{D} = \text{Dose} \end{array}} \tag{1.2}$$

1.3.2 Linear No Threshold Model

Humans are exposed to radiation in a number of different ways, depending on where they live and their occupation. Therefore, the risk associated with various levels and types of exposure needs to be determined. Much information about the response of humans to radiation, most importantly in the development of cancer, has been learned from the various types of exposures. These exposures include relatively low doses, such as medical or occupational exposures, to extremely high doses such as those received by the survivors of the atomic bombs in Japan or the Chernobyl accident. The Linear No Threshold (LNT) model is the current model from which radiation risks to humans are determined, and was first proposed by the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) in 1958. It has been developed using data from human exposure to high doses of radiation, such as the atomic bomb survivors. The results of these studies determined that there are indeed serious biological effects of high dose exposures, and there was an observable dose - response relationship. However, due to the fact that these are all high doses of radiation and the majority of the population will never receive this type of dose, the risks must also be determined for low doses and low rates of radiation exposure. This was done by extrapolation from the data from the Japanese survivors back to zero. This is considered a conservative estimation method as it assumes that there is always a risk with exposure to radiation, no matter how low the dose. Many of the worlds governments adopted this model, as they believe that there are no underestimations of the risks involved.

There is however, much controversy in the field as to whether the LNT model is an accurate one. Preston [2003] said in his paper titled 'The LNT model is the best we can do – today' that this model is the most accurate at this time, as it fits with the vast majority of the data available for both low and high doses. He proposed that the non–targeted effects discovered relatively recently such as the bystander effect, or the adaptive response do not involve a departure from this model. In contrast, Higson [2004] states that there is no evidence to support the theory that there are harmful effects of radiation at very

low doses, and that the LNT model has helped to foster an unwarranted fear of low level radiation. He concludes that the LNT model should only be used for doses above a few tens of mSv a year. In a more recent debate about the LNT model and the possible alternatives, Martin [2005] states that unless there is clear and unambiguous evidence of no risk associated with low dose radiation that the LNT model should be kept in place, as it is conservative and individuals are unlikely to be exposed to unnecessary risk. In relation to the possibility of a threshold model, Martin claims that implementation of such a model may cause many political and practical complications.

However, with the ever increasing body of data on the biological effects of low dose radiation, many scientists believe that the recently discovered non-targeted effects discussed below, need to be taken into consideration when determining the risks involved in radiation exposure.

1.3.3 The Repair Theory

The repair theory was first put forward by Power [1962], and was a slight deviation from the target theory. This model allows for the ability of the cells that are damaged by radiation to recover from this damage by repair of DNA. This theory explained the shoulder region of the survival curve which occurs in the low dose region. The central idea behind the theory was that all irradiated cells were capable of repair however, at high doses, the ability of the cell to repair all damage becomes saturated and so mutations leading to cell death can occur. As this theory developed, the ideas that the ability of the cell to repair itself was associated with the cell cycle, and that repair enzymes were only expressed in certain stages of the cycle, were incorporated into the model, [Sinclair, 1972, Alper, 1984].

1.4 DNA Damage and Repair

As mentioned above, post exposure to radiation DNA damage can manifest in either of two ways. The first is a single strand break (ssb), where one of the DNA strands is damaged. This type of damage can be repaired by proteins within the nucleus designed to proof read the DNA strands. The other is the double strand break (dsb), where both of the strands are damaged at the same point. This type of damage is a more serious break than the ssb, as the DNA becomes fragmented and so aberrations in the DNA strands can occur when these recombine. The point in the cell cycle that the damage occurs determines if it is a chromosomal or chromatid type abberation. If the whole chromosome is hit early in the cell cycle then this damage will be present in both chromatids at mitosis, and is called a chromosomal abberation. However, if the exposure occurs later in the cell cycle, after the chromosomes have condensed to form chromatids, the damage will only be present in one of the chromatids and therefore it is known as a chromatid abberation. Various types of abberations occur post exposure to radiation due to the recombination of these broken pieces of the DNA strand. These include dicentric and acentric fragments, reciprocal translocations, interstitial and terminal deletions (see figure 1.6 for examples). Abberations can be termed stable or non-stable. The stable forms are symmetrical and so allow mitosis to continue, thus this damage is transmitted to the daughter cells. The unstable forms are asymmetric, so cells are no longer able to complete mitosis and are quickly removed from the dividing population, [Steel, 1997, Nias, 1998].

The way in which a cell copes with exposure to radiation varies greatly depending on what stage of the cell cycle it is in. As mentioned above, the cell may suffer chromosome aberrations if irradiated early in the cell cycle or chromatid aberrations if exposed late in the cycle, post DNA synthesis. Protein such as cdk2 and p53 can control the progression of a cell through the cycle and so are capable of arresting the cycle to allow for the repair of damage to the DNA before the cell commits to mitosis, thus attempting to prevent the cell from passing any mutations suffered onto the progeny. In some cases, if the DNA cannot be repaired, proteins such as p53 can accumulate and induce apoptosis, discussed

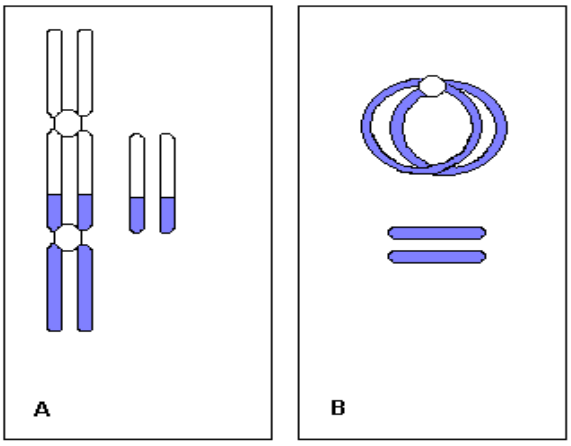

Figure 1.6: Examples of chromosome aberrations. Panel A: Dicentric and acentric fragment. Panel B: Ring. *Adapted from Hall [2005]*

in section 1.5.2 [Nias, 1998].

Depending on the type of damage, the cell may be able to completely repair the DNA correctly and progress to mitosis. However, the DNA may be incorrectly repaired, resulting in the formation of stable aberrations with the possible propagation of the damage to the progeny. Unstable aberrations may also occur, in which case the cell is unable to complete mitosis and dies. In the case of the ssb, the damage is repaired quite easily and accurately using the opposite strand as a template. This is done by excision and removal of the area around the damaged point, synthesis of new DNA in this region and ligation of this new DNA to the original strand. This process is completed by a number of genes involved in DNA repair, such as RAD51 [Nias, 1998, Steel, 1997].

In the case of the dsb, the repair process is a much more complex one, which may result in serious mutations within the cell's DNA. There are two basic forms of repair of double strand breaks, homologous and non-homologous recombination. If it is a chromatid abberation, there is an identical sister chromatid alongside the damaged one, and so this can be used as a template for the repair of the other chromatid. The damaged chromatid invades the helix of the sister chromatid and new bases are added onto both strands until the gap is filled (figure 1.7 panel A). This type of repair is called homologous recombination

and is error free, and is shown to be dependent on cell cycle, and the extent of the damage involved. [Hall, 2005, Golding et al., 2004].

The second type of repair is non-homologous recombination, which is much more common in mammalian cells but quite error prone and therefore leads to numerous types of aberrations. In this case, there is no sister chromatid present, and so no template for the repair of the strand. Some of these aberrations are shown in figure 1.6; panel A illustrates a dicentric and acentric fragment both of which are a result of a dsb occurring in two chromosomes and the illegitimate rejoining of the incorrect strands. When replication occurs, the sister chromatids in one portion are joined by two centrosomes leaving the other portion without centrosomes. Panel B illustrates overlapping rings, also the result of illegitimate rejoining, however in this case there is only one chromosome involved, with two dsb. The two ends of the chromosome join to form a ring, usually resulting in the formation of an acentric fragment, as with the dicentric. Post replication there are two overlapping rings and two acentric fragments [Hall, 2005].

As radiobiology advanced, much has been learned about the way in which a cell copes with radiation exposure. The development of fluorescence in-situ hybridisation (FISH) has lead to the discovery of the complex interaction of various DNA strands post irradiation, and so the dicentric has become a marker of radiation damage in many of the current models. Another common marker of DNA damage is the activation of the repair protein γH2AX, which binds to the DNA helix when damaged. It is now known that there are hundreds of proteins capable of repair of damaged DNA post exposure, with many of the mechanisms elucidated. These mechanisms include base excision, nucleotide excision and mismatch repair.

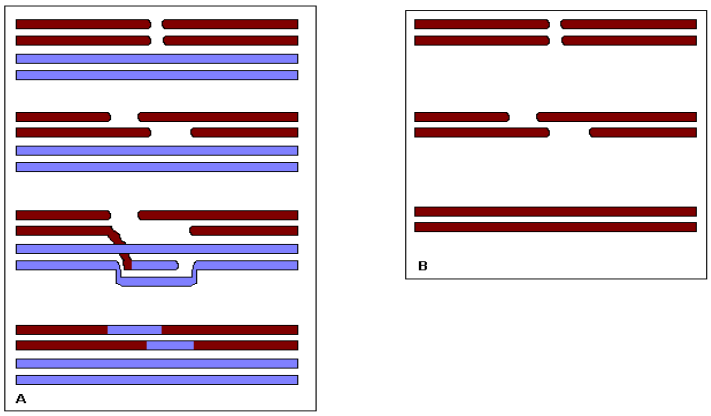

Figure 1.7: Schematic of two types of DNA repair. Panel A: Homologous Recombination. Panel B: Illegitimate Recombination. *Adapted from Hall [2005].*

1.5 Cell Death

Cell death has been the subject of numerous and intensive investigations since the discovery of so called programmed cell death and its importance in tissue development in the embryo and the protection of tissue from cells that have suffered mutation post exposure to an insult such as radiation. Much has been learned about the methods of cell death and the pathways involved, some of which are discussed below. There are two main types of cell death; necrosis and apoptosis, see figure 1.8. Necrosis usually results from a gross insult to the cells, the breakdown of intracellular communication and the rupture of the membranes resulting in inflammation around the cell in question. Apoptosis which is also known as programmed cell death occurs when the cell has suffered an insult, but the intracellular communication remains intact, and the cell itself takes the decision to commit suicide. A third type of cell death is mitotic cell death, which refers to cells that have completely lost the ability to proliferate, and so while the cell remains alive, mutations cannot be passed onto future generations.

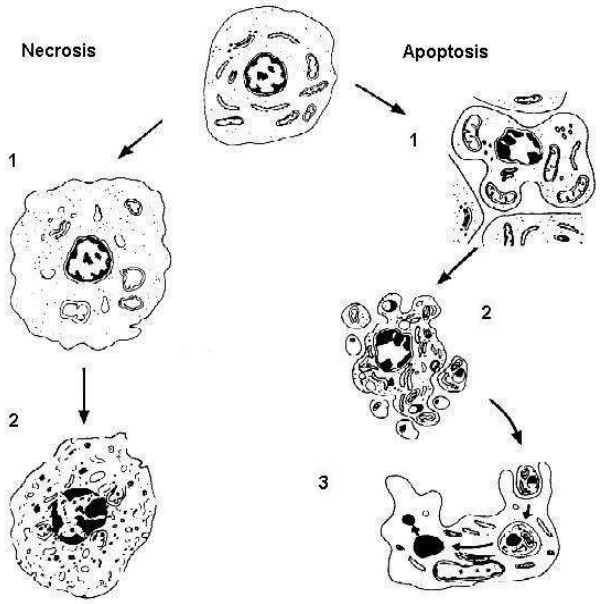

Figure 1.8: Schematic of cell death, necrosis and apoptosis. Necrosis 1: The cell swells. 2: Releases contents into surrounding area, resulting in inflammation. Apoptosis 1: Cell shrinks, detaching from neighbours, plasma membrane remains intact. 2: Chromatin condenses at the nuclear membrane and the cell disintegrates into apoptotic bodies. 3: These are taken up by neighbouring cells. *Adapted from W.M. Blom, 2000*

1.5.1 Necrosis

Necrosis is cell death that only occurs following the receipt of a serious insult causing the failure of the normal functions within the cell. It is favourable for the cell and more importantly for the tissue around it, to control the cell death and prevent inflammation and the possible escape of toxins from the dying cell. However, this is not always possible. If the cell suffers a serious insult, the majority of the systems within the cell will simply cease to function. Due to the lack of control of this type of death, the events commonly associated with necrosis do not always follow the same pattern. Characteristics of necrosis include mitochondrial failure and rupture, loss of the ion pumps and hence osmotic control resulting in rupture of the plasma membrane. At this point the contents of the cell trigger the invasion of macrophages to remove the debris, leading to inflammation of the area [Lockshin and Zakeri, 2004].

1.5.2 Apoptosis

Apoptosis is widely known as programmed cell death, induced by a sequence of events within the cell, and as such is a form of cell suicide. Apoptosis can be induced in cells to allow the proper development of a fetus or tissue, or in cells that have been injured and present a threat to the integrity of the organism. Depending on the proteins involved, apoptosis can be characterised as caspase dependent or independent. As the name suggests, caspase dependent apoptosis involves the activation of a cascade of site-specific caspases, and the activation of proteases and nucleases in the nucleus to destroy DNA. The characteristics of caspase-dependent apoptosis are condensation and blebbing of the cytoplasm, condensation of the chromatin in the nucleus and the degradation of DNA.

The caspases involved in apoptosis can be divided into two groups, the initiator and effector caspases. The initiator caspases, 8 and 9, found at the cell and mitochondrial membrane activate the effector caspases, 3 and 7, present in the cytoplasm. In the case of caspase 8, activation occurs via an extrinsic mechanism at the plasma membrane as a result of activation of the death receptor by a member of the TNF-α family. Caspases

3 and 7 are activated by caspase 8, and attack essential cytoplasmic proteins and critical enzymes within the cell. In contrast to this, caspase 9 activation is via an intrinsic mechanism as it is located on the mitochondrial membrane. Degeneration of the health of the mitochondria resulting in the depolarisation of the membrane leads to the leaking of cytochrome c into the cytoplasm, which then binds to Apaf-1 (apoptosis protease activating factor-1). The combination of proteins present at this point aggregate to form apoptosomes, which bind to and activate caspase-9. Caspase 9 in turn activates caspases 3 and 7, leading to attack of the cytoplasmic protein and enzymes critical to the cell. Mitochondria, as a reflection of the health of the cell are of great importance in this pathway, and the sensitivity of the mitochondria a measure of the insult received. This sensitivity can be adjusted by proteins which can be recruited to the mitochondrial membrane, such as Bcl2 to stabilise or Bax to destabilise the membrane [Lockshin and Zakeri, 2004].

Caspase independent apoptosis involves another protein present in the mitochondria, apoptosis-inducing factor (AIF), which when released from the mitochondria migrates to the nucleus and binds to the DNA, signalling the destruction of the cell. Therefore, mitochondria are also of critical importance in this pathway.

There are many proteins involved in the induction and regulation of apoptosis, including Bcl2, cMyc, BAX, and p53. Many of the recent investigations into the apoptotic pathway have involved examining the levels of these proteins, along with close monitoring of the mitochondria, which is now accepted as the main 'control center' of apoptosis [Brenner et al., 1998, Marzo et al., 1998, Li et al., 1999]

1.5.3 Mitotic Cell Death

Mitotic cell death is that which occurs when the cell can no longer complete mitosis, and is usually the type of death exploited by radiotherapy. The affected cells may complete one or two mitoses post irradiation, however damage to the DNA cause the cells to fail in the attempt to complete mitosis soon after exposure. All the functions of the cell can still be carried out, including the replication of organelles in preparation for mitosis, however

mitosis cannot be completed. Thus one of the common features of mitotic death is an increased cell volume due to the replication of the organelles but the lack of division. Because of this many cells that have undergone mitotic cell death are quite large, and are also termed 'giant cells' [Steel, 1997, Castedo et al., 2004, Hall, 2005].

1.6 The Shifting Paradigm

Many of the ideas and models described above are those that were derived from experiments performed in the early to middle 20th century, and as the field of radiobiology has advanced, some of the data these models are based on has been questioned by conclusions drawn from experiments completed with more sophisticated techniques. The discovery of non-targeted effects in particular have thrown the target theory into doubt. Bystander effects, genomic instability, adaptive response and low dose hypersensitivity are among the key phenomena associated with radiobiology late in the last century, and their discovery have lead to intense investigation worldwide. This has resulted in a paradigm shift within radiobiology, to one that has more scope than the classical models. However, much investigation is still required to elucidate the mechanisms and consequences of non-targeted effects that predominate at low dose, and low dose rate radiation exposure. A more complete understanding of these effects will allow for the development of more comprehensive and accurate models for low dose risk assessment and the possible exploitation of these phenomena for use in radiotherapy [Mothersill et al., 2003, Prise et al., 2005].

1.6.1 The Bystander Effect

History

The first indication of a deviation from the target theory was the discovery of bone marrow damage in children receiving radiation to the spleen for treatment of leukaemia by Parsons et al. [1954]. Over the next few years more evidence of 'abscopal' or 'out of field' effects began to appear in the literature. Souto [1962] showed that rats exposed to the plasma or ultrafiltrates of blood from irradiated rats or sheep developed mammary tumors at a significantly higher level than controls. Hollowell and Littlefield [1968] showed chromosomal damage to lymphocytes in culture which were exposed to the plasma of radiotherapy patients. The aberrations included dicentrics, chromatid and chromosome breaks. Pant and Kamada [1977] showed the presence of a factor in the plasma of atomic

bomb survivors 31 years after exposure. Emerit and Cerutti [1981] found that plasma from radiotherapy patients contained clastogenic factors, which were of low molecular weight, (1000 - 10,000 Da), and that their production involved lipid peroxidation and oxidative stress pathways.

And so it became apparent as the body of evidence increased that radiation did not only cause damage to the tissue that was directly exposed, but also to tissue in the surrounding area, and possibly tissues which are remote from the treated area, if 'factors' were released into the bloodstream. Therefore, doubt was cast on the established dose-relationships and models of radiation damage, as they did not account for these newly discovered effects.

Current Research

One of the first investigations to examine this newly discovered phenomenon was performed by Nagasawa and Little [1992]. The induction of sister chromatid exchanges (SCE) in Chinese hamster ovary cells was used as a measure of radiation damage post exposure to extremely low doses of α particles. Although 1% of the nuclei suffered a direct hit, 30% displayed an increase in SCE. In the case of X–rays, a dose of 2Gy was needed to induce the same effect as that seen post exposure to α particles. However, these important results indicated that this phenomenon can be induced by both high and low LET radiation.

Deshpande et al. [1996] examined the level of SCE following exposure of lung fibroblasts to α particles, and found a large discrepancy between the level of predicted damage using the target theory, and the levels actually observed in the exposed cells. The cells expressed an 8.6 fold higher level of SCE than that estimated by the target theory at low doses, suggesting the presence of an extranuclear target for radiation that can lead to DNA damage. The difference between the expected and observed SCE reduced as the dose increased, suggesting that the extranuclear target is of more importance at low than high doses.

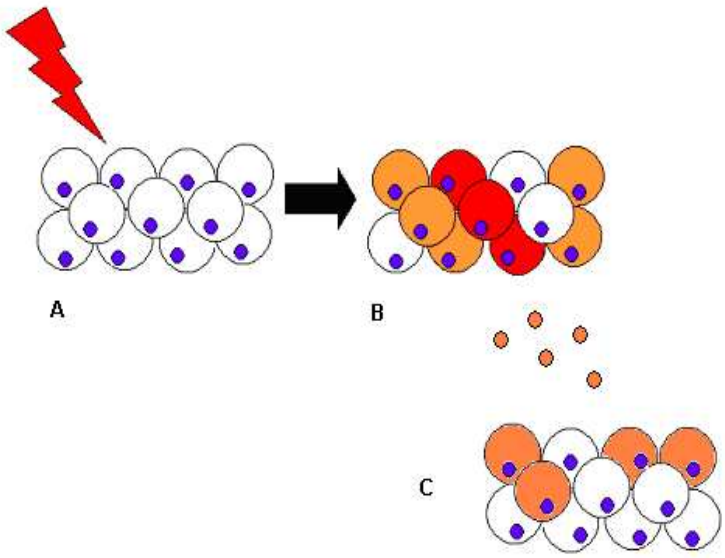

Figure 1.9: Schematic of the bystander effect. A: Cells exposed to direct irradiation. B: Cells shown in red are those directly traversed by the track; cells in orange are bystander cells. Those cells in white are unaffected. C: Cells remote from the exposed cells affected by the bystander signal.

In their paper examining the response of normal human cells to α particles, Lehnert et al. [1997] found that exposed cells produced transmissible factors, and when these were transferred to unexposed cells the level of SCE increased to that seen post direct exposure. It was found that this effect could be inhibited by superoxide dismutase (SOD), a free radical scavenger. The authors concluded that the target for radiation damage may well be larger than the nucleus and indeed the cell itself, since these factors can effect 'un–hit' cells. This was also the first paper to suggest a role for free radicals in the bystander effect, as the effect could be inhibited by a free radical scavenger.

Narayanan et al. [1997] investigated the response of normal human lung cells to α particles and determined the level of reactive oxygen species (ROS) post exposure. In this study, cells exposed to α particles were found to have an elevated ROS level. Un–hit cells exposed to medium from irradiated cells or irradiated medium also displayed an increased level of ROS, however there were important differences between the two. In the case of the cell free medium, the effect seen in the exposed cells was a short lived one, however the effect post exposure to medium from irradiated cells was longer and more pronounced. These effects were inhibited by free radical scavenger SOD and NADPH–oxidase inhibitor diphenyleneiodonim, (DPI). It was suggested that the DNA damage seen as a result of α particle exposure was mediated by ROS, via NADPH–oxidase activation, and that this mechanism may account for the higher than expected levels of damage in unexposed cells.

Mothersill and Seymour [1997b] suggested a role for medium borne factors released into the medium by irradiated cells post exposure to γ rays. A medium transfer technique was used, removing medium from irradiated cells and transferring it to unirradiated cells seeded at cloning densities. In this case the unirradiated cells suffered a significant loss in survival post exposure to medium from irradiated epithelial cells but not from fibroblasts. Both cell types showed a loss in survival post exposure to the medium from irradiated epithelial cells, which was dependent on the number of cells present at the time of irradiation, and could be induced by medium removed from the irradiated cells 30 minutes

post exposure. Apoptosis was suggested as a mechanism of cell death, as there were morphological signs of this in the unirradiated cells.

One of the most significant developments in the investigation of this phenomenon following high LET exposure was the microbeam, which allowed the targeting of individual cells in the field. Using this technology Prise et al. [1998] irradiated primary human fibroblasts with α particles and helium–3 particles. For comparison cells were also exposed to X–rays. In cell populations that were exposed to conventional radiation, a dose response relationship was demonstrated. With increased dose, there was an increase in micronucleated and apoptotic cells. However, when the microbeam was used to target four cells in a population with α particles, an unexpected amount of cells were micronucleated and apoptotic. A localisation of damaged cells around the originally irradiated cells was also noted.

As more evidence of this non–targeted effect accumulated, many questions were raised about the nature of the factor causing the effects in the un–hit cells. In the absence of a concrete answer, the term 'bystander effect' was used, and is defined as the detection of responses in unirradiated cells that can reasonably be assumed to have occurred as a result of exposure of other cells to radiation [Mothersill and Seymour, 2001], see figure 1.9. With the development of new tools and more questions than answers, the field split into two separate areas of investigation, high and low LET exposure, some of the results of which are discussed below.

Bystander Effects Induced By High LET Radiation

Since the discovery of the bystander effect, numerous methods of investigating the phenomenon *in-vitro* have been developed. Two of the most frequently used methods of studying the effect induced by high LET radiation are the microbeam and very low fluences of α particles. As mentioned above, the microbeam was one of the most important tools for investigation of the bystander effect induced by high LET radiation, and much has been learned from investigations using this method. The microbeam targets individ-

ual cells in the population with high LET particles such as α particles or neutrons, thus the bystander effect may be examined in the cells that were not exposed, but were present at the time of irradiation. The second method, very low fluences of α particles, involves the population being exposed to a very small number of α particles, and so only a small percentage of cells in the population are actually traversed. Two less commonly used methods of investigation are the medium transfer protocol, which involves the transfer of the culture medium from exposed cells to cells that have never been exposed, and the double mylar dish, which allows for growth of two separate populations of cells (irradiated and non-irradiated) in the same culture medium.

Using the medium transfer protocol Lehnert et al. [1997] showed that there was a significant increase in the level of sister chromatid exchanges (SCE) in cells exposed to medium from irradiated cells. Just prior to exposure, medium was removed from the cells and replaced immediately after exposure. Increased levels of SCEs was observed in cells treated with media removed from the irradiated cells immediately after exposure, and at various time points following irradiation. No significant difference in the level of SCEs was observed between the unirradiated cells exposed to the medium and those that were directly irradiated. To determine if irradiated culture medium would induce a similar response, cell free medium was exposed to α particles and incubated with unirradiated cells. When the medium was immediately placed on the unirradiated cells, a similar increase in SCEs was observed, however at later time points post exposure, no increase was noted. Also of interest in this investigation was the finding that serum (FBS) free media incubated with irradiated cells and subsequently used to treat unirradiated cells did not cause the increase in SCEs observed when serum was present in the media. The factor in the media causing the increase in SCEs was also found to survive a freeze–thaw cycle, and temperatures up to 53°C, however, was destroyed at 93°C, leading the authors to suggest that the factor was proteinaceous in nature. The authors concluded that following α particle exposure, both cells and culture media produce a signal that can cause an increase in SCE in unexposed cells to the level of that seen in directly irradiated

cells. However, while the signal derived from cells appeared to be a long lived one, the signal from media is short lived, and only capable of inducing damage immediately after irradiation.

In a similar investigation, Iyer and Lehnert [2000] used the medium transfer protocol and α particle exposure to measure bystander effects in the cells exposed to medium from directly irradiated cells. Cells were exposed to very low doses of α particles or the medium from irradiated cells, and the growth was monitored for 3 days. In both cases, increased proliferation was observed. The authors attributed this growth to increased levels of transforming growth factor β 1 (TGF–β 1) found in the medium of irradiated cells. Significantly increased levels of ROS, proliferating cell nuclear antigen (PCNA) and cell cycle protein CDC2 were observed in the medium treated cells along with decreased levels of TP53 and CDK1NA. It was suggested that the increased levels of ROS induced by irradiation resulted in the increased concentrations of TGF–β 1 in the culture medium, and that this led to the decreased expression of TP53/CDKN1A, possibly via the activation of NADPH–oxidase. The authors conclude that this 'decreased TP53/CDKN1A bystander effect', which was abolished by the inhibition of TGF– β 1, was responsible for the increased proliferation, and that this could contribute to carcinogenic processes.

Using the microbeam to target single human fibroblasts with α particles, Belyakov et al. [2001] demonstrated the presence of a high LET radiation induced bystander effect. Three days post exposure, a 2–3 fold increase in damage to the population was observed. The increase in the damage was unaffected if the number of cells that were targeted increased, or if the number of α particles increased. In a separate investigation using porcine urothelial explants Belyakov et al. [2002, 2006] showed premature differentiation be an effect of exposure to the bystander signal. Post exposure of $2\mu\text{m}^2$ of the explant area to α particles, there was a significant increase in the level of terminal differentiation. It was suggested that this could be a protective mechanism of the bystander effect, as the cells undergoing premature differentiation would be unable to propagate damage within the population. It was suggested that the number of cells expressing damage compared to

those irradiated points to a cascade mechanism, where the bystander signal released from the irradiated cells induces the release of a secondary bystander signal from the bystander cells. Also of importance in this study was the confirmation of the bystander effect in a multicellular system, where various cell groups are at different stages of the cell cycle. In a similar system using human and porcine samples, Belyakov et al. [2003] showed a proliferation dependent bystander effect. When 10 actively dividing cells at the periphery of the explants were exposed to α particles, an increase in the level of apoptotic and micronucleated cells was observed. However, there no significant increase in apoptotic and micronucleated cells when 10 differentiated cells at the centre of the explant were exposed, strongly indicating that the generation of the bystander signal is proliferation dependent.

Using a charged particle microbeam Sawant et al. [2002] showed that when 10% of a Chinese hamster V79 cell population was traversed with α particles more cells were unable to form colonies than were hit. To ensure that the cells forming colonies were non–hit cells only, the irradiated cells were removed post irradiation, and so only non-hit cells remained. Due to the cell density at the time of exposure, it was suggested that the bystander signal was enhanced and possibly transmitted through gap junction intercellular communication.

Shao et al. [2003a] also demonstrated the bystander effect in human fibroblasts following microbeam irradiation, using micronucleus induction as an endpoint. By treating the cells with DMSO, a scavenger of ROS, a significant reduction in the effect was noted. This was more pronounced when the gap junction inhibitor, 20-pentahydro-xytiglia-1, 6-dien-3-one 12β -myristate 13-acetate (PMA), was also used to treat the cells. Shao et al. [2003b] also found that adding the nitric oxide scavenger, c-PTIO, to the medium blocked the effect. Therefore, roles for ROS, gap junction intercellular communication, and nitric oxide were indicated.

By exposing a population of cells to very low fluences of α particles, Azzam et al. [1998] demonstrated the bystander effect by monitoring the level of TP53/CDKN1A gene

expression. In this case, only about 2% of nuclei in the population would have been traversed, however, an increase in gene expression occurred in more cells than predicted. The increase in the TP53/CDKN1A expression was reduced significantly with the addition of lindane, a gap junction inhibitor, and in low density populations. This would indicate that, for this type of exposure at least, cell to cell contact is required for the transmission of the bystander factor. This was confirmed in a second study by the same author, [Azzam et al., 2001] comparing the bystander response as measured by $p21^{Waf1}$ induction in cell population with and without GJIC. In cell populations with GJIC, a significant bystander effect was recorded, however this response was absent in cells that were GJIC null, thus providing clear evidence for a role for this method of bystander signal transmission in this model. In a paper investigating the effect of direct irradiation with low and high fluences of α particles on cell cycle arrest in G1, Azzam et al. [2000] found a significant increase in transient and permanently arrested cells when the cell population was exposed to doses as low as 1cGy. Although only 9% of the cells were traversed, 15-20% of the population were arrested in G1. The authors suggested that the bystander effect contributed to this low dose response.

In a study investigating the role of oxidative metabolism in the bystander effect, Azzam et al. [2002] found that superoxide and hydrogen peroxide were involved in the activation of stress response pathways and induction of micronuclei in bystander cells, post very low dose exposure of the population to α particles. Numerous proteins involved in stress response such as p53, $p21^{Waf1}$ and nuclear factor κB were increased in the bystander cells. This effect was inhibited by the addition of the superoxide scavenger, SOD and catalase, which converts hydrogen peroxide to water and oxygen. A role for NADPH-oxidase was also suggested as there was a significant reduction in the effect when the NADPH-oxidase inhibitor DPI was included in the medium. Interestingly, the effects were only significantly inhibited in populations exposed to very low doses, 1-2cGy, and the addition of inhibitors at the higher doses had no effect. It was suggested that this result reflects the importance of ROS in bystander signal mediation, as at low doses, the

majority of the cells displaying damage are bystander cells that did not suffer a direct hit, and so scavengers inhibited the effect. However, at higher doses, where the majority of the cells displaying damage did suffer a direct hit, the scavengers had little or no effect.

In an investigation into the role of membrane signaling in the bystander effect, Nagasawa et al. [2002] monitored the induction of sister chromatid exchanges and HPRT mutations in CHO cells exposed to very low fluences of α particles. A significant increase in both SCE and HPRT mutations was observed following 0.17 cGy and 0.50 cGy in the control group of cells, however when the cells were incubated with Filipin, an agent that disrupts lipids rafts and therefore membrane signaling, the increase in mutations was absent. When the dose was increased so that the majority of the damage would be direct rather than bystander, incubation with Filipin had no protective effect. The authors concluded that membrane signaling is an integral part of the bystander effect, however it does not play a part in the direct damage caused by α particle exposure. In a similar investigation Nagasawa et al. [2003] exposed a repair deficient cell line, xrs-5 to very low fluences of α particles, resulting in less than 1% of nuclei in the population being irradiated. Interestingly a much larger bystander effect was observed when compared to that seen in the CHO cell line, suggesting that unrepaired or misrepaired dsb contribute significantly to the large bystander effect seen in these cells.

In another study, Zhou et al. [2001] also showed that gap junction communication is important in the transmission of a damage signal post exposure to α particles. When less than 20% of the population was exposed to α particles, the fraction of cells displaying damage was similar to that displaying damage when the entire population was exposed. This effect was significantly reduced when the gap junction inhibitor octanol was used to treat the cells.

Little et al. [2002] showed a significant bystander effect in both epithelial and fibroblasts post exposure to very low fluences of α particles. A significant increase in the number of micronuclei was observed in the bystander cells, along with changes in p53, p21 and MDM2. The effect was inhibited by incubation with SOD and NADPH–inhibitors, indi-

cating a role for oxidative stress. Increased activation of stress related kinases, such as JNK and ERK1/2 were also found to be associated with the effect. The authors concluded that there are multiple signal transduction pathways contributing to the bystander effect, arising from both the plasma membrane and DNA damage.

In a study involving a novel approach using a double-mylar dish, Zhou et al. [2002] showed that post exposure of the cells on one side of the dish to α particles, the unirradiated cells on the other side of the dish showed increased level of cell death, however there was no increase in the mutagenic yield. When media only was irradiated, there was no change in the survival of the cells. This showed that for this system cytotoxic factors are released into the medium, and that gap junction communication is not the sole method of transmission of the factor, post exposure to α particles. In a similar study using the same double-mylar dish, Suzuki et al. [2004] showed that there was an increased number of chromaid aberrations in the cells on the side of the dish that were not irradiated. When medium only was irradiated there was no effect on the unirradiated cells. Therefore, it is clear that different mechanisms may be used for different types of damage expression, or end points of bystander effect.

Using an altered version of the mylar dish where the cells to be directly exposed were grown on a 6μm mylar, and the bystander cells grown on 38μm mylar striped insert, Zhou et al. [2005] showed that the cyclooxygenase–2 (COX–2) signaling cascade, which is integral to the inflammatory process, plays a part in the bystander effect. When COX–2 was suppressed, a significant reduction in the bystander effect was observed. When the mitogen–activated protein kinase (MAPK) pathways, extracellular related kinase (ERK), c–Jun N-terminal kinase (c–JNK) and p38 were suppressed in the cells, the bystander effect was also significantly reduced. The activation of these pathways are known to be essential to the activation of the COX–2 pathway, and so the authors concluded that this is further evidence that the COX–2 signaling cascade may be critical to the bystander effect.

Bystander Effects Induced By Low LET Radiation

As mentioned above Mothersill and Seymour [1997b] used a medium transfer technique to investigate the bystander effect of low LET radiation and found that the irradiated cell conditioned medium (ICCM) from epithelial cells reduced the survival of the exposed cells. To determine if cell contact was an important factor in producing this effect, Mothersill and Seymour [1998a] irradiated cultures of single cells, microcolonies and confluent monolayers. The results showed that the level of cell to cell contact was not a factor in the release of the bystander signal into the growth medium. Only cell density was an important factor. When the gap junction inhibitor, PMA, was included in the medium of the irradiated cells, bystander cell killing actually increased. This then suggests that for low–LET radiation, in this case γ rays, gap junction intercellular communication is not an important factor in the production or transmission of the signal.

Further to this investigation, Mothersill et al. [2000] used mutant cell lines to determine if energy metabolism and REDOX biochemistry was involved in the bystander effect. Medium from a glucose-6-phosphate dehydrogenase (G6PD) null cell line, E89, did not produce an effect in cells receiving this medium, however, when the G6DP gene was transfected back in, the cells receiving the medium showed a significant drop in survival. Apoptosis inhibitors and anti-oxidants L-lactate and L-deprenyl reduced or prevented the bystander effect. It was suggested that bystander signal production and response may be separate processes, as they could be independently modulated. The authors concluded that energy metabolism affects the ability of a cell line to produce a bystander signal, however, it is not a factor in the ability of a cell line to respond to this signal. Apoptosis was suggested to be crucial to bystander induced cell death, and that the balance of oxidative metabolism is key to this phenomenon. Using a similar experimental design, Mothersill et al. [2004] showed that repair deficient cell lines had a larger bystander effect in terms of cell kill when compared to repair proficient cell lines. It was suggested that this was a protective mechanism, as the increase in cell death would remove more of the damaged or incorrectly repaired cells from the population.

Using a tissue culture and reporter cell line system, Mothersill et al [2001, 2002b] found that exposure of explants to γ rays also produces a bystander factor. Irradiated tissue conditioned medium (ITCM) obtained from irradiated human and mouse urothlial specimens was used to treat cells that were never exposed to radiation. Some of the effects observed were cell death by both apoptosis and necrosis, reduced cloning efficiency and induction of proteins involved in cell death. Individual variation between samples was also observed when dealing with primary cultures from normal human urothelium. Gender, smoking status and an existing malignancy were shown to influence the production of a bystander signal from these samples.

Using the medium transfer approach post exposure to γ radiation, Lyng et al. [2000, 2001] showed that one of the first responses to the bystander signal was a transient increase in intracellular calcium. This occurred within 30 seconds of the exposure to the ICCM and the calcium levels returned to normal within 60 seconds. An increase in ROS was also noted from 1 to 24 hours post exposure. Six hours post exposure a decrease in the mitochondrial membrane potential was also observed. Both changes in the mitochondrial membrane potential and an increase in ROS have been associated with apoptosis, [Garland and Halestrap, 1997, Green and Reed, 1998] along with the activation of caspases, which are heavily involved in the apoptotic process. In a similar study, Lyng et al. [2002a,b] showed that the bystander signal released by the irradiated cells is also released by their progeny, without any decrease in the potency of the signal. Medium from cells up to 7 passages post irradiation induced transient calcium increase, ROS increase and mitochondrial membrane potential decrease in unexposed cells, suggesting that the bystander factor is also released by the progeny of the irradiated cells. Using a similar system Maguire et al. [2005] showed a dose dependent increase in the level of anti-apoptotic protein Bcl2 and an increase in mitochondrial mass post exposure to irradiated cell conditioned medium (ICCM) in human epithelial cells.

Lyng et al. [2006b] also showed that the mitogen–activated protein kinase (MAPK) pathways ERK and JNK had a role in the bystander signaling process using the medium

transfer protocol. Cells exposed to ICCM from γ irradiated cells had significantly increased levels of activated JNK and ERK proteins. Interestingly, when the JNK pathway was blocked in the bystander cells, there was a decrease in bystander related apoptosis, however when the ERK pathway was blocked, an increased bystander related apoptosis was observed. The authors concluded that further investigation of these pathways, and their influence on the bystander signaling process may lead to the identification of novel therapeutic targets.

In an investigation into the bystander responses seen in the human keratinocyte cell line, HPV-G, used by Mothersill and Seymour [1997b] and Lyng et al. [2001] described above, Lyng et al. [2006a] used the microbeam (see section 'Bystander Effects Induced By High LET Radiation') to determine if a similar bystander effect would be seen in the cells following exposure of a certain cells in the population to direct irradiation. Increased ROS and a decrease in mitochondrial membrane potential were observed 6 hours post exposure, as well as increases in apoptosis related proteins, bcl–2 and cytochrome–c, 24 hours following exposure. Increased apoptosis was also noted. Interestingly, these results were consistent with the response of HPV-G cells following exposure to ICCM derived from HPV-G cells exposed to γ irradiation [Lyng et al., 2000, 2001]. The authors concluded that this indicated there were very similar characteristics of the bystander effect evident in bystander populations that were and were not in physical contact with the directly irradiated cells.

In a study investigating the response of X–ray irradiated cells in terms of the cell surface and vesicles released into the medium, Albanese and Dainiak [2000] found that there was an increase in the expression of the 'death' ligand, TNFSP6, on both the plasma membrane and the surface of the vesicles released from the cells. However, when assayed for biological effectiveness the TNFSP6 harvested from the control cells induced the highest level of cell death, with the lower of the dose points (4Gy) inducing a significant but lower, reduction in survival. Despite having the highest level of this ligand, there was no increase in cell kill in cells exposed to TNFSP6 harvested from cells exposed to 10Gy.

The authors suggested that the increase in TNFSP6 post exposure to ionising radiation may be a mechanism of bystander and other abscopal effects.

A role for nitric oxide in the bystander effect was again shown in a study on wild type and mutated TP53 human gliobalstoma cell lines by Matsumoto et al. [2001]. They showed that nitric oxide may be one of the factors mediating the bystander effect, as bystander medium containing high levels of NO was shown to induce accumulation of TP53 and HSP72 in unirradiated cells. This then resulted in a increased radioresistance in the cells to the conditioned medium.

In similar experiments to those performed by Prise et al. [1998] using the charged particle microbeam, Schettino et al. [2003] used targeted ultrasoft X-rays to irradiate single cells in a population using microbeam technology. When all cells in the population were exposed, a linear dose-response was observed and low dose hypersensitivity was noted. However, when one cell was exposed, there was a 10% increase in cell killing. There was evidence of a dose-response relationship up to 200mGy, after which a plateau was reached. Interestingly, there appeared to be a localisation of the un–hit damaged cells around the hit cells, a result consistent with findings from Prise et al. [1998].

And so late in the 90's a picture of the bystander effect evolved, and the phenomenon was gradually accepted in the radiobiological community. The effect is seen following exposure to high or low–LET radiation and although the end points of the effect may be the same, the mechanisms seem to be different [Mothersill and Seymour, 2001]. The exact nature of the proposed 'bystander factor(s)' is also yet to be determined, although many candidate molecules have been suggested.

Mechanisms of Bystander Effects

The question of the transmission of the bystander effect is one of the most controversial issues yet to be fully resolved. Although many high LET studies display evidence that gap junction intercellular communication is essential [Azzam et al., 1998, Shao et al., 2003a, Hu et al., 2006] many of the low LET studies showed bystander effects in the absence

of GJIC, or indeed cell contact [Mothersill and Seymour, 1998a, Mothersill et al., 2000, Lyng et al., 2000, Schettino et al., 2003]. These authors suggest the release of 'factors' into the medium, which are then transferred to the unirradiated cells. However, there is some evidence of the involvement of medium borne factors in high LET induced bystander effect [Lehnert and Goodwin, 1997, Zhou et al., 2002, Suzuki et al., 2004]. It seems that there are numerous ways of bystander signal transmission, and the cell type, radiation type, and proximity of the unirradiated cells being monitored are mitigating factors in the conclusions drawn from the investigations completed at this time. Bystander effects have now been observed in cells neighbouring the hit cells, in those remote from the site of exposure, and in cells not present at the time of irradiation. It is interesting that one of the commonly used gap junction inhibitors, lindane, has also been found to affect other aspects of the cells treated. Changes in intracellular calcium and mitochondrial membrane potential, both involved in the bystander response [Lyng et al., 2000] are altered by the addition of lindane [Perocco et al., 1995]. As Mothersill and Seymour [2001] pointed out in a review of the field, closing gap junctions may also prevent the bystander factor secretion from the irradiated cells, thus providing a second explanation for the absence of the bystander effect in many systems with closed or absent GJIC. Therefore there may not need to be two separate mechanisms involved in the induction of the bystander effect. Although cells may well make use of GJIC for communication it may not be required for the transmission of the signal. Indeed, the two major modes of transmission of the bystander effect known at this time, signaling molecules and GJIC, may not be mutually exclusive, and it is likely that there are may up-stream events that are common to both mechanisms [Zhou et al., 2005, Hei, 2006].

Azzam et al. [1998] suggested a role for the cell cycle related proteins TP53 and CNKN1A in the bystander effect, as a significant increase in these proteins was noted in cells exposed to very low fluences of α particles. In contrast to this finding, Iyer and Lehnert [2000] found a decrease in the levels of both TP53 and CDKN1A in cells exposed to medium from α irradiated cells, which resulted in an increase in the growth of the

bystander cells. Iyer suggested that this contrast may be due to the difference in the protocol used in each investigation, and stated that an increase in TP53 and CDKN1A was also noted in cells exposed to low doses of α particles in their study, but dropped 24 hours following exposure. However, the use of GJIC was eliminated in the medium transfer study by the nature of the protocol itself, and so the two studies were monitoring two different populations of bystander cells. This then leads to the interesting possibility of the bystander signal eliciting different responses from cells, depending on the mode of transmission. However, it seems from both investigations that the level of TP53 and CDKN1A have a role to play in the bystander effect.

The question of whether the bystander effect is beneficial or harmful is another issue yet to be determined. Many of the endpoints examined thus far seem to be detrimental to the bystander cell, i.e. chromosomal abberations, increase in ROS, cell death, premature differentiation [Nagasawa and Little, 1992, Mothersill et al., 2001, Prise et al., 1998, Belyakov et al., 2002, Suzuki et al., 2004]. However, when these effects are considered at the level of the tissue as opposed to the cell, many are beneficial rather than harmful. If direct or bystander damage has occurred in cells within the tissue, then an increased level of bystander induced cell kill will remove more of the damaged cells from the population, resulting in a lower risk of mutation and carcinogenesis within the tissue. The same is true of premature differentiation [Belyakov et al., 2002] and the increase in the induction of apoptosis [Lyng et al., 2001, 2002b]. Although it has been shown that there is an increase in the level of the anti-apoptotic protein Bcl2 following exposure to the bystander signal, [Maguire et al., 2005], it is clear that there is a higher level of apoptosis in the exposed population [Mothersill et al., 2000, Suzuki et al., 2004], thus suggesting that the delicate balance of pro– and apoptotic proteins within the cell ultimately determines the fate of the cell.

Identity of the 'Bystander Factor'

In conjunction with the mode of transmission studies, there have been numerous attempts to determine the nature of the factor(s) involved in the bystander signal. In one of the earlier studies into the bystander effect, Mothersill and Seymour [1998a] investigated the nature of the signal by manipulation of the ICCM that was used to treat unirradiated bystander cells. Freezing the medium removed from irradiated cells at –20°C did not result in any reduction in the toxicity of the signal however, when the medium was heated to 70°C, there was a significant attenuation in the ability of the medium to reduce the plating efficiency of the unirradiated cells. The authors suggested that the signal is protein–like in nature, however, due to the speed at which the signal is released, *de–novo* protein synthesis was unlikely to be involved. In a similar study using the medium transfer protocol and α particle exposure, Lehnert and Goodwin [1997] also found that a signal in culture media capable of inducing a bystander response in unirradiated cells survived a freeze thaw cycle, and temperatures up to 56°C, however once heated to 93°C the response was abolished. These authors also suggested that the signal was proteinaceous in nature.

Many studies have suggested the involvement of ROS and NO in the transmission or production of the signal [Lehnert et al., 1997, Narayanan et al., 1997, Lyng et al., 2001, Shao et al., 2003a,b, Matsumoto et al., 2001, Azzam et al., 2002] along with the activation of NADPH–oxidase [Narayanan et al., 1997, Azzam et al., 2002]. It has been suggested that the increase in the short lived ROS results in the induction of secondary, longer lived radicals which may be involved in the bystander signal [Azzam et al., 2002, 2003]. In a study investigating the response to oxidative stress in smooth muscle cells, Li et al. [2001] showed that NAD(P)H- oxidase enzymes can be activated by hydrogen peroxide, resulting in increased ROS production, leading to increased NAD(P)H activation and thereby creating a feed–forward mechanism. This mechanism could also be applied to the bystander effect, as the exposure to direct irradiation has been shown extensively to increase ROS levels within the cells, and so the feed forward mechanism described by Li et al. [2001] could possibly be responsible for the bystander signal and the propagation of

ROS in the un-hit cells.

Using α particle exposure and the medium transfer protocol, Iyer and Lehnert [2000] found that increased concentration of TCF– β in the medium from irradiated cells resulted in a bystander effect in unirradiated cells. Inhibition of TCF– β abolished the response, suggesting a role for the protein in the bystander effect.

In their study on the cell surface and the vesicles released into the medium following direct irradiation, Albanese and Dainiak [2000] found an increase in the death ligand TNFSP6 on the surface of both the cells and the vesicles released. The authors suggest this ligand as a possible mediator of the bystander effect. In an investigation into the importance of MAPK and COX–2 pathway, Zhou et al. [2005] found that suppression of these pathways resulted in a significant reduction in the bystander effect of normal human lung fibroblasts. The authors suggested both that the COX–2 pathway, integral to the inflammatory process, and its up–stream activator MAPK, are essential to bystander signal generation. Interestingly, this is consistent with the findings of Lorimore et al. [2001], who suggested the inflammatory process as a possible mediator of the bystander effect, as a persistent increase in the number of macrophages was observed following whole body irradiation (discussed below in 'Bystander Effects in *in–vivo* models')

While there are many candidate molecules, the exact nature of the factor(s) has yet to be determined, although it is likely that there are numerous methods of signal generation and transmission, and therefore types of factor, and so it is unlikely that there is only one molecule responsible for the bystander effect. However, if the identity of the bystander factor were to be determined, many questions regarding the possible link between the bystander effect, genomic instability and the adaptive response could be more accurately addressed, and the mechanisms for these phenomena elucidated [Little, 2006].

While much useful and important information may be gained investigating the bystander effect in the *in-vitro* models described above, there is a great need to explore this phenomenon *in-vivo*. It seems on the one hand that the bystander effect is harmful by inducing chromosomal abberations and on the other, is protecting the tissue from these

mutations by increasing differentiation and cell death. Whether it is the bystander signal inducing these seemingly protective mechanisms, or they are simply a consequence of bystander induced damage is unknown. To further investigate this and determine the relevance of the bystander effect *in-vivo*, investigators have begun to design new experimental models that can reveal more about the nature of this phenomenon, some of which are discussed below.

Bystander Effects in *in-vivo* models

Using a multicellular model, Bishayee et al. [1999] used centrifugation to form three–dimensional clusters of Chinese hamster V79 cells, with various percentages of the population radiolabeled with various types of radiation. Evidence of a pronounced bystander effect was observed in the unlabeled cells. In a second study using the same model, Bishayee et al. [2000] used various types of radioactivity including ^{3}H and ^{131}I, β emitters, ^{125}I, an Auger electron emitter, and ^{210}P, an α particle emitter, incorporated into the clusters. Due to the short range of these particles, only the labeled cells were directly exposed, without cross irradiation of the bystander cells. The ability of the free radical scavenger DMSO to inhibit the bystander response in the clusters was examined. DMSO was effective in the reduction or abolition of the bystander effect in all cases except ^{210}P, possibly suggesting a different type of signal generation following α particle exposure. Continuing from this Bishayee et al. [2001] examined the bystander response in the cell clusters with various levels of ^{3}H labeled cells in the population. Using cell death as an endpoint to examine the response of the unlabeled cells, a significant bystander effect was observed when 50% or 10% of the cells in the clusters were labeled. Both the free radical scavenger DMSO and GJIC inhibitor lindane could protect the unlabeled cells, however, lindane had a greater protective effect. The two combined produced the maximum protective effect. Interestingly, the survival of the cells when 10% were radiolabeled was greater than that when 50% were labeled. It was suggested that this reflects the importance of the bystander effect in multicellular systems, where there are non-uniform

distributions of radioactivity incorporated into tissues. The authors suggested that ROS were important initiators of the bystander effect observed in this model which was likely mediated by GJIC.

A similar study by Howell [2002] showed a significant bystander effect in V79 cell clusters with certain fractions of the population with ^{125}I incorporated into the DNA. As with Bishayee et al. [2001], there was a significant difference between the survival of cells when 10% and 50% of the population were radiolabeled. However, both showed a significant bystander response, again showing the presence of the bystander effect in this model. The survival curves of both cases showed a two-component slope, beginning with a steep slope becoming a shallower one. It was suggested that the first component of the curve represents death in the labeled cells, with the latter part of the curve reflecting death within the bystander cells.

Persaud et al. [2005] also used the three-dimensional clusters to examine the bystander effect in Chinese hamster ovary (CHO) cells and the human–hamster hybrid A_L. The CHO cells were labeled with β emitter ^{3}H dTTP (tritiated thymidine) and clusters were formed with both CHO and A_L cells. After 24 hours incubation, the cells were isolated and examined for the presence of mutations. A significant increase in the level of mutation in the unlabeled A_L cells was observed. DMSO and lindane both inhibited the increase in mutation, suggesting a role for both ROS and GJIC, consistent with the findings of Bishayee et al. [2001]. It was also suggested in this paper that the bystander signal released from the labeled cells could induce the production of free radicals in the bystander cells, and subsequently induce a secondary bystander signal from these unlabeled cells, a mechanism also suggested by Belyakov et al. [2006]. Due to the nature of the damage observed in the bystander cells, the authors propose a bystander signal induced production of free radicals in the A_L cells, as the damage is consistent with that seen in labeled cells, although the free radicals induced in these cells would not be capable of traversing the distance to the unlabeled cells.

These studies of cell clusters, although not in actuality *in-vivo* studies, have more in

common with an *in-vivo* environment than those described in the sections above. Due to the three–dimensional structure of the clusters, there is greater cell to cell contact between the exposed and unexposed cells than in those studies completed using a two–dimensional cell layer, with much less dilution of any signals released by the exposed cells. The exposure time in the three–dimensional clusters is much longer than that in the *in-vitro* studies where the irradiated cells are usually exposed to a defined dose in less than one minute. This then more accurately reflects the types of response involved in environmental or occupational exposure. The model also provides information on the response of the surrounding tissue when radioactivity is incorporated into the tissue, as in the case of certain nuclear medicine imaging techniques such as positron emission tomography (PET).

Using whole body irradiation Lorimore et al. [2001] investigated the response of haemopoietic tissues in mice following exposure to γ rays. A significant increase in the number of activated macrophages and neutrophil infiltration was noted within the tissues. Enhanced superoxide generation, resulting in a respiratory burst, was also observed. It was found that the increase in macrophage activation was due to the presence of apoptotic cells in the tissue as a result of radiation exposure. However, this inflammatory-type response was unexpectedly sustained after the removal of these apoptotic cells. Interestingly, this response was found to be genotype dependent, as it was absent in p53 null mice. The authors concluded that the sustained activation of macrophages could explain bystander effects *in-vivo*, as these cells are known to release molecules such as superoxide and NO, capable of damaging DNA in neighbouring cells.

Using another type of *in-vivo* model Belyakov et al. [2005] combined the microbeam and an *in-vitro* three-dimensional normal human tissue system. This allowed the quantitative measurement of the effects in the unirradiated cells of the tissue to exposure of the mid-line of the tissue to α particles. Two types of 3-dimensional tissue were used, a reconstructed epidermis and a full-thickness skin. Both were irradiated at the mid-line, and slices of the tissue at specific distances from the center were examined for the presence

of apoptotic bodies and micronuclei. Increases in apoptotic bodies and micronuclei were found up to 1mm away from the site of exposure in both models. The authors suggests that this indicated a significant role of the bystander effect *in-vivo* as it was seen in this multicellular model, and that the bystander effect, particularly at low doses, needs to be taken into account in treatment planning and risk assessment.

Using the medium transfer technique along with the explant and reporter cell assay, Mothersill et al. [2005] demonstrated the presence of a bystander signal derived from bladder tissue harvested from mice following total body irradiation. Two types of mice were examined for bystander signal generation *in-vivo*, C57BL/6 and CBA/Ca. One hour post irradiation, the bladder was harvested and explants established. A clear bystander signal was generated from the C57BL/6 mice, as the ITCM generated from these bladder explants reduced the survival and mitochondrial membrane potential of HPV-G reporter cells. A transient calcium increase was also noted in the cells exposed to this medium, an effect known to be one of the earliest in the response to the bystander signal [Lyng et al., 2000]. However, the ITCM generated from the CBA/Ca mice did not induce any effect in the cells exposed, indicating that the generation of a bystander signal was influenced by genetic factors. CBA/Ca mice were found to have an increased level of Bcl2 expression, and ITCM from CBA/Ca mice induced a similar increase in explants from either mouse exposed to this medium. The authors concluded that the CBA/Ca mice had strong anti-apoptotic signals, and that this may be a reason for the absence of a bystander effect in these mice, however it was also suggested that the anti-apoptotic signal may itself be a different bystander effect generated from the CBA/Ca mice.

Using a novel approach to the investigation into the bystander effect *in-vivo* Xue et al. [2002] radiolabeled tumor cells and injected them into the hind legs of mice. Various levels of tumor cells with ^{125}I bound to DNA were injected with unlabeled or dead cells, and the growth of the tumor was monitored. A significant reduction in tumor size was recorded in those tumors with radiolabeled cells, indicating the presence of an *in-vivo* bystander effect. Due to the nature of the radioactivity, an Auger electron emitter, direct damage

could only occur in the radiolabeled cells, and so the reduction in the growth seen in the tumors was attributed to an *in-vivo* bystander effect in this system. The author suggested that Auger electron emitters such as ^{125}I may present an opportunity for exploitation of the bystander effect in therapy.

The 'abscopal effect' is a term used to describe effects seen at a site distant to that irradiated within the same organism [Mole, 1953]. To investigate this effect, Camphausen et al. [2003] irradiated non-tumor bearing legs of mice that had tumors at a distant site. Animals received 5 x 10Gy fractions or 12 x 2Gy fractions of irradiation, and the tumor volumes were compared to mice that received no treatment. In both irradiated groups there was a significant reduction in tumor volume when compared to the unirradiated mice, and at day 15, this was dose dependent. Interestingly when this effect was examined in p53 null mice, or mice treated with p53 blocker pifithrin-α no reduction in tumor volume was observed, indicting a role for p53 in the abscopal effect. Although the authors suggest this effect is distinct from the bystander effect, it is likely that there are similarities between the two. This study strongly suggests the release or up–regulation of certain factors or proteins at the site of exposure that result in reduced tumor cell growth at a distant site, therefore providing evidence for a signal generated by irradiated tissue that effects the survival of un-hit cells *in-vivo*.

Models of the Bystander Effect

In an attempt to create a model of the bystander effect induced by α particles, Brenner et al. [2001] used *in-vitro* data available and found that the effect seemed to be a binary 'all or nothing' response that was only relevant at low doses (< 0.2Gy), and that it may dominate over the direct response at very low doses. This is consistent with the findings of many studies that reported a bystander effect at low doses with no increase in the effect at higher doses, [Prise et al., 1998, Albanese and Dainiak, 2000, Belyakov et al., 2003]. This model incorporates both the bystander and direct (BaD) action of radiation, thus combining both types of exposure, making this model relevant to risk assessment.

Mothersill and Seymour [2003] proposed a speculative 'chaos' or 'bifurcation' model in order to describe the effects of low dose radiation and the bystander effect. In contrast to the target theory, where the model of radiation damage and progression to cancer was a simple one (dose →DNA break →mutation) this model allows for genetic and epigenetic factors to influence the outcome of a insult. As the amount of chaos in the system increases, so does the activity. i.e. following exposure to radiation or the bystander effect, the amount of activity within the cell increases sharply as the cell deals with this insult. This chaos is dissipated when the system reaches a 'bifurcation point' where the cell decides its outcome, i.e. live, die or mutate with the possibility of carcinogenesis. However, this is a multi–step process and there are many options that each cell could choose. Chaos theory predicts that the same cause will not always produce the same effect, therefore this model incorporated the many different responses that occur post exposure to low dose radiation. The authors conclude that due to the chaotic nature of the response to both low dose radiation and the bystander signal, accurate risk estimations of carcinogenesis at low doses are not possible.

Nijkoo and Khvostunov [2002, 2004] designed a bystander diffusion model, BSDM, to investigate the bystander effect, and the theory that it is transmitted via a protein 'factor' of low molecular weight. The model assumes that this factor spreads in culture medium by Brownian motion, and that this signal switches the cells into a state of cell death or induced oncogenic transformation. From the data generated by this model, the authors conclude that the bystander factor is a protein-like molecule, and that extrapolation from high to low dose exposures to determine risk may underestimate the probability of carcinogenesis. This model also predicts that the bystander effect is not only relevant at low doses, but is also present following high dose exposure.

Little et al. [2005] developed a stochastic model based on the data to date that accounts for many of the characteristics of the bystander effect seen in experimental systems. Importantly this model allows for spatial position and the effects of cell turnover, making it more relevant to an *in-vivo* system. Also incorporated into the model is the saturation

of the bystander response with increasing time after exposure, augmentation of the signal with fractionation of relativity large doses seen in some experimental systems [Mothersill and Seymour, 2002a], and decreasing slope with increasing dose rate in the high dose rate region of the dose response curve.

1.6.2 The Death Inducing Effect, DIE

Nagar et al. [2003a] used the medium transfer technique to test the effect of chromosomal instability on stable cells. The unstable clones were derived from a parent cell exposed to various doses of ionising radiation. No stable cells exposed to medium from the unstable clones survived, even after titration of the medium. When cells were exposed to medium from irradiated stable clones, as in the studies of Mothersill et al [1998a, 2000], no bystander effect was detected. The phenomenon was termed the death inducing effect, DIE, and the authors suggest it to be separate to the bystander effect.

In a follow up investigation, Nagar et al. [2003b] monitored cells exposed to medium from the unstable clones to determine the mechanisms of cell death involved in the DIE. It was shown that the cells exposed to this medium had a significant increased level of DNA double strand breaks, micronuclei, and apoptosis. The authors concluded that the DIE is a result of cytotoxic factor(s) secreted from the unstable cells, and that the presence of double stand breaks and micronuclei indicated a role for mitosis–linked cell death and apoptosis in the high levels of cell death associated with this phenomenon. In a similar study, Nagar et al. [2005] increased the amount of cells exposed to the medium from the unstable clones until some of these exposed cells survived. These cells were then clonally expanded and examined for chromosomal instability. Increased micronuclei and HPRT mutation frequency was observed in these clones. The authors concluded that the DIE is likely the result of secreted factor(s) in the medium that is lethal to the majority of cells exposed to it, and can induce delayed genomic instability in those that do survive.

1.6.3 Genomic Instability

Genomic instability is another non-targeted effect of radiation, and is defined as an elevated occurrence of genetic mutations in the progeny of irradiated cells. Some of the characteristics of genomic instability include delayed *de–novo* non-clonal chromosomal aberrations [Kadhim et al., 1992], induction of mutations in specific genes [Chang and Little, 1991], increased micronucleus frequency [Belyakov et al., 1999], and lethal mutations, or delayed cell death [Mothersill and Seymour, 1997a]. The type and dose of radiation have been found to affect the amount of instability, along with the genotype and cell type originally exposed, [Kadhim et al., 2004]. Although similar to the bystander effect in certain characteristics, genomic instability occurs not in cells present, or remotely connected to the exposed cell, but in the progeny of the cells that were previously thought to have survived irradiation and repaired completely, rendering them indistinguishable from cells never exposed to radiation. However, as many studies now show, the progeny of these cells incur damage in a non–clonal fashion that can lead to an unstable phenotype.

Evidence of genomic instability first appeared in the late 1980's when Seymour et al. [1986] found that progeny of irradiated cells had a consistently reduced plating efficiency when compared to controls. This phenomenon was termed 'lethal mutation' or 'delayed cell death' and has been to occur up to 400 population doublings post exposure [Mothersill and Seymour, 1997a]. There are many theories as to the mechanism of this increased cell death. These include a drop in the level of the anti–apoptotic protein bcl–2 a number of generations following exposure thus allowing cell death to occur via apoptosis, latent damage in the exposed cells that is transmitted to the progeny which can manifest as a lethal mutation in any subsequent generation, or a permanent change in the sensitivity of the population to mutation, thus increasing the chance of the cells suffering a lethal mutation. However, due to the nature and lethality of the mutations in the progeny, it is not possible that these lesions are transmitted from one generation to the next as the cell could not survive and divide carrying such damage [Mothersill and Seymour, 1998b].

Another early investigation into genomic instability was preformed by Kadhim et al.

[1992], who reported an increase in chromosomal instability in haemopoietic stem cells following α particle but not γ ray exposure. The abberations occurred at a high frequency in a non–clonal fashion in the progeny of the exposed cells. The authors suggest that lesions present as a result of α particle exposure could transmit the damage to the daughter cells, and that this unexpected instability may have implications for radiation risk assessment.

In a series of investigations into the progeny of irradiated cells, Chang and Little [1991, 1992a] examined the cloning efficiency and cell cycle of the progeny of X–irradiated CHO cells. A significant reduction of cloning efficiency and an increase in cell cycle time was observed, in cells up to 23 generations following irradiation. An increased level of giant cells was also noted in the colonies, indicating reproductive failure within the colony. This phenotype was found to be a dominant trait with hybrid clones displaying persistently depressed cloning efficiency. Chang and Little [1992b] also observed a two fold increase in the level of HPRT mutations in the progeny of X–irradiated cells, and suggested that this persistently elevated frequency of spontaneous mutations were a result of a mutator phenotype induced by irradiation, which lead to genetic instability, and delayed reproductive cell death.

To determine if oxidative stress was involved in the transmission of damage signals to the progeny of irradiated cells, Clutton et al. [1996] studied the levels of superoxide and intracellular oxidants in the progeny of bone marrow cultures exposed to low dose of neutrons. A persistent increase in intracellular oxidants was observed in these cells, indicating a role for oxidative stress in genomic instability. The possible generation of the aggressive hydroxyl radical with the involvement of lipid peroxidation in these cells resulted in levels of oxidants that the cell was unable to cope with, and so DNA damage was likely. The authors concluded that radiation induced oxy metabolism in the progeny of irradiated cells could account for the chromosomal instability observed in the progeny of irradiated cells.

In an investigation to determine if α particles could induce chromosomal instability

in–vivo, Watson et al. [1996] irradiated haemopoietic stem cells, and transplanted them into mice. An increased level of chromosomal instability was observed in the bone marrow, indicating that the instability was transmitted *in-vivo*. To determine if this was genotype dependent, this technique was employed using various types of mice, and a significant difference was found between 'sensitive' and 'resistant' strains of mice, although instability was observed in both. Interestingly, an increased level of superoxide was noted in the 'sensitive' strain when compared to the 'resistant' stain, again indicating a role for oxy metabolism in genomic instability *in-vivo* [Watson et al., 1997].

Examining the human keratinocyte cell line, HPV-G, for 45 population doublings post exposure to γ rays, O'Reilly et al. [1994] found that where was a persistent decrease in the survival of the progeny, which was found to be dependent on dose for the first two passages, but then became more independent. The decrease in survival was found to be approximately 15% at each cell division. In a similar investigation, Lyng et al. [1996] observed morphological changes suggestive of apoptosis in the progeny of both HPV-G and CHO-K1 cells up to 45 populations doubling following exposure to radiation. The authors concluded that this was indicative of a generalised instability in the population resulting in an altered phenotype, as if this change was clonal, the affected clones would die out.

Little et al. [1997] examined the progeny of CHO cells exposed to both X–rays and α particles for 23 population doubling following exposure. A significant amount of delayed mutations was found in the HPRT locus following both types of exposure, with no significant difference found between the two. Of the delayed mutations, the majority were similar to those that arise spontaneously, in contrast to those that occurred immediately after exposure.

In an investigation examining the effect of α particle irradiation on haemopoietic stem cells, Lorimore et al. [1998] irradiated cells with and without a grid that shielded the cells from irradiation. In the case of cell survival post irradiation, there was a level of survival consistent with that expected from the reduced number of cells hit by an α particle,

however when the level of chromosomal instability in the population was examined there was no difference between the shielded and unshielded cells. The authors describe this instability in the progeny of the unirradiated cells as an unexpected interaction between the irradiated and unirradiated cells, and suggest that the target for irradiation induced damage is greater than the nucleus of the hit cell. Although not highlighted in this paper, this study is one of the first linking genomic instability to the bystander effect, as the 'unexpected interaction' between irradiated and unirradiated cells is very similar to that proposed in many of the bystander effect investigations described above.

To determine if γ rays had the capacity to induce genomic instability, and compare it to that induced by α particles in a population of human fibroblasts, Belyakov et al. [1999] measured survival, micronucleus formation and apoptosis in the population for 30 days following exposure. While there were signs of genomic instability after both α particles and γ rays, a significant difference between the two was noted, with α particles inducing a higher level of all three endpoints measured.

In one of the first investigations performed to determine if there was a link between genomic instability and the bystander effect, Watson et al. [2000] used the transplantation of both neutron irradiated, and non-irradiated bone marrow stem cells into unirradiated mice, and monitored the instability in the population for 13 months. Due to the nature of the transplanted cells, and the population itself, it was possible to distinguish both the irradiated and non–irradiated transplanted cells, and be confident that the cells present in the bone marrow after 13 months were not those originally transplanted. A significant increase in the amount of chromosomal instability in the bone marrow was observed, in both cells that were progeny of the originally exposed cells, and those that were never irradiated. The authors concluded that this reflects two possible mechanisms for the propagation of instability in the system, a direct one from the irradiated cells to the progeny, and an indirect, or bystander mechanism from the irradiated cells, and their progeny to the unirradiated cells. This then is evidence of involvement of a bystander mechanism in the transmission of genomic instability.

Using the transplantation technique, Watson et al. [2001] also demonstrated that long term chromosomal instability can be induced by both low and high LET radiations *in-vivo*. A similar long–term instability was observed following whole body irradiation. The number and type of aberrations were recorded, and no correlation between cells expressing stable and unstable aberrations was found.

In a series of investigations, Limoli et al. [2001, 2003, 2003] found that cells incubated with free radical scavengers when irradiated had a reduced level of genomic instability, that cells exposed to chronic levels of free radicals display increased instability, and that there was an elevated level of free radicals in unstable cells derived from irradiated cells, along with an increase in dysfunctional mitochondria. These results indicate the importance of sustained increased levels of free radicals in the propagation of genomic instability with the populations, and suggest a role for mitochondria in the response.

Many investigations have at this point identified clear characteristics of genomic instability in various models both *in-vitro* and *in-vivo*. Whether genomic instability and the bystander effect are linked and have common mechanisms is not known or fully understood, however there has been much speculation that there are connections between the two [Lorimore and Wright, 2003, Morgan, 2003, Kadhim et al., 2004]. As pointed out in a recent review of the field, it may be that separate terms are needed for these effects until they are completely understood, at which point the non-targeted effects of radiation will be in the same category [Kadhim et al., 2004]. Many similar mechanisms have been proposed to explain the two phenomena, including the release of signaling molecules [Lorimore and Wright, 2003] and increases in oxidative metabolism [Clutton et al., 1996, Watson et al., 1997, Limoli et al., 2003]. The importance and contribution of epigenetic factors such as methylation, oxy metabolism and phosphorylation in the propagation of an instable phenotype has been suggested in many investigations into genomic instability, some of which are also associated with cancer [Kadhim et al., 2004]. However, as with the bystander effect, the significance of genomic instability in radioprotection and risk assessment is not yet known, but with the development of new techniques, and new models

of non-targeted phenomena emerging, a greater understanding of genomic instability and its contribution to carcinogenesis can be elucidated.

1.6.4 Adaptive Response, Low Dose Hypersensitivity and Increased Radioresistance

Olivieri et al. [1984] first discovered the adaptive response in human lymphocytes that were incubated with ^{3}H prior to exposure to a higher dose of radiation. The level of abberations in these cells was significantly less than that observed in cells that were not incubated with thymidine before irradiation. The author compared this to the adaptive response seen following exposure to alkylating agents, where treatment with a low dose over a long time period reduces the damage caused subsequently by a higher dose. In the many investigations into the adaptive response since this discovery, the phenomenon has been observed several models and a number of characteristics have been elucidated, including possible mechanisms [Kadhim et al., 1992, Bonner, 2003] and the the significance of the adaptive response with respect to the LNT model [Ballarini and Ottolenghi, 2002]. Some of the characteristics seen in populations displaying an adaptive response following a low priming dose and subsequent challenging dose include increased cloning efficiency [Smith and Raaphorst, 2003], decreased mutation frequency [Zhou et al., 2003] and decreased levels of micronuclei [Broome et al., 2002]. In the course of these investigations, another phenomenon, low dose hypersensitivity/increased radioresistance, (HRS/IRR) was also discovered. This refers to the increased sensitivity of certain populations of cells to very low doses of radiation, followed by increased resistance to radiation up to a threshold, at which point the survival returns to the expected levels. There is much speculation that there are similar mechanisms involved in these two phenomena, with a significant roles for the increased efficiency and amount of DSB repair implicated in both adaptive response and HRS/IRR [Joiner et al., 1996, 2001, Bonner, 2004].

To establish if there was an adaptive response associated with the bystander effect, many recent studies have monitored the two phenomena in the same system or used the bystander signal as a priming dose to determine if an adaptive response was induced. Using α particle exposure and the medium transfer protocol described above (see 'Bystander Effects Induced By High LET Radiation'), Iyer and Lehnert [2002a] showed a

significant adaptive response induced by exposure to a bystander signal. When cells were incubated with medium from 1 cGy irradiated cells and subsequently exposed to a direct dose of 10 or 19 cGy, there was a significant increase in cell survival when compared to that of cells that had not been incubated with the medium from the irradiated cells. An increase in the DNA base excision repair enzyme AP–endonuclease, was also noted in the cells incubated with the conditioned medium. The authors concluded that the factor(s) present in the medium as a result of irradiation induced a significant adaptive response in the unirradiated cells prior to exposure to a challenging dose. In a similar investigation, Iyer and Lehnert [2002b] also showed a low–LET induced adaptive response in normal human lung fibroblasts, following incubation with medium from γ irradiated cells prior to a challenging dose. As with the high–LET investigation, increased survival and levels of AP–endonuclease was noted in the cells that were incubated with the conditioned medium, suggesting that low–LET irradiation can also produce factor(s) that induce radioadaptation in unexposed cells prior to direct irradiation.

Zhou et al. [2003] found that pre–treating A_L cells with X rays 4 hours before exposing 10% of the population to α particles significantly decreased a bystander effect seen in populations not previously exposed to X rays. When the level of mutations in the population was assessed, there were significantly lower mutations in the pretreated cells when compared with the untreated cells. However, when the mutations in each population were examined, it was found that there was a significantly higher level of complex mutations in those cells that received both X rays and α particles. Interestingly, when the types of radiation were reversed, i.e. cells were pretreated with α particles to 10% of the populations, and subsequently exposed to X rays, the opposite effect occurred. In these cells there was an increase in the level of mutation when compared to the cells that were directly exposed. The authors suggested that an inducible protein may be triggered at low doses, that resulted in the increased resistance to a subsequent exposure to a higher dose, although it was only activated by pretreatment with X rays, and not α particles, or the bystander signal produced by α particle exposure.

To determine if there was a connection between the bystander effect and HRS/IRR, Mothersill et al. [2002] examined both phenomena in 13 different cell lines. A weak inverse relationship was found between the two, cell lines that produced a strong bystander signal did not display HRS/IRR, and *vice versa*. The authors suggested that this may be related to the malignancy status of the cells, and that these cells are unable to communicate damage signals effectively.

As with the other non-targeted effects described above, adaptive response and HRS/IRR are still not fully understood, and so there is no clear picture of their contribution to the response to radiation, although many studies point to the importance of these effects at low doses. However, as investigations into these non–targeted effects continue, the links between bystander effects, genomic instability, adaptive response and HRS/IRR can be elucidated, and their contribution to risk assessment and carcinogenesis understood.

1.7 Aims

Due to the various levels of exposure and response seen throughout the literature discussed above, there is clearly a need to examine the effect of radiation both at lower and higher levels of complexity. In this thesis an attempt is made to do this by examining the response of cell lines, individual tissues exposed *in-vitro*, and individual tissues following whole body irradiation. Thus a comparison can be made between the response of the single cell and that of the whole tissue. Depending the level of complexity in the experimental model used, the effects seen can be viewed as positive or negative. As mentioned in the chaos theory proposed by Mothersill and Seymour [2003], what seems like a detrimental effect at the cellular level, i.e. cell death, may be seen as a protective mechanism at the tissue level. A greater understanding of the effects seen in the *in-vitro* cell and tissue culture models in use today and most importantly their relevance to the *in-vivo* response is essential if the area of low dose radiation exposure is to be fully understood, and the risks associated with low dose exposure determined. However, as is proposed in much of the current literature, there is great complexity in the response of various cells or tissue to radiation, and so a simple solution to the problem of risk estimates and the response predicted at low doses is unlikely.

Early in this investigation, a simple model of the bystander effect is used to determine the relevant importance of the bystander signal versus the response seen in the bystander cells. Using the medium transfer method and a matrix design experiment, various cell lines were exposed to ICCM generated from different sources, thus allowing a comparison of the response to each bystander signal. The study then expands to include primary tissue culture in conjunction with cell culture, in order to determine if there is a difference between the bystander signal generated from different tissues, the bladder and oesophagus. The response of the cytoskeleton and the status of differentiation with the primary tissue culture are also investigated following both direct γ ray exposure and incubation with bystander medium (ITCM).

The final section of this study is devoted to the effect of a specially formulated anti-

oxidant diet, originally designed to ameliorate the effects of cognitive decline in transgenic mice, on bystander signal generation following both *in-vitro* and *in-vivo* irradiation. In the first part of this study, bladder and oesophagus were harvested from mice that have been on the diet since birth, γ irradiated and the bystander signal compared to that generated by normal tissue using the reporter system described above [Mothersill et al., 2001]. In the second part of the study, the mice were whole body irradiated, and the bladder and oesophagus removed one hour following exposure. The tissues were then processed for primary culture, and the culture medium harvested and used to treat the reporter cell line. Thus, the bystander signal generated *in-vivo*, can be monitored *in-vitro*, and compared to the results from the *in-vitro* investigation, allowing a comparison of the two and so determining if results seen *in-vitro* are reflected in an *in-vivo* environment.

Chapter 2

Bystander Signal versus Response

2.1 Introduction

The bystander effect is now a well established phenomenon, and has been extensively characterised in numerous cell lines and tissues [Prise et al., 2003, Morgan, 2003, Coates et al., 2004, Prise et al., 2005, Little, 2006, Mothersill and Seymour, 2006]. However, although many candidate bystander factors and modes of action have been implicated in the literature, the exact mechanism of this signalling process is not yet known. Some studies have cited the importance of gap junction intercellular communication (GJIC) in the transmission of the bystander signal [Azzam et al., 2001, 2003, Hu et al., 2006, Shao et al., 2003a], while many others display evidence of the bystander effect in the absence of GJIC, or indeed cell contact [Lehnert et al., 1997, Mothersill and Seymour, 1998a, Mothersill et al., 2000, Lyng et al., 2000, Schettino et al., 2003, Prise et al., 1998]. In these investigations, the authors suggest the release of medium borne factors into the culture medium, and thus the transmission of the bystander signal to the unexposed cells. The nature of the "factor(s)" is as yet unknown, however many candidates have been proposed to be involved, such as ROS [Shao et al., 2003a, Lyng et al., 2000, Schettino et al., 2003, Narayanan et al., 1997], NADPH-oxidase [Narayanan et al., 1997, Azzam et al., 2002] and nitric oxide, [Matsumoto et al., 2001, Shao et al., 2003b]. In order to

learn more about the nature of the signal and its mode of action, cell lines with well established and different bystander responses were used in this study to investigate the relative importance of the signal produced by the irradiated cells versus the response in the bystander cells. It is important to establish whether it is the signal or the response that determines the ultimate magnitude of the bystander effect in the affected cells, as this will help elucidate possible mechanisms and points of action of bystander factors. If it is the signal itself that determines the effect, then all cells capable of showing a response will display the same magnitude of response. However, if an individual response of each cell type determines the final effect, then various cell lines will respond in different ways to a bystander factor produced from the same source.

To investigate this, a matrix style experiment was designed with three different cell lines. The cell lines used in this study were HPV-G, CHO-K1, and one of its daughter cell lines E89. The HPV-G line was chosen as it has an established bystander response, and has been used as a reporter system in previous studies to test irradiated cell conditioned medium (ICCM) from other cell lines [Mothersill et al., 2000, Lyng et al., 2000]. It shows approximately 40% reduction in cell survival post exposure to autologous ICCM [Mothersill et al., 2000]. CHO-K1 is a Chinese hamster ovary cell line, and has a much higher doubling time than the HPV-G line, and so is useful as a second reporter system in the matrix experiment. It has a significantly different response to autologous ICCM (approximately 15% reduction in survival, [Mothersill et al., 2004]), therefore making it useful to compare the effect of CHO-K1 ICCM on cell survival to that of HPV-G ICCM. The E89 cell line is derived from CHO-K1 and is glucose 6-phosphate dehydrogenase (G6PD) null [Stamato et al., 1989]. Due to the crucial involvement of G6PD in the mitochondrially located hexose monophosphate pathway (HMP shunt, also known as the pentose phosphate pathway), this pathway is compromised in the E89 cell line. The HMP pathway reduces NADP+ to NADPH, providing the cell with a reducing agent, which is required in the biosynthesis of numerous molecules including fatty acids and nucleotides, and oxidises glucose-6-phosphate to ribulose 5-phosphate, a sugar which is a

component of RNA, DNA and ATP. Therefore, if NAD(P)H is involved in the production of, or response to the bystander effect, interference with this pathway should alter the production of the bystander factor. Our group has previously shown that E89 cells have no significant response to the bystander factor, but when the DNA sequence coding for the G6PD gene is reintroduced, the cells show a bystander effect [Mothersill et al., 2000]. It was suggested that the HMP pathway (and the production of NAD(P)H) is an important step in the production of and response to the bystander effect and that the production of the bystander signal is a separate process to the response of the cells exposed to that signal.

Numerous other studies have suggested a role for NAD(P)H, and NAD(P)H-oxidase in the bystander effect, [Azzam et al., 2003, Narayanan et al., 1997, Azzam et al., 2002]. Narayanan et al. [1997] showed an increase in both superoxide and hydrogen peroxide post exposure to alpha particles. A role for NAD(P)H-oxidase was suggested, as the response was inhibitable by diphenylene iodonium, a selective inhibitor of NAD(P)H-oxidase. Cells incubated with medium from irradiated cultures in that study also displayed an increase in ROS, indicating a role for free radicals and NAD(P)H-oxidase in the transmission of the bystander effect. Azzam et al. [2002] found increased levels of reactive oxygen species, (ROS), an effect commonly associated with the bystander signal, [Lyng et al., 2000, Narayanan et al., 1997]. The authors suggested that the initial production of short lived radicals post exposure to direct radiation or the bystander signal, leads to the production of secondary longer lived radicals, possibly involving the activation of NAD(P)H-oxidase [Azzam et al., 2003, 2002]. Li et al. [2001] have shown that ROS such as hydrogen peroxide can activate NAD(P)H-oxidase, leading to the generation of more ROS. Taken together these papers suggested that NAD(P)H-oxidase may have a significant role in the production of the bystander signal, and so a cell lacking the ability to produce NAD(P)H may have weaker bystander signal production, as seen previously in the case of the E89 cell line [Mothersill et al., 2000].

2.2 Methods and Materials

2.2.1 Cell Culture

Cell lines used in this study were HPV-G, CHO-K1, and E89. The HPV-G cell line is a human keratinocyte line, which has been immortalised by transfection with the HPV virus. The cell line was originally a kind gift from Dr. J. Di Paolo, NIH, Bethesda, MD [Pirisi et al., 1988]. The E6–protein of the HPV virus eliminates approximately 70% of the p53 function. They grow in culture to form a monolayer, display contact inhibition and gap junction intracellular communication. They are maintained in Dulbecco's MEM: F12 (1:1), containing 10% fetal calf serum (Gibco), 5000 IU/ml penicillin streptomycin solution (Gibco), 2mM L-glutamine (Gibco), 0.025 mol/L HEPES buffer (Gibco) and 1μg/ml hydrocortisone (Sigma). CHO-K1 is a long established hamster cell line, and is normally maintained in Ham's Nutrient Mixture (F12), 5000 IU/ml penicillin streptomycin solution (Gibco), 2mM L-glutamine (Gibco), 0.025 mol/L HEPES buffer (Gibco) and 1μg/ml hydrocortisone (Sigma). The E89 cell line is a line derived from the CHO-K1 line, and is maintained in the same medium. For the purposes of this investigation, both the CHO-K1 and E89 lines were adapted to Dulbecco's MEM: F12 (1:1), so that medium transfer could be performed between these and the HPV-G cell line. The plating effiencies for each cell line in both media is shown in table 2.1. Supplements for media were all obtained from Gibco Biocult Ltd. Irvine, Scotland and Sigma, Dorset, UK. All lines were maintained in an incubator at 37°C in an atmosphere of 5% CO_2. Subculture was routinely performed using a 1:1 solution of trypsin (0.2%) and Versene (0.02%).

2.2.2 Irradiation

Cells were irradiated in T-25 (NUNC, Uden, Denmark) containing 5ml culture medium at room temperature using a cobalt-60 teletherapy source at St. Luke's Hospital, Dublin, at a flask to source distance of 80cm. The dose rate during the experiment was approximately 1.8Gy/min. Cells were irradiated at 0.5 and 5Gy. Control flasks were sham irradiated.

Cell Line	Media	
	Hams F12	DMEM
CHO-K1	70.88 ± 1.95	71.00 ± 1.24
E89	74.77 ± 1.48	45.48 ± 1.87

Table 2.1: A comparison of CHO-K1 and E89 cell lines plating efficiencies (± SEM) in Ham's Nutrient Mixture (F12) and Dulbeccos MEM: F12.

Post exposure, all flasks were returned to the incubator.

2.2.3 Harvesting of ICCM

Subconfluent flasks that had a medium change the pervious day were chosen. Donor flasks were set up with 5 x 10^5 cells per flask, in 5ml culture medium, 6 hours before irradiation. 1 hour post exposure, the medium was removed for the donor flasks, and filter sterilised with a 0.22μm Nalgene filter to ensure that no cells remained in the medium.

2.2.4 Media exchange experiments

Using the clonogenic assay technique of Puck and Marcus [Puck and Marcus, 1956], cells were plated for treatment with ICCM. Recipient flasks were set up with 100 cells (CHO-K1 and E89 cell lines) or 400 cells (HPV-G cell line) per flask, in 5mls of culture medium. All flasks used were T-25 (NUNC), and were incubated at 37°C, in 5% CO_2 in air. HPV-G, CHO-K1 and E89 cell lines were all used to generate ICCM, and were treated with each type of ICCM, including autologous medium in this matrix design experiment. 6 hours post plating the original culture medium was removed from the recipient flasks, and replaced with ICCM. Cells were then allowed grow to until colony formation (14 days for HPV-G, 7 for CHO-K1/E89) at which point they were stained with carbol fuschin (BDH, Poole, UK) and the cell survival determined.

2.2.5 Statistical Analysis

Each cell line was treated with each type of ICCM at least 3 times in independent experiments, with 3 replicate flasks per experiment. One way ANOVA or students t-test was used where appropriate to determine the significance in each case, with n=3 in all cases.

2.3 Results

2.3.1 Direct Irradiation

The survival of all cell lines following direct irradiation is shown in figure 2.1, and the actual plating efficiencies are shown in table 2.2. In the HPV-G cell line, there is a 28.3% and 76.16% decrease in survival post exposure to 0.5Gy and 5Gy respectively. CHO-K1 displayed a similar response with a 30.34% and 75.92% reduction in survival at the same doses; while the E89 line showed a 19.18% and 74.08% decrease in survival post exposure to 0.5 and 5Gy respectively.

Cell Line	Dose		
	0Gy	0.5Gy	5Gy
HPV-G	17.55 ± 0.46	12.41 ± 0.45	4.09 ± 0.31
CHO-K1	70.88 ± 1.95	48.66 ± 1.64	16.88 ± 0.93
E89	74.77 ± 1.48	59.88 ± 1.45	19.44 ± 0.68

Table 2.2: Plating Efficiency for all cell lines post exposure to direct radiation.

Both the CHO-K1 and E89 lines were adapted from Ham's Nutrient Mixture (F12) to Dulbecco's MEM: F12 (1:1), to allow for medium transfer between these lines and the HPV-G cell line. The plating efficiency of theses lines in both types of media is shown in table 2.1. No change in the plating efficiency of CHO-K1 was observed, although a reduction in the plating efficiency of the E89 cell line was noted. However, as this was consistent throughout the investigation, the cell line was included in the study.

2.3.2 Bystander Signal vs. Response

In figures 2.2 - 2.4 the response of cell lines to all types of ICCM generated in this study is shown. Table 2.3 shows the actual plating efficiencies for each set of data. Figure 2.2 shows the response of the cell lines to HPV-G ICCM. Both HPV-G and CHO-K1 cell lines have a very similar response to this medium at 5Gy ICCM (34.05% $\pm$ 3.83 and 35.96% $\pm$ 1.24 reduction in HPV-G and CHO-K1 cell survival respectively). No change in E89 survival was observed post exposure to this ICCM.

In figure 2.3, the response of cell lines to CHO-K1 ICCM is shown. The HPV-G cell line has a significantly different response to this medium compared to HPV-G ICCM, (18.26% $\pm$ 2.74 reduction in survival). The CHO-K1 cell line also responds differently, and shows a 15.99% $\pm$ 2.36 decrease in survival. The E89 cell line again shows no significant response to this ICCM.

The response to E89 ICCM is shown in figure 2.4. No change in survival was observed in any cell line post exposure to this ICCM.

The data in table 2.3 shows the survival fraction of each cell line post treatment with each type of ICCM generated in the study.

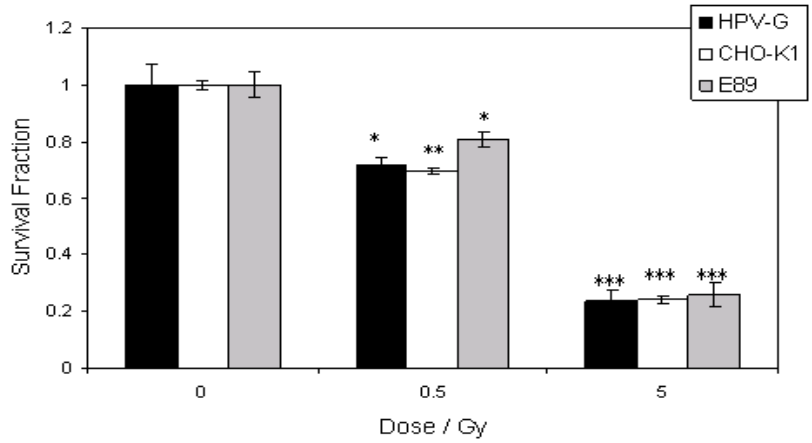

Figure 2.1: Survival of all cell lines post exposure to direct radiation. ($*$ p < 0.05, $**$ p < 0.001, $***$ p < 0.0001)

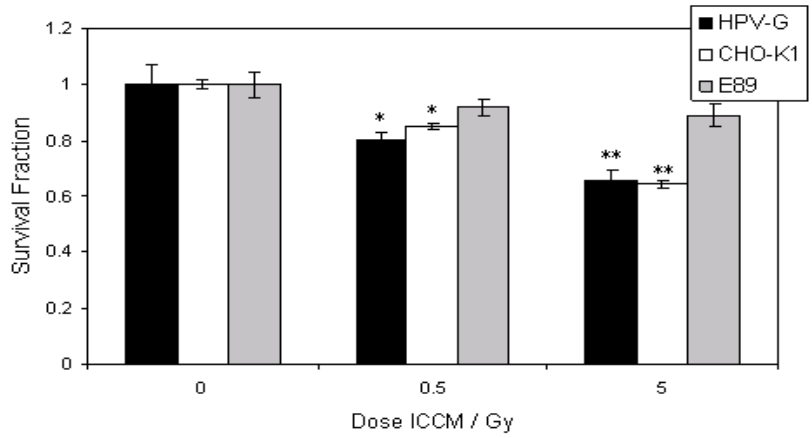

Figure 2.2: Clonogenic survival of cell lines post exposure to ICCM generated from HPV-G cells. ($*$ p < 0.05, $**$ p < 0.001)

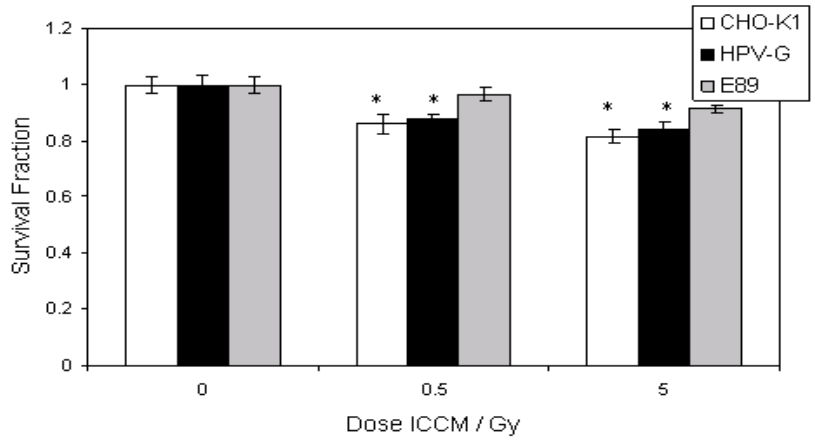

Figure 2.3: Clonogenic survival of cell lines post exposure to ICCM generated from CH0-K1 cells. ($*$ p < 0.05)

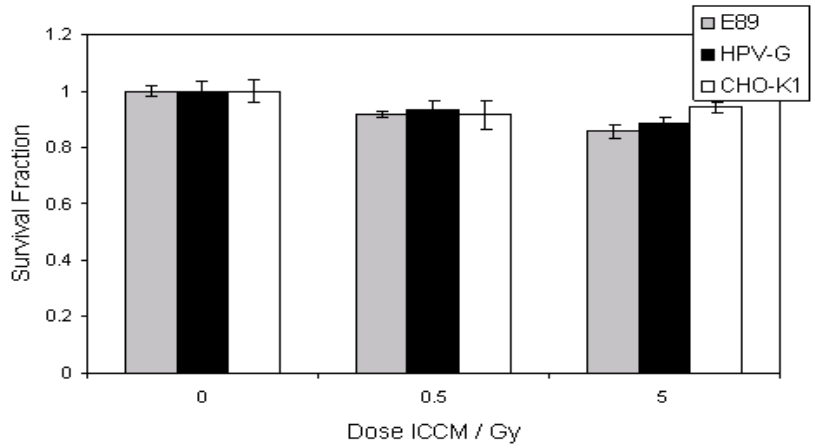

Figure 2.4: Clonogenic survival of cell lines post exposure to ICCM generated from E89 cells.

Cell Line (donor-recipient)	Dose ICCM 0Gy	0.5Gy	5Gy
HPV-G - HPV-G	17.86 ± 1.71	14.11 ± 0.56	11.77 ± 0.74
HPV-G - CHO-K1	71.00 ± 0.63	61.33 ± 0.30	45.22 ± 0.49 †
HPV-G - E89	45.22 ± 1.87	41.59 ± 1.22	40.92 ± 2.13
CHO-K1 - CHO-K1	68.89 ± 1.99	60.56 ± 2.43	57.89 ± 1.57
CHO-K1 - HPV-G	17.19 ± 1.20	14.96 ± 2.03	14.02 ± 0.85 †
CHO-K1 - E89	72.68 ± 1.73	69.92 ± 1.53	66.50 ± 0.86
E89 - E89	75.11 ± 1.34	69.22 ± 0.81	64.66 ± 1.85
E89 - HPV-G	15.92 ± 0.47	14.92 ± 0.61	14.34 ± 0.34 †
E89 - CHO-K1	72.66 ± 2.93	67.77 ± 3.01	69.16 ± 1.45 †

Table 2.3: Plating Efficiency for ICCM treatment of all cell lines. † indicates that the response is significantly different from that of autologous ICCM treatment.

2.4 Discussion

It has been suggested that bystander signal production is a separate process to that of the subsequent response seen in the exposed cells [Mothersill et al., 2000]. To investigate this hypothesis, the present study was designed to separate the bystander signal from response. By testing individual cell lines with ICCM generated from various lines and comparing the results, the relative importance of the signal versus the response was determined. The cell lines tested in this study responded differently to ICCM generated from different sources, suggesting that that bystander signal production and response are indeed separate processes, and that in the case of HPV-G and CHO-K1 cell lines it is the signal that determines the toxicity, and hence the magnitude of the response in the exposed cells. Post exposure to autologous 5Gy ICCM, HPV-G cells showed a 34.05% reduction in survival, however, when treated with CHO-K1 5Gy ICCM, the reduction in survival was only 18.26%. The ICCM generated from E89 cells had no significant effect on the survival of

HPV-G cells, again underlining the different types of response in one cell line, depending on the source of the ICCM. CHO-K1 cells showed a 15.99% reduction in cell survival when treated with autologous 5Gy ICCM, but showed a 35.96% reduction in survival when treated with HPV-G 5Gy ICCM. Similar to HPV-G cells, when treated with E89 ICCM they maintained survival at a control level. The E89 cell line showed no significant difference in survival post exposure to any ICCM generated in this study. However, there was a significantly reduced plating efficiency in the control E89 cells exposed to 0Gy HPV-G ICCM when compared to those exposed to autologous ICCM, indicating that the HPV-G 0GY ICCM is in some way cytotoxic to the E89 cells. However, this is not seen in the parent cell line, CHO-K1, possibly suggesting that lack of G6DP causes the E89 cells to be more sensitive to the HPV-G ICCM. Similar results were obtained for 0.5Gy ICCM in all cases.

From these results it is clear that there are different responses to ICCM from different sources. Each cell line appears to generate its own characteristic bystander signal. ICCM generated from HPV-G cells at 5Gy caused a greater than 30% reduction in survival in HPV-G and CHO-K1 cells. E89 cells showed no response, however, due to the compromised HMP pathway in this cell line, this result is not surprising. Exposure to CHO-K1 ICCM resulted in approximately a 15% reduction in survival in exposed cells, with again the exception of the E89 line, where there is no response. E89 ICCM failed to generate a bystander effect, with no significant decrease in survival in either HPV-G or CHO-K1 cells. These data are consistent with previous findings by Mothersill et al. [2004], where the extent of the bystander effect generated from certain cell lines was determined by the cell line originally irradiated. Also, in the present study, the response to the bystander effect saturated at a relatively low dose, i.e. there was no significant increase in cell death with increased dose, a common characteristic of the bystander effect. This was consistent across all cell lines tested, and so provides further evidence that it is the bystander signal that determines the response, as the response seen in each cell line depends on the type of ICCM it is exposed to, irrespective of the dose.

In a similar study investigating the bystander effect, and the ability of different cell lines to cope with the signal, Kashino et al. [2004] found that different cell lines responded differently to the bystander signal generated from the same cell line (CHO-K1). However, a crucial difference between the findings of this study and those presented here is the nature of the cell lines being investigated. The cell lines tested in that investigation were repair deficient, and so the varying responses to the same bystander signal can be attributed to the fact that some cell lines were able to repair the damage, while others were not. In the present study, all the cell lines exposed were equally capable of repair, and so different responses to different types of ICCM seems to reflect the varying toxicity of the bystander signal generated from different sources.

It has previously been suggested that the bystander signal generation is energy dependent, [Mothersill et al., 2000]. It is interesting that the E89 cell line, a line developed from the CHO-K1 line [Mothersill et al., 2004], produces no bystander signal. Considering that this cell line is G6PD null, the data suggest that bystander signal production is dependent on the HMP pathway. Absence of G6DP compromises this pathway, and so the absence of the bystander signal from this line could be linked to the reduced ability of the cell to produce NAD(P)H. The balance of NAD/NAD(P)H within the cell is critical in driving metabolism, and NAD(P)H-oxidase has been implicated in numerous studies examining bystander signal production [Azzam et al., 2003, Narayanan et al., 1997, Azzam et al., 2003].

The variation of the bystander signal production between the cell lines was also reflected in the direct irradiation data. At the higher dose of 5Gy, all the cell lines showed very similar responses, however at the lower dose of 0.5Gy, there were significant differences between the cell lines. The HPV-G cells and CHO-K1 cells which show significant bystander effects, showed a similar reduction in survival post exposure to 0.5Gy direct radiation (28.30% and 30.34% respectively). However, E89 cells, which showed little or no bystander effect, show a much greater survival at 0.5Gy direct radiation, (19.18% reduction in survival). This suggests, as many other studies have [Schettino et al., 2003,

Seymour and Mothersill, 2000] that the bystander effect is dominant at low doses, and may contribute to a greater reduction in the survival in the cell lines that produce a bystander signal.

Due to the varying response of each cell line to ICCM generated from different sources, it appears to be the signal produced by the irradiated cell line, and not the individual response of the treated cell line, that determines the overall bystander effect in cell lines capable of responding to the signal. However, in those lines that are not capable of response, or that respond to a different type of signal, exposure to the bystander signal has no effect.

Chapter 3

Bystander Effect in Epithelial Tissue

3.1 Introduction

While important information can be learned about the bystander effect from cell lines, it is imperative that multicellular systems are also examined so that the relevance of the bystander effect can be determined *in-vivo*. Study of various tissues has revealed much information about how bystander signal generation differs in a multicellular environment where cells are at various stages of the cell cycle. Numerous models have been used to study this issue, both *in-vitro* and *in-vivo*. Using a primary tissue culture model and a reporter cell assay, Mothersill et al. [2001, 2002b, 2005] have shown bystander effect generation by both human and murine bladder samples following *in-vitro* and *in-vivo* irradiation. Unirradiated cells exposed to medium from explants irradiated *in-vivo*, or *ex-vivo* were shown to have significantly lower survival to those that were exposed to medium from unirradiated explants. In the case of the human cultures, significant variation was observed between samples, and the bystander effect was found to be influenced by gender, smoking status, and the presence of a pre-existing malignancy in the bladder tissue. Watson et al. [1996] found evidence of a bystander effect *in-vivo* when irradiated hemopoietic stem cells were transplanted into a mouse. An increase in chromosomal aberrations in the progeny of unirradiated stem cells was observed, suggesting a link between the bystander

effect and genomic instability *in-vivo*.

Lorimore et al. [2001] investigated the response of haemopoietic tissues in mice exposed to whole body gamma radiation. A significant increase in macrophage activity was noted in these tissues, a response which was sustained after the removal of all apoptotic bodies by these cells. The authors suggest that this inflammatory response may provide a mechanistic pathway for bystander signal transmission *in-vivo*, as activated macrophages are known to release molecules such as superoxide and NO, capable of damaging DNA in neighbouring cells.

Belyakov et al. [2002, 2006] found a bystander-induced increase in apoptosis and terminal differentiation using a primary tissue culture model and suggested a protective mechanism for the bystander effect in multicellular systems. Belyakov et al. [2003] also found evidence of a proliferation-dependent bystander effect in a primary tissue model using porcine and human urothelial explants. It was observed that irradiation of a very small number of single cells at the periphery of the explant culture, where the cells were actively dividing, produced a potent bystander signal. However, when the fully differentiated cells at the centre of the culture were irradiated there was no significant difference between the irradiated samples and the controls. Using an *in-vitro* 3D tissue culture model, Belyakov et al. [2005] also showed evidence of bystander effects, with increased apoptotic bodies and micronuclei up to 1mm away from the site of exposure.

The tissues being investigated in this study are the bladder and oesophagus of male Wistar rats, using the primary tissue culture model and reporter cell assay described in Mothersill et al. [2001] above. The microstructure of both these tissues are quite similar. Both consist of epithelium, adapted to the specific function of the tissue. The oesophagus is smooth walled, with thick protective stratified squamous epithelium. Stratified squamous epithelium has a variable number of layers, with a cuboidal basal layer, which becomes progressively flatter as it travels towards the lumen of the oesophagus. It is the basal cuboidal layer that is actively dividing. The epithelium in the bladder is slightly different, and is known as transitional epithelium. Due to the nature of its environment

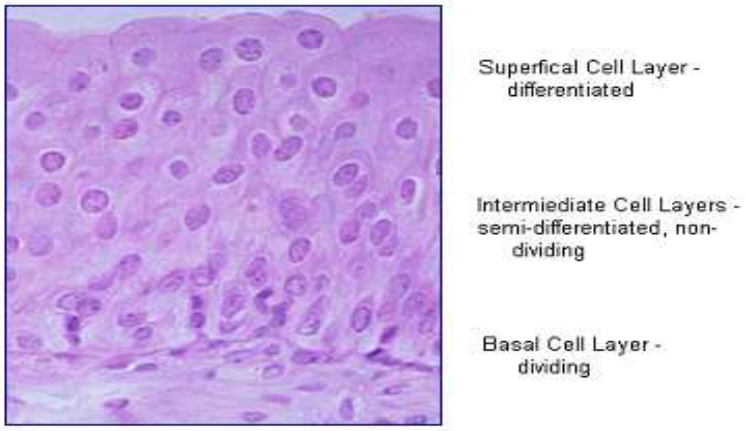

Figure 3.1: Microstructure of Epithelia (From Wheater's Functional Histology)

this epithelium is adapted to withstand huge degrees of stress and toxicity. Similar to the oesophagus, it is the basal layer that is activity dividing. The general structure of these epithelia can be seen in figure 3.1.

The basal cell layer closest to the lamina propria *in vivo*, is the dividing cell layer and is located on the outer most part of explants. The intermediate layer is usually 2 - 3 cells thick, and consists of semi-differentiated cells that are not dividing. Finally, the superficial cell layer consists of fully differentiated cells, and is located next to the lumen *in vivo*, [Young and Wheater, 1993]. When cultured *in-vitro* as explants, the growth around the tissue fragment forms a two dimensional representation of the three dimensional structure *in-vivo*. It is the dividing cell layer that is the outermost part of the explant, with the superficial cell layer located in the inner circle.

The outgrowth of these explants are also investigated for expression of proteins linked with apoptosis, Bcl2 an anti-apoptotic protein, and cMyc an apoptotic protein, also linked with proliferation. The Bcl2 protein is an integral inner mitochondrial membrane protein that is a potent inhibitor of cell death, both programmed and accidental. It is thought that it inhibits mitochondrial permeability transition, thus preventing cytochrome C release. The release of cytochrome C is a very important step in the apoptotic pathway, and so prevention of its release terminates the apoptotic pathway [Shimizu et al., 1998, Kowal-

towski et al., 2002]. Other studies indicate Bcl-2 is also capable of inhibiting cytocrome c release pathways independent of mitochondrial permeability transition, therefore adding to the ability of the protein to inhibit apoptosis [Polster et al., 2001]. Harney et al. [1995] showed a significant increase in Bcl2 expression in individual urothelial patient cultures exposed to direct irradiation, however, there was significant variation between different samples. Maguire et al. [2005] showed that there was a significant increase in Bcl2 expression in human keratinocytes post exposure to autologous irradiated cell conditioned medium.

cMyc was also examined in these explants, a transcription factor that is tightly regulated by both external signals, such as growth factors, and internal signals, such as the cell cycle. Resting cells, in interphase, will express a low background level of cMyc, whereas actively dividing cells will express higher levels of the protein. This increase in expression is maintained in the cell cycle, and returns to basal levels once the cell is in interphase. An over expression of cMyc is involved in apoptosis, via a p53 dependent pathway. However, in cancerous cells, it is now thought that cMyc rushes the cell through the cell cycle, and so various check points are overlooked, resulting in the production of mutant cells. In a certain amount of these cells, the apoptotic pathway will remove the cells, although in the presence of anti-apoptotic proteins and increased proliferation, due to the cMyc elevation, the cancer persists [Gardner et al., 2002]. Using the primary tissue culture model, Mothersill et al. [1991] showed post exposure to direct irradiation a persistent increase of cMyc in groups of cells within the culture led to morphological change and increased proliferation. In another study by Mothersill et al. [1994] where both uroepithelium and oeosphageal epithelium were investigated a significant increase in c-Myc was observed post exposure to direct radiation in discrete pockets of cells within the outgrowths .

The aim of this study was to test cell lines with irradiated tissue conditioned medium (ITCM), generated from different tissues, to determine the effect of multicellular systems on bystander signal production. The cell lines chosen were HPV-G as they have a well established response to the bystander effect, and HaCaT, a second human keratinocyte

cell line, which has been previously used for similar radiation exposure and bystander effect investigations [Mothersill and Seymour, 1997c, Banerjee et al., 2005]. The lines lines chosen in the previous chapter, CHO-K1 and E89 were not used in this investigation as attempts to convert them to the correct medium used for primary cultures, (RPMI-1640, Sigma) failed. Apoptotic and anti-apoptotic protein expression post exposure to direct irradiation was examined in both bladder and oesophagus explants. These proteins were also examined post exposure to ITCM in bladder explants, however oesophagus tissue was unavailable, and so the response to the bystander effect in this tissue could not be measured.

3.2 Materials and Methods

3.2.1 Animals

All tissue used in these experiments was prepared from male Wister rats. The tissue samples were obtained from Dr. Philip Nolan's Cardiovascular Laboratory, Physiology Department, UCD (bladder and oesophagus, direct exposures) and Dr. John O'Connor's Neurophysiology Laboratory, Conway Institute, UCD (bladder, bystander exposures).

3.2.2 Tissue Culture

The bladder and in some cases the oesophagus were removed from the rat and processed according to the protocol developed by Mothersill et al. [2001]. The tissue was transported to the Dublin Institute of Technology Laboratory, St Luke's Hospital in RPMI 'start up' medium. This contained 12% fetal calf serum (Gibco), 8% horse serum (Gibco), 1000 IU 1% penicillin streptomycin solution (Gibco), 2mM L-glutamine (Gibco), 0.025 mol/L HEPES buffer (Gibco), 1μg/ml hydrocortisone (Sigma), 0.051 IU/ml human recombinant insulin. Tissue samples were trimmed of all fat and connective tissue, and cut into segments of approximately 2-3 mm^2, for explantation. Samples were then digested with a trypsin - collagenase solution at 37°C for 10 minutes. The solution was then inactivated

with RPMI medium, and seeded in 3 mls RPMI in T24 tissue culture flasks. These were allowed to attach for 24 hrs, before irradiation, after which the medium was changed to KGM (Keratinocyte Growth Medium). KGM was used as it was developed by Mothersill et al. [2001], as a method of eliminating fibroblast growth in the explants. The RPMI medium removed from the explants was filter sterilised using 0.22um Nalgene filters to remove any cells or debris and used as ITCM. The explants were allowed grow a further 7 days, and were then fixed in 10% buffered formalin for further investigation using immunohistochemistry. A whole explant can be seem in figure 3.2 and a haematoxylin and eosin stained section of the outgrowth in 3.3.

3.2.3 Cell Culture

Cell lines used in this study were HPV-G and HaCaT. Details of the HVP-G line are contained in Chapter 2, section 2.2.1. The HaCaT cell line is a human keratinocyte line, which is immortal but not tumourigenic. It has an aberrant p53 expression with a deletion on one allele and a point mutation on the other. These cells can stratify in three-dimensional culture to give differentiated skin-equivalent architecture, and are proficient in gap junction intercellular communication. Both these lines are grown in Dulbecco's MEM: F12 (1:1), containing 10% fetal calf serum (Gibco), 5000 IU/ml penicillin streptomycin solution (Gibco), 2mM L-glutamine (Gibco), 0.025 mol/L HEPES buffer (Gibco) and 1μg/ml hydrocortisone (Sigma). For the purposes of this study both HPV-G and HaCaT cell lines were adapted to RPMI 1640, to allow from medium transfer between the tissue and the lines. This contained 12% fetal calf serum (Gibco), 8% horse serum (Gibco), 1000 IU 1% penicillin streptomycin solution (Gibco), 2mM L-glutamine (Gibco), 0.025 mol/L HEPES buffer (Gibco), 1μg/ml hydrocortisone (Sigma), 0.051 IU/ml human recombinant insulin. For adaptation cell lines were grown in alternative medium for 1-2 weeks, and no significant difference was noted in the plating efficiencies of either lines in the alternate media, (HPV-G: DMEM 17.55% (see table 2.2), RPMI 21.31%; HaCaT: DMEM 39.8% [Herzog et al., 2007], RPMI 34.11%) Supplements for media were all obtained from Gibco

Biocult Ltd. Irvine, Scotland. All lines were maintained in an incubator at 37°C in an atmosphere of 5% CO_2. Subculture was routinely performed using a 1:1 solution of trypsin (0.2%) and Versene (0.02%).

3.2.4 Irradiation

Explants were irradiated in T-24 (NUNC, Uden, Denmark) containing 3ml culture medium at room temperature using a cobalt-60 teletherapy source at St. Luke's Hospital, Dublin, at a flask to source distance of 80cm. The dose rate during the experiment was approximately 1.8Gy/min. Explants were irradiated at 0.5 and 5Gy. Post exposure, all flasks were returned to the incubator.

3.2.5 Reporter Cell Assay

1 hour post exposure medium was then removed, and filtered for exposure to cell lines. HPV-G and HaCaT cell line adapted to RPMI medium were chosen and using the clonogenic assay technique of [Puck and Marcus, 1956] cells were plated for treatment. The HPV-G cell line was plated at 400 cells per flask, the HaCaT cell line plated at 600 per flask. Both were treated with the ITCM 6 hours post seeding. Cells were allowed to grow until colony formation, 10 - 12 days post exposure, at which point they were stained with carbol fuschin (BDH, Poole, UK), and the cell survival determined.

3.2.6 Tissue ITCM exposure

Bladder explants were plated as described above for exposure to ITCM harvested from directly irradiated explants. Both sets of tissue were harvested and plated on the same day. One set was directly irradiated on day 2, the medium removed and filter sterilised using 0.22μm Nalgene filters 1 hour post exposure. The original medium from the unexposed explants was removed, and replaced with the ITCM generated from the directly irradiated explants. 24 hours post treatment, the ITCM was removed from these explants, replaced

with KGM, and the explants were allowed to grow for a further 7 days and were then fixed in 10% buffered formalin for further investigation using immunohistochemistry.

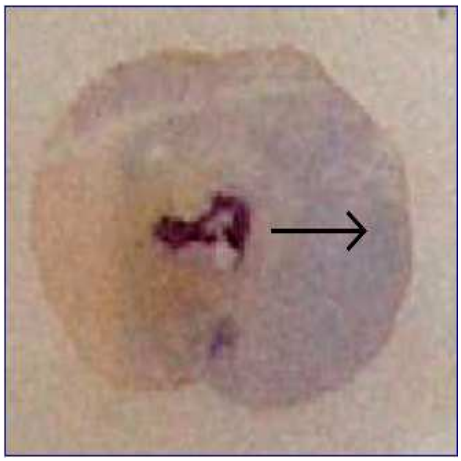

Figure 3.2: The explant outgrowth showing direction of growth, and the explant itself in the centre. The basal cell layer is located at the periphery, and fully differentiated cells are at the centre, next to the explant tissue (equivalent to the superficial cell layer *in vivo*).

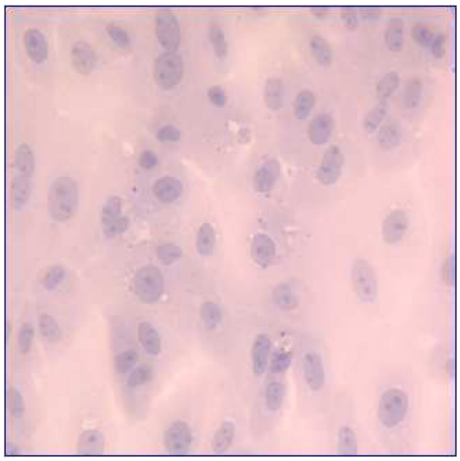

Figure 3.3: Haematoxylin and Eosin stained section outgrowth from a bladder explant

3.2.7 Immunohistochemistry: Strepavidin Peroxidase Method

Formalin was removed from all flasks, and explant outgrowth rehydrated with PBS. The upper section of the flask was removed, and the explant itself carefully taken away with a forceps.

For morphological examination haematoxylin and eosin staining was used. The culture was treated with haematoxylin for 1 minute. The slide was placed in tap water, to remove excess haematoxylin from the tissue. 1% acid alcohol was placed on the slide for 15 seconds for differentiation. The culture was then washed with tap water. Eosin was then applied for 30 seconds, and washed off with tap water. The culture was then dehydrated by placing it into increasing levels of alcohol (50% - 100%), and the slide mounted in glycergel

Immunohistochemical analysis was performed using the streptavidin peroxidase method for cell culture, with the Vectastain ABC kit. To quench endogenous peroxidase activity, the culture was treated with 3% hydrogen peroxide for 3 minutes, which was then washed away with PBS. 1% bovine albumin serum (blocking serum) was applied to the tissue for 20 minutes to prevent false positives and was again washed away with PBS. The primary antibody, Bcl2 (1:50) or cMyc (1:500) was then applied for 60 minutes. A negative control, where no primary antibody was applied was included in each run. The culture was washed again with PBS, and biotinylated anti-mouse regent was applied for 30 minutes. After washing with PBS, strepavidin peroxidase was applied for 30 minutes. The culture was washed again with PBS, and then treated with the chromagen - DAB (3,3-diaminobenzidine) in darkness for 10 minutes. The culture was then washed with distilled water, and counterstained with Harris haematoxylin for approximately 10 seconds. The slides were then placed into running hot water, to blue the culture and remove excess haematoxylin. At this point each explant was examined under the microscope to access the level of haematoxylin staining present. If this level was too high, the culture was differentiated in 1% acid alcohol for approximately 10 seconds, to reduce the level of staining. Slides were then mounted with glycergel.

3.2.8 Outgrowth Area Measurement

The outgrowth area of each explant culture was determined in DAB stained cultures using a transparent sheet of 1 mm^2 grid paper. The number of 1 mm^2 grids covered by the stained explant was counted.

3.2.9 Statistical Analysis

Each cell line was treated with each type of ITCM from 3 different animals at least 3 times in independent experiments, with 3 replicate flasks per experiment. For the protein expression study, 3 animals were scored, in 3 random fields with approximately 500 cells per field. One-way ANOVA was used to determine the significance in each case.

3.3 Results

3.3.1 Bystander signal versus response

Figure 3.4 shows the response of HPV-G cells and HaCaT cells to ITCM generated by bladder tissue. HPV-G cells showed a 20.09% ± 2.41 and 29.11% ± 5.52 drop in survival when treated with 0.5Gy and 5Gy ITCM respectively. However, the reduction displayed by HaCaT cells was 3.26% ± 12.09 and 10.91% ± 9.62 when treated with 0.5Gy and 5Gy ITCM. The response of the cell lines to oesophagus ITCM is shown in figure 3.5. Similar to the response to bladder ITCM, when treated with ITCM generated from oesophagus the HPV-G cells show a 10.04% ± 5.35 and 24.35% ± 4.59 reduction in survival to 0.5Gy and 5Gy ITCM respectively, while HaCaT cells display a 8.56% ± 7.35 and 13.81% ± 7.64 drop in survival post exposure. The survival fraction of both cell lines post exposure bladder and oesophagus ITCM is displayed in table 3.1

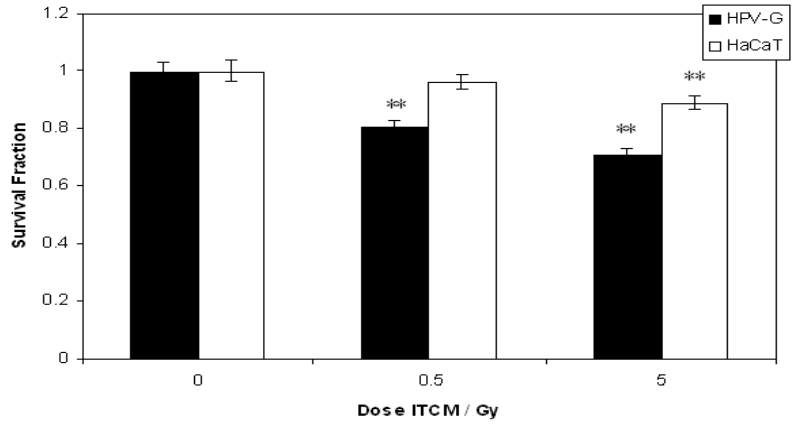

Figure 3.4: HaCaT and HPV-G cell survival post exposure to bladder ITCM. ($* * p < 0.01$)

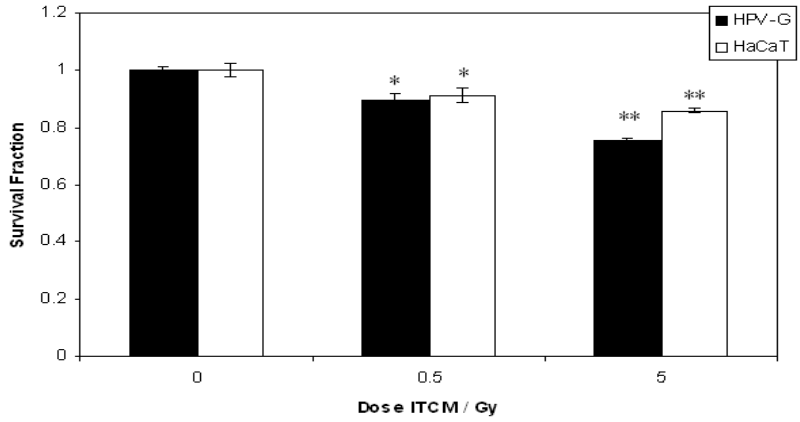

Figure 3.5: HaCaT and HPV-G cell survival post exposure to oesophagus ITCM. ($* p < 0.05 * * p < 0.01$)

Treatment	Dose ITCM		
	0Gy	0.5Gy	5Gy
Bladder ITCM			
HPV-G	20.47 ± 1.39	16.36 ± 0.49	14.51 ± 1.13
HaCaT	34.13 ± 3.64	33.02 ± 4.12	30.41 ± 3.29
Oesophagus ITCM			
HPV-G	22.14 ± 1.18	19.91 ± 1.18	16.75 ± 1.02
HaCaT	34.08 ± 2.54	31.17 ± 2.51	29.37 ± 2.61

Table 3.1: Plating efficiency of HPV-G and HaCaT post treatment with ITCM from bladder and oesophagus.

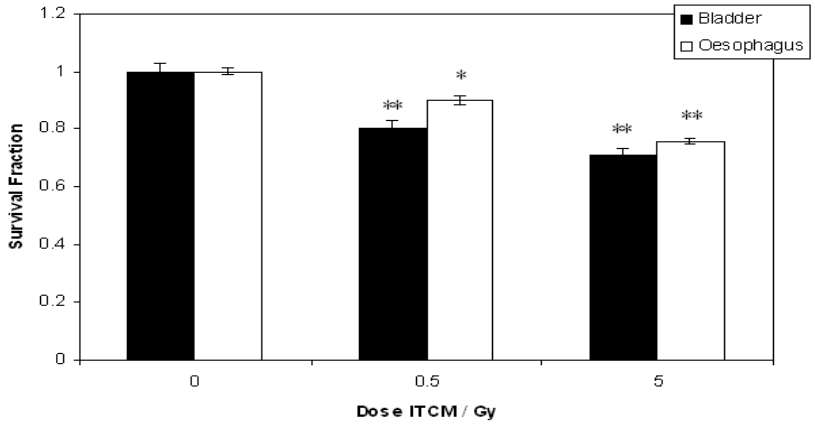

Figure 3.6: HPV-G cell survival post exposure to bladder ITCM and oesophagus ITCM. ($(* \text{p} < 0.05 * * \text{p} < 0.01)$

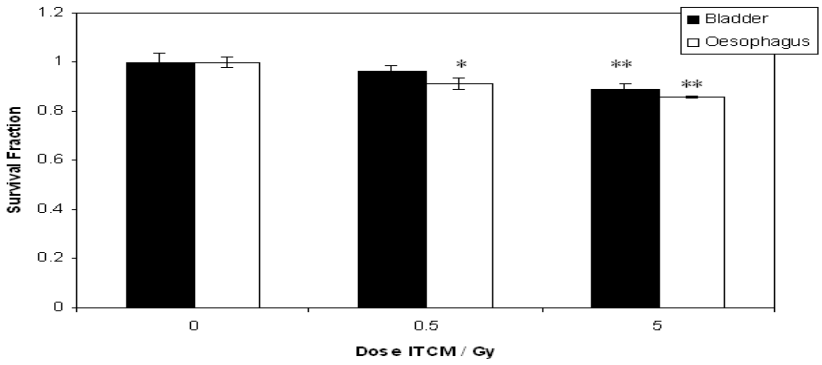

Figure 3.7: HaCaT cell survival post exposure to bladder and oesophagus ITCM. ($* \text{ p } < 0.05 * * \text{ p } < 0.01$)

3.3.2 Bystander Signal: Organ specific vs. Systemic

Figures 3.6 and 3.7 compare the response of a cell line to ITCM generated from the two tissues, bladder and oesophagus. Figure 3.6 displays the response of HVP-G cells; figure 3.7 that of HaCaT cells. As can be seen in both cases, the response to the oesophagus ITCM differs very little from that of the bladder ITCM. At 5 Gy, the HPV-G cells show a reduction to 79.91% $\pm$ 5.2 survival when treated with bladder ITCM, and 75.65% $\pm$ 4.5 survival when treated with oesophagus ITCM. In contrast to this, the HaCaT cells display a smaller decrease in both cases, but a similar response is seen post exposure to both bladder and oesophagus ITCM, with survival at 89.09% $\pm$ 9.62 and 86.19% $\pm$ 7.64 respectively.

3.3.3 Outgrowth Area and Protein Expression

Figure 3.8 displays the area of bladder and oesophagus explants post exposure to either direct irradiation, or in the case of bladder explants, autologous ITCM. No significant difference was found between the control and the exposed explants in any group. However the area of the oesophagus explants was notably smaller that that of the bladder, and

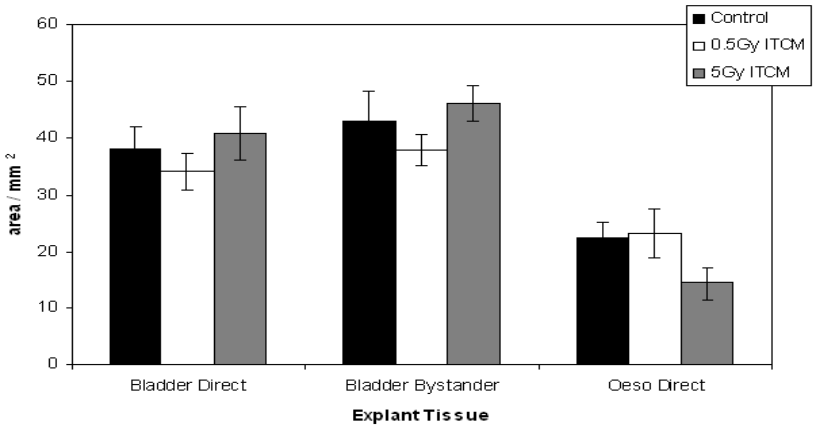

Figure 3.8: Area of bladder and oesophagus explants post exposure to direct radiation and autologous ITCM ($* \ p \ < 0.05$)

there was an insignificant but clear decrease in the area of oesophagus explants post exposure to 5Gy radiation.

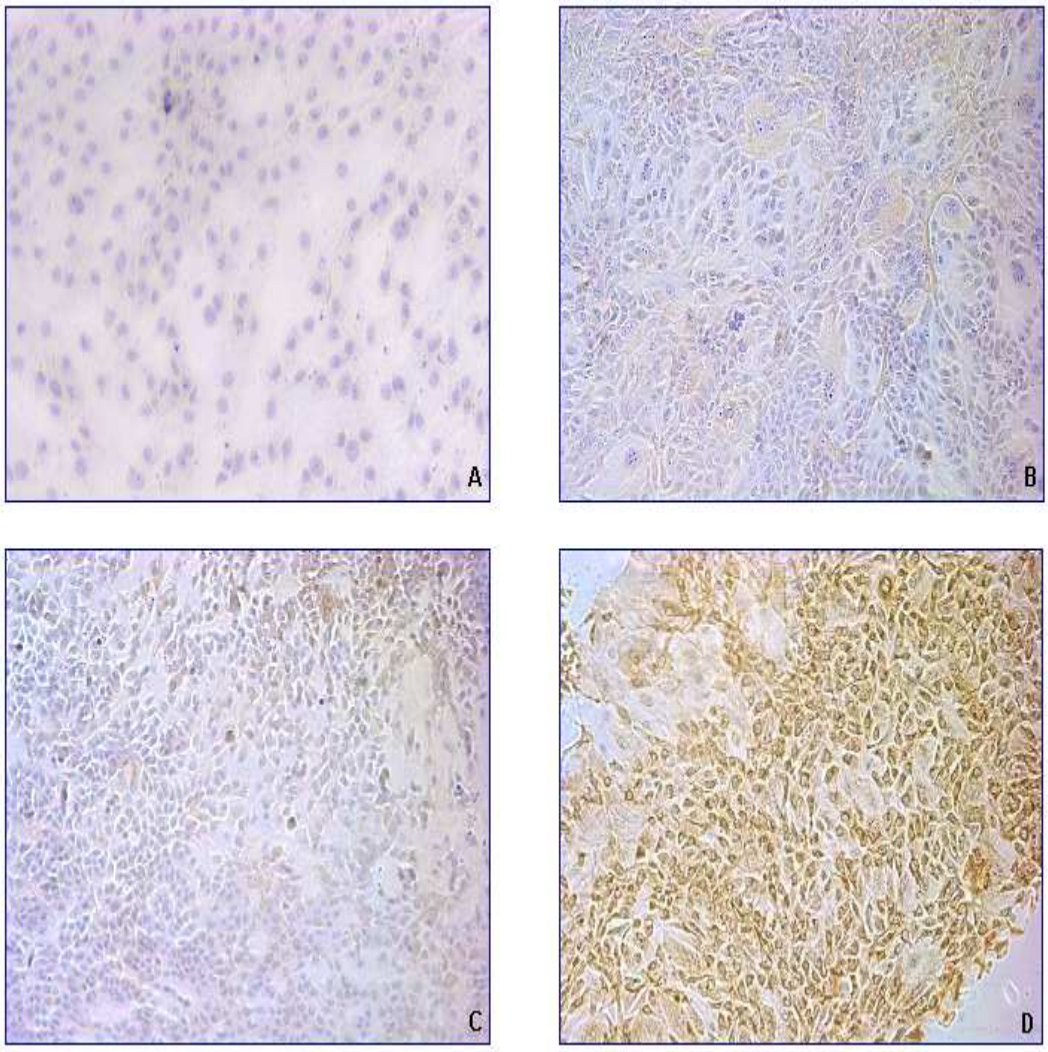

Figure 3.9: Bladder explant outgrowth stained for Bcl2, post direct irradiation. Brown staining indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

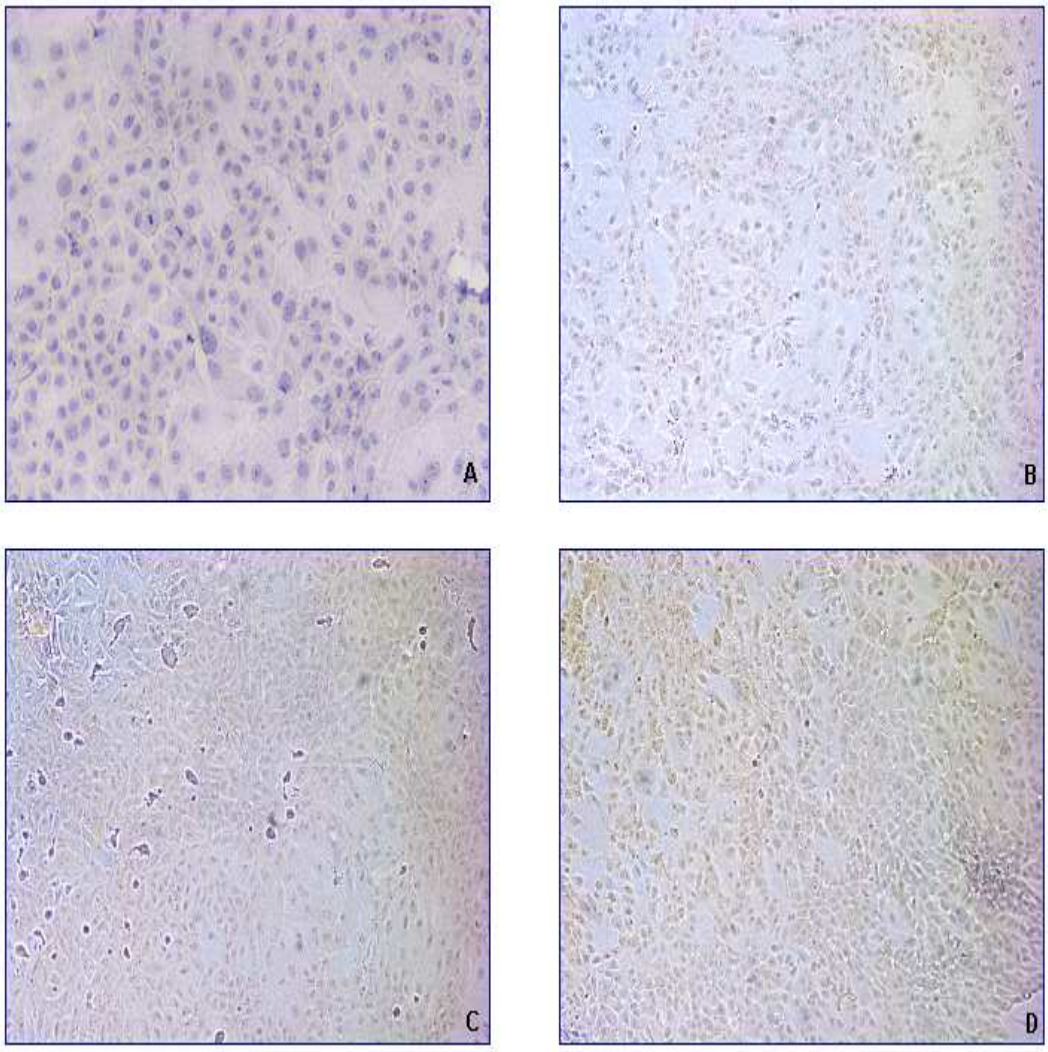

Figure 3.10: Bladder explant outgrowth stained for cMyc, post direct irradiation. Brown staining indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

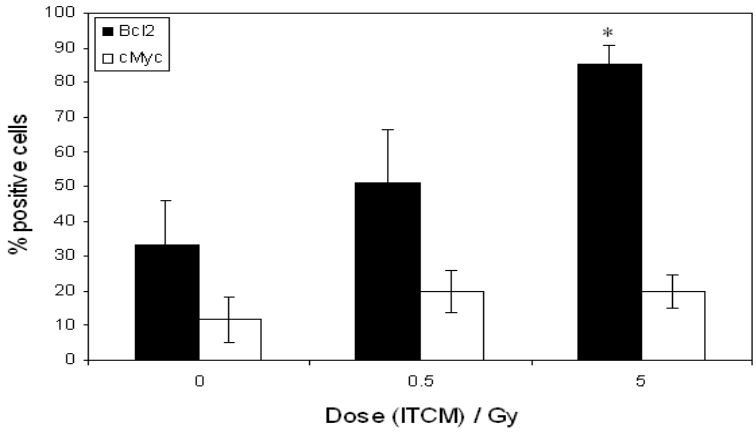

Figure 3.11: Bcl2 and cMyc expression in directly irradiated bladder explants. 3 fields of 500+ cells were counted per mouse for 3 independent experiments ($** p < 0.01$)

Bladder and oesophagus cultures were stained for Bcl2 and cMyc protein expression using immunocytochemistry. In all cases, positivity of the protein is indicated by the brown cytoplasmic staining. Figure 3.9 above shows Bcl2 expression with increasing dose of direct radiation, with 75.58% $\pm$ 4.32 of the population expressing Bcl2 at 5Gy, (graph figure 3.11). A small, but insignificant increase in expression was observed at 0.5Gy. Figure 3.10 shows the expression of cMyc, which remained unchanged post exposure to direct irradiation (graph figure 3.11).

Figure 3.12 shows expression of Bcl2 in the oesophagus with increasing dose. Significant increases were observed at both 0.5 and 5Gy however, the background level of stain (i.e. the control explants, see figure 3.14) is slightly higher here than for bladder. Figure 3.13 shows cMyc expression, 85.79% $\pm$ 4.5 of the population expressed cMyc at 5Gy (graph figure 3.14). There was no significant increase at 0.5Gy. Again the control levels of cMyc were higher than for bladder.

Figure 3.15 shows the expression of Bcl2 and cMyc in bladder explants post exposure to autologous ITCM. In the case of Bcl2, there is a significant increase at both 0.5 and 5Gy with 83.28% $\pm$ 8.86 of the population expressing Bcl2 at 5Gy. There is no significant

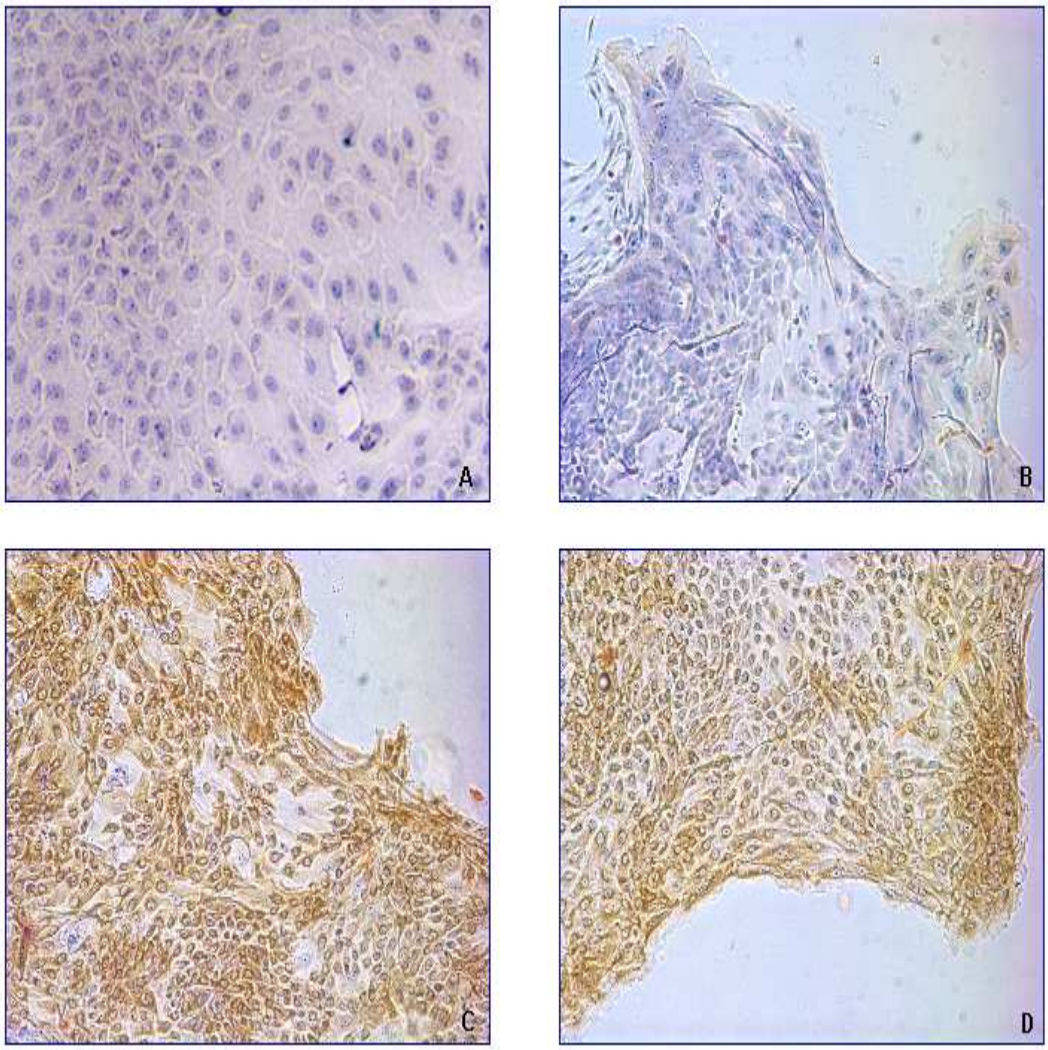

Figure 3.12: Oesophagus explant outgrowth stained for Bcl2, post direct irradiation. Brown staining indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

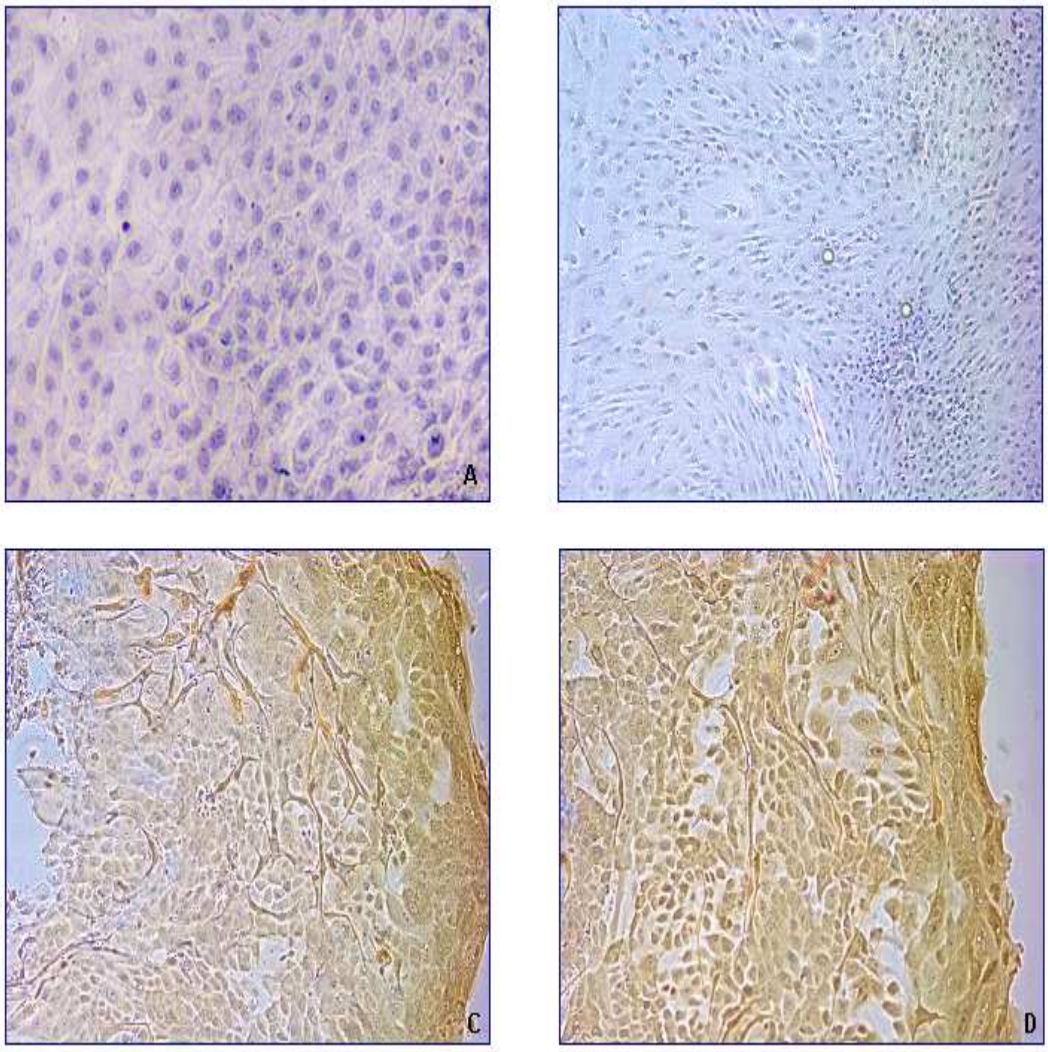

Figure 3.13: Oesophagus explant outgrowth stained for cMyc, post direct irradiation. Brown staining indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

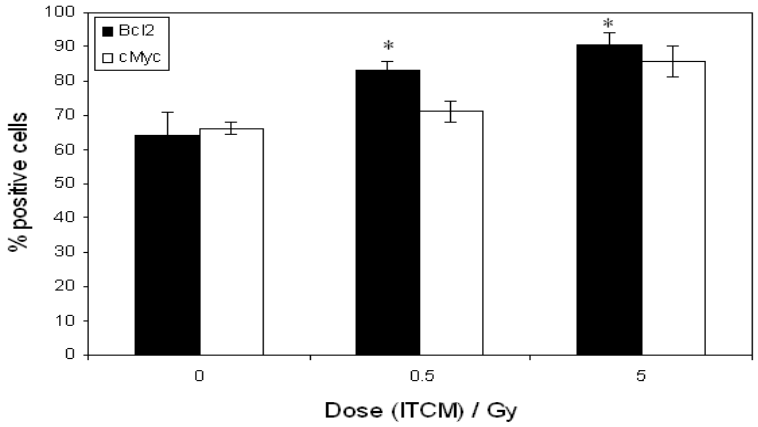

Figure 3.14: Bcl2 and cMyc expression in directly irradiated oesophagus explants. 3 fields of 500+ cells were counted per mouse for 3 independent experiments ($*$ p < 0.05)

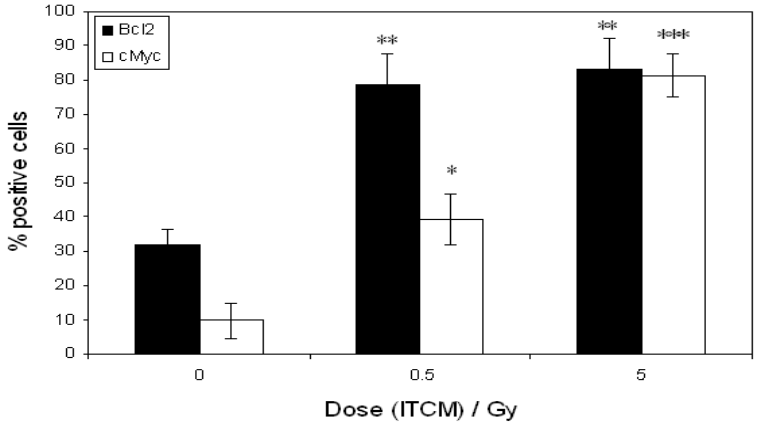

Figure 3.15: Bcl2 and cMyc expression in bladder explants exposed to autologous ITCM. 3 fields of 500+ cells were counted per mouse for 3 independent experiments ($*$ p < 0.05 $**$ p < 0.01 $***$ p < 0.001)

difference between the expression at 0.5 and 5Gy. However, in the case of cMyc, although there were significant increases at both 0.5 and 5Gy, there was also a significant difference between the doses. Post exposure to 0.5Gy ITCM, 39.48% ± 7.4 of the population were positive for cMyc, while 81.28% ± 6.3 were positive at 5Gy ITCM.

3.4 Discussion

While much information has been learned about the radiation-induced bystander effect in the last decade, there are still many unanswered questions regarding this phenomenon. Various mechanisms and pathways have been proposed, however there is yet to be a definitive answer regarding the exact mechanism of the bystander effect, and more importantly its relevance *in-vivo*. One of the main reasons for this is that it is likely that the bystander effect is different in each system examined, and is dependent on many factors involved in the process, including type and dose of radiation, stage of cell cycle and cell type. In order to examine the bystander signal generated in a multicellular environment, bladder and oesophagus tissue was explanted and irradiated *ex-vivo* in order to generate ITCM. This ITCM was tested using cell line and primary tissue culture models.

Continuing from the investigation in the previous chapter, two cell lines were exposed to ITCM from both tissues and the cell survival determined. From the above results it is clear that the bystander signal produced from the bladder and oesophagus is quite similar, however they produce different responses in the cell lines exposed. HPV-G cells showed approximately a 30% reduction in survival post exposure to both types of ITCM, while HaCaT cells displayed a much smaller decrease in survival, approximately 10%. This is in direct contrast with the findings in the previous chapter, where it was shown that the source of the bystander signal determined the magnitude of the response. However, when the sources of the ITCM tested in this study are considered, it is not surprising that there were differences in the response of the cell lines. In the case of examining signal versus response using cell lines, the bystander signal was produced by a very specific type of cell,

and so the model used was a relativity simple one. There was a clear trend that indicated it was the irradiated cell line that determined the magnitude or potency of the signal produced. In contrast to this, the signal investigated here was generated by a section of tissue from the bladder or oesophagus and so there were many different cell types present, at various stages of the cell cycle. Some of the cells would be actively dividing, while others are differentiated, resulting in a more complex model than the cell line one, and so there were more factors influencing bystander signal production. In this case, it seems that the cell lines tested respond to bystander signals generated from different tissues in a similar way. What is clear from these results is these tissues produce a bystander signal capable of significantly reducing the survival of cell lines.

When the levels of Bcl2 and cMyc are examined, it is clear that the response of each tissue to direct irradiation is quite different, with the oesophagus seeming to be the more radiosensitive of the two. This was also reflected in the survival of the explants, with bladder tissue showing no loss in survival, while the oesophagus showed an insignificant, but notable reduction in outgrowth area, at 66% of the control value. At 0.5Gy, there is a significant increase in the expression of Bcl2 in the oesophagus, while there was no change in the expression in bladder explants. There is little difference between the tissues in protein expression at 5Gy in the case of Bcl2, however when cMyc expression was examined, the oesophagus again shows increased radiosensitivity with significantly higher expression at 5Gy. The bladder showed no significant increase in cMyc at either dose. When the controls for each tissue are compared, the bladder expressed lower levels of both proteins. There are a number of possible explanations for this, the most probable being related to the physiological function of the two tissues, and the nature of their environment. However, the success in culture maybe be another factor causing the tissue to express higher levels of both proteins. When the average area of the oesophagus is compared to that of the bladder, it is clear that the bladder explants were significantly larger than that of the oesophagus (Bladder explant control area: 38.16 ± 3.7 ; Oesophagus explant control area: 22.33 ± 2.8). Since there is no apparent difference in the ability of the bystander

signal produced by these tissues to reduce cell survival, it would seem that the level of Bcl2/cMyc expression is not directly connected to bystander factor production.

A number of interesting differences between directly irradiated bladder cultures and those exposed to ITCM were observed. No change in the levels of Bcl2 was noted after exposure to 0.5Gy direct irradiation, however there was a significant increase in this protein at 5Gy. In contrast to this, there was a significant increase in Bcl2 at both doses in the ITCM exposed cultures, with no difference between the two doses. This type of response has frequently been observed in bystander effect investigations, has been termed an 'all or nothing' response, and is indicative of a saturation of the bystander effect at low doses, [Prise et al., 1998, Albanese and Dainiak, 2000, Belyakov et al., 2003]. Bcl2 is an anti-apoptotic protein, and so this increase at lower doses post exposure to ITCM may indicate that the tissue is attempting to save cells, and allow them time to recover. While this can be viewed as a protective mechanism for the cells in question, it is probably not for the tissue, as preventing cells that have been damaged from entering the apoptotic pathway may have detrimental consequences for the tissue as a whole. As was pointed out by Mothersill and Seymour [2003] in a paper modelling the bystander effect, what may be seen as a negative effect at one level, may be positive at another, and vice-versa. What needs to be considered here however, is the level of expression at both doses of ITCM. The majority of the cell population express Bcl2 post exposure to ITCM, and so this may reflect a co-ordinated response of the tissue to limit damage and cell loss. Levels of Bcl2 only increased significantly in directly irradiated explants at 5Gy, possibly indicating a similar effort of the tissue to repair some of the damaged population, and minimise cell loss. This is consistent with the findings of Mothersill et al. [1999] where there was a proposed threshold of approximately 1Gy for the induction of Bcl2 in directly irradiated cultures. However, with respect to the bystander effect, this threshold seems to be lower in the current study.

The levels of cMyc in both directly irradiated and ITCM exposed cultures also differ significantly. There is no change in cMyc expression at either dose post exposure to direct

irradiation, however there is a dose-dependent increase in this protein post exposure to ITCM. As mentioned above, it is quite common for there to be a saturation of the bystander effect at low doses, however this response indicated that in the case of cMyc activation, there is a significant increase or alteration in the bystander signal generated at 0.5 and 5Gy. cMyc is linked to both apoptosis and proliferation [Gardner et al., 2002], and is thought to be a marker of neoplastic transformation [Mothersill et al., 1991]. Whether this increase is reflective of an increase in apoptosis, a common characteristic of the bystander effect, [Mothersill et al., 2001, Suzuki et al., 2004, Belyakov et al., 2006, Lyng et al., 2006b] or an increase in proliferation in this case is unknown. However as there was no change in the area of the explant outgrowth it may be possible that cMyc plays a dual role in the response of the tissue as a whole. If the response was purely apoptosis or proliferation related, it should be reflected in the area of the explant as an increase or decrease in the area. Taken with the Bcl2 expression results, it is possible that the balance of Bcl2 and cMyc in each cell significantly contributes to whether the cell lives or dies by apoptosis. As mentioned above, Maguire et al. [2005] also observed a dose-dependent alteration of the bystander signal at 0.5Gy and 5Gy in HPV-G cells. It was suggested that this may reflect a controlled response of the population of cells to radiation, and possibly resulted in increased survival of cells at higher doses of exposure.

Taken together, it seems that post exposure to ITCM, the response of bladder explant cultures as a whole is for maintenance of the tissue. The increases in Bcl2 and cMyc reflect an attempt by the tissue to remove or regulate the damaged cells within the population. The dose dependent response of cMyc expression coupled with the 'all or nothing' expression of Bcl2 indicates a complex production of and response to a bystander signal in a multicellular, *in-vivo*–like environment.

From the results described above, it is clear that the bystander effect generated from explanted tissue is a significantly more complicated one than that seen in cell lines. The signal had different effects on different cell lines, and altered protein expression in exposed tissues in a both dose dependent and independent manner. Interestingly, there

was no significant difference between the bystander signal generated from the bladder and oesophagus, although these tissue did respond differently to direct radiation, with the oesophagus being the more sensitive of the two. Significant differences between the response of the bladder tissue to direct irradiation and the bystander effect were also observed. This then reflects the need to examine the bystander effect in *in-vivo*–like systems, as the effects seen in these systems and cell lines seem to differ considerably.

Chapter 4

Bystander Effect in Epithelial Tissue II

4.1 Introduction

Continuing from the findings presented in the previous chapter, this section of the study further investigates the response of epithelial tissue to both direct irradiation and the bystander effect. While the previous section focused mainly on proteins involved in apoptotic regulation, this study examines the differentiation of the tissue, damage to the nucleus, and the effect on the actin component of the cytoskeleton. Therefore a comparison can be made between what has been described as protective (differentiation, [Belyakov et al., 2006]) and damaging (nuclear fragmentation and alterations in cytoskeletal components [Azzam et al., 2002, Little et al., 2002, Belyakov et al., 2005, Lisi et al., 2006]) for the tissue.

The majority of the effects of both direct radiation and the bystander signal tend toward mutation or cell death, however, Belyakov et al. [2002, 2006] reported premature differentiation in a tissue culture model post exposure to a small number cells in the population being irradiated. In this study pig ureters were explanted and allowed to grow to form a 2D culture of the tissue. A small number of cells within the population were

exposed to α particles using a microbeam, after which the tissue was tested for positivity for the urothelial terminal differentiation marker, uroplakin III. A significant increase in this protein was found, and it was suggested the premature differentiation induced by exposure to the bystander signal was a protective mechanism to remove cells which may be damaged from the proliferating population of the tissue. Whether this is a response that can be associated with a low-LET induced bystander effect is not known, and so in the current study premature differentiation was examined in the same primary tissue culture model investigated in the previous chapter.

There have been some reports in the literature of actin damage post exposure to radiation, and of a link between differentiation and the actin component of the cytoskeleton. Woloschak et al. [1990] investigated the effect of various types of radiation (neutrons, gamma rays, and x-rays) on the mRNA coding for beta-actin, gamma-actin, and alpha-tubulin in Syrian hamster embryo cells. It was found that both low and high LET radiation caused alpha-tubulin mRNA to accumulate, a result which was mirrored by gamma-actin. However, beta-actin mRNA showed the opposite effect, and so it was concluded that both high and low LET radiation decrease the ratio of beta-actin:gamma-actin. A role for changes in actin- and tubulin-mRNA expression for radiation-mediated transformation was proposed, as these changes are similar to those seen in cell lines treated with tumor promoters. In a further investigation, Woloschak and Chang-Liu [1991], compared the effect of radiation on both resting and proliferating cells, and found differences in the expression of mRNA encoding for actin, and that the level of expression of these genes in proliferating cells was much greater than that in resting cells, post exposure to neutrons.

In a study investigating the effects of direct γ radiation on the hematopoietic tissue of the rainbow trout, Olwell et al. [2005] found that there was a significant reorganisation of the actin cytoskeleton, and decreases in the phagocytotic efficacy of cells cultured from this tissue post exposure. There were also a change in the shape of the cells from rounded to stretched forms in the exposed culture, an effect attributed to the alteration of actin within the cells.

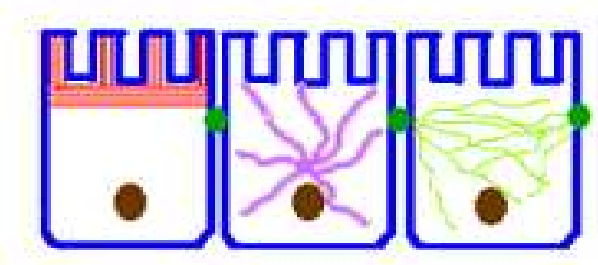

Figure 4.1: Epithelial cells showing the three parts of the cytoskeleton, and their position within the cell. Red: microfilaments, (f-actin), Purple: microtubules, (tubulin), Green: Intermediate filaments (vimentin and desmon). Adapted from *www.biology.arizona.edu*

In a recent study investigating the effect of electromagnetic radiation on the HaCaT cell line, Lisi et al. [2006] found alterations in the distribution of actin in cells post exposure. A significant increase in the differentiation of the cells was also noted, as indicated by involucrin positivity. In another study linking differentiation and the cytoskeleton, Vijayakumar et al. [1999] found that there was a remodeling of the cytoskeleton in intercalated epithelial cells depending on the presence of a protein, hensin, in the extracellular matrix (ECM) and the seeding density of the cells. If the cells were seeded at low densities, the cells retained hensin within vesicles in the cytoplasm, had an apex devoid of actin and had sparse microvilli. However, at high seeding density, these cells released the hensin into the ECM, the apex had high levels of actin, and the cells developed numerous microvilli. The authors suggest that this type of differentiation was induced by the release of hensin, and mediated by the remodeling of the actin cytoskeleton. In a study on the terminal differentiation of osteoblasts to osteocytes in bone, Kamioka et al. [2001] found a critical role for actin and the actin binding proteins. It was reported that osteocyte shape was dependent on actin filaments. Actin binding proteins, the control elements in the organisation of the actin cytoskeleton, were dramatically altered in the differentiation of osteoblasts to osteocytes. It was suggested that the actin cytoskeleton plays a critical role in the development and differentiation of osteoblasts to osteocytes.

The cytoskeleton is composed of three distinct, co-dependent sections. These are

microfilaments, intermediate filaments and microtubules, as shown in figure 4.1. Microfilaments are thin filaments 8nm in diameter, composed of actin and are located primarily underneath the plasma membrane of the cell, providing it with both protection and strength. Intermediate filaments are slightly larger, with a diameter of 15nm, are located round the nucleus and throughout the cytoplasm and are composed of several different proteins, including vimentin and desmon. Microtubules are much larger, at 25 nm in diameter, and are hollow fibres located throughout the cytoplasm and are composed of tubulin. This investigation focuses on the effect of direct radiation and the bystander signal on the actin in the cytoskeleton and how the filaments are altered post exposure.

Much is known about the properties of actin both *in vitro* and *in vivo*. Monomeric actin, globular or g -actin is usually found in pools in the cytosol, with one ATP molecule bound per monomer. For a filament to begin formation, three of these come together, a process called nucleation, and begin the strand. This is a slow process, but it is thought to be aided by nucleation sites that are located on the inner plasma membrane of cells that need to alter their cytoskeleton for movement in a short time interval. Post nucleation g-actin is attached to the strand quite rapidly, with the hydrolysis of ATP to ADP and phosphorus (pi). However, it has been shown that g-actin will polymerise without the hydrolysis of ATP as they will still attach when there are ADP or non-hydrolyzable ATP bound. The filament is polarised, and g-actin will attach to the positive end until there is a limiting concentration of g-actin remaining in the cell. This is the critical concentration, and it is thought that at this point the g-actin is being added to the positive end of the filament at the same rate as it is dissociating from the negative end. Thus the ratio of g to f actin in the cell is maintained in a dynamic balance and is crucial to the efficient operation of the cell, and thus the tissue, [Bonder et al., 1983, Korn et al., 1987].

Once the filament is formed, numerous stabilizing proteins become associated with the filaments, allowing them to form into bundles to carry out their various functions. As mentioned above, they are located primarily just under the membrane, with some distribution through the cytoplasm. The area just underneath the membrane is known

as the actin rich cortex, and is of huge importance to cells, especially those involved in movement, (Korn et al. [1987])

In conjunction with the investigation into f-actin, the presence of micro-nuclei, or nuclear fragmentation was also determined in these cultures. Micronuclei have been associated with radiation damage and the bystander effect in numerous investigations [Azzam et al., 2002, Little et al., 2002, Belyakov et al., 2003, 2005, Marozik et al., 2007], and are thought to be damaging for the cell and tissue as a whole, as they result in propagation of errors, and often cell death in the progeny of exposed populations. It should be noted that the micronucleus assay using cytochalasin B was not preformed here. The presence of cells containing nuclear fragments within the cell population not undergoing mitosis was determined, similar to the method described in Belyakov et al. [2003].

This study investigated whether premature differentiation is induced by a low–LET radiation induced bystander effect, and if so whether it is linked to changes in the actin cytoskeleton. By correlating these results with the nuclear fragmentation data and the findings of the previous chapter, an attempt is made to determine if the bystander effect may be termed protective or damaging in a *in-vitro* primary tissue culture model.

4.2 Methods and Materials

4.2.1 Tissue culture

All the tissue used in these experiments were prepared from male Wister rats, weighing 50-100g, and where obtained from Dr. John O'Connor's Neurophysiology Laboratory, Conway Institute, University College Dublin. The bladder tissue processed as described in Chapter 3, section 3.2.2, [Mothersill et al., 2001].

4.2.2 Irradiation

Explants were irradiated as described in Chapter 3, section 3.2.4

4.2.3 Exposure to ITCM

Bladder explants were plated as described above for exposure to ITCM harvested from directly irradiated explants. Both sets of tissue were harvested and plated on the same day. One set was directly irradiated on day 2, the medium removed and filter sterilised using 0.22mm Nalgene filters 1 hour post exposure. The original medium from the unexposed explants was removed, and replaced with the ITCM generated from the directly irradiated explants. 24 hours post treatment, the ITCM was removed from these explants, replaced with KGM, and the explants were allowed to grow for a further 7 days. All explants were then fixed in methanol or 4% buffered paraformaldehyde for further investigation using immunohistochemistry.

4.2.4 Immunocytochemistry: Strepavidin Peroxidase Method

Samples were processed as described in Chapter 3, section 3.2.7. The primary antibody, Uroplakin III was applied in a 1:5 dilution.

4.2.5 Immunofluorescence Staining

Paraformaldehyde was removed from the flasks, and a triple wash with PBS was then performed. The culture was then permeabilized with 0.1% triton - X - 100 in PBS for four minutes, at room temperature. Culture was washed again three times, with PBS. Phalloidin - TRITC, 1:40 in PBS was then applied to the culture, and allowed to incubate at 37°C for 45 minutes, in the dark. The culture was again washed 5 times with PBS. The culture was treated with 0.5 μg/ml DAPI, and allowed incubate for 15 minutes, in darkness. Excess dye was then drained from the culture. Finally, a coverslip was mounted onto the culture upside-down with Antifade mountant. To determine damage to the f-actin, 3 fields of 500 cells were scored, within 3 samples. Simultaneously, the number of micro-nuclei present in these fields was also determined.

4.2.6 Statistical Analysis

For all experiments, 3 animals were scored, in 3 random fields with approximately 500 cells per field. The students t-test or one way ANOVA were used where appropriate to determine the significance.

4.3 Results

4.3.1 Terminal Differentiation

Figure 4.2 below shows a bladder explant culture stained for uroplakin III, a marker of terminal differentiation in urothelial tissue. The level of expression is shown in figure 4.4. In the control explants, there was a very low percentage of cells expressing the protein, while at 0.5Gy there was an increase in the expression. However, this increase was only significant in the bystander explants, where the expression increased to $17.88 \pm 5.35\%$ of the cell population. Much of the positive cells, in both types of exposure were located toward the centre of the explant. This is consistent with the theory that there is a 2-D reconstruction of the 3-D tissue structure in this *in-vitro* system [Belyakov et al., 2002]. When the tissue was exposed to 5Gy direct radiation or 5Gy ITCM, the level of expression had returned to control levels in both cases.

4.3.2 Actin Disruption and Nuclear Fragmentation

Figure 4.5 shows the response of the f-actin post exposure to both direct irradiation and ITCM. Although there is a slight increase in the number of cells with actin disruption with increased dose in both treatments, no significant difference was observed between the control and any treated groups. An example of the actin distribution in the explant cultures is shown figure 4.7, panel A.

The presence of cells containing nuclear fragments, or micronuclei, within the culture were scored in both directly irradiated and ITCM treated cultures, shown in figure 4.6.

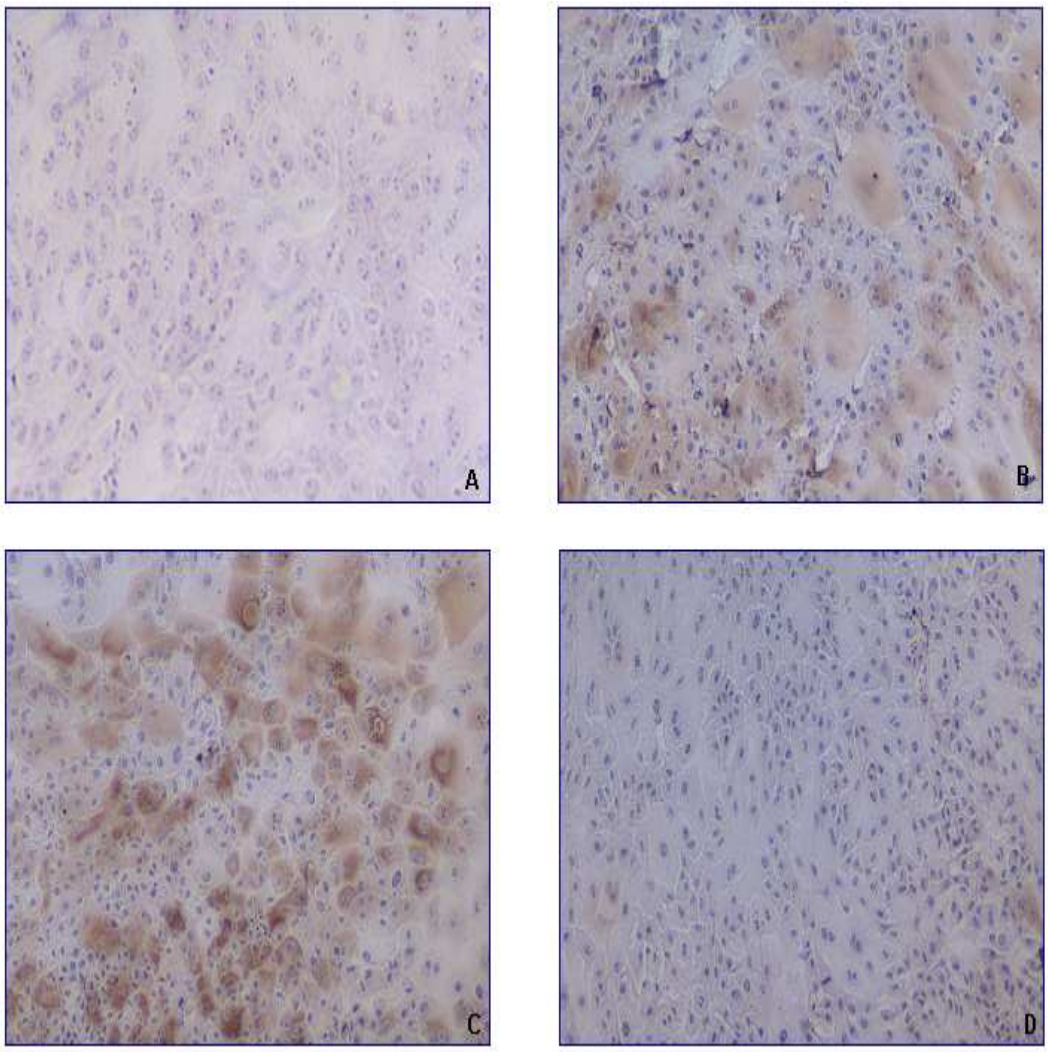

Figure 4.2: Bladder explant, stained with an anti-body for uroplakin III post exposure to direct irradiation. Brown stain indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

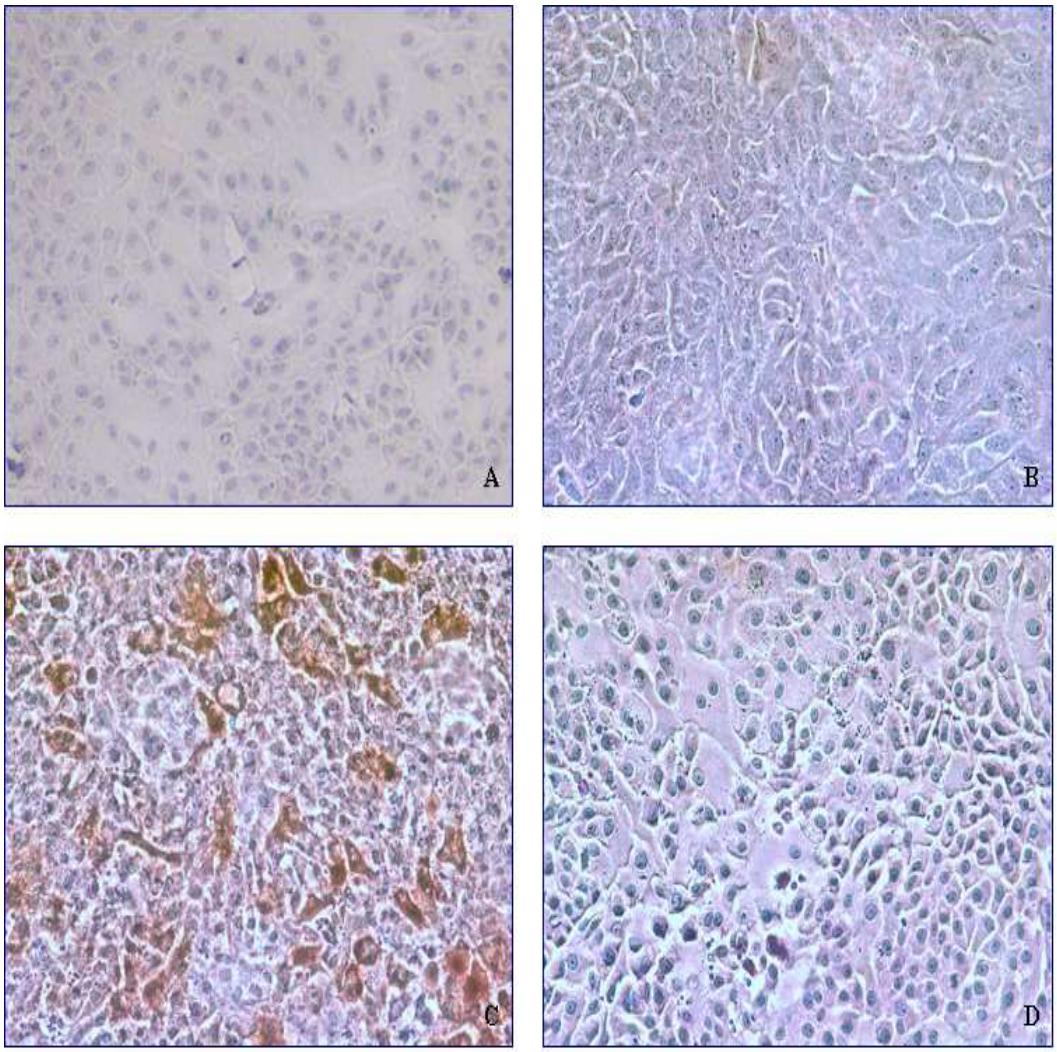

Figure 4.3: Bladder explant, stained with an anti-body for uroplakin III post exposure to ITCM generated from irradiated bladder explants . Brown stain indicates positivity. A: Negative Control B: Control C: 0.5Gy D: 5Gy

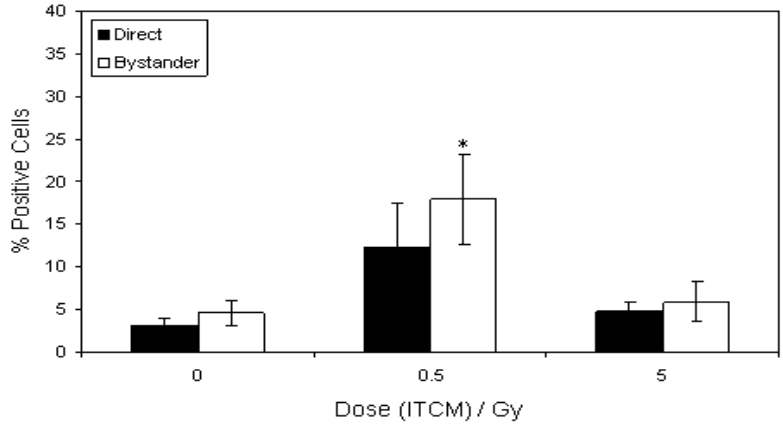

Figure 4.4: % Expression of uroplakin III in bladder explants exposed to direct radiation and the bystander signal. $*$ p < 0.05, $*$ indicates significantly different to control.

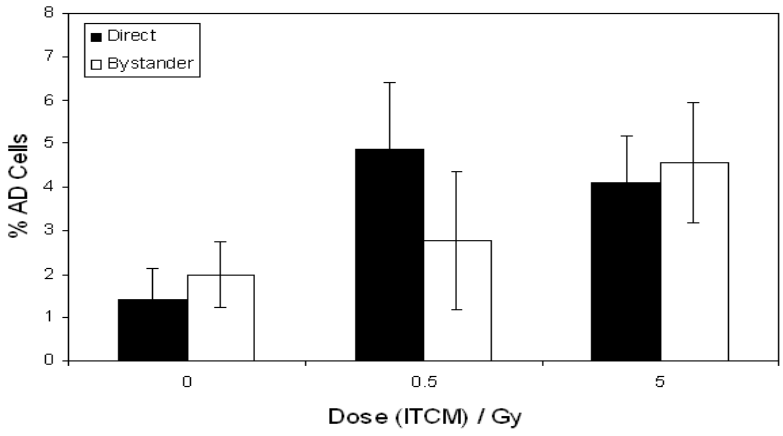

Figure 4.5: % Cells containing actin disruption in bladder explants exposed to direct radiation and ITCM.

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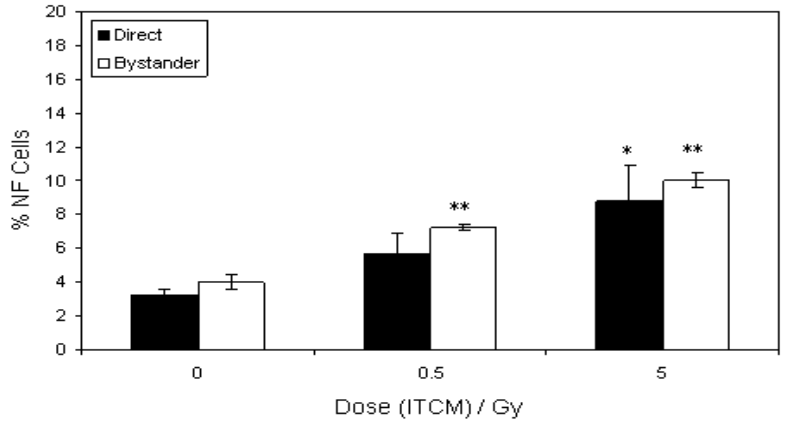

Figure 4.6: % Cells containing nuclear fragmentation in bladder explants exposed to direct radiation and ITCM. $*$ p < 0.05, $*$ indicates significantly different to control.

The level of cells containing nuclear fragments did not increase post 0.5Gy direct irradiation, however, there was a significant increase at 5Gy direct exposure from 3.22% $\pm$ 0.33 to 8.82 $\pm$ 2.16 of the population. In ITCM cultures there was a significant increase at both doses, from 3.96% $\pm$ 0.43 to 7.23% $\pm$ 0.16 and 10.03% $\pm$ 0.43 at 0.5 and 5Gy ITCM. An example of a cell containing nuclear fragments is shown in figure 4.7, panel b.

4.4 Discussion

Levels of uroplakin III, used as a marker of terminal differentiation in uothelial cells, were measured in both directly irradiated and ITCM exposed cultures. As seen in the previous chapter, there were significant differences in the response to direct radiation and ITCM. There was no significant change in the level of differentiation in directly irradiated cultures at either dose, however in ITCM exposed cultures, there was a significant increase in uroplakin III at 0.5Gy ITCM. This increase was absent at 5Gy ITCM. The increase seen at 0.5Gy ITCM is reflective of an increased level of differentiation within the culture. This has also been shown in an investigation into the bystander effect induced by high

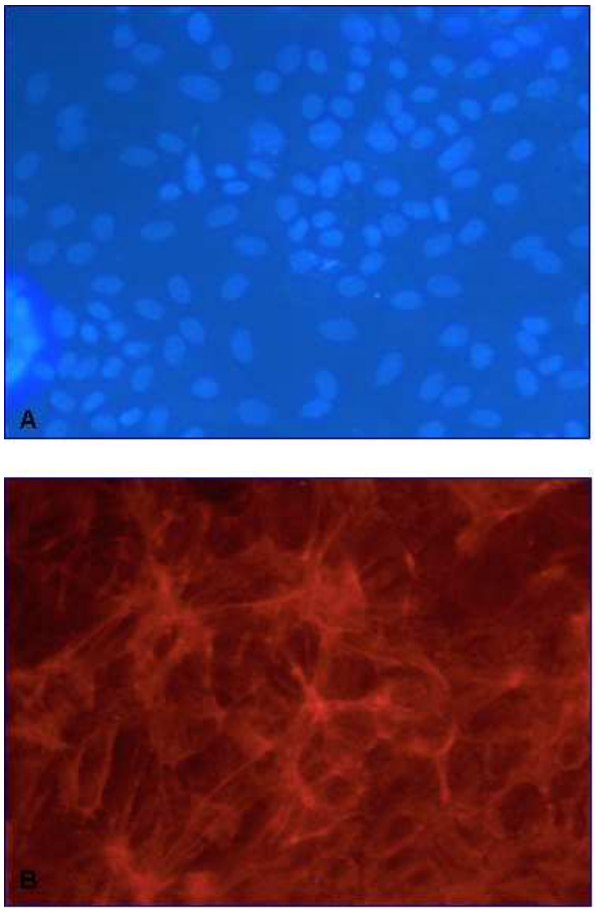

Figure 4.7: Explant stained with phalloidin and DAPI. A: F-Actin B: Nuclei

LET radiation, [Belyakov et al., 2002, 2006]. The authors suggest that this is indicative of a protective role for the bystander effect, as premature differentiation would result in the removal of these cells from the proliferating population of the tissue. The results presented here are the first indication of this effect post exposure to a bystander signal induced by γ radiation, in a model where there was no physical contact between the directly irradiated cells and the bystander cells. Interestingly, there is only a significant increase in differentiation at 0.5Gy ITCM. At 5Gy ITCM there is no change from control levels. This may indicate that the bystander effect at this dose is more toxic to the tissue, or that an active protective mechanism is turned at 0.5Gy but not 5Gy. This also provides further evidence for differences in the bystander signal generated at different doses of direct radiation. Therefore, low doses of ITCM, or bystander signal generated *in-vivo*, or a multicellular environment may be protective for the tissue, however at higher does, the bystander effect may be purely damaging. The levels of cMyc seen previously (figure 3.15) may also be responsible for the lack of differentiation seen at 5Gy ITCM. cMyc has been associated with proliferation and an escape from cell cycle, [Gardner et al., 2002], and so it is possible that the high levels of cMyc seen at 5Gy ITCM are at least partially responsible for the absence of differentiation at this dose.

The levels of uroplakin III in the directly exposed culture did not show any significant changes in expression, but a similar pattern to that seen in the ITCM treated cultures was observed. There was a slight increase in 0.5Gy irradiated cultures, which may suggest there is a similar mechanism involved here attempting to protect the tissue, however it was not as evident as in the ITCM treated cultures.

There was no significant change in f–actin distribution post either direct irradiation, or ITCM treatment. However, there were slight increases in both treatments, which may indicate that there is some link between f-actin disruption and differentiation. It also suggests that while f-actin may be quite resistant to radiation and the bystander signal, there may be some changes to its conformation post exposure. Although there have been reports of actin being sensitive to UV radiation, [Rafferty et al., 1993, Grzanka et al.,

2006] and reorganisation of f–actin has been shown post exposure to direct γ radiation [Olwell et al., 2005], the slight increase in disruption in this model is not significant.

The presence of cells containing nuclear fragments, also known as micronuclei were examined in the cultures. There was a significant increase in nuclear fragmentation post exposure to 5Gy direct radiation, although at 0.5Gy there was no change from the control cultures. However, as with cMyc and uroplakin III, there seems to be an difference in the response to the bystander effect at 0.5 and 5Gy ITCM when the levels of nuclear fragmentation were examined. There were significant increases at both doses, with significant differences between the levels at each dose. As mentioned previously, micronuclei have been observed in many bystander effect investigations, [Azzam et al., 2002, Little et al., 2002, Belyakov et al., 2003, 2005, Marozik et al., 2007] and are an indication of damage within the population.

Taken together, it seems that post exposure to ITCM the response of the bladder explant cultures as a whole is for maintenance of the tissue. The increases in Bcl2, cMyc and uroplakin III all reflect an attempt by the tissue to remove or regulate the damaged cells within the population. However, the increased levels of nuclear fragmentation indicate that there is a damaging as well as a protective role to the bystander effect. Further investigations into the nature of the bystander effect, and its overall role in a tissues response to direct radiation are needed to fully understand the implications of radiation exposure, and whether the bystander effect contributes to the damage caused by exposure, or protects cells from it.

Chapter 5

The influence of an Anti-Oxidant Diet on Bystander Signal production *in - vitro*

5.1 Introduction

As discussed in Chapter 3, much of the earlier work into the bystander effect has been performed using cell lines, and while a great deal of information has been obtained using this method, there is a need to determine the relevance of the effects seen *in-vitro* in an *in-vivo* environment. One method to monitor the bystander effect in an *in-vivo* like system is the explantation of tissue. Bystander signal production and response in the tissue can then be examined *in-vitro*. Bystander responses have been measured in cells from explanted tissue which were not directly irradiated but were present at the time of exposure using microbeam technology [Belyakov et al., 2003, 2006], and in cells and explants that were exposed to medium harvested from directly irradiated explants, using the medium transfer approach [Mothersill et al., 2001, Mothersill and Seymour, 2002b]. Some of the responses include increased apoptosis and micronucleus formation [Belyakov et al., 2003], premature differentiation [Belyakov et al., 2006], decreased cell survival

[Mothersill and Seymour, 2002b] and increased Bcl2 and cMyc expression [Mothersill et al., 2001].

Since the discovery of the bystander effect, there has been intense investigation into the nature of the signal that is thought to be produced by the irradiated cell, and transmitted to the unirradiated cells. One of the most notable processes discovered in these investigations is the generation of ROS in both irradiated and bystander cell populations [Narayanan et al., 1997, Lyng et al., 2000, Azzam et al., 2002, Shao et al., 2003a]. This has been measured directly, by examining ROS levels in bystander cells [Narayanan et al., 1997, Lyng et al., 2000, 2001], and indirectly by measuring bystander effect in cells which have been treated with free radical scavengers such as SOD and DMSO [Lehnert et al., 1997, Shao et al., 2003a]. It has been suggested that indirect effects of radiation involve induction of short lived ROS, which then leads to the production of secondary longer lived radicals, creating a type of feed forward system [Azzam et al., 2002]. A role for NAD(P)H in the propagation of ROS has also been suggested because when this enzyme is inhibited the bystander effect is abolished [Narayanan et al., 1997]. It has been shown to be activated by ROS, which in turn leads to increased ROS production [Li et al., 2001], a finding which strongly supports the feed forward system suggested by Azzam et al. [2002]. Nitric oxide has also been implicated in the bystander effect, with increased levels of NO found in cells exposed to bystander medium [Shao et al., 2003b] and increased nitrite in the medium itself [Matsumoto et al., 2001]. A significant reduction in the observed bystander effect was noted post treatment with the NO scavenger c–PTIO in both cases.

Markers of apoptosis, such as loss of mitochondrial membrane potential, have been found to be associated with the bystander effect, with significant decreases in membrane potential in epithelial cells exposed to bystander medium from 1 to 24 hours post exposure, [Lyng et al., 2000, 2001]. Loss of mitochondrial membrane potential is an integral part of apoptosis, and results in the release of cytochrome c, which leads to the activation of caspases involved in apoptosis [Green and Reed, 1998, Garland and Halestrap, 1997] (for a full discussion see section 1.5.2).

Inflammatory–type responses have also been associated with the bystander effect *in-vivo* [Lorimore et al., 2001]. An initial increase in macrophages was noted in haemopoietic tissues in mice following whole body exposure to γ rays, and was suggested to be as a result of an increased level of apoptotic cells in the area. However, the high levels of macrophages was sustained, and so this inflammatory–type response was proposed as a mode of transmission of the bystander effect *in-vivo*.

Gender has also been shown to influence the response to radiation and the bystander effect. In an investigation to determine the effect of gender, smoking status and existence of tumor on the bystander effect, Mothersill et al. [2001] found a lesser bystander effect in males, and in particular male smokers when compared to the females. When examining oncogenic signalling in males and females exposed to low dose radiation, (0.5Gy), Besplug et al. [2005] found significant differences in protein expression between the two post both acute and chronic exposure. Expression of proteins such as the Ras superfamily, protein kinase C group and AP–1 factor components were examined in the muscle, liver and spleen of the irradiated animals. For example, the level of p–PKC expression in muscle in females was significantly reduced post both acute and chronic exposure, however in males a significant decrease in expression was only observed post chronic exposure. When p–PKC was examined in the liver, there was no change in male or female post acute exposure, and an increase in expression post chronic exposure in the females. The authors conclude that this response to whole body irradiation is a complex one, and is dependent on the tissue and the sex of the animal exposed.

In the current study, bystander effects were investigated in mice that had been on an anti–oxidant diet since birth, and compared to effects in mice that had been on an a 'normal' diet. The anti–oxidant diet has previously been shown to abolish age – related cognitive decline in transgenic mice expressing elevated free radical processes [Lemon et al., 2003]. The diet was designed to reduce reactive oxygen and nitrogen species, reduce inflammation, promote membrane and mitochondrial integrity, and increase insulin sensitivity. Hence, the diet aims to preserve many of the cellular processes that are altered

by the bystander effect. A comparison of the bystander signal generated by male and female mice was also performed.

5.2 Methods and Materials

5.2.1 Animals

All animal experiments were conducted at McMaster University, Ontario Canada. The mice were C57BL/6J male x SJL female hybrids, kindly donated by Dr. C D Rollo, Life Sciences Dept, McMaster University. Four mice were maintained per cage, (27 x 12 x 15.5 cm) bedded with wood chip. A stainless steel hopper provided food ad libitum (LabDiet ^{TM} 5001, PMI Feeds) and supported a water bottle. The housing room was maintained with a 12:12 hour light:dark photoperiod, at 22 $\pm$ 2°C. Animals on the AO diet were fed mid-way through the photo period each day, all details of the diet can be found in Lemon et al. [2003], the ingredients are listed in figure 5.1. All protocols adhered to the Canada Council guidelines on animal care.

5.2.2 Tissue Culture

All mice were anaesthetised with isoflorine, and killed by cervical dislocation. The bladder and oesophagus were removed, and maintained in RPMI 1640 complete culture medium until processed, which was carried out as described in Chapter 3, section3.2.2, [Mothersill et al., 2001]. ITCM was transported to Ireland at 4°C, aliquoted, and frozen at –20°C. Aliquots were thawed and used as required for the study.

5.2.3 Cell Culture

The reporter cell line used in this study was the HPV-G cell line, which has been used routinely in the laboratory for this purpose [Mothersill et al., 2001, 2005]. Details of the HVP-G line are contained in Chapter 2, section 2.2.1.

Vitamin B12[b]	0.72 mg/day	Flax Seed oil[h]	21.6 mg/day
Vitamin B1[b]	0.72 mg/day	Folic Acid[b]	0.01 mg/day
Vitamin B3[b]	0.72 mg/day	Garlic[b]	21.6 mcg/day
Vitamin B6[b]	0.72 mg/day	Ginger[h]	7.2 mg/day
Vitamin C[b]	3.6 mg/day	Gingko Biloba[h]	1.44 mg/day
Vitamin D[b]	2.5 IU/day	Ginseng (Canadian)[h]	8.64 mg/day
Vitamin E[b]	1.44 IU/day	Green Tea Extracts[f]	7.2 mg/day
Acetyl L-Carnitine[e]	14.4 mg/day	L-Glutathione[a]	0.36 mg/day
Alpha-Lipoic Acid[e]	0.72 mg/day	Magnesium[b]	0.72 mg/day
ASA[d]	2.5 mg/day	Melatonin[g]	0.01 mg/day
Beta Carotene[b]	50.0 IU/day	N-Acetyl Cysteine[e]	7.2 mg/day
Bioflavinoids[h]	4.32 mg/day	Potassium[b]	0.36 mg/day
Chromium Picolinate[i]	1.44 mg/day	Rutin[h]	0.72 mg/day
Cod Liver Oil[b]	5.04 IU/day	Selenium[h]	1.08 mcg/day
CoEnzyme Q10[h]	0.44 mg/day	Zinc (chelated)[b]	0.14 mg/day
DHEA[g]	0.15 mg/day		

Vitamin Brands: [a]Cell Life; [b]Jamieson vitamins; [c]Jarrow Formulas; [d]Lifebrand; [e]Natural Factors; [f]Naka; [g]Promatrix; [h]Swiss Vitamins; [i]Vitamin Power Inc.

Table 5.1: The formulation of the AO Diet, from Lemon et al. [2003] with permission.

5.2.4 Irradiation

Irradiation of explants took place 24 hour post explantation. Cultures were sealed, and irradiated at room temperature, using a Caesium (γ) source in McMaster University, at a dose rate of 0.5Gy/min. The source to flask distance was 50 cm, and the field size was 6 x 6 cm. Explants were irradiated at 0.05Gy and 0.5Gy. Flasks were returned to the incubator immediately after irradiation. Control flasks were removed from the incubator, and handled under the same conditions as the irradiated explants.

5.2.5 Chemicals

2,7-dichlorofluoresin diacetate and rhodamine 123 (Sigma) (Molecular Probes) were dissolved in DMSO. All dilutions were made in buffer solutions (Ca^{2+}/Mg^{2+}PBS buffer, containing 130mM NaCl, 5mM KCl, 1mM Na_2 HPO_4, 1mM $CaCl_2$, 1mM $MgCl_2$ and 25 mM Hepes (pH 7.4)) so that the final concentration of DMSO was 0.1%. This volume of DMSO was added to controls and was shown to have no effect.

DMEM F-12, without phenol red, for the Alamar Blue ^{TM} assay was made in dH_2O using DMEM F-12 (15.6 g/L) and $NaHCO_3$ (1.2 g/L), pH 6.9 $\pm$ 0.2.

5.2.6 Mitochondrial Membrane Potential

HPV-G cells were seeded at 20,000 cells per well, in a 96 well micro-plate (Nunc, Denmark), and maintained at 37°C for 24 hours. This was found to be the optimal cell number to achieve the desired confluency (80-90%) of the cell culture post 24 hours incubation. To negate the effects of medium evaporation from the outer wells of the microplate, only the internal 60 wells were used in the study. The original medium was removed, and 50 μl ITCM was placed in each test well, for 6 hours. Post exposure ITCM was removed and the cells stained with 5 μM rhodamine 123 for 20 minutes at 37°C. The dye was then removed, and the cells washed with Ca^{2+}/Mg^{2+} PBS buffer. The florescence intensity in each well was measured with excitation wavelength at 485nm and emission at 535nm,

with a TECAN GENios microplate reader. The resulting fluorescence intensities were normalised with regard to the control for each plate.

5.2.7 Reactive Oxygen Species

Induction of reactive oxygen species by certain groups in the study was initially measured using the confocal microscope and 2,7–dichloroflourescein diacetate, (DCF). DCF emits green fluorescence when oxidized by reactive oxygen species [Yang, 1998]. 10,000 cells were seeded in glass bottomed petri dishes, with 1 ml fresh RPMI and were maintained in the dishes for 24 hours. Cultures were washed twice with a buffer containing Ca^{2+}/ Mg^{2+}PBS buffer. The original medium was removed from cultures, and 200 μl ITCM placed on the cells. Post 6 hrs exposure, the ITCM was removed, and the cells were loaded with 5μM DCF for 20 minutes at 37°C. The culture was with washed with Ca^{2+}/Mg^{2+} PBS buffer. DCF was excited at 488 nm and fluorescence emission at 525 nm was recorded using a Zeiss LSM confocal microscope. The fluorescence intensities in 4 areas per dish was used as a quantitative measure of ROS levels in control and treated cells.

However, due to the volumes of media required for the ROS assay on the confocal microscope, it was preferable to use the microplate reader for this assay, as 50 μl of ITCM was required per well on a 96 well plate, compared to 200 μl per dish when using the confocal microscope. Therefore quantitative results for the ROS assay were measured using a microplate reader. 96 well plates were set up as for the mitochondrial membrane potential assay described above. Post a 6 hour incubation with 50μl ITCM, the test medium was removed, and the cells loaded with DCF. The cells were incubated for 20 minutes at 37°C, the dye removed, and the cells incubated with Ca^{2+}/Mg^{2+} PBS for a further 20 minutes. The plates were then read in the TECAN GENios ^{TM} microplate reader, at an excitation wavelength of 488nm, and an emission wavelength of 525 nm. The resulting fluorescence intensities were normalised with regard to the control for each plate.

5.2.8 Cell Viability Assay

HPV-G cells were seeded at 2,000 cells per well, in a 96 well micro-plate (Nunc, Denmark), and maintained at 37°C for 24 hours. This was found to be the optimal cell number to achieve the desired confluency (80-90%) of the cell culture post 96 hours incubation. To negate the effects of medium evaporation from the outer wells of the microplate, only the internal 60 wells were used in the study. The original medium was removed, and 100 μl ITCM was placed in each test well, for 96 hours. Post exposure, ITCM was removed, and the cells washed with PBS. 5% Alamar Blue ^{TM} in DMEM F-12 which did not contain phenol red, was added to the cells for 3 hours. Alamar Blue ^{TM} is a dark blue non-fluorescent resazurin dye which is reduced to a pink and highly fluorescent resorufin dye through a redox reaction in the cytoplasm, the level of which can be correlated to cellular proliferation and metabolism [O'Brien et al., 2000, Slaughter et al., 1999]. Post 3 hours exposure, Alamar Blue ^{TM} fluorescence was measured using the TECAN Genios ^{TM} microplate reader at an excitation wavelength of 540 nm and emission of 595 nm. The resulting fluorescence intensities were normalised with regard to the control for each plate.

5.2.9 Statistical Analysis

All statistical significance was determined using the Students t-test. 60 mice were used in this study, with 15 mice per group. Each assay was preformed in triplicate at minimum in 4-7 independent experiments.

5.3 Results

5.3.1 Mitochondrial Membrane Potential

Mitochondrial membrane potential was recorded in cells that had been exposed to ITCM from unirradiated and irradiated tissue after 6 hours. When exposed to ITCM generated

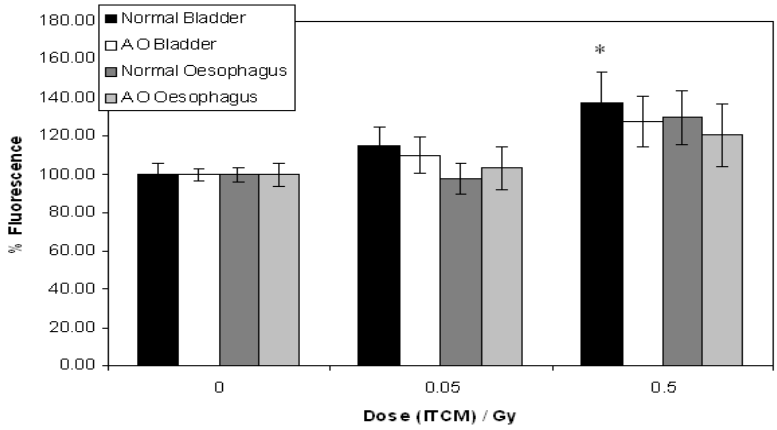

Figure 5.1: Rhodamine 123 fluorescence in HPV-G cells post 6 hour exposure to ITCM from male normal and AO bladder and oesophagus explants. $*$ indicates $p < 0.05$.

from normal male bladders, cells showed a small increase in fluorescence at 0.05Gy ITCM and a significant increase at 0.5Gy ITCM, to 129.59% $\pm$ 14.36 ($p = 0.045$) of control values, shown in figure 5.1. This increase in fluorescence intensity is indicative of a hyperpolarisation of the mitochondrial membrane potential. In the cells exposed to ITCM generated from the bladders of mice on the AO diet a similar increase in fluorescence intensity was evident at 0.5Gy ITCM, 124.11% $\pm$ 11.94 ($p = 0.045$) of control values. Although the increase from the 0.5Gy AO ITCM is slightly lower than that seen at 0.5Gy normal ITCM, there was no significant difference between the two.

When cells were exposed to ITCM generated from male normal and AO oesophagus, a similar increase in fluorescence was observed, see figure 5.1. In both cases, there was no significant difference between control cells and cells exposed 0.05Gy ITCM. However, at 0.5Gy normal ITCM there was a significant increase to 136.19% $\pm$ 12.01 ($p = 0.011$). Although there was also an increase in fluorescence in the cells exposed to AO ITCM (129.15% $\pm$ 15.41, $p = 0.059$), it was not significant.

In cells that were exposed to ITCM from normal bladder or oesophagus from female mice, there was no change in fluorescence from controls at either dose. The same was

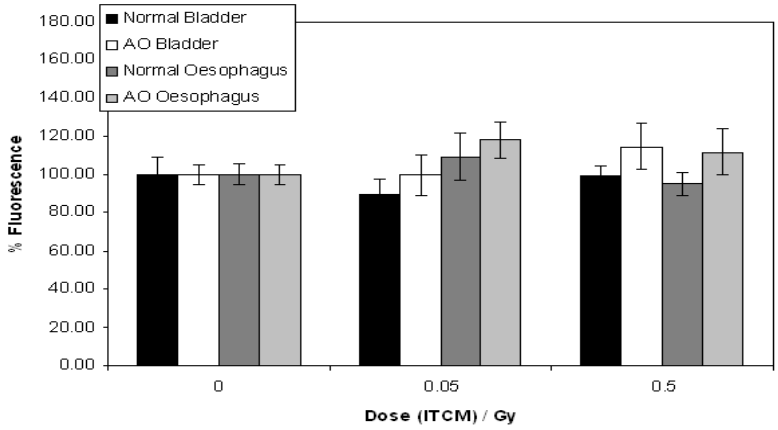

Figure 5.2: Rhodamine 123 fluorescence in HPV-G cells post 6 hour exposure to ITCM from female normal and AO bladder and oesophagus explants.

true of cells treated with ITCM from AO female mice, which also showed no change, see figure 5.2.

5.3.2 Reactive Oxygen Species

ROS was recorded in cells exposed to ITCM using the confocal microscope for initial tests, and the microplate reader for the definitive tests. When exposing cells to ITCM for the confocal microscopy assay, a higher volume of ITCM was need per petri dish (200μl) when compared to that required for the microplate reader (50μl), therefore the DCF fluorescence assay was performed using the microplate reader, as the volume of ITCM per group was limited. The initial results from the confocal microscope suggested a large increase in ROS in cells exposed to ITCM from male normal and AO bladder however, a significantly lower increase in ROS was observed for the ITCM from the AO mice (figure 5.3).

However, when ROS was measured using the microplate reader, no increase in DCF fluorescence was recorded in any male or female groups, as shown in figures 5.4 and 5.5.

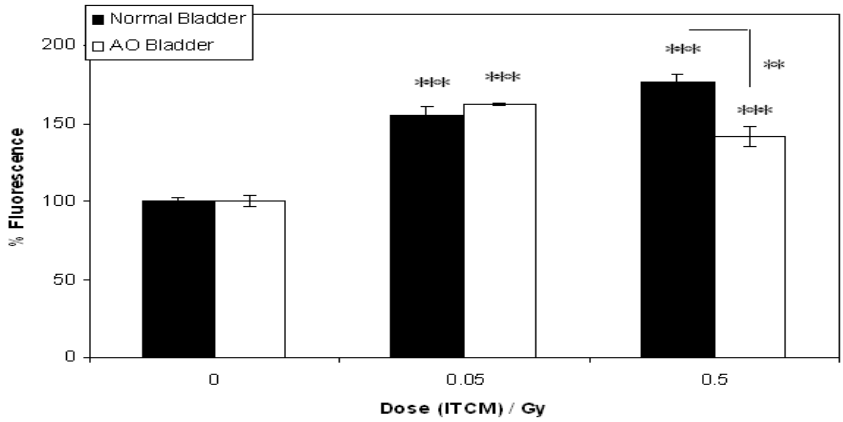

Figure 5.3: Initial ROS results using confocal microscopy. DCF fluorescence in HPV-G cells post 6 - hour exposure to bladder ITCM generated from male mice.

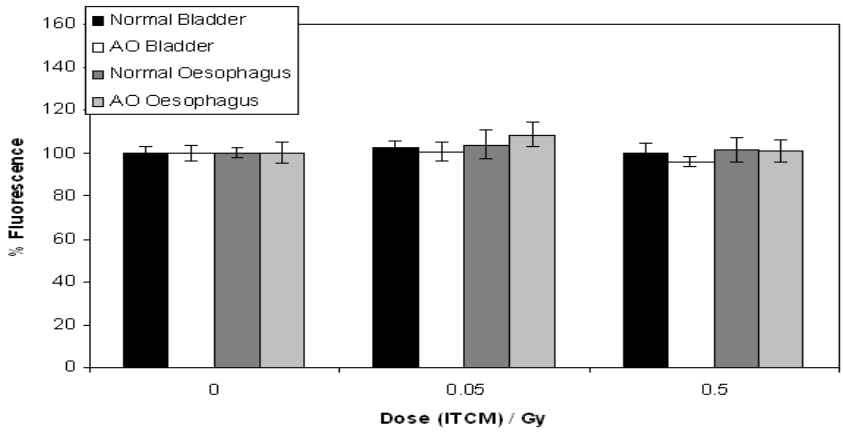

Figure 5.4: DCF fluorescence in HPV-G cells post 6 - hour exposure to bladder and oesophagus ITCM generated from male mice.

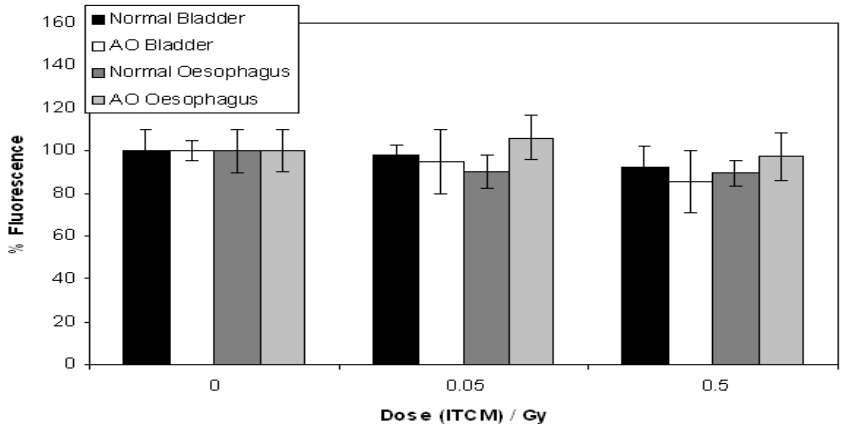

Figure 5.5: DCF fluorescence in HPV-G cells post 6 - hour exposure to bladder and oesophagus ITCM generated from female mice.

5.3.3 Cell Viability

Figure 5.6 and 5.7 show the viability of HPV-G cells post 96 hours exposure to male and female ITCM generated from normal and anti-oxidant bladder explants. There was a small but significant increase in cell viability when cultured in 0.5Gy ITCM from male normal bladder explants (105% $\pm$ 1.47, p = 0.026), however this increase was absent in cells cultured in ITCM from the AO explants, as shown in figure 5.6. In contrast to this, there was no significant change in viability when the cells were cultured in ITCM from either the normal or AO oesophagus explants (figure 5.6).

As with the mitochondrial membrane and ROS assays, the response to ITCM generated from female bladder explants was different to that of the male bladder explants in the Alamar Blue ^{TM} assay. Interestingly there was no change in cells exposed to 0.05 and 0.5Gy ITCM from female normal bladders, however when exposed to 0.5Gy ITCM from AO bladder, there was a significant decrease in viability to 90.8% $\pm$ 2.25 (p = 0.008). As with the male oesophagus ITCM, there was no change in cell viability at either dose of ITCM from normal or AO female oesophagus, see figure 5.7.

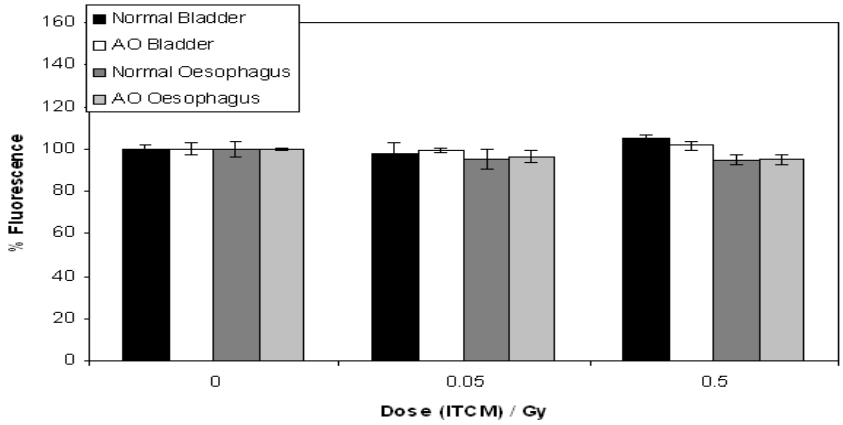

Figure 5.6: Alamar Blue ^{TM} fluorescence in HPV-G cells post exposure to ITCM from male bladder explants. $*$ indicates p < 0.05

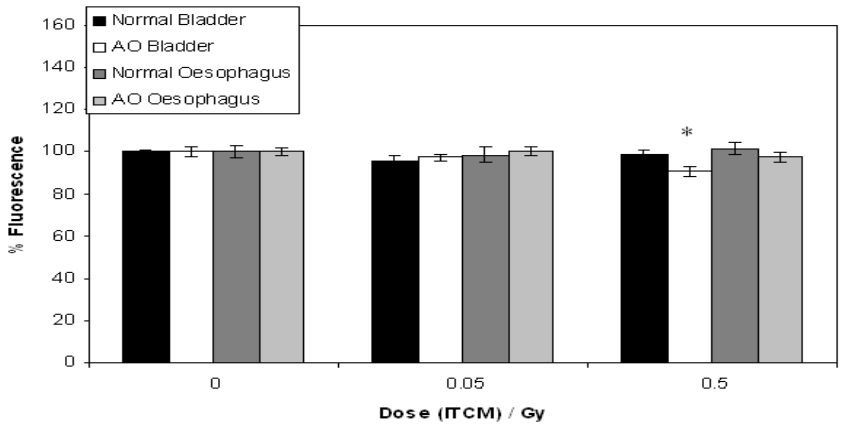

Figure 5.7: Alamar Blue ^{TM} fluorescence in HPV-G cells post exposure to ITCM from female bladder explants.

5.4 Discussion

When the results from ITCM generated from animals not on the AO diet are examined, it is clear that the ITCM from some of the groups elicited a bystander response in the HPV-G cells. However, both the signal generated, and the subsequent response to this signal is complex. The type of signal produced is shown to be dependant on both the type of tissue irradiated and the gender of the animal.

Using rhodamine 123 fluorescence as an index of mitochondrial membrane potential, a significant hyperpolarisation of the membrane post exposure to male bladder ITCM at 0.5Gy was observed. Hyperpolarisation of the mitochondrial membrane potential has been associated with very early stages of apoptosis and is thought to be a transient increase occurring prior to depolarisation [Marzo et al., 1998, Scarlett et al., 2000, Khaled et al., 2001]. In an investigation into staurosporine–induced apoptosis, Scarlett et al. [2000] found that there was an initial increase mitochondrial membrane potential, which was followed by depolarisation, release of cytochrome–c and apoptosis. When examining the effect of interleukin–3 (IL–3) withdrawal from a IL–3 dependent cell line to elucidate the mechanisms of apoptosis, Khaled et al. [2001] found an increase in the mitochondrial membrane potential within two hours of withdrawal. This increase was not associated with an increase in ROS, as this did not occur until 20–24 hours later. In the current study mitochondrial membrane potential increased significantly to 129.57% $\pm$ 14.3 of the control value, within 6 hours of exposure to ITCM from male bladder explants. Interestingly, this was not associated with an increase in ROS over the same time period. The increase in mitochondrial membrane potential could be associated with early signs of apoptosis, although many previous studies into the bystander effect have found a decrease in potential at 6 hours [Lyng et al., 2000, 2001, Mothersill et al., 2005]. However, in these investigations, the bystander signal may have been a stronger one, and was capable of inducing a calcium flux in bystander cells 30 seconds post exposure. In preliminary experiments in this study, no significant increases in calcium were observed post exposure (data not shown). A significant increase in ROS was also noted in these investigations,

however when the ROS was measured in this investigation using the microplate reader no increase was noted, although in initial experiments using confocal microscopy, a significant increase in ROS was observed. This may be due to the increased number of cells being monitored in the microplate, and so any increase in ROS was diluted in a greater population of cells and not detected. To determine if individual cells had increased levels of ROS, the complete study would have to be conducted on the confocal microscope however as mention above, due to the limited availability of ITCM, and the volume required for the investigation to be performed using the confocal microscope, the study was carried out using the microplate reader.

For similar reasons, it was decided to use a cell viability assay which measured cellular metabolism and proliferation over a 96 hour period, rather than the standard clonogenic assay. In this case, the volume of media used in the viability assay is substantially less than that used in a clonogenic assay (100μl compared to 5ml). The Alamar Blue ^{TM} assay was performed over 96 hours in an attempt to measure mitotic cell death as well as apoptosis. Therefore, the assay was similar to the clonogenic assay, however is a much faster and media economic means of measuring mitotic cell death.

Cells exposed to ITCM generated from male normal oesophagus did not show any bystander effects in any of the endpoints measured. This suggest that there were significant differences between the two tissues, an effect noted in an earlier investigation in this project (see Chapter 3). This may indicate that the oesophagus is more sensitive to direct irradiation as it does not seem to be producing a bystander signal, which may be a protective mechanism for the tissue, [Belyakov et al., 2002, 2006, Mothersill and Seymour, 2003].

The hyperpolarisation of the mitochondrial membrane observed in cells post exposure to male normal bladder ITCM, was absent in cells exposed to ITCM from the AO treated group. In fact, there was no evidence of a bystander effect from the male AO group from either the bladder or oesophagus. This suggests that the bystander effect that was present in the normal group was abolished by the AO diet, and possibly indicates the

importance of ROS in the response to direct radiation and the production of a bystander signal, despite the lack of ROS in the cells treated with normal ITCM. However, it is also possible that the anti-inflammatory properties of the AO diet contributed to the lack of a bystander effect, as this has also been shown to be play a significant role in bystander signal production [Lorimore et al., 2001, Lorimore and Wright, 2003].

Comparing these results with those obtained from the female ITCM, it is clear that there is a significant difference between the genders, a phenomenon also noted by Mothersill et al. [2001]. There was no significant difference between the controls and exposed cells with either normal or AO bladder or oesophagus ITCM. However, when the results from the Alamar Blue ^{TM} cell viability assay were examined, there was a significant drop in viability post exposure to AO bladder ITCM. There was no change in viability in any of the other groups. This again shows a clear difference between the genders, as this was the exact opposite of the effect seen in the males, and points to a completely different bystander signal being generated by the AO female bladders. The main response in the male was the hyperpolarisation of the mitochondrial membrane, indicative of early apoptosis, while the only measurable alteration of any endpoint in the females is decreased metabolism, possibly pointing to mitotic cell death as opposed to apoptosis. Furthermore, the fact that this decrease was seen only post exposure to the AO bladder ITCM indicates that the diet may somehow sensitise the bladder to radiation, leading to production of a weak bystander signal.

When the results from the normal and AO exposed cells are compared, it seems that the AO diet was extremely effective in decreasing or abolishing the bystander signal generated by male bladder tissue. However, there was no measurable effect on either the male or female oesophagus, as these tissues failed to produce a signal. In the case of the of female bladder, the only evidence of an effect of the AO diet is the production of a bystander signal that resulted in a reduction in cell viability. The reason normal females lack a bystander effect may be due to the different levels of hormones in the males and females. Oestrogen has been suggested as an anti-apoptotic hormone [Meda et al., 2000]

and has been shown to be a strong anti–oxidant [Behl et al., 1997, Behl, 2002], thus enabling the body to be more capable of dealing with insults. The level of oestrogen in the female tissues may allow them to deal with radiation better than the males, leading to a decreased bystander effect. If the bystander effect is seen as protective, [Belyakov et al., 2006, Mothersill and Seymour, 2003], the females may be more susceptible to long term damage, as damaged cells would not be removed from the tissue.

Chapter 6

The influence of an Anti-Oxidant Diet on bystander signal generation in whole body irradiated mice

6.1 Introduction

In the previous chapter the bystander effect from *in-vitro* irradiated bladder and oesophagus was examined, and a comparison between mice on a normal or anti-oxidant diet was made. Although that study and others [Mothersill et al., 2001, Mothersill and Seymour, 2002b, Belyakov et al., 2006, 2005], more accurately reflect an *in-vivo* environment when compared to studies using cell lines, the tissue fragments were exposed to radiation *in-vitro*, and therefore the results do not reflect the response of the tissue or generation of a bystander signal post whole body radiation. However, as mentioned by Mothersill et al. [2001], it is very difficult to measure a bystander effect *in-vivo*, as it may be masked by other cellular processes within the body, and it would be extremely difficult to determine if any response measured was due to a bystander signal generated from exposed cells. Therefore, to determine what bystander signal is generated post whole body irradiation, a similar technique as used in chapter 5 was used, except that the tissues were harvested

and explanted post whole body radiation. Mothersill et al. [2005] employed this method to examine the bystander effect generated by two different strains of mouse, to determine if genetic factors influence bystander signal production. Significant differences between the two strains were observed, with CBA/Ca mice, inducing no response in the endpoints examined, while a significant bystander effect was observed in cells exposed to ITCM from the C57BL/6 mice.

Other groups have developed various methods to examine the bystander effect *in-vivo*, or in an *in-vivo* like environment. Lorimore et al. [2001] examined the bystander effect in the haemopoietic tissue of mice post exposure to whole body irradiation. Significant increases in macrophages and neutrophils were observed, along with increases in superoxide generation. The authors suggest that this inflammation may provide a mechanism for the bystander effect *in-vivo*, as macrophages are known to release superoxide capable of DNA damage.

Cell clusters have also been used to attempt to determine the relevance of the bystander effect in an *in-vivo*–like environment [Bishayee et al., 1999, Howell, 2002, Persaud et al., 2005]. Many of these investigations have shown bystander effects in cell clusters containing radiolabeled cells. Some of the responses noted include increased cell death [Bishayee et al., 1999, Howell, 2002] and increased levels of mutations [Persaud et al., 2005]. Belyakov et al. [2005] used an *in-vitro* 3–dimensional tissue culture system, which allowed bystander effects to be measured in the unirradiated portion of the tissue, post exposure to α particles from a microbeam. An increase in both micronuclei and apoptosis was measured in cells up to 1mm away from the site of exposure, indicating that a strong bystander signal was generated within the tissue.

As in the previous chapter, bystander effects generated from both male and female mice either on a normal or an anti–oxidant diet were examined. The results were also compared to those obtained in chapter 5, so that the bystander effect induced by *in-vitro* and *in-vivo* irradiation could be examined, and the effect of the anti–oxidant diet in both groups assessed. The anti–oxidant diet was found to be more effective in reducing the bystander

effect in the male oesophagus than the bladder *in-vitro*, although the small increase in viability noted in cells treated with ITCM from male normal bladder was indeed absent in cells treated with AO ITCM. However, in the case of the oesophagus, there was a large increase in the mitochondrial membrane potential in cells treated with ITCM from normal mice, and no change from the controls in cells treated with AO ITCM, suggesting that the AO diet abolished this effect in these cells. The present chapter will investigate the effect of the AO diet on bystander signal generation post whole body exposure, and determine if the diet was more effective post *in–vitro* or *in–vivo* irradiation.

6.2 Methods and Materials

6.2.1 Animals

All animals were maintained as described in Chapter 5, section 5.2.1.

6.2.2 Irradiation

Mice were exposed to 0.05Gy or 0.5Gy total body γ –irradiation at a dose rate of 0.5Gy/min, using a Caesium irradiator in McMaster University, Canada. Animals were placed into specially designed housing for exposure, and controls were sham irradiated. 1 hour post exposure, the bladder and oesophagus were removed and processed for explantation.

6.2.3 Tissue Culture

All mice were anaesthetised with isoflorine, and killed by cervical dislocation. The bladder and oesophagus were removed, and maintained in RPMI 1640 complete culture medium until processed, which was carried out as described in Chapter 3, section3.2.2, [Mothersill et al., 2001]. ITCM was transported to Ireland at 4°C, aliquoted, and frozen at –20°C. Aliquots were thawed and used as required for the study.

6.2.4 Cell Culture

The reporter cell line used in this study was the HPV-G cell line, which has been used routinely in the laboratory for this purpose [Mothersill et al., 2001, 2005]. Details of the HVP-G line are contained in Chapter 2, section 2.2.1.

6.2.5 Chemicals

All chemicals used are as described in Chapter 5, section 5.2.5. Fluo 3 and Fura Red acetoxymethyl (AM) ester (Molecular Probes) were dissolved in DMSO.

6.2.6 Mitochondrial Membrane Potential

This assay was performed as described in Chapter 5, section 5.2.6.

6.2.7 Ratiometric measurement of Calcium Levels.

Cells were seeded in glass bottomed petri dishes, with 1 ml fresh RPMI. They were maintained in the dishes for 24 hours, and then prepared for the calcium measurements. Intracellular calcium levels were measured using two visible wavelength calcium sensitive dyes, Fluo 3 and Fura Red. Fluo 3 exhibits an increase in green fluorescence upon binding to calcium, whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The Fluo 3 / Fura Red ratio is a good indicator of intracellular calcium levels [Lipp and Niggli, 1993]. Initially, cells were loaded with the calcium sensitive dyes by incubation with 3μM Fluo 3 and 3μM Fura Red acetoxymethyl esters for 1 hour in Ca^{2+}/ Mg^{2+}PBS buffer at 37°C. The dye was removed, and the culture washed with Ca^{2+}/ Mg^{2+}PBS buffer. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm, and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Zeiss LSM confocal microscope. The culture was scanned for 30 seconds to determine a base line, ITCM was added to the culture and the scan was continued for 270 seconds, [Lyng et al., 2000]

6.2.8 Reactive Oxygen Species

This assay was performed as described in Chapter 5, section 5.2.7 on the TECAN GENios ^{TM} microplate reader.

6.2.9 Cell Viability Assay

This assay was performed as described in Chapter 5, section 5.2.8

6.2.10 Statistical Analysis

One–way ANOVA was used to determine statistical significance in each assay, and the Students t-test was used to determine significance between individual doses in different experimental groups. 60 mice were used in this study, with 15 mice per group. Each assay was performed in triplicate at minimum in 3-10 independent experiments, except in the case of the calcium measurements where the calcium level was measured in two separate samples in 3 independent experiments.

6.3 Results

6.3.1 Mitochondrial Membrane Potential

Mitochondrial membrane potential was recorded in cells that had been exposed to ITCM from explanted tissue from unirradiated and irradiated animals for 6 hours. When exposed to 0.05Gy ITCM from male normal bladder, HPV-G cells showed a significant drop in mitochondrial membrane potential to 84.74 % $\pm$ 4.1. This depolarisation of the membrane was absent in cells exposed to 0.5Gy ITCM from the same group. In cells exposed to ITCM from the AO bladder no change from the control levels were observed at either dose, see figure 6.1.

When exposed to ITCM from normal oesophagus, cells showed no change from control values at 0.05Gy, however at 0.5Gy ITCM an increase in fluorescence to 118.16% $\pm$ 9.04

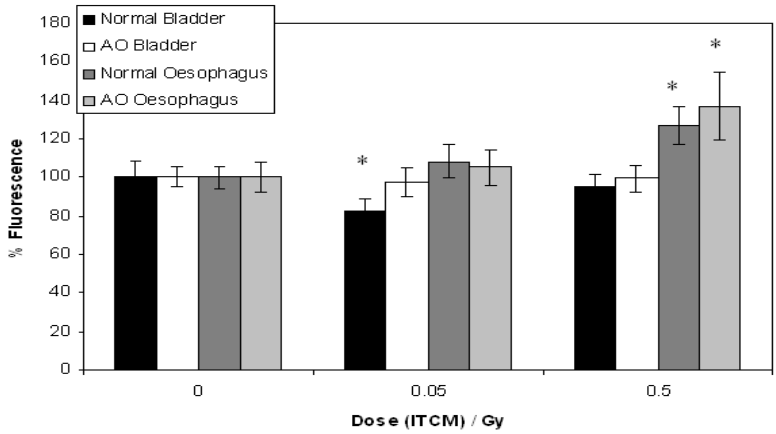

Figure 6.1: Rhodamine 123 fluorescence in HPV-G cells post 6 hour exposure to ITCM from male normal and AO bladder and oesophagus explants. Error bars indicate SEM, $*$ $p < 0.05$.

($p = 0.047$) of control values was observed. A similar hyperpolarisation was observed post exposure to 0.5Gy ITCM generated from AO oesophagus, with the fluorescence values rising to 130.8% $\pm$ 13.59 of controls.

No significant change in fluorescence was observed in cells exposed to ITCM from any female group (figure 6.2)

6.3.2 Reactive Oxygen Species

ROS was recorded in cells post 6 hours exposure to ITCM from explanted tissue from unirradiated and irradiated animals. When exposed to male normal or AO bladder ITCM, no significant increase in ROS was observed at either dose, (figure 6.3). However, when exposed to ITCM from normal oesophagus, there was a significant increase at both doses, 122.11 $\pm$ 5.5% and 113.29 $\pm$ 7.3% at 0.05 and 0.5Gy ITCM respectively, figure 6.3). This response was absent in cells exposed to AO oesophagus ITCM.

As observed for the mitochondrial membrane potential assay, no significant change in

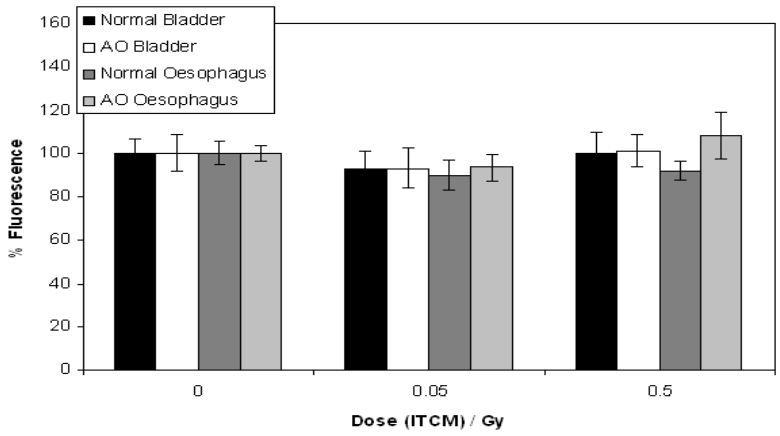

Figure 6.2: Rhodamine 123 fluorescence in HPV-G cells post 6 hour exposure to ITCM from female normal and AO oesophagus explants. Error bars indicate SEM.

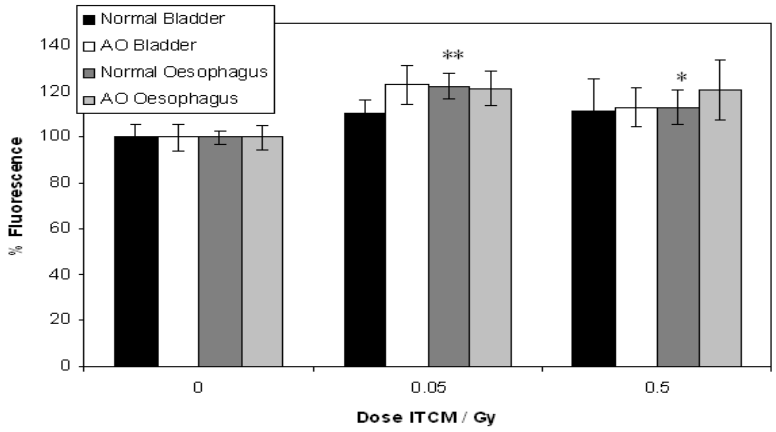

Figure 6.3: DCF fluorescence in HPV-G cells post 6 hour exposure to ITCM from male normal and AO bladder and oesophagus explants. Error bars indicate SEM $*$ indicates p < 0.05, $**$ indicates p < 0.001.

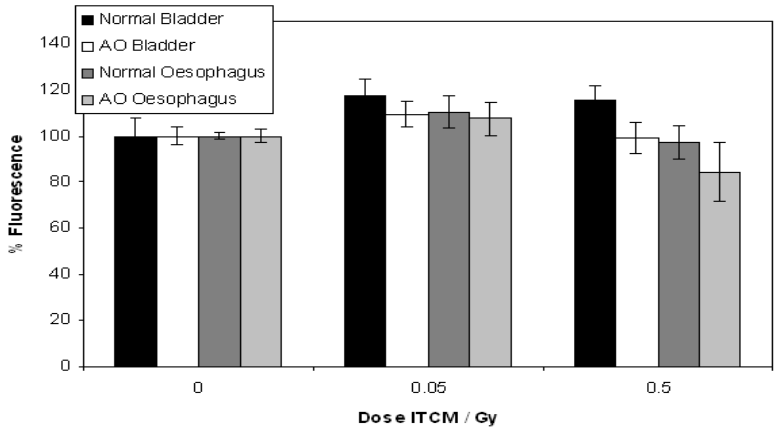

Figure 6.4: DCF fluorescence in HPV-G cells post 6 hour exposure to ITCM from female normal and AO oesophagus explants. Error bars indicate SEM.

ROS levels was observed from ITCM generated for any female group (figure 6.4).

Group	*Fluo 3/Fura Red*			
	t0	t30	t120	t300
Male Normal Bladder				
0Gy	0.48 ± 0.03	0.51 ± 0.02	0.47 ± 0.03	0.47 ± 0.03
0.05Gy	0.45 ± 0.004	0.48 ± 0.04	0.49 ± 0.004	0.52 ± 0.02
0.5Gy	0.50 ± 0.03	0.55 ± 0.03∗	0.55 ± 0.01	0.51 ± 0.02
Male AO Bladder				
0Gy	0.49 ± 0.05	0.53 ± 0.07	0.47 ± 0.05	0.47 ± 0.05
0.05Gy	0.45 ± 0.04	0.48 ± 0.09	0.42 ± 0.05	0.43 ± 0.08
0.5Gy	0.40 ± 0.04	0.53 ± 0.08	0.43 ± 0.04	0.40 ± 0.04

Table 6.1: Ratiometric calcium measurements (± SEM) in HPV-G cells post exposure to ITCM generated from male normal and AO bladder explants. ∗ indicates $p < 0.05$.

6.3.3 Calcium Measurements

Table 6.1 shows the ratiometric values that indicate intracellular calcium levels in cells at 0, 30, 120 and 300 seconds post exposure to ITCM from male normal and AO bladder. No change was noted in cells exposed to 0.05Gy normal ITCM, however increased calcium levels were observed in the cells exposed to 0.5Gy normal ITCM at 30 seconds. No increases were observed in the AO ITCM treated cells.

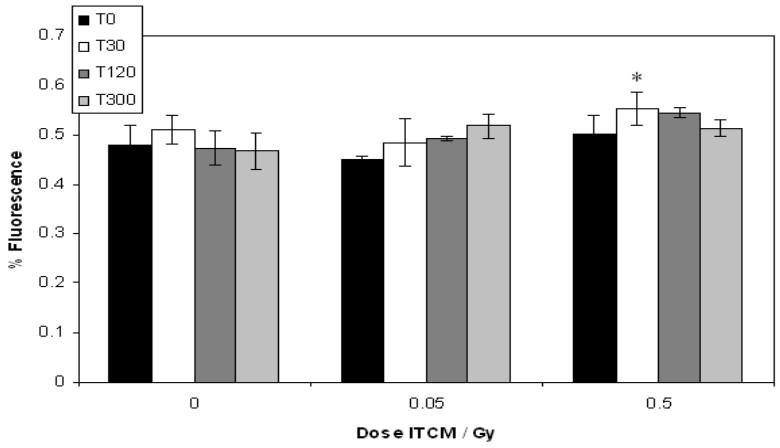

Figure 6.5: Ratiometric calcium measurements in HPV-G cells post exposure to ITCM generated from male normal bladder explants at selected time points. Error bars indicate SEM $*$ indicates p = < 0.05.

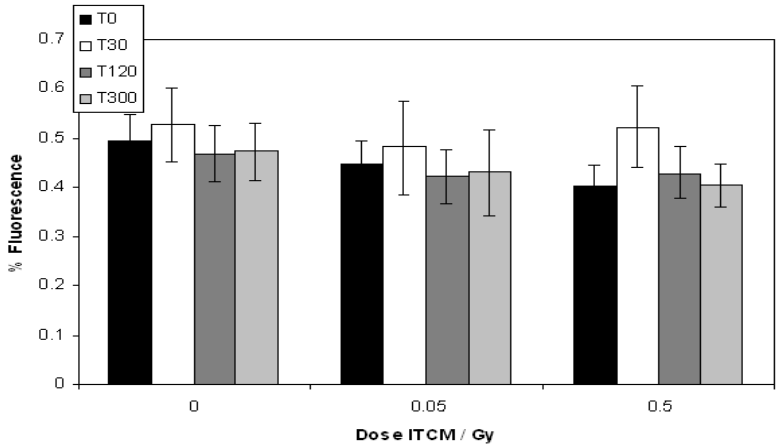

Figure 6.6: Ratiometric calcium measurements in HPV-G cells post exposure to ITCM generated from male AO bladder explants at selected time points. Error bars indicate SEM.

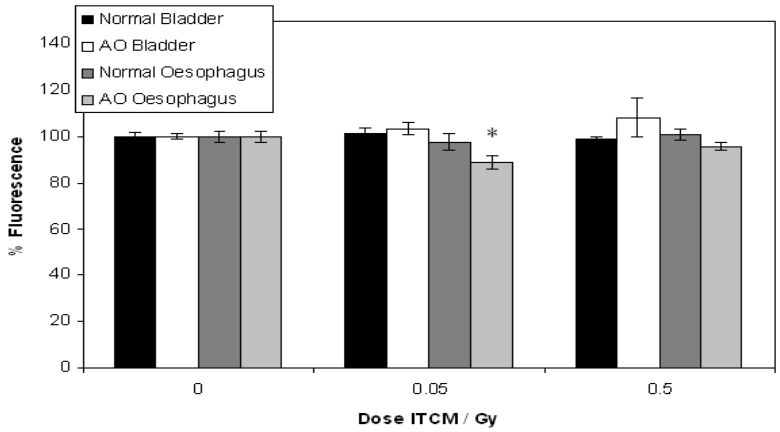

Figure 6.7: Alamar Blue fluorescence in HPV-G cells post 6 hour exposure to ITCM from male normal and AO bladder and oesophagus explants. Error bars indicate SEM.

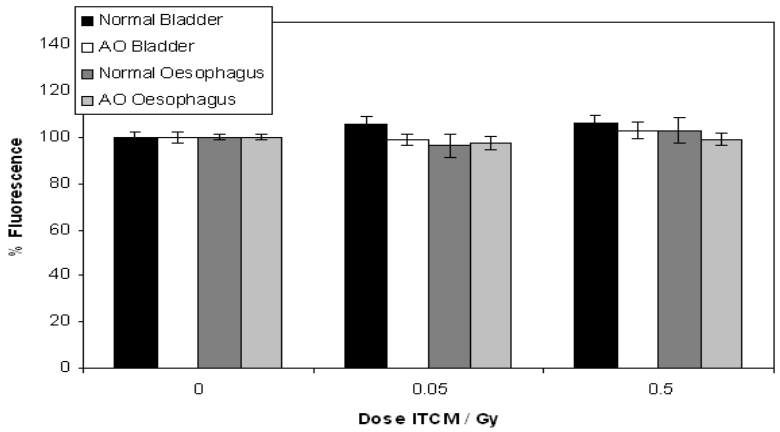

Figure 6.8: Alamar Blue fluorescence in HPV-G cells post 6 hour exposure to ITCM from female normal and AO oesophagus explants. Error bars indicate SEM.

6.3.4 Cell Viability

Cell viability, measured using the Alamar Blue ^{TM}assay, for cells exposed to ITCM from male tissues is shown in figure 6.7. There was no significant difference in the normal or AO bladder, or in the normal oesophagus. However, in the case of cells exposed to ITCM from male AO oesophagus, there was a significant decrease in viability to 89% ± 2.65 % of control values.

When cells were exposed to ITCM generated from normal or AO female tissues, no change in viability was noted in any group (figure 6.8).

6.4 Discussion

From the results shown above, it is clear that a bystander signal was generated by individual tissues post whole body exposure to low doses of γ radiation. There were also significant differences between both the normal and AO, and male and female mice. In the case of the normal male mice, there was a bystander effect generated by both bladder and oesophagus. Cells treated with 0.05Gy ITCM generated from normal male bladders showed a significant decrease in mitochondrial membrane potential, a response which has been observed in cells exposed to bystander medium previously [Lyng et al., 2000, 2002a, Mothersill et al., 2005], and is a marker of apoptosis, [Brenner et al., 1998, Marzo et al., 1998]. In addition, an increase in calcium was observed in cells treated with 0.5Gy ITCM, a response which has also commonly been associated with the bystander effect, [Lyng et al., 2000, 2002a, 2006b, Mothersill et al., 2005]. However, the large calcium flux seen in these studies was not observed here, instead a small increase was noted immediately post exposure, similar to that seen in cells exposed to medium from CBA/Ca mice in an investigation into the influence of genetic factors on bystander signal generation post whole body irradiation [Mothersill et al., 2005]. This suggests that the bystander effect produced from the mice in this study, although clearly causing a response in the cells treated, is different from that seen in other *in-vivo* models, again highlighting the influence of genetic background on bystander signal production. No change in ROS levels or viability was observed in cells exposed to male bladder ITCM of either dose, indicating that the bystander effect can be transduced via different pathways, as increased ROS and reduced viability is often associated with loss of mitochondrial membrane potential. Whether this indicates that the bystander signal generated from the bladder of these animals is a different one, does not follow the same pathway, or whether there are effects that have not been measured in the endpoints examined here is not known.

In contrast to this, cells exposed to ITCM generated from AO male bladder showed no indication of a bystander effect at all. The loss of mitochondrial membrane potential, and increased calcium were absent in these cells, suggesting that the AO diet reduced

the bystander signal production significantly in these animals. This is similar to the response seen in the *in-vitro* section of the study (figure 5.1), and suggests that the levels of anti-oxidants in the body at the time of exposure had a significant influence on bystander signal production. As mentioned previously, addition of anti-oxidants to cells exposed to the bystander signal (via single cell irradiation, or medium transfer) has been shown to abolish the bystander effect in both *in-vitro* models [Lehnert et al., 1997, Narayanan et al., 1997, Azzam et al., 2002, Shao et al., 2003a] and *in-vivo*–like systems, [Bishayee et al., 2000, Persaud et al., 2005]. It is interesting to note that the AO diet was capable of reducing the bystander effect generated from the bladder in both the *in-vitro* and *in-vivo* exposures, even though the response seen in the normal animals was quite different (hyperpolarisation at 0.5Gy ITCM *in-vitro* versus depolarisation at 0.05Gy ITCM *in-vivo*). This suggests that the higher levels of anti-oxidants in the animals were protective of the mitochondria regardless of the strength of the insult. The fact that anti-oxidants controlled the bystander effect despite the fact that this effect does not seem to be associated with an increase in ROS indicates that there are many unknown aspects of bystander signal transduction pathways at present.

In the case of the normal male oesophagus, there was increased ROS in cells exposed to 0.05Gy ITCM and a significant increase in both MMP and ROS in cells post exposure to 0.5Gy ITCM. While there was a bystander signal released from the oesophagus at both doses, there were slight differences between the two, as there was no alteration of the mitochondrial membrane potential at the lower dose. However, the increase in ROS was greater in the cells exposed 0.05Gy ITCM (122.11% at 0.05Gy vs. 113.29% at 0.5Gy), although there was no significant difference between the two. Cells exposed to ITCM generated from male AO oesophagus also displayed evidence of bystander signal production. A small but significant decrease in viability was observed at 0.05Gy ITCM, while hyperpolarisation of the mitochondrial membrane potential was seem at 0.5Gy ITCM. This suggests that while the AO diet was capable of reducing the level of ROS in these cells, it did not result in the protection of the mitochondria, or prevention of cell

death. This again points to the fact that the bystander effect produced by these animals is not one that is solely mediated by ROS production, as seen in many other studies, [Narayanan et al., 1997, Lyng et al., 2000, 2002a, Azzam et al., 2002].

In contrast to the results described above for the whole body irradiated male mice, the results from cells treated with ITCM generated from female mice suggest a complete lack of bystander signal generated from both normal and AO mice. There was no change in any of the endpoints measured, at any dose in either normal or AO ITCM treated cells. This is similar to the result seen in the *in-vitro* section of this study, where the only effect measured was a slight loss of viability in AO bladder ITCM treated cells. Gender related differences have previously been reported in the literature with respect to bystander signal production, [Mothersill et al., 2001], oestrogen has been suggested to be an anti-apoptotic hormone [Meda et al., 2000] and has been shown to be a strong anti–oxidant [Behl et al., 1997, Behl, 2002], thus enabling the body to be more capable of dealing with insults. The level of oestrogen in the female tissues may allow them to deal with radiation better than the males, leading to a decreased bystander effect. However, if the bystander effect is seen as protective, [Belyakov et al., 2006, Mothersill and Seymour, 2003], the females may be more susceptible to long term damage, as damaged cells would not be removed from the tissue.

Overall, it seems that in the case of the cells treated with ITCM generated from male mice, there was a bystander effect which was significantly altered by the AO diet, however, whole body irradiated female mice did not produce a bystander effect which altered any of the endpoints measured above. If the lack of a bystander effect from the female mice is as a result of a hormone related difference, and whether this can be viewed as damaging or protective is difficult to determine from this investigation, as the effect of direct radiation on the animals was not examined. It is possible that the females may display less cell death but higher levels of mutations post exposure to direct irradiation. In fact, oestrogen has been associated with breast cancer, and many of the current treatments for this disease are aimed at limiting the production of this hormone [Subramanian et al., 2007].

6.4.1 A comparison of groups from both *in-vitro* and *in-vivo* exposure

Table 6.2 shows all endpoints examined in cells post exposure to ITCM generated from *in-vitro* and *in-vivo* irradiation of male bladder and oesophagus, and 6.3 that of the female counterparts.

Normal versus Anti-Oxidant (AO)

For the majority of endpoints examined post exposure to ITCM generated from male mice both post *in-vitro* and *in-vivo* exposures, there was a significant difference between the normal and AO treated groups. Cells treated with ITCM from *in-vitro* and *in-vivo* exposed normal bladder displayed significant alterations in mitochondrial membrane potential, and in the case of *in-vivo* generated ITCM, increased calcium levels. However, cells treated with ITCM from mice on the AO diet showed no evidence of bystander signal production. This strongly suggests that the AO diet abolished bystander signal production in the bladder of these animals. Treatment of cell models with anti-oxidants or free radical scavengers have frequently been shown to be capable of reducing or abolishing the bystander effect, [Lehnert et al., 1997, Narayanan et al., 1997, Bishayee et al., 1999, Mothersill et al., 2000, Azzam et al., 2002, Shao et al., 2003a, Kashino et al., 2007]. However, in many of these investigations the anti-oxidant treatment is on both direct and bystander cells, or on the bystander cells alone. In the present study, the anti-oxidant treatment is purely of the directly irradiated tissue, thus indicating the importance of ROS in the production of, as well as response to, the bystander signal.

The results from cells exposed to ITCM generated from oesophagus tissue were more complex, in that there was no evidence of a bystander effect in cells treated with ITCM from either normal or AO mice post *in-vitro* exposure, but there was a significant effect in both groups post *in-vivo* exposure. There were slight differences between the results from both groups. The increase in mitochondrial membrane potential was evident in both normal and AO ITCM treated cells, however the increased ROS levels seen at both doses

post exposure to ITCM from normal mice was absent in the AO treated group. This would suggest that the diet did alter bystander signal production in these animals, if not completely abolishing it. However, a loss of cell viability was also noted in the AO treated group, which was absent in the normal group. Whether this was due to a sensitisation of the tissue to radiation by the AO diet, similar to that seen in the female AO bladder group, or the activation of some other pathway that results in a decrease in viability is not known, although the diet does seem to have altered the tissues response to radiation in some way. What this does point to is that while ROS increases, which have frequently been shown to be associated with the bystander effect [Narayanan et al., 1997, Iyer and Lehnert, 2000, Azzam et al., 2002, Lyng et al., 2000, 2001, Shao et al., 2003a], they are not absolutely required for the generation of a bystander signal, suggesting the involvement of other pathways which may result in a loss of cell viability.

In the case of cells treated with ITCM from female mice, there is little difference between the normal and AO treated groups as there was no real evidence of a bystander effect in the endpoints measured. The exception to this is the reduction in viability in cells treated with ITCM from AO bladder post *in-vitro* exposure. It is interesting that this effect was seen in the AO treated mice, while there was no sign of an effect from normal ITCM, however as mentioned above, it seems that the AO diet has in some way sensitised the bladder tissue to radiation, resulting in the production of a bystander signal seen here.

Male versus Female

What is the most obvious and interesting result from the comparison of the male and female data is the almost complete lack of a bystander effect in the endpoints measured in cells exposed to ITCM from female mice. In stark contrast to this, the cells exposed to ITCM from male mice display a number of notable effects including alternation of mitochondrial membrane potential, ROS levels and some changes in cell viability. Why there is such an obvious distinction between the two genders is not known, although

gender differences have been reported previously [Mothersill et al., 2001, Mothersill and Seymour, 2002b]. As mentioned above, one of the reasons for the lack of a bystander signal from the female mice may be to do with the anti-apoptotic and anti-oxidant properties of oestrogen, [Meda et al., 2000, Behl et al., 1997, Behl, 2002]. Since the bystander signal is known to induce increases in apoptosis [Mothersill et al., 2001, Mothersill and Seymour, 2002b, Suzuki et al., 2004, Lyng et al., 2006a,b] and ROS [Narayanan et al., 1997, Iyer and Lehnert, 2000, Azzam et al., 2002, Lyng et al., 2000, 2001, Shao et al., 2003a] is it likely that oestrogen would reduce these effects, resulting in a significantly different response to radiation and therefore bystander signal production. This maybe be particularly relevant when considering the role that ROS is thought to have in the propagation of the bystander signal. It has been suggested that one the mechanisms of bystander signal propagation is increased ROS levels which activates NADPH-oxidase, and results in an increase in ROS, thus a positive feed back loop is created [Azzam et al., 2002, Li et al., 2001]. However, if oestrogen were to significantly reduce the levels of ROS in directly irradiated tissue, this pathway would not be activated, and so the propagation of the signal in this way prevented.

As the lack of bystander signal generation from female mice in this study is evident in both *in-vitro* and *in-vivo* exposures, in the two tissues examined and under the two diets, the question of whether females are capable of producing an alternative type of bystander effect that is not being measured here is an interesting one. And if not, does direct irradiation of female tissue result in an increase or decrease in damage to the tissue due to the lack of a bystander effect? Whether this result has any effect on the treatment of female patients in a clinical setting cannot be accessed here, as evidence of the phenomenon in human models would be required. However when Mothersill et al. [2001, 2002b] conducted a similar *in-vitro* study using bladder samples from both male and female patients, the direct opposite result was found. While both genders did produce a significant bystander effect, it was the irradiated female tissue that produced the stronger response in the same reporter cell line that is used here. One possible explanation for this

the that the endpoint examined in that study was clonogenic survival, which measures mitotic cell death, as opposed to early stage apoptosis and oxidative stress which was investigated here. While female ITCM did not induce changes in MMP, ROS, or in most cases cell viability, it is possible that there are other pathways activated, which may result in increased in mitotic cell death. It is interesting that the one sign of a bystander effect from the female mice is indeed a reduction in cell viability, albeit in mice on the AO diet. Therefore, it may be that males and female respond to radiation in different ways, resulting in an alteration of bystander signal production, and thus different cell death pathways.

in-vitro versus *in-vivo*

While much of the early investigations in the bystander effect were on cell lines, many studies in the last number of years have been concerned with providing evidence of the bystander effect in an *in-vivo*–like, or *in-vivo* environment. Numerous models have been designed to examine *in-vivo* bystander effects, including cell cluster investigations [Bishayee et al., 1999, 2000, 2001, Howell, 2002, Persaud et al., 2005], 3D tissue models [Belyakov et al., 2005], *ex-vivo* irradiation [Mothersill et al., 2001, Mothersill and Seymour, 2002b], whole body exposures [Lorimore et al., 2001, Camphausen et al., 2003, Mothersill et al., 2005], and transplantation of radiolabeled cells [Watson et al., 1996, Xue et al., 2002]. However, few studies have compared the bystander signal generated from tissue that has been irradiated under *in-vitro* conditions (i.e. explanted tissue) and that generated during whole body exposure. This comparison allows the *in-vitro* results to be accessed with regard to their relevance *in-vivo*, and addresses whether the two sets of data relate to each other.

Numerous conclusions may be drawn from the above data, the most notable being that there does not seem to be a good correlation between the results of the *in-vitro* investigation and the *in-vivo* one. For example, the increase in mitochondrial membrane potential seen in cells exposed to 0.5Gy ITCM from male normal bladder irradiated *in-vitro* was

absent in cells exposed to ITCM from the same tissue irradiated *in-vivo*. However, there was a significant decrease in mitochondrial membrane potential in cells exposed to 0.05Gy ITCM from *in-vivo* irradiated tissue. This trend was reflected strongly in the results from cells exposed to ITCM from irradiated oesophagus. While cells exposed to ITCM from *in-vitro* irradiated oesophagus showed no measurable bystander effects, those exposed to ITCM from *in-vivo* irradiated tissue displayed increases in both mitochondrial membrane potential and ROS, along with a loss of viability in the AO group.

While differences between the *in-vitro* and *in-vivo* was not unexpected, the fact that the results point to a complete different bystander signal being produced post different types of exposures is surprising. The bystander signal produced from male bladder tissue in both cases may be argued to be similar, as both involve alteration of the mitochondrial membrane potential, albeit at different doses, however that of the oesophagus seems to be completely different in both cases. It appears that interaction with the surrounding environment *in-vivo* is essential for bystander signal production in the oesophagus, a characteristic that was not altered by the AO diet.

Why *in-vivo* irradiation alters the production of a bystander effect from the oesophagus so significantly is not known, however it does raise interesting questions about the nature and importance of intercellular communication between tissues and the response to an insult within the body. The overall damage limitation that the exposed tissues could be attempting may result in a bystander signal being produced *in-vivo* and not *in-vitro*. Perhaps signaling mechanisms switched on by the insult in the surrounding tissues results in the activation of repair or protective mechanisms in the oesophagus, which remain active post harvesting, and lead to the presence of the bystander signal in the culture medium.

However, the most important conclusion from this investigation is that while *in-vitro* tissue and cell line investigations are clearly useful in exploring the bystander effect, and have given important insights into the mechanisms of the bystander effect, *in-vivo* studies are imperative if the phenomenon is to be fully understood, as the influence of surrounding

tissues and organs on the generation of a bystander signal cannot be underestimated.

Group	Ca^{2+}	MMP	ROS	Viability
Normal Male				
Bladder *in-vitro*	–	↑; 0.5Gy	NC	NC
Bladder *in-vivo*	↑; 0.5Gy	↓; 0.05Gy	NC	NC
Oesophagus *in-vitro*	–	NC	NC	NC
Oesophagus *in-vivo*	–	↑; 0.5Gy	↑; 0.05Gy ↑; 0.5Gy	NC
AO Male				
Bladder *in-vitro*	–	NC	NC	NC
Bladder *in-vivo*	NC	NC	NC	NC
Oesophagus *in-vitro*	–	NC	NC	NC
Oesophagus *in-vivo*	–	↑; 0.5Gy	NC	↓; 0.05Gy

Table 6.2: A comparison of the bystander effect generated by male bladder and oesophagus irradiated *in-vitro* and *in-vivo*. NC = no change, – not measured.

Group	MMP	ROS	Viability
Normal Female			
Bladder *in-vitro*	NC	NC	NC
Bladder *in-vivo*	NC	NC	NC
Oesophagus *in-vitro*	NC	NC	NC
Oesophagus *in-vivo*	NC	NC	NC
AO Female			
Bladder *in-vitro*	NC	NC	↓; 0.5Gy
Bladder *in-vivo*	NC	NC	NC
Oesophagus *in-vitro*	NC	NC	NC
Oesophagus *in-vivo*	NC	NC	NC

Table 6.3: A comparison of the bystander effect generated by female bladder and oesophagus irradiated *in-vitro* and *in-vivo*. NC = no change.

Chapter 7

Discussion

The bystander effect, first discovered by Parsons in 1954, has now become an area of intense investigation, with laboratories worldwide attempting to discover the 'key' to this phenomenon. Numerous models have been developed, ranging from relatively simplistic ones involving cell lines [Narayanan et al., 1997, Mothersill et al., 2000], to complex studies involving *in-vivo* exposure and measurement of the bystander response [Lorimore et al., 2001, Mothersill et al., 2005, Xue et al., 2002, Camphausen et al., 2003]. In the work presented in this thesis, an attempt has been made to examine the bystander effect from various angles, involving a development of the project from cell lines, to primary tissue, and finally to an *in-vivo* study. Many of the aspects of the bystander effect were investigated, including the importance of the signal versus response, the expression of various proteins related to apoptosis and premature differentiation, and the effect of an anti-oxidant diet on the production of the bystander signal in mice.

As mentioned previously, cell line models have provided excellent insights into the mechanisms of the bystander effect, as they are extremely easy to manipulate, examine, and are capable of generating a huge amount of data in a short space of time. Many of the first clues to the generation of a bystander signal and the effects of exposure to that signal, which have since been shown in many different models came from studies using cell lines, such as the role of ROS, [Narayanan et al., 1997, Lehnert et al., 1997], the production

of chromosomal abberations [Nagasawa and Little, 1992], the reduction in clonogenic cell survival [Mothersill and Seymour, 1997b], and the increases in micronucelei and apoptosis [Prise et al., 1998]. Since some of these now 'classic' characteristics of the bystander effect had been established, many studies have used cell lines to manipulate both bystander signal production and the response to that signal. The use of free radical scavengers, [Lehnert et al., 1997, Narayanan et al., 1997, Shao et al., 2003a, Azzam et al., 2002], have shown that ROS generation plays a critical role in the response to the bystander signal, the use of GJIC blockers such as lindane have highlighted the importance of intercellular communication [Azzam et al., 2001], investigations using cell lines that have been compromised in some way have shown the role that various DNA repair processes and proteins play in the bystander effect [Iyer and Lehnert, 2000, Mothersill et al., 2000, 2004, Kashino et al., 2004, Zhou et al., 2005]. However, while many of these investigations used multiple cell lines, and examined various endpoints, there has been no direct examination of bystander signal generation and the response to that signal as completely separate processes. Therefore, a matrix–type experiment was designed using three different cell lines, which involved the generation of a bystander signal from multiple sources and the examination of the response to that signal in various cell lines. This study confirmed that bystander signal and response are indeed separate processes, which had been eluded to in previous studies [Mothersill et al., 2000] and that in the case of cell lines capable of generating and responding to this signal, it was the signal that determined the magnitude of response in the exposed cells. This finding may shed new light on the bystander effect, but more importantly, allow better investigation of the phenomenon in future work, as the bystander signal and response can be separated and manipulated independently resulting in a more thorough analysis of each process.

Many proteins involved in numerous different processes and pathways in the cell have have been implicated in the bystander effect. In particular, those proteins involved in apoptosis have been shown to play a role in the bystander effect in many studies, [Iyer and Lehnert, 2000, Azzam et al., 1998, 2002, Maguire et al., 2005, Mothersill and Sey-

mour, 2002b, Lyng et al., 2006a]. Progressing from the finding in the first section of this project, an attempt was made to determine if the bystander effect was organ specific or systemic, and to analyse two proteins related to apoptosis in the tissue post both direct and bystander exposure. It was shown that while the bystander signal generated from the bladder and oesophagus was quite similar, there were significant differences in both tissue expression of Bcl2 and cMyc post direct radiation, with the oesophagus being the more sensitive of the two. This indicates that while these proteins play an important role in the response to direct radiation, they may not effect bystander signal production, as there were different levels of expression but a similar bystander signal generated from both. In an investigation into the bystander effect produced post α particle exposure of urothelial tissue, Belyakov et al. [2002, 2006] found that there was a significant increase in the amount of differentiation with the explant outgrowth. In the many investigations into the bystander effect, it has been shown that the bystander effect generated from high LET exposure and low LET exposure can often differ in the mode of action, while the endpoints may be similar. Many high LET studies studies suggest that GJIC is essential for the propagation of the bystander effect [Azzam et al., 1998, Shao et al., 2003a, Hu et al., 2006], while many low LET studies disagree [Mothersill and Seymour, 1998a, Mothersill et al., 2000, Lyng et al., 2000, Schettino et al., 2003]. This is mainly as a result of the nature of the investigations in each case, as high LET studies tend to irradiate one cell in a population, and low LET studies often use the media transfer protocol to examine the phenomenon. Nevertheless, there have been studies in both cases that have employed the method commonly associated with the other type of exposure and shown similar results [Schettino et al., 2003, Mothersill and Seymour, 1998a, Mothersill et al., 2000, Lyng et al., 2000, Schettino et al., 2003]. However, what is interesting about the increase in premature differentiation found by Belyakov et al. [2002] is that it seems to be a protective mechanism employed by the tissue, and as such, it would be expected that intercellular communication would be the most likely method of propagating this message to the tissue as a whole. Since this is a rare finding in a bystander effect investigation,

and this has never been examined in a low LET model, this effect was investigated here using a similar tissue model, but low LET radiation and the media transfer protocol to determine if this effect may be induced by media borne factors. A significant increase in differentiation was noted at the lower doses examined, suggesting that this effect can indeed be induced by media borne factors, and is prominent at low doses where the bystander effect has been shown to dominate [Narayanan et al., 1997, Nagasawa et al., 2002, Azzam et al., 2002, Brenner et al., 2001, Mothersill and Seymour, 2003].

In the final part of the project, the influence of an anti-oxidant diet on bystander signal production post both *in-vitro* and *in-vivo* exposure was investigated. This part of the project posed a number of important questions regarding the bystander effect, and the relevance of the many *in-vitro* studies that are conducted within this field. For example, can the bystander effect be altered *in-vivo* by increased anti-oxidants in the diet, does a diet have similar effects on different tissues in the body, and most importantly, would the results from an *in-vitro* investigation mirror those from the same investigation *in-vivo*? As mentioned previously, there are many models currently in use to examine the bystander effect in an *in-vivo* system, including cell cluster investigations [Bishayee et al., 1999, 2000, 2001, Howell, 2002, Persaud et al., 2005], 3D tissue models [Belyakov et al., 2005], *ex-vivo* irradiation [Mothersill et al., 2001, Mothersill and Seymour, 2002b], whole body exposures [Lorimore et al., 2001, Camphausen et al., 2003, Mothersill et al., 2005], and transplantation of radiolabeled cells [Watson et al., 1996, Xue et al., 2002]. However, no one study has compared the bystander effects produced by the same tissue post *in-vitro* and *in-vivo* exposure. The most interesting and surprising result of this investigation was the fact that the results from the two exposures differed greatly, which in some way, throws into question the relevance of many *in-vitro* studies. However, anti-oxidants were indeed shown to significantly alter bystander signal production, and in some cases to abolish it completely, an effect which has been noted in many other bystander effect investigations [Lehnert et al., 1997, Mothersill et al., 2000, Bishayee et al., 2000, Narayanan et al., 1997, Shao et al., 2003a, Azzam et al., 2002, Kashino et al., 2007]. Another interesting result of

this investigation was the lack of a bystander signal generated by the female mice. This gender dependence has been previously reported in the literature, [Mothersill et al., 2001, Mothersill and Seymour, 2002b], however, in that case it was the females that produced the stronger bystander signal. This may indeed be due to the differing endpoints examined in the two studies, however, the extreme difference between the male and female shown here was unexpected. One possible reason for the lack of bystander signal production from the female mice in this study is oestrogen, which has been found to have both anti-oxidant and anti-apoptotic properties, Meda et al. [2000], Behl et al. [1997], Behl [2002]. Further investigation of this result may lead to a much greater understanding of bystander signal generation, with the possibility of exploiting this difference for therapies in the future.

Therefore, this project has displayed many different aspects of the bystander effect, in various different models. The relevance of the bystander effect is now unquestionable, as more and more evidence is presented with more accurate models of *in-vivo* systems available each year. With current technological advances and the wealth of knowledge that is now available to researchers, the bystander effect may well become a useful tool in a clinical setting for individual radiotherapy treatment planning in the future.

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Appendix

Appendix

List of publications and conferences

- Bystander Signal Production and Response are independent processes which are cell line dependent. AM Vines, FM Lyng, B McClean, C Seymour and CE Mothersill. International Journal of Radiation Biology, 2007 *in press*.

- Bystander effect induced changes in apoptosis related proteins and terminal differentiation in in-vitro murine bladder cultures. AM Vines, FM Lyng, B McClean, C Seymour and CE Mothersill. International Journal of Radiobiology, 2007 *submitted*.

- The effect of gender and an anti-oxidant diet on bystander signal production in *in-vitro* and *in-vivo* irradiated murine bladder and oesophagus cultures. AM Vines, FM Lyng, J Lemon, C Seymour and CE Mothersill. Radiation Research, 2007 *submitted*.

- LOWRad Conference, Hamilton, Canada 2005
  Oral presentation : A dietary supplement influences bystander signal production post exposure to gamma radiation *in-vitro*.

- RRS Annual Conference, St. Louis, USA 2004
  Poster : Investigations into the Bystander Effect: Cytoskeletal Response.

- ICRR Conference, Brisbane, Australia 2003 – ICRR Travel Award
  Oral presentation : Investigations into the Bystander Effect : Signal vs. Response.

Data for Figure 2.2:

Data set from Experiment 1 : HPV-G ICCM to E89 bystander exposure

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Bystander Control							
0Gy ICCM	100	37	42	53	44	44 ± 4.72	100
Bystander Exposure							
0.5Gy ICCM	100	32	44	38	38	38 ± 3.46	86.36
5Gy ICCM	100	42	42	38	40.67	40.66 ± 1.33	92.42

Data set from Experiment 2 : HPV-G ICCM to E89 bystander exposure

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Bystander Control							
0Gy ICCM	100	55	59	59	57.67	57.66 ± 1.33	100.00
Bystander Exposure							
0.5Gy ICCM	100	52	55	51	52.67	52.66 ± 1.2	91.33
5Gy ICCM	100	55	43	63	53.67	53.66 ± 5.81	93.06

Data set from Experiment 3 : HPV-G ICCM to E89 bystander exposure

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Bystander Control							
0Gy ICCM	300	88	111	114	104.33	34.77 ± 2.73	100.00
Bystander Exposure							
0.5Gy ICCM	300	102	101	104	102.33	34.11 ± 0.29	98.08
5Gy ICCM	300	97	73	86	85.33	28.44 ± 2.31	81.78

Data set from Mean Experiment : HPV-G ICCM E89 to bystander exposure

Treatment	Plated	Mean PE 1	Mean PE 2	Mean PE 3	Mean Count	Mean PE ± SEM	% Survival
Bystander Control							
0Gy ICCM	NA	44	57.66	34.77	NA	45.48 ± 6.64	100
Bystander Exposure							
0.5Gy ICCM	NA	38	52.66	34.11	NA	41.59 ± 5.64	91.92
5Gy ICCM	NA	40.66	53.66	28.44	NA	40.93 ± 7.28	89.09

Data for Figure 2.1 and 2.4:

Data set from Experiement 1 : E89 Direct / Bystander

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Direct Control							
0Gy	100	93	84	87	88	88 ± 2.64	100.00
Direct Irradiated							
0.5Gy	100	65	58	71	64.67	64.66 ± 3.75	73.48
5Gy	100	23	22	26	23.67	23.66 ± 1.20	26.89
Bystander Control							
0Gy ICCM	100	82	75	71	76	76 ± 3.21	100.00
Bystander Exposure							
0.5Gy ICCM	100	62	66	67	65	65 ± 1.52	85.53
5Gy ICCM	100	74	64	61	66.33	66.33 ± 3.96	87.28

Data set from Experiement 2 : E89 Direct / Bystander

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Direct Control							
0Gy	100	70	62	66	66	66 ± 2.30	100.00
Direct Irradiated							
0.5Gy	100	60	61	55	58.67	58.66 ± 1.85	88.88
5Gy	100	19	15	17	17	17 ± 1.15	25.76
Bystander Control							
0Gy ICCM	100	79	78	79	78.67	78.67 ± 0.33	100.00
Bystander Exposure							
0.5Gy ICCM	100	84	80	80	81.33	81.33 ± 1.33	103.40
5Gy ICCM	100	74	63	74	70.33	70.33 ± 3.66	89.40

Data set from Experiement 3 : E89 Direct / Bystander

Treatment	Plated	Count 1	Count 2	Count 3	Mean Count	Mean PE ± SEM	% Survival
Direct Control							
0Gy	100	65	74	72	70.33	70.33 ± 2.72	100
Direct Irradiated							
0.5Gy	100	58	57	54	56.33	56.33 ± 1.02	80.09
5Gy	100	20	17	16	17.67	17.66 ± 1.20	25.12
Bystander Control							
0Gy ICCM	100	66	74	72	70.67	70.66 ± 2.40	100.00
Bystander Exposure							
0.5Gy ICCM	100	60	64	60	61.33	61.33 ± 1.33	86.79
5Gy ICCM	100	57	60	55	57.33	57.33 ± 1.45	81.13

Data set from mean Experiment : E89 Direct / Bystander

Treatment	Plated	Mean PE 1	Mean PE 2	Mean PE 3	Mean Count	Mean PE ± SEM	% Survival
Direct Control							
0Gy	NA	88	66	70.33	NA	74.77 ± 6.72	100.00
Direct Irradiated							
0.5Gy	NA	64.66	58.66	56.33	NA	59.88 ± 2.48	80.82
5Gy	NA	23.33	17	17.66	NA	19.44 ± 2.11	25.92
Bystander Control							
0Gy ICCM	NA	76	78.67	70.66	NA	75.11 ± 2.35	100.00
Bystander Exposure							
0.5Gy ICCM	NA	65	81.33	61.33	NA	69.22 ± 6.14	91.90
5Gy ICCM	NA	66.33	70.33	57.33	NA	64.66 ± 3.84	85.93