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The Influence of Telomerase on Induction and Repair of Targeted and Non-targeted Radiation Effects

Otilia Nuta
Technological University Dublin

The influence of telomerase on induction and repair of targeted and non-targeted radiation effects

Otilia Nuță, B.Sc., M.Sc.

A thesis submitted for the Degree of Doctor of Philosophy to the Dublin Institute of Technology

Under the supervision of Prof. Klaus-R. Trott (GCI), Dr. Firouz Darroudi (LUMC), Dr. Fiona M. Lyng (DIT)

Radiation and Environmental Science Centre, Dublin Institute of Technology, Republic of Ireland

Toxicogenetica, Leids Universitair Medisch Centrum, Nederland

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I certify that this thesis which I now submit for examination for the award of Degree of Doctor of Philosophy, 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.

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Abstract

The main aim of the project is to investigate the role of the telomere / telomerase system in the bystander effect.

Pilot experiments were carried out on broad field X-ray and γ-ray- induced bystander effect in normal BJ and immortalised BJ1-hTERT human foreskin fibroblasts. This work led to finding increased clonogenic inactivation and chromosomal damage in cells directly hit by ionising radiation and provided direct evidence for medium –mediated bystander responses (micronuclei and cell inactivation) in fibroblasts irradiated with low LET radiation.

Later, this work on targeted and non-targeted effects of radiation was extended as a number of different responses appeared which suggested expanding the scope of the studies. Therefore, different cytogenetic responses and their relationship were studied. Connections between bystander effects and other non-targeted effects of radiation, such as low-dose hypersensitivity/ increased radio-resistance phenomenon were considered. There was an indication that the bystander effect may play a role in cell inactivation at low radiation doses.

Formation of DSBs induces the phosphorylation of the tumor suppressor protein, histone H2AX and this phosphorylated form, named γ-H2AX, forms foci at DSB sites. Although γ-H2AX foci were observed in exponentially growing cells containing media conditioned on X-irradiated cells, it is not clear if X-irradiation leads to double strand breaks in bystander cells. The data in this study suggest that lesions other than DNA double strand

breaks are involved in the bystander effect and that different mechanisms are responsible for the production of γ-H2AX foci in direct irradiated and bystander cells.

No induction of foci of γ-H2AX in bystander confluent cells was observed and this result is discussed.

Our findings rule out any major involvement of the telomerase in the bystander effect and propose that telomerase may have other physiological functions associated with the protection of chromosomes from breakage.

Abbreviations List

IR- ionising radiation

LET- linear energy transfer

SSBs- single strand breaks

DSBs- double strand breaks

NER- nucleotide excision repair

BER- base excision repair

HR- homologous recombination

NHEJ- non homologous end joining

GGR- global genome repair

TCR- transcription-coupled repair

GI- genomic instability

SCEs- sister chromatid exchanges

TGF-β1- tumor growth factor β1

TNFα- tumor necrosis factor α

IL-1, IL-8- interleukin 1, 8

TP53- tumor suppressor protein p53

GJIC- gap junction intercellular communication

LHR- low-dose hyper-radiosensitivity

IRR- increased radio-resistance

MN- micronuclei

PCC- premature chromosome condensation

CA- Calyculin A

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Introduction

One of the most important progresses of radiobiology, i.e. the study of the effects of radiation on living cells was the development of the cell culture technique by Puck and Marcus in 1956. This technique is now known as the clonogenic assay, and has been used to investigate the effects of a wide range of treatments.

Cells which fail to form colonies may undergo a limited number of divisions forming small colonies with a cell number that almost never exceeds 50 even after a long time of incubation. Those cells which do not succeed in producing 50 progeny in two weeks are still able to proliferate for some time (Trott, 2001). On the other hand the proliferative efficiency of the cells which succeed in producing 50 cells may be seriously compromised, as was shown by Puck and Marcus (1956). The loss of the ability to retain reproductive integrity (i.e. colony forming efficiency), as a function of radiation dose is described by the dose survival curve. A typical example is shown below in Figure 1.1.

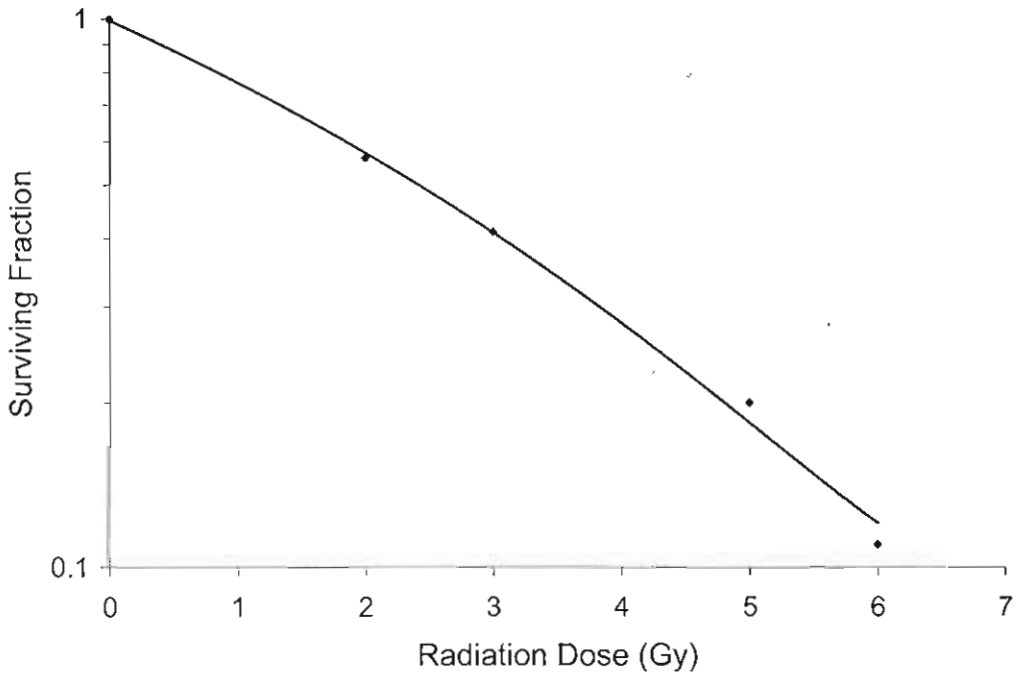


Figure 1.1: The survival curve of BJ cells, fitted to a linear-quadratic dose response relationship

The loss of colony forming ability is the main mechanism of cure of cancer by radiotherapy and is also involved in the recovery from acute radiation injury in patients. However, its role on the pathogenesis of other effects, such as late radiation damage and radiation-induced cancer is controversially discussed.

Developing alongside, the clonogenic assay was the target theory which explained how the radiation interacted with the cells, and depending on which areas were traversed, how much damage could be expected. The target theory of radiation assumes that there is a critical volume in the cell that must be hit in order to induce cell death (Lea, 1946; Marshall, 1970). It is generally accepted that nuclear DNA is the critical target for radiation induced reproductive cell death. Early experiments demonstrated that damage to the DNA is more effective in sterilising of cells in vitro than membrane damage (Warters and Hofer, 1977). Yet, there is evidence suggesting that the cell membrane might also be a target to induce cell death in some instances (Haimovitz-Friedman et al, 1994; Gillies, 1997).

The target theory was first thrown into question when in 1954 Parsons et al, showed that children that received radiation to the spleen for treatment of leukaemia, showed damage in their bone marrow. This was the first evidence to suggest that damage from radiation was not confined to the cells and tissue that had been exposed. Over the next years more studies were performed to investigate this newly discovered effect. Souto et al (1962) showed that rats injected with plasma or ultrafiltrates of blood from irradiated rats or sheep developed mammary tumours at a significantly higher level than controls. Work on this effect continued with Hollowell and Littlefield (1968) who showed that lymphocytes in culture which were exposed to the plasma of radiotherapy patients developed chromosomal aberrations including dicentrics, chromatid and chromosome breaks. Further reports of the chromosome-damaging effects of plasma from irradiated individuals came from Japan, where heavily exposed A-bomb survivors were studied.

Clastogenic activity persisted up to 31 years after radiation exposure (Pant and Kamada, 1977). Clastogenic factors were also found in Chernobyl accident recovery workers (often referred to as liquidators) in a study by Emerit et al (1994).

Thus, as more evidence was collected, it became apparent 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. This was called abscopal radiation effect.

It was concluded that, the established dose-relationships should be re-examined to include this extra damage caused by this effect which is now called the bystander effect.

1.1. Direct and Indirect Effects of Irradiation

Interaction of ionising radiation with living cells and tissues results in a range of biological endpoints, including DNA damage, chromosomal aberrations, mutations, cell transformation, inflammation, tissue injury, carcinogenesis and death. The initial step in this interaction of radiation with biological material is the deposition of energy to atoms and molecules which results in ionisation and excitation. The lethal total body dose of X-ray irradiation to humans is about 4 Gy. This dose is very small, and the equivalent heat energy of this dose would raise body temperature by only 1/1000°C. The lethality of this small quantity of energy from radiation exposure results from the non-uniform deposition of energy and through biochemical processes that amplify damage. These processes

include the formation of free radicals and other reactive species at the molecular level and the release of biological mediators at the cellular and tissue levels.

Biological responses to ionising radiation can be divided into early and late effects. Early effects are those which become apparent within milliseconds to days following exposures. Figure 1.2 shows a schematic of temporal stages of radiation action. Radiation injury begins with the physical processes of ionisations and excitations. These two processes lead to molecular damage by direct interaction of the radiation with the target and indirectly through the generation of free radicals, oxidising agents and other molecular species. Molecular damage is expressed through alteration of biochemical processes and is amplified by biological mediators. Depending on the biological end-point examined, biological effects attributed to the radiation exposure may become evident within femtoseconds to years. In addition, damage may persist which is not generally visible or clinically detectable, leading to genetic instability.

If radiation interacts with the atoms of the DNA molecule, or some other cellular component critical to the survival of the cell, it is referred to as a direct effect. Such an interaction may affect the ability of the cell to reproduce and, thus, survive. If enough atoms are affected such that the chromosomes do not replicate properly, or if there is significant alteration in the information carried by the DNA molecule, then the cell may be destroyed by "direct" interference with its life-sustaining system.

If a cell is exposed to radiation, the probability of the radiation interacting with the DNA molecule is very small since these critical components make up such a small part of the cell. However, each cell is mostly water, therefore, there is a much higher probability of

radiation interacting with the water that makes up most of the cell's volume.

When radiation interacts with water, it may break the bonds that hold the water molecule together, producing fragments such as hydrogen (H) and hydroxyls (OH). These fragments may recombine or may interact with other fragments or ions to form compounds, such as water, which would not harm the cell. However, they could combine to form toxic substances, such as hydrogen peroxide (H_2O_2), which can contribute to the destruction of the cell.

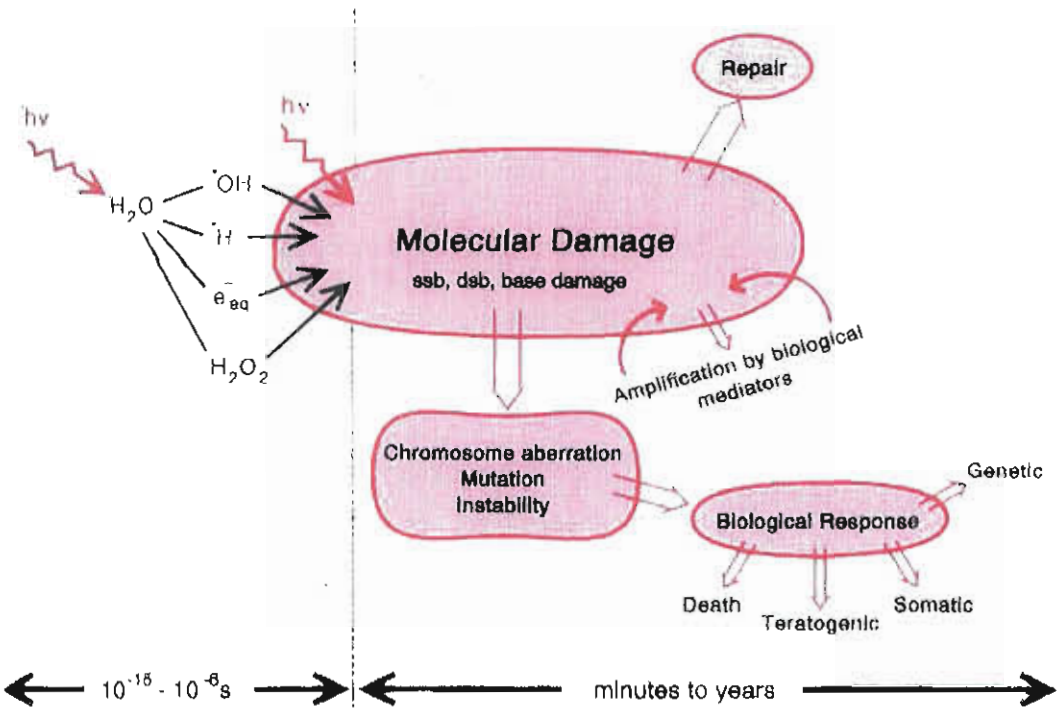


Figure 1.2: Time course of radiation action in biological systems.

In general, high-LET radiation such as α-particles would induce primarily direct damage, whereas low-LET radiation such as γ radiation will mostly induce indirect damage (Hall, 2000).

1.2. Types of DNA Damage, Its Repair and Its Effects on Chromosomes

There are several major types of DNA damage that can be produced by ionising radiation. Single strand breaks (SSBs) occur due to the deposition of energy on one strand of DNA. Double strand breaks (DSBs) can be formed by a single ionising event or by the coincidence of random single strand breaks on the complementary strands, DNA base damage occurs when radiation damages the purine and pyrimidine bases of DNA. Finally DNA-DNA and DNA-protein crosslinks may occur and lead to significant biological effects (Ward, 1988).

Radiation induced DNA damage can be repaired. There are three types of repair: error free, error prone and incomplete repair. Error free repair includes excision repair and generally does not result in mutations or lethality, error prone repair may result in lethal or non lethal mutations and incomplete repair does not result in the re-establishment of continuity in the DNA sequence and thus may be considered lethal (Hall, 2000).
The main DNA maintenance mechanisms operating in mammals are nucleotide and base –excision repair (NER and BER), homologous recombination (HR), non homologous end joining (NHEJ), mismatch repair and telomere metabolism.

NER deals with the wide class of helix-distorting lesions that interfere with base pairing and generally obstruct transcription and normal replication. Small chemical alterations of bases are targeted by BER. These lesions may or may not impede transcription and replication, although they frequently miscode. BER is therefore particularly relevant for preventing mutagenesis. Most lesions which are dealt with by NER arise from exogenous sources (except for some oxidative lesions), whereas BER is mostly but not exclusively, concerned with damage of endogenous origin such as oxidative stress. Lesions for these two repair processes affect only one of the DNA strands. The injury is removed and the resulting single-stranded gap is filled using the intact complementary strand as template. Of all repair systems, NER is the most versatile in terms of lesion recognition. NER is comprised of two different sub-pathways: global genome repair (GGR), responsible for repairing DNA damage throughout the whole genome and transcription -coupled repair (TCR), which preferentially repairs the DNA lesions in the transcribed strand of active genes.

DSBs are most problematic as both strands are affected. To properly repair such breaks the cell has to know which ends belong together. Two pathways, homologous recombination and end-joining (and presumably additional back-up systems), are responsible for solving the DSB problem. Homologous recombination seems to dominate in late S and G_2 phases of the cell cycle when the DNA is replicated, providing a pristine second copy of the sequence (sister chromatid) for aligning the breaks. In contrast, the more error prone end joining is active in all cell cycle phases, but appears to play a major

role in eliminating spontaneous and IR-induced DSBs during the G1 phase of the cell cycle, when a second copy is not available (Takata et al, 1998; Rothkamm et al, 2003).

After DSB detection, a complex cascade of reactions is triggered aimed at halting the cell cycle machinery and recruiting repair factors (Zhou and Elledge, 2000; Khanna and Jackson, 2001; Karran, 2001). One of the early initiators is the ataxia telangiectasia mutated (ATM) protein kinase. Other proteins such as NBS1, Mre11, BRCA1 play a role in DNA repair itself. The NBS1 protein, which can be activated by ATM in response to DSBs, and the Mre11 protein, which is activated independently of ATM, are part of a complex consisting of three proteins: Rad50, Mre11 and NBS1, involved both in homologous recombination and in end-joining (reviewed by Haber, 1998; d'Amours and Jackson, 2002). In response to DSB inducing agents, Rad50-Mre11-NBS1 (RMN) complexes rapidly form nuclear foci colocalizing with H2AX, the DSB-inducible isoform of histone H2A (Chen et al, 2000; Paull et al, 2000). Although its function is currently not known, the BRCA1 protein colocalises both with the RAD51 protein involved in HR and with the Rad50-Mre11-NBS1 foci in response to ionising radiation, thus suggesting that BRCA1 might be involved both in the homologous recombination and end-joining pathways (Khanna and Jackson, 2001)

Finally, some single repair proteins directly reverse certain injuries, such as O^6 - methylguanine methyltransferase, which removes O^6 -methyl guanine. This highly mutagenic lesion permits base pairing with both C or T and is capable of fooling the

mismatch repair system into triggering ineffective rounds of mismatch removal and subsequent reincorporation of the erroneous base by repair replication.

Non repaired DNA breaks may lead to chromosome aberrations by illegitimate joining of free extremities (Bender et al, 1974). Many types of aberrations are produced; some of them are unstable aberrations such as dicentrics, rings, fragments which ultimately lead to loss of clonogenic survival, and some non lethal (stable aberrations like reciprocal translocations) which may lead to oncogenesis. Unstable aberrations may result in the formation of micronuclei, which are the consequences of separation of acentric fragments (or whole chromosomes) from the mitotic spindle and are clearly visible in the cellular cytoplasm at the first post irradiation mitosis (Muller et al, 1996).
Micronuclei are much more readily quantified than aberrations and they serve as an important biological criterium for detecting the genotoxic effect of physical or chemical agents in cultured cells and in intact organisms.

The nature of radiation induced structural chromosome alterations at first metaphase following exposure depends on the phase of the cell cycle at the time of exposure. Chromosome-type aberrations which involve both chromatids of a chromosome at the same location are characteristic of damage sustained in G_0/G_1 cells, which is converted into breakage/ exchange events prior to chromosome replication. The aberrations induced include: terminal and interstitial deletions, inversions, fragments, acentric and centric rings, dicentrics and tricentrics (asymmetrical interchanges) and reciprocal translocations.

Chromatid-type aberrations are characteristic of damage in single chromatids which arises from exposure of cells in S phase and the post-synthesis phase (G_2) to S-independent agents such as ionising radiation and radiomimetic drugs, which can induce chromosomal aberrations at all stages of the cell cycle. Chromatid-type aberrations can also be observed at the first metaphase following exposure of cells in G_1 to S-dependent agents such as UV light and alkylating compounds, which need an intervening S phase for the formation of aberrations (Evans, 1977). The aberrations induced include: achromatic lesion or gap, deletions, inversion, centric and acentric rings, isochromatid aberrations, chromatid exchanges.

In addition to DNA breaks and their repair, cells may respond rapidly to irradiation, through a number of biological pathways by the initiation of signal transduction pathways, the activation of gene transcription and cell cycle specific growth arrest. These early events precondition and influence the later consequences of radiation.

1.3. Non-Targeted Radiation Effects

The abscopal effects described before, together with many other effects such as bystander effects (Nagasawa and Little, 1992; Mothersill and Seymour, 1997; 1998), genomic instability (Wright, 1998; Wright, 2000), adaptive response (Wolff, 1998), low dose hyper-radiosensitivity (HRS) (Joiner et al, 2001), delayed reproductive death (Seymour et al, 1986) and induction of genes by radiation (Amundson et al, 2001) are today termed

"non-(DNA)-targeted" effects of radiation. An essential feature of non-targeted effects is that they are particularly significant at low doses.

It has been known for more than 50 years that cells including bacteria, yeast and mammalian cells can produce signals that affect other cells (Puck et al, 1956; Bonner, 1977), hormones being a prime example. Irradiated feeder cells have been used to stimulate the growth of co-cultivated, non-irradiated cells (Fisher and Puck, 1956). Growth stimulation by this "conditioned medium" did not require direct cell to cell contact. Such early experiments demonstrated that irradiated cells could affect non-irradiated cells, a clear example of a non-targeted effect.

1.3.1. Radiation-Induced Genomic Instability

In addition to damage directly induced by the deposition of energy in the nucleus of the irradiated cell, consideration must now be given to the extranuclear effects of radiation and their role at low doses which have been reported as bystander effects (Mothersill and Seymour, 1997; 1998). An irradiated cell can send out a signal and induce a response in a cell whose nucleus was not hit by radiation. This might result in genetic damage or genomic instability or might be lethal to non-irradiated cells. These non-targeted effects in essence amplify the biological effectiveness of a given radiation dose.
Radiation is capable of inducing genomic instability in mammalian cells. Genomic instability in general is thought to be the driving force responsible for radiation

carcinogenesis. Genomic instability (GI) is a term used to describe a phenomenon that results in the accumulation of multiple changes required to convert a stable genome of a normal cell to an unstable genome characteristic of a tumour. Radiation-induced instability is observed in cells at delayed times after irradiation and may persist in the progeny of exposed cells many generations after the initial insult.

At the cellular level, genomic instability is characterized by diverse changes that include large-scale chromosomal rearrangements, amplification of genetic material, changes in ploidy, micronucleus formation, microsatellite instability and gene mutations (Pierce and Preston, 2000; Nowell, 1976). There are multiple pathways for initiating and perpetuating induced instability (Coleman and Tsongalis, 1999; Lengauer et al, 1998), and the relative contributions of the different pathways depend upon the genetic background of the target cell or organism (Sankaranarayanan and Chakraborty, 1995; Ullrich and Ponnaiya, 1998).

The capacity of radiation to induce genomic instability depends on radiation quality or LET and dose. There appears to be a low dose threshold above which no additional genomic instability is induced. Low doses of both high and low LET radiation are capable of inducing genomic instability whereas DNA damage observed immediately following exposure to an agent generally demonstrates a clear dose response. However, genomic instability, which is characterised by delayed presentation of multiple end points, may not demonstrate a dose response compatible with target theory.

Understanding the molecular, biochemical and cellular events that initiate and perpetuate instability is critical to radiation risk assessment in terms of clinical, occupational or accidental exposure.

1.3.2. The Bystander Effect

The bystander effect describes the ability of cells affected by an agent to convey manifestations of damage to other cells not directly targeted by the agent (Whitehouse et al, 1998; Little et al, 2002). In vitro bystander effects are the result of a signal generated by irradiated cells which then interacts with non-irradiated cells. They are not the result of radiation-induced changes in the culture medium (Whitehouse and Tawn, 2001).

Different effects are observed in different cell types and depend on the type of cell producing the bystander signal after irradiation and the type of cell receiving the bystander signal. They include induction of apoptosis (Mothersill at al, 1997), chromosomal aberrations (Lorimore at al, 1998; Watson et al, 2000), senescence (Belyakov at al, 2002), micronucleus formation (Belyakov et al, 1999), alteration of gene expression (Azzam et al, 1998; Hickman et al, 1994; Azzam et al, 2000; Azzam et al, 2001), transformation (Lewis et al, 2001; Sawant et al, 2001); mutations (Nagasawa and Little, 1999; Zhou et al, 2001) and sister chromatid exchanges (Nagasawa and Little, 1992; Deshpande et al, 1996).

A schematic representation of the bystander effect can be seen in Figure 1.3 below.

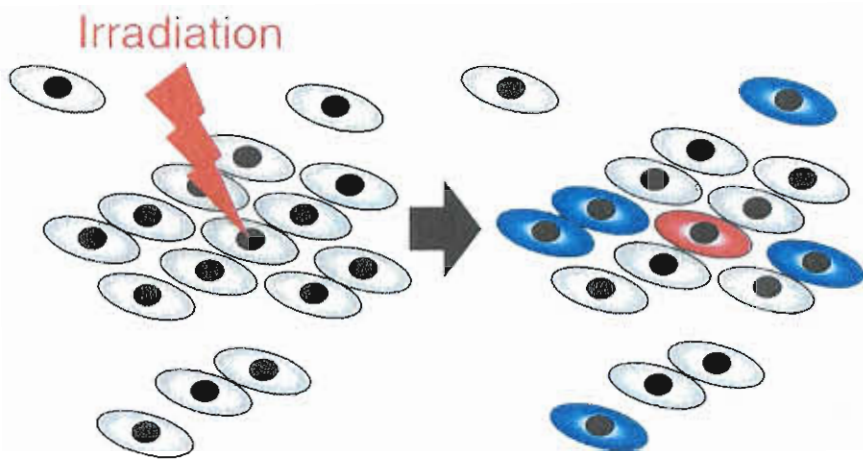


Figure 1.3: Scheme of the bystander effect. The directly damaged cell is marked in red; bystander damaged cells are marked in blue.

In the last few years a large number of articles were published demonstrating evidence for the radiation induced bystander effect (Iyer and Lehnert, 2000; 2002; Mothersill and Seymour 2001; 2002; 2003; 2004; 2005; Prise et al, 2003; Azzam and Little, 2004; Schettino et al, 2003; Hei, 2006).

Although bystander effects have been demonstrated *in vitro* with a variety of biological endpoints in both human and rodent cell lines (as well as in 3D tissue samples), the mechanisms of the phenomenon remain obscure.

It is known that the bystander effect is cell type dependent (Mothersill and Seymour, 1997), depends on cell proliferative state (Belyakov et al, 2002) and that energy/REDOX

metabolism may be involved in the expression of a radiation induced bystander response (Mothersill et al, 2000). However, it is clear that bystander signal production and cellular response may involve different pathways (Grosovsky, 1999). Bystander signaling is a complex system which most likely involves more than one messenger and is connected with tissue microenvironment signaling (Barcellos-Hoff, 1998; Barcellos-Hoff and Brooks, 2001).

Although gap junction communication and the presence of soluble mediator(s) are both known to play important roles in the bystander response, the precise signaling molecules have yet to be identified.

The data available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in unirradiated cells. Experiments involving the transfer of medium have demonstrated a significant reduction in the plating efficiency of unirradiated cells that received culture medium from irradiated cultures (Mothersill and Seymour, 1997; 1998; 2000; 2001). Under this protocol supernatant from irradiated cells was transferred to test "reporter" cell cultures which can be analysed using the Puck and Marcus clonogenic assay or for presence of apoptotic cells. This approach to study bystander effects will be described in more detail in the Materials and Methods chapter as it was used in the experiments described in this dissertation.

Medium transfer experiments have also demonstrated bystander effects for cell lethality, chromosomal aberrations and cell cycle delay (Mothersill et al, 2000).

The type of cell, epithelial or fibroblast appears to be important. Experiments suggest that the effect is due to a molecule secreted by irradiated cells (the so- called bystander factor), which is capable of transferring damage signals to distant cells (Mothersill and Seymour, 1997; 2000; 2001) and cannot be eliminated by media filtering. This factor or factors can increase neoplastic transformation (Lewis et al, 2001), or induce genomic instability (Seymour and Mothersill, 1997) in non-irradiated cells. Not all cells are capable of producing the toxic bystander factor, nor are all cells capable of receiving and responding to the secreted signal (Mothersill and Seymour, 1997; Mothersill et al, 2001).

Lyng et al (2000; 2002) have showed that the first response of the unexposed cells after transferring the medium containing the bystander factor is a calcium influx within two minutes of exposure, followed 30-120 minutes later by changes in mitochondrial membrane permeability and by the induction of reactive oxygen species (Lyng et al, 2000)

The majority of bystander experiments involving medium transfer have been studying the effects after low-LET X or γ- rays (Mothersill et al, 2001).

A related class of effects was demonstrated by other groups which have reported the transmission of protective factors from non-irradiated to irradiated cells. For example, in thymocytes, interactions between different types of cells led to different degrees of radiation induced apoptosis, via the production of soluble autotoxic mediators. Underlying this response may be the involvement of lipo-oxygenase products (Korystov

et al, 1993). When irradiated thymocytes are mixed with non-irradiated thymocytes, less interphase-induced cell killing was observed than would be predicted on the basis of ratios of the cells mixed together. This protective effect was not observed when the medium from non-irradiated cells was added to the irradiated cells.

Nitric oxide has also been shown to play a role as a signalling agent in bystander responses (Shao et al, 2003; 2005) and has been reported to lead to radioresistance in neighbouring cells (Matsumoto, 2001), potentially having a protective value.

Many publications on bystander effects describe studies using alpha particle irradiation delivered from conventional low dose broad field sources (Metting et al, 1995). They suggested another possible mechanism in which the irradiated cells secrete cytokines or other factors that act to increase intracellular levels of reactive oxygen species in unirradiated cells (reviewed in Morgan, 2003).

Nagasawa and Little in 1992 described the bystander effect measured as an increase of sister chromatid exchanges (SCEs). Chinese hamster ovary cells were irradiated with low doses of alpha particles from a conventional broad field source. Alpha particles at low fluences do not traverse every cell in the irradiated cell population. Yet, experimental results suggest that many more cells show SCEs than would be predicted on the basis of the number of cell nuclei being traversed by an alpha track. The authors proposed the hypothesis that cell irradiation induces some indirect effects within neighbouring cell via free radical cascades or signal transduction pathways.

This observation was later confirmed and extended in a series of papers from the Los Alamos National Laboratory (Deshpande et al, 1996; Lehnert and Goodwin, 1997). High

levels of SCEs were observed in cell cultures of primary human fibroblasts after low dose alpha particle irradiation (Deshpande et al, 1996). Lehnert and Goodwin in 1997 demonstrated that the culture medium harvested from cells irradiated with low fluences of alpha particles could induce an increase in sister chromatid exchanges when incubated with unirradiated test cells providing convincing evidence for the production of extra cellular factors released into cell culture medium It was found that the activity of this factor(s) could be inhibited by superoxide dismutase, that it survives freezing and thawing but not heating. The authors suggested a role for reactive oxygen species (Lehnert and Goodwin, 1997; Narayanan et al, 1997; 1999). Later, the same group (Iyer and Lehnert, 2000; 2002) observed a cell growth-related bystander response in cells exposed to supernatants from alpha particle irradiated cultures. Upregulation of ROS in bystander cells mediated by the redox-activated TGF-β1 cytokine has also been reported (Iyer and Lehnert, 2000; Iyer et al, 2000).

The alpha-particle induced increase in reactive oxygen species appears to be temporally linked to enhanced production of tumor necrosis factor alpha (TNFα) and interleukin 1, which in turn operate in an autocrine manner to up-regulate interleukin 8 (Narayanan et al, 1999).

In rat lung epithelial cells changes in tumor suppressor protein p53 (TP53) expression after exposure to low doses of alpha particles were measured and more cells demonstrated increased TP53 expression than were directly hit by alpha particles (Hickman et al, 1994).

More recently, the gold standard for investigating the bystander effects is the application of single particle microbeams, which allow specific cells to be irradiated and biological effects studied in their neighbours. Using this method bystander effects were demonstrated for chromosomal aberrations, cell lethality, mutation and oncogenic transformation. When cells are in close contact, allowing gap junction communication bystander effects are much larger in magnitude than the same phenomenon in medium transfer experiments (Azzam et al, 2001).

Based on earlier studies which demonstrated that the target for chromosomal damage is larger than the nucleus on the basis of calculations of the fraction of micronucleated Chinese hamster V79 cells after alpha particle irradiation (Manti et al, 1997) experiments with a charged-particle microbeam were performed at the Gray Cancer Institute which provided direct evidence of bystander effects in AG01522B normal human fibroblasts (Belyakov et al, 1998; 2001; Shao et al, 2005). Irradiation of a single fibroblast with a single $^{3}He^{2+}$ particle delivered by the microbeam through the nucleus gave a significant rise of bystander damaged cells measured as apoptotic and micronucleated cells (Figure 1.3).

Other groups also utilised microbeam approaches to study bystander effects (Randers-Pehrson et al, 2001; Suzuki and Tsuruoka, 2004; Zhou et al, 2004; Frankenberg et al, 2006).

Evidence for the existence of extra nuclear targets for radiation induced effects was observed when the cytoplasm of human-hamster hybrid A_L cells was irradiated avoiding

traversal of the cell nucleus (Wu et al, 1999). Cytoplasmic irradiation led to considerable mutagenesis; the mutations found were similar to those of spontaneous origin but entirely different from those observed after targeted nuclear irradiation. Since cytoplasmic irradiation initiated the generation of reactive oxygen species, it was concluded that cytoplasmic irradiation by alpha particles may be more mutagenic and carcinogenic than nuclear irradiation, as bystander mutagenicity is associated with negligible killing of the target cells (Wu et al, 1999). Cytoplasmic irradiation also may induce chromosomal bystander responses using the micronucleus assay as endpoint (Shao et al, 2004).

Azzam et al (1998) demonstrated that bystander effects may be dependent on gap junction intercellular communication (GJIC) in confluent cultures of different primary human diploid fibroblast lines exposed to low fluences of alpha particles. They showed that TP53 and CDKN1A expression are activated in bystander cells after low dose alpha particle irradiation. Importantly, they also observed clustering of expression in neighbouring cells. Treatment of cells with lindane, which inhibits gap junction intercellular communication, led to a marked reduction in the increase in the levels of TP53 and CDKN1A. Another paper from the same authors (Azzam et al, 2001) provided direct evidence for the participation of gap junction intercellular communication in the transmission of damage signals from irradiated to non-irradiated cells. The bystander effect was also significantly reduced in cells pre-treated with octanol, which also inhibits gap junction mediated intracellular communication (Zhou et al, 2001). The same paper also reports that the bystander effect was suppressed in cells carrying a dominant negative connexion 43 vector, which is part of the connexon complex forming the gap junction.

Little is known concerning the chemical nature of the signals which may be transferred via gap junction intercellular communication. Gap junctions allow ions, secondary messengers and small molecules to pass between cells and modification of connexon proteins, by phosphorylation can open or close the pores. Whether the junctions are specifically opened or specific signal molecules are transmitted between the cells, as part of the bystander response needs to be addressed.

Various studies showed that intercellular communication plays a critical role in mediating the bystander phenomenon (Zhou et al 2000; 2001).

Using AL cells, Zhou et al (2000) demonstrated a bystander mutagenic effect after alpha –particle microbeam irradiation. They showed that irradiated cells could induce a bystander mutagenic response in non exposed neighbouring cells. Furthermore, the types of mutations observed in bystander cells were significantly different from those of spontaneous origin and the ones observed after cytoplasmic irradiation, suggesting that different mutagenic mechanisms are involved in the process (Zhou et al, 2000). The bystander effect was not modulated by addition of dimethyl sulfoxide (a free radical scavenger), unlike bystander effects following cytoplasmic irradiation (Wu et al, 1999) or low fluences of alpha particles (Lehnert and Goodwin, 1997; Narayanan et al, 1997; Narayanan et al, 1999).

When cells were treated with lindane- an inhibitor of gap junction intercellular communication - the bystander effect was reduced, but not completely eliminated.

Another study from the same group (Zhou et al, 2001) concluded that when 10% of the cell population was irradiated with a single alpha particle, the mutation yield was similar to that observed when 100% of cells were irradiated. This effect was reduced by an

inhibitor of gap junction-mediated intercellular communication, or in cells carrying a dominant negative connexion 43 vector.

An important question is whether the bystander effect may contribute to carcinogenesis. Lewis et al (2001) tested the response of non-irradiated cells when these were exposed to medium from X-irradiated human CGL1 hybrid cells. They reported a significant rate of neoplastic transformation after treatment with medium from irradiated cells. Sawant et al (2001), using the Columbia University microbeam demonstrated that the transformation rate in C3H 10T1/2 cells was similar when every cell or when only 10% of the cells were exposed to the same number of alpha particles.

In contrast to the cell inactivation effects observed after medium transfer from γ-irradiated cultures such as those reported by the Mothersill and Seymour laboratory, cell inactivation reported by Lehnert and colleagues (Iyer and Lehnert, 2000) was quite different in human fibroblast cells cultured in supernatants from alpha-particle- irradiated fibroblasts. Radiation induced bystander effects may produce not only damage but other effects which may be interpreted as neutral or beneficial. For example it was reported that exposure of normal lung fibroblasts to a low dose of alpha particles enhances their proliferation *in vitro* (Iyer and Lehnert 2000, 2002). When uniraddiated cells were treated with media from cell cultures irradiated with alpha particles, decreased basal levels of TP53 and CDKN1A in unirradiated cells were observed. These decreases were accompanied by increases in proliferating cell nuclear antigen and CDC2, apparently mediated by TGF-β1 and by the induction of intracellular reactive oxygen species

(Narayanan et al, 1997). These results are in contrast to the increased levels of TP53 and CDKN1A described by Hickman et al (1994) and Azzam et al (2001).
These bystander effects described by Iyer and Lehnert (2000) correlate with increased cell proliferation. If these effects are involved in radiation induced carcinogenesis bystander induced cell death may actually be a protective mechanism by eliminating damaged cells.

1.3.3. Messengers

The exact nature of bystander signalling is not known. Two mechanisms of transmission from an irradiated cell to an unirradiated neighbour have been proposed as described above. A bystander messenger can be either a soluble factor transmitted into the cell culture media from the irradiated cells or be directly transmitted by gap junction intercellular communication (GJIC) between hit and non hit cells (Grosovsky, 1999).

Based on this distinction it can be speculated that at least two types of bystander messenger might exist. A primary messenger may be excreted by the targeted cell. It may be short lived, not very stable, travels through gap junctions, should be water soluble and most likely is not a protein. Suitable candidates here could be organic radicals. Such radicals could have lifetimes of up to 20 hours (Watanabe et al, 1990; Koyama et al, 1998). Among other candidates for GJIC mediated bystander messengers are antioxidants

(thiols) (Prise, 2002), Ca^{2+} (Lyng et al, 2001), and cAMP (Lehnert, 1975), which is an important secondary messenger involved in Ca^{2+} metabolism.

Secondary bystander messengers emitted by activated not directly traversed cells should be more stable and are likely proteins or peptides.

Suitable candidates here would be lipid hydroperoxidases (Lehnert et al, 1991), ceramide (Haimovitz-Friedman et al, 1994), death ligand (TNFSF6) produced from exfoliation (Albanese and Dainiak, 2000). Other evidence supports a role for cytokines such as TNF-α (Ramesh et al, 1996; Khan et al, 1998), TGF- β (Iyer and Lehnert, 2000; Barcellos-Hoff and Brooks, 2001) or IL-1 (Khan et al, 1998), as key signalling molecules in the transfer of bystander damage.

There is a range of possible candidates for bystander effect mediators, which are medium borne and could be either primary or secondary messengers. Reactive oxygen species including superoxide ($HO_2^{\cdot}/O_2^{\cdot-}$),and hydrogen peroxide (H_2O_2) have been proposed as possible signals involved in bystander responses (Narayanan et al, 1997; Iyer and Lehnert, 2000). Reactive oxygen species can damage cells in many ways: by inactivating proteins, damaging nucleic acids, and altering the fatty acids of lipids, which leads in turn to perturbations in membrane structure and function. Other groups have proposed that nitric oxide (NO) might play a central role in mediation of bystander effect potentially having a protective value (Matsumoto et al 2000; 2001; Shao et al, 2005)

More recently, using the Columbia University charged particle beam in conjunction with a novel strip dish design, Zhou et al (2005) showed that the cyclooxygenase-2 (COX-2)

signalling cascade plays an essential role in the bystander process. Treatment of bystander cells with NS-398, which suppresses COX-2 activity, significantly reduced the bystander effect as well as the induction of the mitogen-activated protein kinase (MAPK) pathways. These results provided the first evidence that the COX-2-related pathway, which is essential in mediating cellular inflammatory responses, is the critical signaling link for the bystander phenomenon (Hei, 2006).

In conclusion, it is most likely that there is no single mechanism underlying the bystander effect and both media borne and gap junction intercellular communication factors are involved in its induction and perpetuation. The mechanisms are probably cell type specific which may account for the current uncertainty in the literature as to the processes involved.

1.4. Low-Dose Hypersensitivity and Increased Radio-Resistance

In cell-culture models, radiation sensitivity has typically been described by a relation in which survival decreases after an initial shoulder exponentially with dose, or in recent years more commonly, by a two component linear quadratic model, in which an initial exponential decrease is followed by increasing steepness with rising dose (Prise et al, 2005). However, the established clonogenic assay usually lacks the precision to measure the radiosensitivity at doses below 1Gy, due to statistical uncertainties associated with cell plating (Boag, 1975).

This low-dose limitation to assess the exact number of cells at risk can be overcome using cell plating approaches that use a microscope cell location technique (Marples and Joiner, 1993) or flow cytometry based methods (Wouters and Skarsgard, 1997; Durand, 1986). Using the cell counting technique, the phenomenon known as low-dose hyper-radiosensitivity (HRS) has been discovered (Marples and Joiner, 1993).

This term refers to the observation that cell survival is more reduced per dose increment for low radiation doses (less than 50 cGy of X- or γ-rays) than for larger doses. On a dose response curve there is a steep initial slope, followed by a flat region (or in some cases even an increased survival with increasing dose over a small dose region), then a shoulder and a subsequent steeper region (Marples and Joiner, 1993; Lambin et al, 1993) (Figure 1.4).

This means that the radiation response to acute irradiation is dose dependent, with a hypersensitive region for doses below about 20 cGy and lower sensitivity for larger doses. Historically, this more delicate change in radiosensitivity over the 20-70cGy dose range has been termed increased radio-resistance (IRR). In other words, small acute doses (below about 20 cGy) are more lethal per absorbed dose than larger doses. This effect is probably not due to variations of radiation response of cells in different phases of the cell cycle (Marples and Joiner, 1993; Lambin et al, 1994; Chadwick and Leenhouts, 1998; Wouters and Skarsgard, 1997; Short et al, 2001).

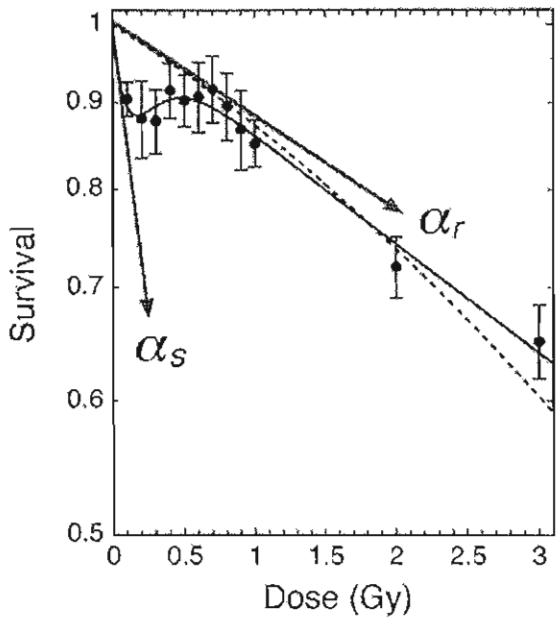


Figure 1.4: Low-dose clonogenic cell survival of V79 hamster fibroblasts irradiated with 240 kVp X - rays (from Marples et al, 2004).

Hypersensitivity to low dose exposure followed by an increased radioresistant region have been characterised by several laboratories in over 40 X-irradiated human cell lines, using different biological end points and different radiation qualities (Wouters and Skarsgard, 1994; Marples et al, 1996; 1997; Wouters et al, 1996; Vral et al, 1998; Skov, 1999; Dionet et al, 2000; Tsoulou et al, 2001; Redpath et al, 2003; Short et al, 2001; 2003; Dey et al, 2003).

The extent of the HRS varies noticeably between different cell lines, being more prominent in malignant cells than in normal tissues (Joiner et al, 2001).
These two effects have also been recorded in cells targeted with individual protons delivered by a charged-particle microbeam. Cell survival in this case was measured by follow-up of each cell measuring the expansion of the clones formed (Schettino et al, 2001). Low dose hyper-radiosensitivity and increased radio-resistance responses have as well been detected after high LET neutrons given at a low dose rate (Dionet et al, 2000). The occurrence of the low dose hyper-radiosensitivity indicates that this observable fact is a different response of cells to small doses of both low-and high-LET irradiation.

There are also a minority of cell lines which do not exhibit HRS. The explanation why some cell lines do not show this phenomenon certainly reflects deregulation of the underlying molecular pathway or the dominance of a subpopulation of cells which do not have such a response (Marples et al, 2003).

A number of hypotheses have been proposed to explain low-dose hyper-radiosensitivity, but the precise mechanism remains unanswered (Marples et al, 1997; Skov, 1999; Marples and Joiner, 2000; Joiner et al, 2001; Marples et al, 2003).

Still it remains to be explained how an organism could develop a cellular repair system which is dependent on the level of damage. It has been argued by Joiner et al that it is the mechanism the organism uses to eliminate small numbers of cells with low levels of damage, thus protecting it from possible repair related mutations. In this case, cells would

proceed through the cell cycle with damaged DNA. DNA DSBs induced after exposure to very low doses seem not to be repaired and the damaged cells could be targeted by P53-dependent apoptosis (Enns et al, 2004) presumably at the G_2 check-point. This suggestion is supported by a study from Rothkamm and Löbrich (2003) which demonstrated that low numbers of DSBs can remain unrepaired for few days.

Another possibility is that the cell will only activate the repair mechanisms when absolutely required. This is because mechanisms such as NHEJ can be detrimental for the genome as it results in deletion or gain of genetic material at the sites of DNA damage. Thus the HRS appears to be the default survival response of cells to radiation damage at doses below 10-20cGy and to all doses in NHEJ repair-deficient mutants (Rothkamm and Löbrich, 2003).

Recent evidence suggests that for certain cell types there are genotype –dependent and cell-type specific modifiers of the responses that influence the efficiency with which a damaged cell initiates an apoptotic response or a growth arrest. These signalling processes are strongly dependent on genetic background. The genetic background which will be more effective in killing the cells by apoptosis would be the less predisposed to consequences of radiation because of elimination of malignant and unstable cells.

The HRS/ IRR response is likely to involve some sort of threshold where a defined level of damage leads to expression of a DNA repair response (Joiner et al, 2001).

1.5. The Relationship between Radiation Induced Bystander Effects, Radiation Induced Genomic Instability and the Low-Dose Hypersensitivity/ Increased Radio-Resistance Phenomenon

There is little information on the relation between radiation induced bystander effects, radiation induced genomic instability and the low-dose hypersensitivity/ increased radio-resistance phenomenon, but the experiments described in this thesis suggest that there might be such a relationship.

Radiation induced bystander effect and genomic instability are both non-targeted effects of irradiation. However, the relationship between the bystander effect and genomic instability is not clear. Genomic instability occurs in the distant descendants of an irradiated cell, whereas the bystander effect occurs almost immediately in the non-irradiated cell receiving the bystander signal. Genomic instability and bystander effect can both be induced *in vivo* and *in vitro* (Watson et al 2000; 2001). It was reported that persistent genomic instability could be induced *in vitro* via a bystander mechanism and that bystander effects may be produced as a consequence of instability (Lorimore and Wright, 2003). Chromosomal instability was demonstrated in the clonal descendants of haemopoietic stem cells after irradiating murine bone marrow with alpha particles (Lorimore et al, 1998). The authors studied the effects by interposing a grid between the cells and the radiation source, so the surviving population consisted predominantly of non –traversed stem cells. It was shown that the number of clonogenic cells transmitting chromosomal instability was greater than the number expected to be hit and survive.

Later, the same group utilised a bone marrow transplantation protocol in which a mixture of irradiated and non-irradiated murine bone marrow cells was transplanted into mice. It was demonstrated that genomic instability could be observed in the progeny of non irradiated stem cells under *in vivo* conditions (Lorimore et al, 2005)

The expression of these non-targeted effects is influenced by cell type and genetic factors and in some cases by the type of radiation exposure (Limoli et al, 2000; Mothersill et al, 2005).

It is likely that the genomic instability is a consequence of the bystander effect, but at this time it is not known to what extent these effects contribute to overall cellular radiation responses.

There is variation in response between the cell lines, suggesting that genetic factors may be involved, as they are in genomic instability or in the HRS phenomenon.

However, the underlying mechanisms of the HRS and BE may be different. HRS is centred on a hypersensitive subpopulation of targeted cells and BE on factors transferred from targeted to non-targeted cells (Bonner, 2004).

1.6. The Telomere/ Telomerase System

There have been suggestions that the telomerase system is involved in non-targeted effects of radiation such as radiation induced genomic instability (Sabatier, 1994; Desmaze, 2003)

The end of linear eukaryotic chromosomes contain specialised structures called telomeres (reviewed in McEachern et al, 2000; Collins, 2000) composed of DNA repeat sequences and associated proteins (Blackburn, 2001). The DNA sequence of human telomeres typically consists of tandem G- rich repeats, (TTAGGG), with a single-stranded 3'-end overhang (Makarov et al, 1997; Wright et al, 1997). Telomeric sequences vary around this theme in most animals and plants (Blackburn, 1991).

Electron microscopy has revealed that the single-stranded 3'- end overhang invades the duplex telomeric DNA repeat array to form a D-loop and T-loop structure in vitro (Greider, 1999; Griffith et al, 1999). Telomere binding proteins function to maintain and regulate this unique structure.

The telomere is involved in several essential biological functions. It protects chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA. It provides a means for complete replication of chromosomes, contributes to the functional organisation of chromosomes within the nucleus, participates in the regulation of gene expression and serves as a molecular clock that controls the replicative capacity of human cells and their entry into senescence (Smeal and Guarente, 1997; Morin, 1997).

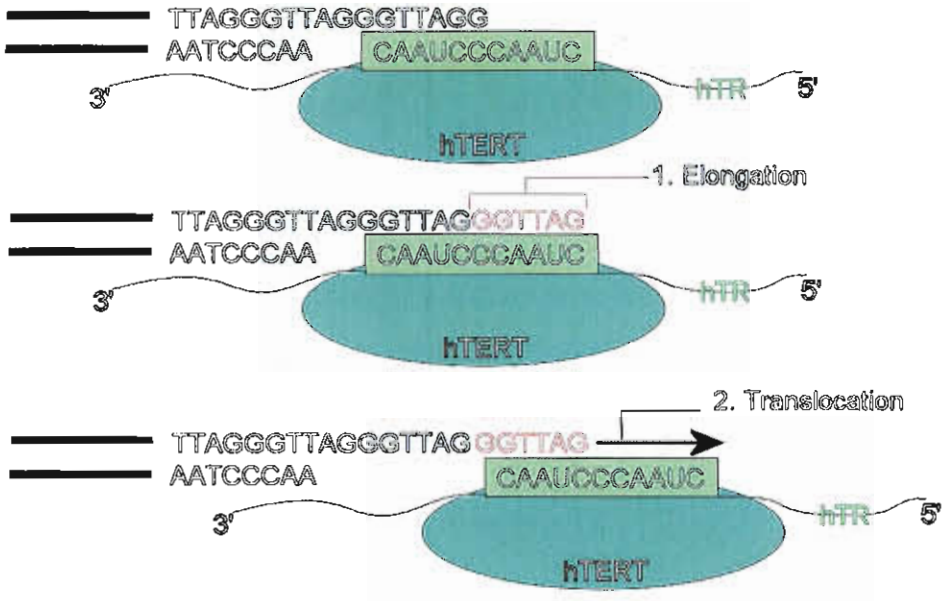


Figure 1.5: Telomerase enzyme. The telomerase enzyme adds telomeric repeats, TTAGGG, in two steps (1) elongation and (2) translocation. The enzyme is composed of two primary parts, hTR, the template RNA unit and hTERT, the catalytic subunit.

The telomere can reach a length of about 15-20 kbp at birth to sometimes less than 5kbp in chronic disease states (Aisner et al, 2002; Forsyth et al, 2002; Collins, 2000). As cells divide, the length of the telomeres shrinks (Allsopp et al, 1995; Harley et al, 1990; Greider, 1990). The rate of decrease varies among different cell lines, but is generally around 50 to 100 base pairs per cell doubling. These findings led to the hypothesis that

human cells undergo a senescence finally leading to growth arrest when their telomeres reach a critically short length (Chiu et al, 1997).

Telomere length is maintained in stem cells, but telomere length can also be maintained in cancer cells by the enzyme called telomerase (Figure 1.5). Telomerase is a reverse transcriptase composed of two primary components, the protein catalytic subunit, hTERT (Nakamura et al, 1997; Nakamura and Cech, 1998), and the template RNA subunit, hTR (Feng et al, 1995). While hTR is expressed in all cells, hTERT is present only in cells with telomerase activity (Nakamura et al, 1997).

Normal fibroblasts and epithelial cells transfected with the catalytic subunit of human telomerase (hTERT), although they are not transformed, do not display replicative senescence when considered as cell populations (Bodnar et al, 1998). In other words, introduction and overexpression of the human catalytic subunit of telomerase (hTERT) in normal fibroblasts and epithelial cells leads to telomerase activity, elongation of telomeres and extension of life span of the cells.

Critically short and unprotected telomeres are supposed to be an initial event of genetic instability in cells lacking telomerase activity (as reviewed in Murnane and Sabatier 2004). Therefore, loss of the capping function of the telomeres can be seen as an early stage in the transformation process from normal to tumor cells. Once telomerase is upregulated, the telomeres should be stabilized and tumor cells protected against telomere length dependent genomic instability. Nevertheless, immortal and tumor cells are similarly prone to genomic instability.

Telomere-associated proteins have been identified that regulate telomere length indirectly. Some of these proteins appear to alter the telomeric structure and hence the ability of telomerase to access the telomere (Campisi et al, 2001; Evans et al, 2000). One such protein is TIN2.TIN2 negatively regulates telomere length in a telomerase dependent fashion but does not act directly on the enzyme (Kim et al, 1999). Thus, telomerase expression alone may not be sufficient to prevent replicative senescence.

Short telomeres may be more prone than long telomeres to structural dysfunction, and telomere function, rather than length, may control cellular senescence. It has been suggested that telomerase can prevent cellular senescence by preferentially capping and acting on the shortest telomeres and that the senescence response is not induced by telomere length but, rather, by a dysfunctional telomere structure (Blackburn, 2000; 2001).

Telomeres may influence the sensitivity of cells and organisms to DNA-damaging agents such as ionizing radiation. There was an inverse correlation between telomere length and chromosomal radiosensitivity in the lymphocytes of some breast cancer patients (Mc Ilrath and Bouffler, 2001), and short telomeres enhanced IR-induced lethality in telomerase-deficient mice (Wong et al, 2000). In addition, cells from organisms with defects in telomere maintenance are frequently radiosensitive (Hande et al, 1999; Samper et al, 2000). Moreover, telomerase has been reported to protect some cells from the

lethality that results from severe damage to telomeres (Lu et al, 2001; Ludwig, 2001; Ren et al, 2001).

Thus, hTERT appears to be the limiting factor for telomerase activity, telomere extension and extension of cellular life span. Telomerase activity permits the maintenance of chromosome ends, cellular immortalisation and a lower chromosome instability is observed after the reactivation of the telomerase (Pommier et al, 1995).

Aims of the study

The main aim of the present work started on the assumption that the telomere / telomerase system might be involved in the bystander effect.

The justification of the experiments is based on the fact that a potential link between radiation-induced GI and BE was indicated by the persistent reduction in the cloning efficiency of non irradiated normal and malignant epithelial cell lines exposed to medium from irradiated cultures. The first link between radiation induced chromosomal instability and BE was provided by the observation that chromosomal instability was expressed in the progeny of more clonogenic haemopoietic stem cells than were traversed by an alpha particle.

It has been reported that in ovarian cancer GI is associated with lack of telomerase activity. Yeast cells with GI have been shown to survive in the absence of telomerase by increased recombination events. Human cell lines that lack telomerase activity have long and heterogeneous telomeres. They might maintain telomeres via a recombination pathway.

During the course of these experiments on targeted and non-targeted effects of radiation, a number of different responses appeared which suggested expanding the scope of the studies. Therefore, different cytogenetic responses and their relationship were studied and the key ultimate question was to try to relate the various responses to each other.

More specifically, the aims of this study were:

- to characterise the response of normal human fibroblasts cell culture system to low LET radiation;
- to investigate the medium –mediated bystander effect in normal human fibroblast cells;
- to explore connections between bystander effects and other non targeted effects of radiation, such as low-dose hypersensitivity/ increased radio-resistance phenomenon;
- to evaluate the contribution of DNA double strand breaks underlying γ-H2AX focus formation in bystander effect.

Materials and Methods

2.1. Characterisation of Cell Lines

Two strains of human foreskin fibroblasts, BJ and VH10 were used for the experiments. Both strains were used either in their native, wild type form or after transfection with hTERT. The BJ strains cells were obtained from Dr. Kevin Prise (Gray Cancer Institute, U.K.) and from Dr. Brian Ponnaiya (RARAF Center for Radiological Research, Nevis Laboratory, Columbia University, NY).

The BJ line was established from skin taken from normal foreskin. The BJ line has longer lifespan (85 to 90 population doublings) in comparison with other normal human fibroblast cell lines (Bodnar et al, 1998). These cells have a reported normal diploid karyotype at population doubling 61 but an abnormal karyotype at population doubling 82 (Morales et al, 1999). They are telomerase negative (Yl et al, 1999). Cells from the current distribution stock (population doubling 22) have the capacity to proliferate for at least an additional 52 population doublings before the onset of senescence.

The hTERT-BJ1 cell line is a telomerase-immortalised BJ cell line. This cell line stably expresses human telomerase reverse transcriptase (hTERT), which allows the cells to live indefinitely while retaining normal phenotype and function (Bodnar et al, 1998; Jiang et al, 1999). Immortal cell lines provide a uniform, stable, and perpetual source of

nonmalignant cells for a wide range of cellular studies- from genetic modifications such as transfections or gene knockouts, to functional assays such as drug screening or toxicity testing. Cells expressing hTERT are also valuable for dissecting the genetic pathways leading to cancer, as they circumvent the barrier of limited lifespan that limits the steps of cancer progression.

Overall, the expression of hTERT in normal cells allows them to grow indefinitely, but does not confer a transformed cell phenotype.

Immortalised cells utilize the hTERT subunit of telomerase for immortality, but are not plagued by the additional genetic disruptions characteristic of viral transformed cells (Jiang at al, 1999; Morales at al, 1999).

Viral transformed cell lines are immortal, do have crisis and the cells tend to be genetically unstable, as they can became aneuploid by losing or gaining whole or partial chromosomes.

Normal human somatic cells have a limited lifespan and then undergo a state of irreversible growth arrest called senescence. Studies show that when telomeres become critically short, senescence is triggered (Harley at al, 1990), possibly because some of the short telomeres are perceived as DNA breaks as part of p53-dependent cell-cycle checkpoint arrest (Morales at al, 1999). It has also been shown that immortal cells do not senesce because their telomeres are maintained by telomerase (Bodnar at al, 1998, Yang at al, 1999, Morales at al, 1999).

hTERT-BJ1 cells express telomerase at a level similar to that of a malignant cell line (Vaziri et al, 1998). In addition, the telomeres of the chromosomes of immortal cell lines are maintained or extended in comparison to the parental cells that do not express the telomerase gene (Bodnar et al, 1998; Yang at al, 1999).

VH10 human foreskin fibroblasts cells were kindly provided by Prof. Bert van Zeeland (LUMC, The Netherlands). They were immortalised in the department while the experiments described in this thesis were performed by transfection of VH10 cells with the pBABE-NEO-hTERT plasmid (Rubio et al, 2002). A calcium phosphate transfection method was used.

Two days prior to transfection, the cells were plated such that they were 50-60% confluent at the time of transfection. One day prior to transfection the medium was removed from the cells and replaced with fresh growth medium.

The Ca-phosphate-DNA precipitate was taken into cells by an endocytic-type mechanism and was made as follows:

In a sterile tube10μg DNA (in total, plasmid DNA and Salmon Sperm DNA) and sterile water to a total volume of 225μl were added. After mixing well, 25μl 2.5 M $CaCl_2$ was added and mixed by vortexing. While vortexing at low speed, 250μl 2X HBS was added dropwise into the tube which was then left for 15-30 minutes at room temperature in the dark. During this time the solution appeared slightly opaque due to the formation of a fine calcium phosphate-DNA coprecipitate.

The 2X HEPES buffered saline (HBS) was made by dissolving 280mM NaCl , 10 mM KCl, 1.5mM $Na_2HPO_4.2H_2O$, 12 mM dextrose and 50mM HEPES. The pH was adjusted to 7.05 with 0.5 M NaOH and the volume to 100 ml. Everything was sterilised with a filter, aliquoted and stored at -20°C.

The DNA $CaPO_4$ precipitate was added slowly drop by drop to the cells (0.5 ml precipitate for a 5 cm Petri dish) mixing well by shaking the dish carefully. The dishes were incubated for 5-6 hours. After the incubation the medium from the dishes was removed and 2.5 ml 10% v/v DMSO in 1X HBS (2X HBS diluted 1:2) was added to the cells and left for 90 seconds. The cells were then washed 3 times with 5 ml 1X HBS and fresh growth medium was given to the cells.
24 hours after transfection fresh medium containing 500µg/ml G418 (neomycin) as a selection marker was given to the cells. Two weeks later clones appeared. One of these clones was used in these experiments.

To test if the hTERT transfected cells used in these experiments possessed telomerase activity, the cells were analysed for telomerase activity using a Telomeric Repeat Amplification Protocol (TRAP). The TRAP assay includes a detergent lysis method that allows uniform extraction of telomerase from a small number of cells. Telomerase activity in a cell extract is determined through its ability to synthesise telomeric repeats onto an oligonucleotide substrate *in vitro*.
In brief, the *Telo*TAGGG Telomerase PCR ELISA assay kit (Roche Applied Science, Germany) used for this study can be separated into the following steps:

In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic P1-TS-primer. In the second step, these elongation products are amplified by polymerase chain reaction (PCR) using the primers P1-TS and P2, generating PCR products with the telomerase- specific 6 nucleotide increments.
In the next step an aliquot of the PCR product is denatured and hybridised to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting product is immobilised via the biotin labeled primer to a streptavidin-coated microplate. The immobilised PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. Finally, the probe is visualised by virtue of peroxidase metabolising TMB to form a coloured reaction product.
As positive control VH10-SV40 transformed cells were used. Negative controls were produced by heat-treatment of the cell extract for 10 min at 85° C prior to the TRAP reaction, in order to inactivate the telomerase protein.
The telomerase activity test was performed for the following cell lines and controls: VH10, VH10- negative control, VH10-SV40, VH10-SV40-negative control, VH10-hTERT, VH10-hTERT-negative control, BJ1-hTERT, BJ1-hTERT-negative control, BJ. Cells were harvested and counted using a hemocytometer, and then 2 X 10^5 cells per single reaction were transferred into a fresh Eppendorf tube. The cells were pelleted at 3000 rpm for 10 min in a refrigerated centrifuge (2-8° C), the supernatant was carefully removed, cells were re-suspended in phosphate buffer solution (PBS) and the centrifugation step was repeated. After removing the supernatant, in order to lyse the cells, the pelleted cells were re-suspended in 200µl Lysis reagent pre-cooled on ice and left on ice for 30 min. The lysate was centrifuged at 16000 rpm for 20 min in a

refrigerated centrifuge.175 µl of each supernatant was transferred to a fresh PCR tube.

The reaction mixture for each sample and the controls was:

For samples (VH10, VH10-hTERT, BJ1-hTERT, BJ): 3 µl cell extract per tube+ 25 µl Reaction mixture + 22 µl sterile water;

For negative controls (VH10, VH10-SV40, VH10-hTERT, BJ1-hTERT, BJ): 3 µl of heat treated cell extract + 25 µl PCR Reaction mixture + 22 µl sterile water;

For positive control (VH10-SV40): 3 µl of the reconstituted solution + 25 µl Reaction mixture + 22 µl sterile water.

Tubes were transferred to a thermal cycler and a combined primer elongation/ amplification reaction was performed according to the following protocol:

	Time	Temperature	Cycles
Primer elongation	30 min	25° C	1
Telomerase inactivation	5 min	94° C	1
Amplification:			
Denaturation	30s	94° C	
Annealing	30s	50° C	30
Polymerization	90s	72° C	
	10min	72° C	1
Hold		4° C	

The hybridisation and ELISA procedure was performed following the protocol described below:

1. Per sample 20 µl of Denaturation reagent were transferred into sterile Eppendorf tubes;
2. 5 µl of the amplification product was added to the tubes and incubated at room temperature for 10 min;
3. 225 µl Hybridisation buffer were added per tube and everthing spinned down briefly;
4. 100 µl of this mixture were transferred per well of the pre-coated microplate modules supplied with the kit and the wells covered with the self-adhesive cover foil to prevent evaporation;
5. The modules were incubated at room temperature on a shaker (300rpm for 2h);
6. The hybridization solution was removed completely, then the modules washed 3 times with 250 µl of Washing buffer per well for 30 seconds each and the Washing buffer removed carefully;
7. 100 µl Anti-DIG-POD working solution was added per well, the MP modules were covered with a cover foil and incubated at room temperature for 30 min while shaking at 300 rpm;
8. The solution was removed completely, the modules rinsed 5 times with 250 µl of Washing buffer per well for a minimum of 30 seconds each and the Washing buffer removed carefully;

9. 100 µl TMB substrate solution pre-warmed at room temperature was added per well, the wells were covered with foil and incubated for colour development at room temperature for 10 min while shaking at 300 rpm;
10. Without removing the reacted substrate, 100 µl Stop reagent per well were added in order to stop colour development. Addition of the Stop reagent caused the reacted POD substrate to change colour from blue to yellow.

Details on reagents used in this protocol were not provided by the company.

Measurements of the absorbance of the samples at 450 nm were performed within 30 min after addition of the Stop reagent using a Microplate (ELISA) reader (BIO-RAD Model 550).

2.2. Cell Culture

Cells were grown in plastic T75 culture flasks (Greiner bio-one, Holland) at 37°C in a humidified atmosphere of 95% air: 5% CO_2. For all experiments, cells were seeded from stocks in the confluent growth phase. They had a doubling time of about 24 hours and cells were typically used between passage 7 and 12.

For experiments, culture media was removed from the confluent stock flasks and the cells washed with calcium free phosphate buffered saline (PBS). 1 ml of Trypsin (0.05%w/v)/ EDTA (0.02% w/v) (Gibco-Invitrogen, USA) was added and the cells incubated at 37°C for 5 minutes to facilitate dispersal. When the cells were detached, 9 ml of complete

growth medium was added and the cell suspension was then resuspended. The number of cells in the resulting suspension was counted with a coulter particle counter and seeded as appropriate in cell culture flasks or Petri dishes (all from Greiner bio-one, Holland)

BJ cells:

Cells were grown in Eagle's MEM with Earle's BSS medium supplemented with 10% fetal bovine serum, 10mM non-essential amino acids, 100 mM sodium pyruvate, 200mM L-Glutamine and antibiotic/antimycotic (Gibco-Biocult, Irvine, UK).

BJ1-hTERT cells:

Cells were grown in a 4:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Media 199 supplemented with 10% fetal bovine serum, 100 mM sodium pyruvate, 200mM L-Glutamine and antibiotic/antimycotic (penicilin streptomycin solution)(Gibco-Biocult, Irvine, UK).

VH10 and VH10-hTERT cells:

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (penicillin streptomycin solution) (Gibco-Biocult, Irvine, UK).

2.3. Irradiation

Experiments were performed in three different laboratories using different radiation sources.

St. Luke's Hospital, Dublin

Cells were irradiated at room temperature using a cobalt-60 teletherapy unit delivering 1.8 Gy per minute at a source to flask distance of 80 cm. Cells were returned to the incubator immediately after irradiation. Information about the dose rate was provided by the laboratory but not opportunity was provided to check this information.

Gray Cancer Institute

Cells were irradiated in Petri dishes, T75 flasks or T25 flasks according to the design of the experiments. The cells were irradiated from above in a circular aluminium jig using an industrial Pantak IV X-ray unit, set at broad focus with an accelerating voltage of 240kV, with a filtration of 0.5 mm copper and 0.9 mm aluminium and a tube current of 13 mA at a fixed distance, giving a dose rate of 0.73 Gy/ minute. Dosimetry was carried out using a Farmer-Baldwin dosimeter. Dishes or flasks were incubated for different times prior to irradiation to allow cells to attach; the times were dependent on the experiment performed. The cells were then removed from the incubator to adjust to room temperature for a few minutes before being moved to the X-ray room and were returned immediately after irradiation to the incubator. The cells were not out of the incubator for

more than 15 minutes in any experiment. Control dishes were also moved to the X-ray room and sham irradiated for the same time period.

Leiden University Medical Center

Cells were X-irradiated from above at room temperature using an Andrex SMART 255 X-ray machine, operating at 200 kV, with a filtration of 1mm aluminium and a tube current of 4 mA at a fixed distance, giving a dose rate of 2 Gy/min. Dosimetry was carried out using a PTW dosimeter. Cells were used either immediately after exposure or after different incubation time intervals at 37° C. Non-irradiated control cultures were processed in parallel.

2.4. Endpoints

A range of different response criteria (endpoints) was used for studying the direct and bystander effect of radiations in fibroblasts. They will be described in the next paragraphs.

2.4.1. Clonogenic Assay- Direct Radiation Effect

Cell survival assays were used for BJ and VH10 normal human foreskin fibroblast and BJ1-hTERT and VH10-hTERT immortalized fibroblast cell cultures. Cells from the stock

flasks were removed using the method described in section 2.2. When the cells had detached they were gently resuspended in medium using a small volume pipette tip to produce a single suspension and an aliquot was counted using a coulter counter. Appropriate cell numbers were plated for survival using the clonogenic assay technique described by Puck and Marcus (1956), so that about 100 colonies per dish were expected. Both cell lines were plated 15 hours before irradiation from confluent cultures with the same number and type of flasks and the same dose points. For doses of 0, 0.1, 0.3, 0.5, 0.7, 1.0 Gy, 1000 cells were plated, 2000 cells for doses of 2.0, 3.0 Gy and 5000 cells for the doses of 5.0 and 6.0 Gy. Cells were grown in 5 ml of culture medium as described above (cell culture section). For each dose point three T25 flasks were used.

The cells were allowed to grow for 14 days (BJ and VH10 cells) and 17 days (hTERT cells) respectively until the colonies became visible. Each flask was removed from the incubator and the medium was withdrawn. The resulting colonies were stained with carbol fuchsin (1:15) or with 2% crystal violet to determine the plating efficiencies of the control cells and the surviving fractions of the irradiated cells.
Colonies containing more than 50 cells were scored as representing cells with clonogenic potential. The mean proportion of plated cells, which gave rise to a colony, was calculated as the plating efficiency of the respective dose. The surviving fraction was calculated by dividing the colony forming efficiency of irradiated cells by the plating efficiency of the respective unirradiated control cells. The mean surviving fraction of the three independent dishes from each dose point and their standard error was calculated and plotted on logarithmic scale as a function of dose on a linear scale.

2.4.2. The Bystander Effect on Clonogenicity

Flasks were set up one day prior to irradiation from confluent cultures as Donor and Recipient flasks.

The "Recipient" cells were plated out as described in section 2.4.1. with 500 cells per T25 flasks(Nunc, Denmark), three flasks per each dose point.

Flask designed to donate medium were plated in T75 flasks with approximately 200.000 cells. Controls for medium only effects were included in each experiment. These were set up, incubated and irradiated at the same time as the rest of the donor flasks but contained no cells.

15 hours later the cells from the donor flasks were irradiated at doses of 0, 0.1, 0.3, 0.5, 0.7, 1.0, 2.0, 3.0, 5.0, 6.0 Gy.

All flasks were returned to the incubator immediately after the irradiation.

Medium was sucked off donor flasks 2 hours post irradiation using a 20 ml syringe (Terumo Europe N.V., Leuven, Belgium or Becton Dickinson, Ireland). The medium was filtered through a 0.22 μm filter (Corning Costar, USA or Schleicher & Schuell, Germany) used for sterilising solutions to prevent bacterial contamination and to allow the passage of molecules but not cells. The presence of cells in the transfer medium was regularly checked microscopically and no cells were found in the transferred medium for any of the cell lines. Culture medium was then gently aspirated from the recipient flasks and the irradiated filtrate was immediately added with great care to prevent disruption of attached cells to these flasks. A similarly filtered medium change from unirradiated donor

flasks to unirradiated (controls) recipient flasks was performed at the same time. Standard plating efficiency controls were also set up.

The effect of exchanging irradiated medium between cell lines was checked for the BJ and BJ1-hTERT cell lines. Medium from irradiated flasks containing BJ cells was removed 2h post irradiation and then used to replace the medium from flasks containing non-irradiated BJ1-hTERT cells. The reverse experiment whereby medium from irradiated BJ1-hTERT cells was used to replace medium from non-irradiated BJ cells was also performed.

The cells were allowed to grow for 14 days (BJ and VH10 cells) and 17 days (hTERT cells) respectively until the colonies became visible. Each flask had the medium removed and the colonies were stained with carbol fuchsin (1:15) or with crystal violet. Colonies containing more than 50 cells were counted.

2.4.3. Cytogenetic Short-Term Assays

Cytogenetic assays such as sister-chromatid exchanges (SCEs), chromosomal aberrations (CAs) and micronuclei (MN) are widely utilised as biological end points for evaluating genotoxic agents in vivo and in vitro (for reviews see Natarajan and Obe, 1982; Carrano and Natarajan, 1988; McFee et al, 1989). Correlations were found between DNA repair deficiencies and chromosomal alterations in human hereditary disorders with increased

susceptibility to cancer. This confirms the importance of cytogenetic analysis as a biological monitor for evaluating the hazard potential of genotoxic substances.

In standard cytogenetic methods, chromosomes are observed in metaphase, and their aberrations counted. This method provides a wealth of information about the chromosomal lesions, but it has the disadvantage that enumerating and evaluating chromosomal aberrations in metaphase preparation are both complex, expensive and time consuming.

Therefore, initially in this study experiments followed a sequence of increasing complexity starting with simple methods which permitted rapid screening to be followed by various more targeted procedures.

1^0. Micronuclei

The micronucleus assay is a relatively simple and rapid method to evaluate irradiation damage to the chromosomes.

After exposure of cells to DNA damaging agent such as radiation, fragmented chromosome material lacking an intact centromere may not be properly segregated into daughter cells during cell division. The chromosome fragment appears as a micronucleus within the cytoplasm of the irradiated cell or may be randomly segregated into either or both of the daughters. Micronuclei (MN) are formed by chromosomal fragments or

lagging chromosomes in anaphases, which are not included in the nuclei of the daughter cells (Countryman and Heddle, 1976).

After irradiation a characteristic dose response of micronucleus frequency is observed. The frequency of cells with micronuclei as well as the number of micronuclei per cell determined 24 hours after irradiation increases with dose and reaches a peak value of approximately 50% at 4 Gy, decreasing at higher doses to a plateau at around 30% (Jamali and Trott, 1996). When the frequency of cells without micronuclei was plotted against the surviving fraction for each experimental point at doses of < 4 Gy, a linear relationship described the data well ($r = 0.9$). However there was no one-to -one relationship between surviving fraction and cells without micronuclei, which demonstrated that less than half of the sterilised cells express micronuclei at their first division.

Fenech and Morley (1985) developed the cytokinesis block procedure which increased the sensitivity of the micronuclei test. They used cytochalasin B that arrests cytokinesis but does not interfere with karyokinesis. With this protocol it is possible to distinguish between proliferating and nonproliferating cells. Micronuclei are scored only in binucleated cells with intact cytoplasm which had progressed through one division after adding cytochalasin B.

Micronucleus Frequency after Irradiation

Although the micronucleus test appears simple, great care and patience is required to optimise procedures to get reproducible results. Despite extensive pilot experiments varying cytochalasin B concentration (0.3-3 μg / ml), exposure times (18-48h), fixation methods which extended over one year, no reproducible data were obtained in the Dublin laboratory. Experiments were set up again at LUMC where extensive experience in this technique on other cell lines was available.

Early passage cells in exponential growth were seeded (at a density of 10^5 cells/ dish) onto T25 flasks with 5 ml culture medium (same as above) and 3 dishes were plated per dose in each experiment. Following a 24h incubation period, the cells were irradiated with single doses ranging from 0.1 to 6 Gy. Non-irradiated control cultures were processed in parallel.

Immediately after irradiation, cytochalasin B (Sigma-Aldrich Chemie GmbH, Germany) was added to block cytokinesis, but not karyokinesis. This protocol allows non-proliferating and proliferating cells to be distinguished and to exclusively score micronuclei in binucleated cells, i.e. in cells after the first mitosis.

The stock solution of cytochalasin B was prepared by dissolving 1 mg in 1 ml dimethyl sulphoxide (DMSO) (J.T.Backer, Holland) divided into aliquots and stored frozen at -20 ^{0}C. The optimum concentration of cytochalasin B for each cell line was determined by comparing the effect of 1.2, 1.5, 2 μg / ml. In our hands 1.5 μg/ml cytochalasin B was

found to be optimal for human fibroblasts. After 48 h incubation (with cytochalasin B), the cells were collected into centrifuge tubes and centrifuged at 800 rpm for 8 minutes. The duration of incubation was chosen to allow all proliferating cells to accomplish the first karyokinesis, resulting in the formation of binucleated cells for maximum micronuclei yields.

After removing the supernatant until 1cm above the ring of the tube cells were resuspended in 5ml cold hypotonic (KCl 5.6g/l). Cells were then centrifuged at 800 rpm for 8 minutes and the cell suspension fixed in 5 ml fixative (acetic-acid: methanol, 1:3 v/v) (both from J.T.Backer, Holland), while dispersing slowly with a vortex stirrer. Three drops of formaldehyde 37% (Merck, Darmstadt, Germany) were added to the cells and the fixative was changed for 2 more times after centrifugation at 600 rpm for 8 minutes. The slides were prepared by dropping the cell suspension on precleaned object glasses with a drawn-out Pasteur pipette. When drops have maximally expanded and when Newton rings began to appear, the slides were blown gently to accomplish a flattering of the cells.

The slides were stained for 5 minutes in a 2% aqueous Giemsa solution (BDH Laboratory Supplies, Poole, England). After rinsing for a few seconds in distilled water to remove the excess stain, the slides were air-dried at room temperature and mounted in a small amount of DePex mounting medium (BDH Laboratory Supplies, Poole, England). Slides were then coded and stored until required.

For each dose point the parameters defined were the percentage of BN cells with at least one micronucleus (%BNC+MN) and the average number of micronuclei per individual

BN cell (MN/BNC). The percentage of binucleated (BN) cells was scored in a total of 500 cells per dose (in three dishes) for each experiment. The micronucleus (MN) frequency was determined in a total of 500 BN cells per dose in each experiment. Those binucleate cells with more than one micronucleus were also considered. Micronuclei frequency was calculated in two ways: 1) micronuclei per cell i.e. total number of micronuclei including multiple micronuclei per cell divided by the number of counted binucleate cells and 2) cells with micronuclei i.e. cells with one or more micronuclei divided by the number of binucleate cells.
The distribution of micronuclei per binucleate cells was plotted.

All the observations and scoring were carried out at 1000x magnification with an Axioscop (Zeiss, Germany) microscope under oil immersion.
For each cell line, the experiment was repeated 3–6 times. Micronucleus assays for wild type and immortalized cell lines were always prepared in parallel from the same passages.
Mean values and errors (SEM) for micronucleus frequency were calculated from all data obtained for all experiments.
For the media transfer studies, BJ, VH10 and BJ1-hTERT, VH10-hTERT cultures were seeded as described above. The cells designated to donate medium were irradiated with 0.1; 0.5; 1; 3; 5Gy. At 2h after irradiation, the media were removed from the irradiated cultures, filtered as explained on the method to study the bystander effect for clonogenicity and added to the dishes containing the unirradiated bystander cells. Cytochalasin B was added to the "recipient cells" immediately after filtering and

transferring the media from the "donors" to the "recipients" and the protocol described above was followed.

Media transferred from unirradiated cultures and media irradiated in the absence of cells were used as controls.

2^0. Premature Chromosome Condensation Assay (PCC)

The technique of premature chromosome condensation (PCC) first described by Johnson and Rao (1970), in principle, enables preparation of chromosome spreads independent of cell cycle stage and with minimal culturing artefacts. Conventionally, PCC is induced by fusion of test cells with mitotic cells in the presence of virus or fusing agent.

The Premature Chromosome Condensation (PCC) method has been demonstrated to be an excellent tool for the estimation of low dose exposure. The conventional colcemid method often fails to obtain mitotic chromosomes (mitotic index 7%); PCC may overcome this, but the conventional fusion method is technically demanding and the efficiency is very low for fibroblasts. Calyculin A (CA) (inhibitor of protein phosphatses 1 and 2A) was used as new inducer of PCC with high efficiency.

Cells were grown as monolayers in the medium described previously in the cell culture section (2.2). Cells were washed with phosphate-buffered saline (PBS), and fresh medium was added after irradiation and before treatment with CA.

Induction of PCC and Harvesting

Calyculin A (CA) (Sigma-Aldrich Chemie GmbH, Germany) was dissolved in ethanol (J.T. Baker, Holland) as stock solution of 4 mM and stored at -20°C.

The effect of Calyculin A (CA) was investigated with BJ and BJ-hTERT cells in monolayer culture at three different concentrations of 40, 80, and 120 nM for 0.5 hours, 45 minutes, and 1 hour.

The highest frequency of PCC was typically obtained at 80 nM and after 45 minutes treatment. Therefore, CA was used at a final concentration of 80 nM. During the treatment with CA, cells were monitored by use of phase-contrast microscopy. The majority (at least 95%) of the cells were found to round up and detach after 45 minutes treatment with 80 nM CA.

After irradiation fresh medium was given to the cells and the above mentioned amount of CA was added to the cells. Cells were incubated then collected and treated according to standard cytogenetic procedures to obtain chromosome spreads. Cell pellets were collected by centrifugation at 800 rpm for 8 minutes and treated with a prewarmed hypotonic solution of KCl (0.075 M) for 20 minutes at 37°C and fixed in three changes of fixative (acetic acid: methanol, 1:3 v/v). Finally, the fixed cells were dropped by using a 20 µl pipette onto pre-cleaned slides and air-dried. The slides were allowed to dry over night and they were stained with Giemsa 2% solution for 3-5 minutes according to the PCC morphology. The PCC technique can also be combined with Fluorescence in Situ Hybridisation (FISH) using DNA libraries for specific chromosomes to analyse simultaneously, unstable (breaks, dicentrics) as well as stable (translocations) aberrations.

2.4.4. Metaphases Preparations

Approximately 2 X 10^6 BJ and BJ1-hTERT cells were plated in T75 flasks for each X-ray dose of 0, 0.1, 0.5 and 1.0 Gy. Cells were incubated for 24h and irradiated with the above mentioned doses. After irradiation colcemid at a concentration of 0.2 μg/ ml was added to each flask which was then returned to the incubator for 4 hours. Chromosome preparations were performed by accumulating methaphases. Cells were harvested as described before and a single cell suspension was prepared which was collected and transferred to centrifuge tubes. This was followed by centrifugation at 1000 rpm for 6 minutes after which the supernatant was removed. Cells were then treated with prewarmed hypotonic KCl solution (5.6 g / l) and incubated without shaking for 15 minutes in a water bath at 37^0C after which they were spun down at 800 rpm for 8 minutes and the supernatant poured off. Cells were fixed three times in methanol: acetic acid (3:1 v/v) which was added drop wise while dispersing slowly with a vortex stirrer. The slides were made by dropping the cell suspension on pre-cleaned object glasses with a Pasteur pipette. When drops were maximally expanded and when Newton rings began to appear the slides were blown to accomplish a flattering of the cells. The slides were allowed to air dry overnight and they were stained with Giemsa 2% solution for 3-5 minutes according to the metaphases morphology. Finally, they were mounted with cover glasses using DePex mounting medium (BDH Laboratory Supplies, Poole, England). Scoring was carried out in 50 cells/ dose at 1000x magnification with an Axioscop (Zeiss, Germany) microscope under oil immersion.

2.4.5. γ-H2AX Histone Phosphorylation

One of the earliest steps in the cellular response to double strand breaks (DSBs) is the phosphorylation of serine 139 of H2AX, a subclass of eukaryotic histone proteins that are part of the nucleoprotein structure called chromatin (Rogakou et al., 1998).
Using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX), discrete nuclear foci can be visualized at sites of DSBs, either induced by exogenous agents such as ionising radiation (Rogakou et al., 1999; Burma et al., 2001) or generated endogenously during programmed DNA rearrangements (Che at al., 2000; Petersen et al., 2001; Redon et al., 2002). Recently, a direct correlation was observed between the number of foci and the number of DSBs produced by the decay of ^{125}I incorporated into cellular DNA (Sedelnikova et al., 2002), suggesting that each focus may represent an individual break and that each DSB may form a focus. Furthermore, another recent report indicated the possibility of measuring the effects of radiation doses close to 1mGy using the γ-H2AX method (Rothkamm and Löbrich, 2003).

For the experiment the cells were seeded at a high density of 10^4cells/cm^2 on sterilised round glass coverslips (Marienfeld GmbH &Co. KG, Germany) in P6 or P9 Petri dishes (Greiner bio-one, Holland) and grown to confluency during seven subsequent days. Preceding the experiment the coverslips were coated with Alcian Blue solution for 10 minutes, rinsed with 70% ethanol then with water to remove excess Alcian Blue, polished and sterilized by autoclaving.

Confluent cells were X-irradiated with 0.25; 0.5; 1 and 2 Gy. Cells were fixed after different incubation times (10 minutes, 30 minutes, 1h, 4h, 24h, 48h, 72h) at 37° C. Non-irradiated control cultures were processed in parallel.

For immunofluorescence, cells were fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes, washed in PBS for 3 x 5 minutes and permeabilised for 5 minutes on ice in 0.2% Triton X-100 (Sigma-Aldrich Chemie GmbH, Germany). Then the coverslips were removed from the dishes, placed on parafilm to produce a hydrophobic environment and blocked in PBS with 3% Albumin, Bovine Serum (BSA) (Sigma-Aldrich Chemie GmbH, Germany) for 30 minutes at room temperature. All the washing steps were done very gently by using a pipette. The coverslips were incubated with mouse anti-gamma-H2AX antibody (Trevigen, Gaithersburg, MD) for 1h in a wet chamber, washed in PBS containing1% BSA and 0.05% Tween-20 (Sigma-Aldrich Chemie GmbH, Germany) for 3 X 5 minutes, and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) containing 100µg/ ml DAPI for 45 minutes in dark wet chamber at room temperature. Cells were washed in washing buffer (PBS with 1%BSA and 0.05% Tween-20) for 3 x 5 minutes, fixed for 5 minutes with 2% formaldehyde in PBS, washed once with PBS to remove fixative and mounted by using 5 µl of antifade Vectashield mounting medium (Vector Laboratories), Aqua Polymount (Polysciences, Inc., Warrington, PA) or Citifluor (Agar Scientific, U.K.). When Vectashield was chosen as a mounting medium the DAPI was added into it, therefore the incubation with the secondary antibody was done without adding the DAPI. After mounting, the slides were sealed by using nail polish to avoid contact with immersion oil. The sealing was not necessary when mounting with Aqua Polymount.

Fluorescence images were captured and examined by using a Zeiss Axioplan 2 epifluorescence microscope fitted with appropriate filters coupled to an AttoArc HBO 100W adjustable mercury arc lamp, and a Hamamatsu C5935 cooled CCD camera. The pictures were captured and processed with Metasystems (Altlussheim, Germany) ISIS software. The γ-H2AX foci were counted by eye in a blinded fashion in randomly chosen cells. Approximately 1% of the nuclei were substantially larger than normal (possibly indicating the presence of tetraploid cells), and were not considered for evaluation. For each sample cell counting was performed until at least 100 cells. The specific numbers of cells counted per single determination were: control and doses up to 1Gy, 200-600 cells; 1 Gy, 100-200 cells. The error bars in Figures 3.15, 3.16, 3.17, 3.18 in the Results section represent the SEM from the analysis of these numbers of cells from two to three independent experiments. Cells with apoptotic features (bubble like appearance of the nucleus) were not considered for γ-H2AX analysis.

For the media transfer studies, BJ, VH10, BJ1-hTERT and VH10-hTERT cultures were seeded as described above, grown to confluency (about 4 X 10^4cells/ cm^2) during seven subsequent days and irradiated with 0.25; 0.5; 1Gy (BJ and BJ1-hTERT cells) and with 0.1; 0.25; 0.5Gy (VH10 and VH10-hTERT cells). At 2 hours after irradiation, the media were removed from the irradiated cultures, filtered as explained on the method to study the bystander effect for clonogenicity and added to the Petri dishes containing the unirradiated bystander cells. The cultures containing the conditioned media were incubated for various times (1h, 6h, 18h and/ or 30h), fixed and stained for γ-H2AX foci according to the immunofluorescence procedure described. Bystander cells were assessed regardless of their position. Since the BJ and VH10 primary human fibroblasts were

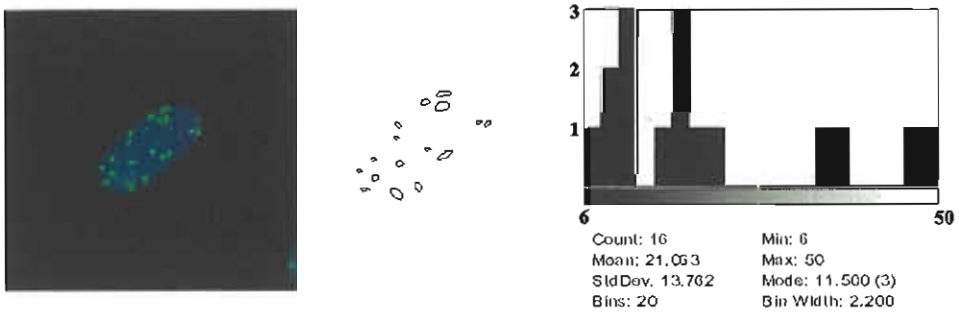
subcultured during all these experiments, the numbers of population doublings changed and correspondingly the background level of γ-H2AX foci varied slightly among experiments.

Media transferred from unirradiated cultures and media irradiated in the absence of cells were used as controls, and both showed no bystander effect.

γ-H2AX foci were also counted automatically using the ImageJ software. An example of the outline is given below in Figure 2.1 and Table 2.1.

With "analyse particles" command foci were counted and measured as objects in binary or threshold images. The objects were measured by entering a value for the size of the particles and particles smaller than that value were ignored.

No differences were found between the automatic and the manual counting.



(a) Original image (b) "Outlines" of the measured particles (c) Particles size distributions

Figure 2.1: Example of the outline of the automatic counting of γ-H2AX foci in an irradiated BJ cell using the ImageJ software.
(a) Original image of one irradiated BJ cell with γ-H2AX foci (green channel). Nuclei were stained with 4,6-diamidino-2-phenylindole (blue channel);
(b) "Outlines" of the measured particles (γ-H2AX foci counted). Digital images were processed by applying the threshold, edge finder and binarise tools prior to automatic particle (γ-H2AX foci) detection;
(c) Size distribution of automatically detected particles (γ-H2AX foci). X-axis represents the values (in pixels) of the sizes of the particles counted. Particles smaller than the minimum value (6 pixels) and greater than the maximum value (50 pixels) were considered artifacts (imaging and/or analysis) and were thus ignored. The Y-axis represents the number of particles counted.

No of particles	Area	X	Y
1	35	98	67
2	22	87	73
3	50	102	74
4	9	126	87
5	15	132	87
6	11	67	89
7	6	66	99
8	12	89	106
9	37	104	108
10	11	50	113
11	17	69	116
12	9	41	121
13	18	51	126
14	21	81	131
15	17	44	134
16	47	64	136

Table 2.1: Summary of results of the automatic counting of γ-H2AX foci in one irradiated BJ cell using the ImageJ software.
The first column enumerates all particles (γ-H2AX foci) counted in this cell;
The 2-nd column represents the area (number of pixels) of each of those particles (γ-H2AX foci);
The 3-rd and the 4-th columns indicate the Cartesian coordinates of each particle (γ-H2AX focus), i.e. their location in the image.

To examine γ-H2AX foci formation in cycling cells, the protocol described above was used. The cells were plated to give sub-confluent cultures at the time of fixation.

2.4.6. Measurement of Reactive Oxygen Species

Induction of reactive oxygen species was measured using 2,7 – dichlorofluorescin diacetate (DCF-DA). Once inside a cell, the acetate group is cleaved by cellular esterases leaving dichlorofluorescein which emits green fluorescence when oxidised by the reactive oxygen species, hydrogen peroxide and nitric oxide. Cultures were washed twice with a buffer containing 130mM NaCl, 5mM KCl, 1mM Na2HPO4, 1mM CaCl2, 1mM MgCl2 and 25 mM HEPES (pH= 7.4). Cells were loaded with 5 microM 2,7 – dichlorofluorescin diacetate for 30 minutes in the buffer at 37 C. Subsequently, the cultures were washed three times with buffer. The cells were then returned to the incubator for 30 minutes and washed once more prior to scoring. Fluorescence images were captured using a Zeiss Axioscope epifluorescent microscope with a BP 450-490 nm excitation filter and LP515 emission filter and cooled CCD camera system (Photonic Science, U.K.)

2.4.7. Rad51 Foci Formation in BJ and BJ1-hTERT Human Foreskin Fibroblasts

The central homologous recombination molecule, Rad51, forms nucleoprotein filaments on DNA and promotes exchange between homologous sequences (Ristic et al, 2005). Furthermore, Rad51 displays a dynamic redistribution into nuclear foci after treatment with DNA damaging agents (Li et al, 1997). These foci are formed at the site of DNA

damage and most probably represent centers for the repair of such lesions (Raderschall et al, 1999).

To examine Rad51 foci formation, BJ and BJ-hTERT fibroblasts were grown in P9 Petri dishes on sterile glass slides, giving sub-confluent cells at the time of fixation. The cells were seeded two days prior to X-irradiation at a density of approximately 5 X 10^4cells/ slide. For Rad51 analysis, cells were either mock-treated or irradiated with 12Gy X-rays. After 8 or 24 hours recovery period, the cells were washed quickly with PBS then fixed immediately with 2% formaldehyde in PBS for 15 minutes at room temperature. Following the fixation, the cells were permeabilised for antibody staining with PBS/ 0.1% Triton X-100 2 X 10 minutes at room temperature. Subsequently, the cells were blocked for 30 minutes in PBS/ BSA (0.5 %) / glycin (0.15%) and thereafter incubated with rabbit anti-HsRad51 antiserum (2307)(a kind gift from Prof Dr Roland Kanaar, Erasmus MC, Rotterdam) for 90 minutes in a humidified atmosphere. The slides were washed 3 times in PBS/ 0.1% Triton X-100 and then incubated with Alexa ™ 488-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 hour at room temperature in a humidified atmosphere. After 3 washes with PBS/ 0.1% Triton X-100 the cells were counterstained with 4', 6-diamino-2-phenylindole (DAPI; 0.1 μg/ ml)in Vectashield mounting medium (Vector Laboratories). Fluorescence microscopy was performed with a Zeiss Axioplan microscope equipped with filters for observation of DAPI, FITC and TRITC. Rad51 foci were analyzed manually or by means of a computerized image analysis system consisting of a Nu200 CCD camera, an Apple Macintosh PowerPC and IPlab software (Scanalytics Inc.).

For each individual experiment 150-600 cells were analysed.

Results

In the results section, experimental data shown as graphs or tables will be presented along with the main conclusions from each of the experiments and some additional comments. The initial experiments involved radiation exposure studies with normal human BJ fibroblasts and immortalised human BJ1-hTERT fibroblasts, which aimed at characterising response of these cell lines to radiation and to understand if the telomere/ telomerase system plays any role in the bystander effect. Later this study was extended to normal human VH10 fibroblasts and immortalised VH10-hTERT fibroblasts.

3.1. Characterisation of Cells with Respect to Telomerase Activity

To test if the extended life span of immortalised BJ1-hTERT and VH10-hTERT cells correlated with telomerase activity, the cells were analysed for telomerase activity using a Telomeric Repeat Amplification Protocol (TRAP) as described in Materials and Methods chapter.

Figure 3.1 shows that primary BJ and VH10 cells were negative by TRAP for telomerase activity, while BJ1-hTERT and VH10-hTERT cells had telomerase activity similar to that of transformed cells.

VH10, VH10-SV40, VH10-hTERT, BJ1-hTERT and BJ heat treated cell extracts were used as negative controls, while the VH10-SV40 transformed cell line was a positive control.

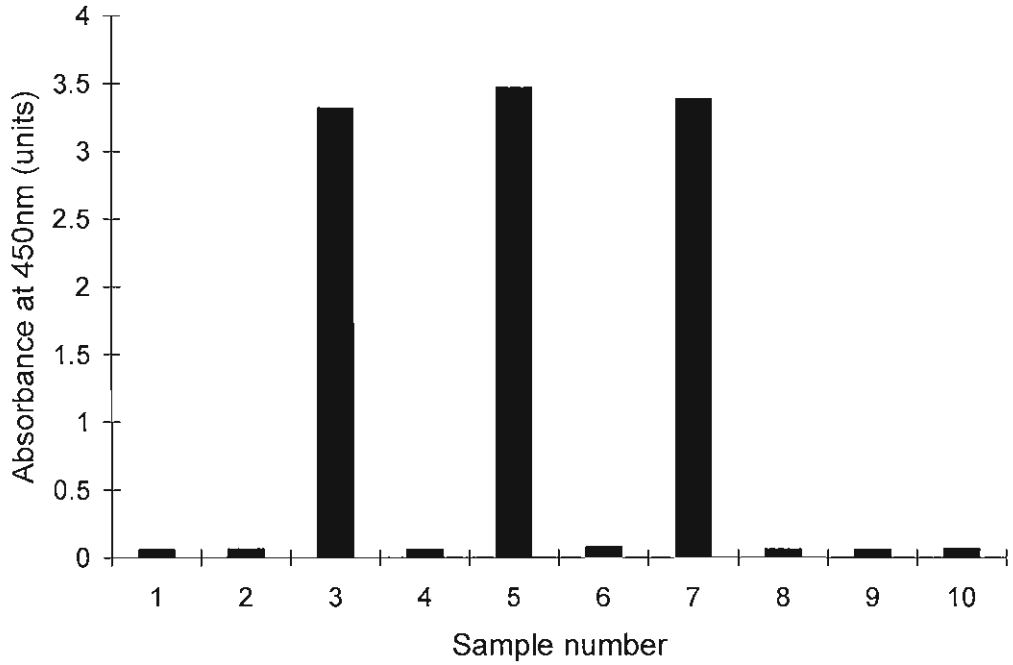


Figure 3.1: Detection of telomerase activity in cell lines using *Telo*TAGGG Telomerase PCR ELISA. Telomerase activity was measured in normal and immortalised human fibroblast cells. Assays were performed according to the kit protocol. Samples tested are as follows: **1**-VH10 normal human foreskin fibroblasts; **2**- VH10 heat at 85^0 C (negative control); **3**- VH10-SV40 transformed (positive control); **4**- VH10-SV40 heat at 85^0 C (negative control); **5**- VH10-hTERT immortalised human foreskin fibroblasts; **6**- VH10-hTERT heat at 85^0 C (negative control); **7**- BJ1-hTERT immortalised human foreskin fibroblasts; **8**- BJ1-hTERT heat at 85^0 C (negative control); **9**-BJ normal human foreskin fibroblasts; **10**- BJ heat at 85^0 C (negative control).

3.2. Dose-Effect Curves

Initial experiments in this work were started with γ rays to test for direct evidence of a bystander effect in normal human fibroblasts. Later this study was updated and a more comprehensive set of data obtained.

Dose-effect curves were obtained for cell survival, micronuclei induction, formation of γ-H2AX foci and chromosomal aberrations for cells irradiated with cobalt-60 γ- rays and/or 250 keV and/or 200 keV X-rays.

3.2.1. Dose Response for Direct and Bystander Effect of Ionising Radiation in Normal Human Fibroblasts and Immortalised Fibroblasts

The results of the experiment to determine clonogenic survival of BJ and BJ1-hTERT cell lines after exposure to γ radiation of increasing dose show that both cell lines responded identically to the radiation (Figure 3.2).

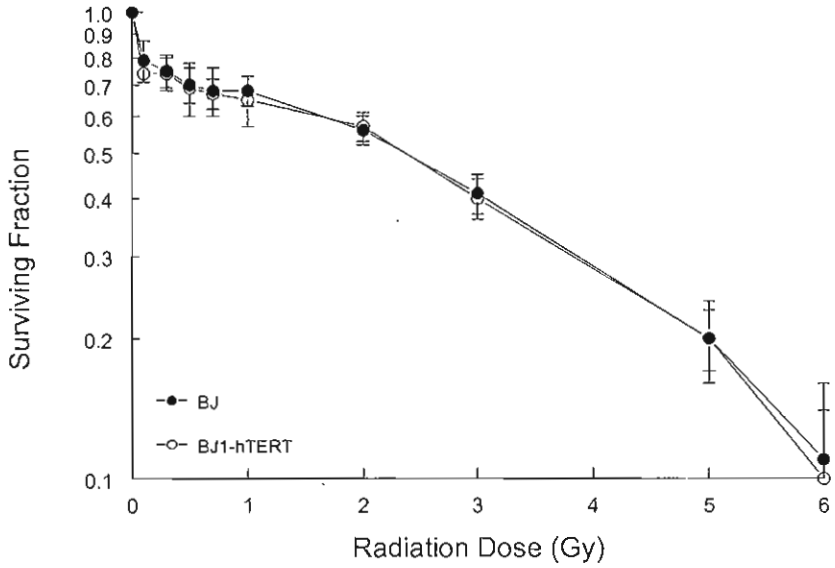


Figure 3.2: Comparison between the radiation dose dependence of the surviving fraction of BJ and BJ1-hTERT cell lines. The points are the means of three independent experiments. The error bars show the standard deviation.

While directly irradiated cells have initially a shoulder then a steady decrease on the survival with increasing dose, the data in Figure 3.3 show that the cloning efficiency of bystander cells decreased when the irradiated donor cells received doses between 0.1 and 6 Gy, but the decrease was almost dose-independent above 1 Gy. Again, the response of the hTERT cell line was not different from that of the BJ cell line.

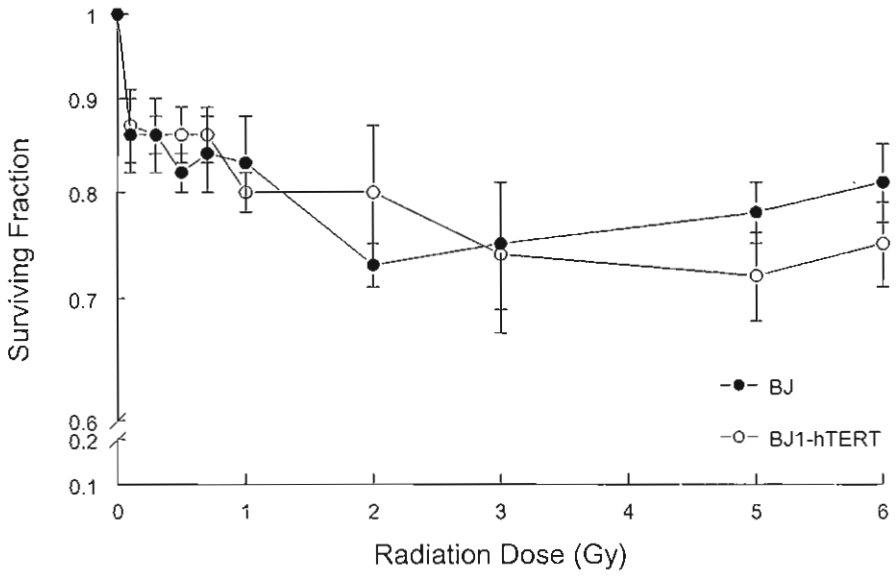


Figure 3.3: Comparison between the bystander survival curves of BJ and BJ1-hTERT cell lines. The points are the means of three independent experiments. The error bars show the standard deviation.

In order to see if the irradiated medium from one type of cells would have any effect when transferred to the other non irradiated cell line, a bystander medium transfer experiment of different cell lines as donors and recipients was performed. Figure 3.4 shows that the cloning efficiency of bystander cells decreased when the irradiated donor cells received doses between 0.1 and 6 Gy and the decrease was dose independent and did not depend on the donor or recipient cell type. The response of the BJ irradiated medium transferred to BJ1-hTERT bystander cells was not different from the response of BJ1-hTERT irradiated medium transferred to BJ bystander cells.

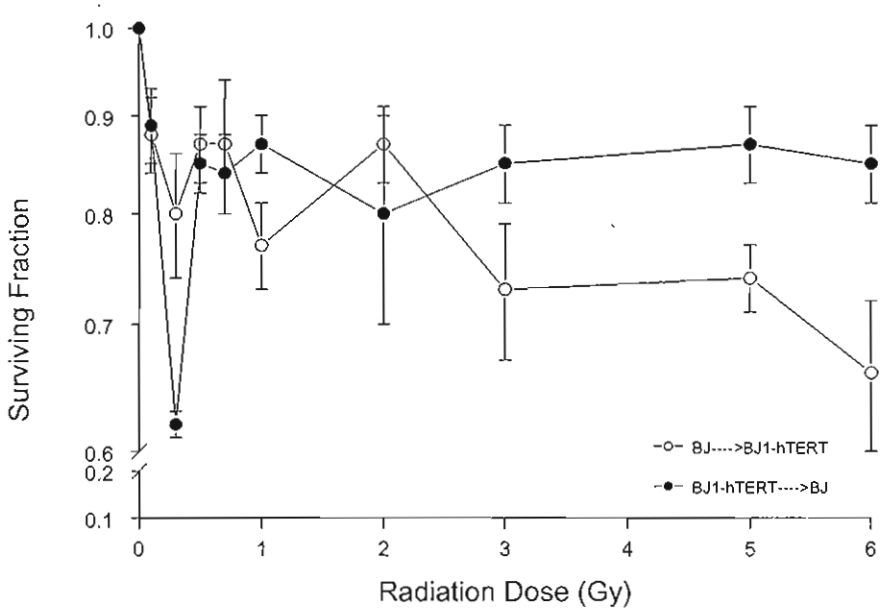


Figure 3.4: Comparison between the bystander survival curves of different cell lines as donors and recipients: BJ irradiated and medium transferred to BJ1-hTERT cells (opened symbols) and BJ1-hTERT irradiated and medium transferred to BJ cells (filled symbols). Data are means ± standard deviations of three independent experiments.

The results of the experiment to determine the clonogenic survival of VH10 and VH10-hTERT cell lines after exposure to 200 keV X-rays of increasing dose illustrate that both cell lines responded identically to the radiation.

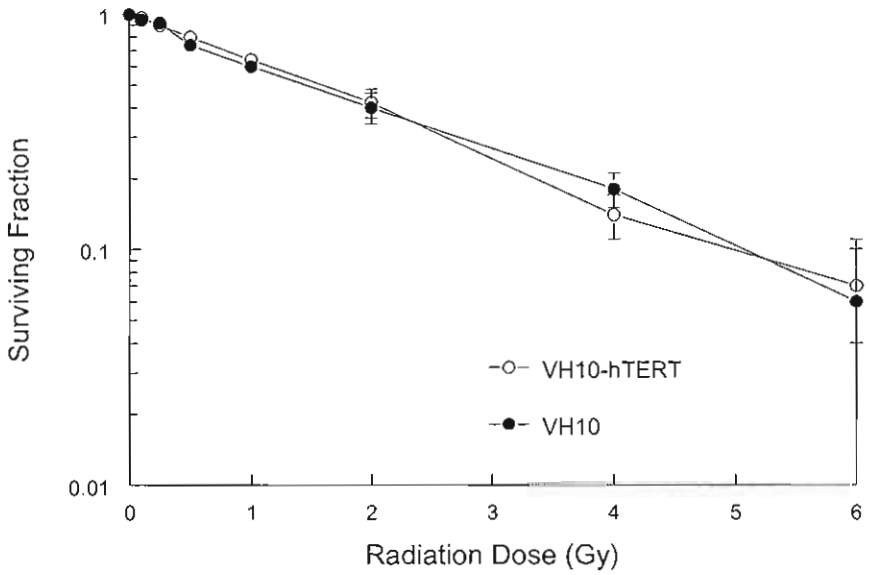


Figure 3.5: Comparison between the radiation dose dependence of the surviving fraction of VH10 and VH10-hTERT cell lines. The points are the means of three independent experiments. The error bars show the standard deviation.

The data in Figure 3.6 show that the cloning efficiency of bystander cells decreased when the irradiated donor cells received doses between 0.t and 6 Gy, but the decrease was dose independent. Again, the response of the immortalised cell line was not different from that of the wild type cell line.

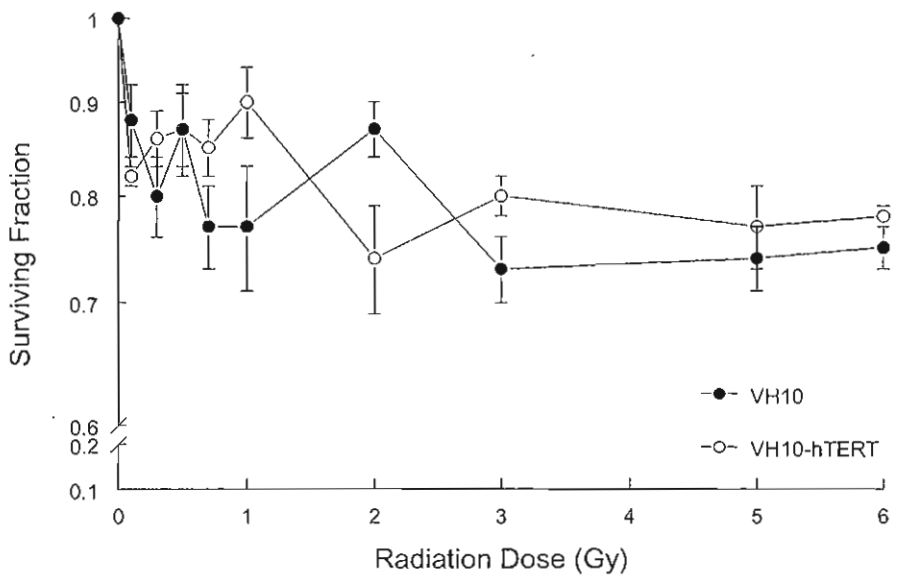


Figure 3.6: Comparison between the bystander survival curves of VH10 and VH10-hTERT cell lines. The points are the means of three independent experiments. The error bars show the standard deviation.

3.2.2. Micronuclei Formation in Directly Irradiated Cells and Bystander Cells

Since the clonogenic survival was reduced, in order to test whether the signals transmitted by irradiated cells could be related to chromosomal damage in nonirradiated cells, the frequency of micronuclei formation in both directly irradiated cells and bystander cells was measured.

The micronucleus (MN) frequency was determined in a total of 500 binucleate cells per dose in each experiment.

Those binucleate cells with more than one micronucleus were also recorded. The micronucleus frequency was calculated in two ways: 1) micronuclei per cell i.e. total number of micronuclei including multiple micronuclei per cell divided by the number of counted binucleate cells and 2) cells with micronuclei i.e. cells with one or more micronuclei divided by the number of counted binucleate cells.

The frequency of binucleate cells with micronuclei, plotted in Figure 3.7 indicates that the induction of micronuclei in directly irradiated cells increased with increasing dose to about 13 times the control level at 1Gy.

There was no difference between BJ and BJ1-hTERT cells.

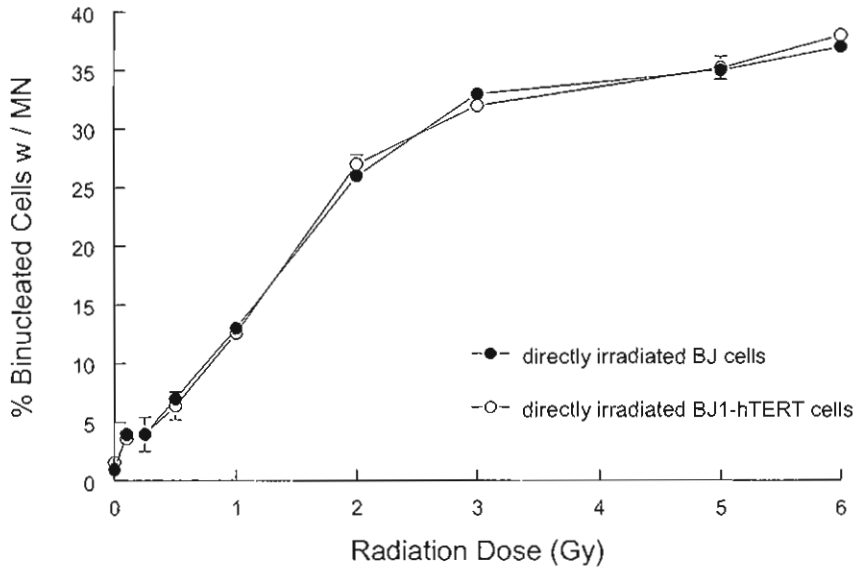


Figure 3.7: Dose dependence of micronucleus (MN) induction: % BNC+MN in BJ human fibroblasts (filled symbols) and BJ1-hTERT human immortalised fibroblasts (opened symbols) after irradiation with 200 keV X-rays. Data points represent the means from three experiments ± SEM.

The data in Figure 3.8 indicate a twofold increase in micronuclei formation in bystander cells at 0.1Gy. There was a slight further increase in micronuclei formation with increasing dose to the irradiated cells. No increase in micronuclei formation was detected when the bystander cells were cultured with medium irradiated in the absence of cells. There was no difference between BJ and BJ1-hTERT cells.

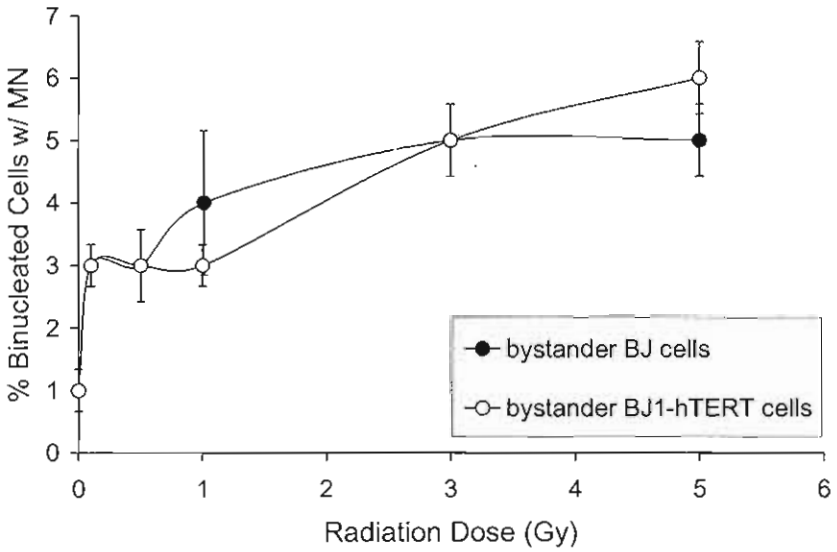


Figure 3.8: Dose dependence of micronucleus (MN) induction: % BNC+MN in bystander BJ human fibroblasts (filled symbols) and bystander BJ1-hTERT human immortalised fibroblasts (opened symbols) after irradiation with 200 keV X-rays. Data points represent the means from three experiments ± standard errors of the means.

The data obtained in the dose response study for each cell line are summarised in Table 3.1. Mean values (+/- SEM) of percentages of binucleate cells with micronuclei and the number of micronuclei per single binucleate cell are tabulated for 200 keV X-ray. The background (0Gy) micronucleus frequencies were 1% for the BJ cell line and 1.6% for the BJ1-hTERT cell line. The number of micronuclei/ cell increases with dose.

Dose (Gy)	Cells with MN (mean)				MN / BNC
BJ cell line					
	0MN	1MN	2MN	3 MN	
0	495	5	0	0	0.01 ± 0.002
0.1	480	19	1	0	0.042 ± 0.007
0.25	480	18	1	1	0.046 ± 0.006
0.5	465	28	5	2	0.088 ± 0.012
1	435	50	10	5	017 ± 0.01
2	370	108	16	6	0.316 ± 0.022
3	335	140	19	6	0.392 ± 0.014
5	325	129	33	13	0.468 ± 0.01
6	315	116	42	21	0.526 ± 0.013
BJ1-hTERT cell line					
	0MN	1MN	2MN	3MN	
0	492	8	0	0	0.016 ± 0.003
0.1	482	17	1	0	0.038 ± 0.008
0.25	480	17	2	1	0.048 ± 0.011
0.5	468	27	3	2	0.078 ± 0.007
1	437	47	10	6	0.017 ± 0.012
2	365	112	17	6	0.328 ± 0.023
3	340	136	19	5	0.378 ± 0.03
5	324	131	32	13	0.468 ± 0.046
6	310	123	45	22	0.558 ± 0.006

Table 3.1: Dose-response data for micronucleus induction after irradiation with 200 keV X-rays. Values are given as means of three experiments ± SEM.

Figure 3.9 shows the dose response relationship for micronucleus induction (micronuclei per binucleate cell) for both cell lines after irradiation with 200keV X-rays.

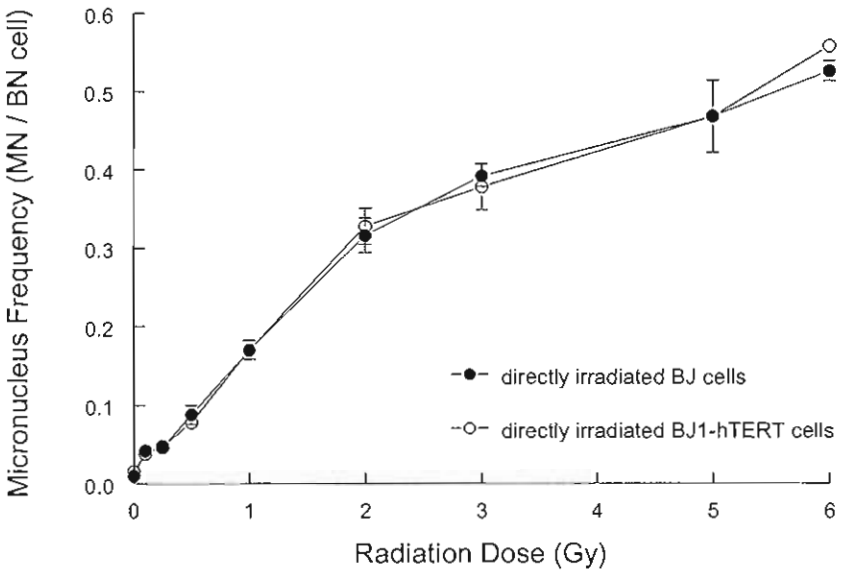


Figure 3.9: Dose dependence of micronucleus (MN) induction: MN / BNC in BJ human fibroblasts (filled symbols) and BJ1-hTERT human immortalised fibroblasts (opened symbols) after irradiation with 200 keV X-rays. Data points represent the means from three experiments ± standard errors of the means.

The fraction of binucleated cells with micronuclei shown in Figure 3.7, as well as the number of micronuclei per binucleate cell represented in Figure 3.9 were found to increase with dose up to 3-4 Gy for the two cell lines and to approach a plateau at higher doses.

The data were analysed in terms of yields of MN per cell. The distributions of MN, which at each dose were tested for the conformity with Poisson statistics, were found to be over dispersed by a mean factor of 1.2. This was taken into account both in the calculation of standard errors on the MN yields and in the fitting procedure. Initially, the MN yields for the two cell lines were fitted separately to a straight line ($Y = C + \alpha D$). The "α" coefficient represents the slope of the curve, D is the dose in Gy and the intercept "C" is the fitted background micronuclei frequency. However, since there is no evidence of any discrepancy at any dose between the BJ and BJ1-hTERT cell lines (see Figures 3.7 and 3.9), the two sets of data were combined and fitting was performed in this case as well. Overall, there is some evidence of saturation of the MN yield at 5 and 6Gy, which would lead to a negative quadratic term (dose squared). Therefore, it was decided to perform the fitting in three cases: up to 2Gy, 3Gy and 6Gy. The results are presented in the Table 3.2. If the 5 and 6Gy points are included the fit is very poor confirming the saturation at those doses. The fits are good for the other data demonstrating that it does not matter whether the 3Gy point is excluded or not. Therefore, the best estimate for α is 0.15 ± 0.01 for both cell lines.

	Up to 2Gy	Up to 3Gy	Up to 6Gy
BJ	C = 0.014 ± 0.004 α = 0.153 ± <u>0.011</u> χ^2 = 3.14 on 4 DF	C = 0.016 ± 0.005 α = 0.138 ± 0.001 χ^2 = 6.0 on 5 DF	C = 0.028 ± 0.012 α = 0.101 ± 0.010 χ^2 = 40 on 7 DF
BJ1-hTERT	C = 0.016 ± 0.003 α = 0.151 ± <u>0.012</u> χ^2 = 1.9 on 4 DF	C = 0.019 ± 0.005 α = 0.135 ± 0.009 χ^2 = 6.1 on 5 DF	C = 0.028 ± 0.011 α = 0.102 ± 0.010 χ^2 = 34 on 7 DF
Combined data	C = 0.015 ± 0.003 α = 0.152 ± 0.008 χ^2 = 4.1 on 4 DF	C = 0.018 ± 0.005 α = 0.137 ± 0.001 χ^2 = 11 on 5 DF	C = 0.028 ± 0.012 α = 0.102 ± 0.010 χ^2 = 74 on 7 DF

Table3.2: Summary of results of radiation dose dependence of micronucleus induction in BJ and BJ1-hTERT cells fitted by the linear quadratic equation.
The data were analysed in terms of yields of MN per cell. The distributions of MN at each dose were tested for the conformity with Poisson statistics. Initially, the MN yields for the two cell lines were fitted separately to a straight line ($Y = C + \alpha D$). The "α" coefficient represents the slope of the curve, D is the dose in Gy and the intercept "C" is the fitted background micronuclei frequency. However, since there is no evidence of any discrepancy at any dose between the BJ and BJ1-hTERT cell lines (see Figures 3.7 and 3.9), the two sets of data were combined and fitting was performed in this case as well. The fitting was performed in three cases: up to 2Gy, 3Gy and 6Gy.
Underlined errors have been enlarged by sq root (DF/ χ^2) due to the fact that χ^2 < DF.

The data in Figure 3.10 point out an approximately three-fold increase in micronuclei formation in bystander VH10 and VH10-hTERT cells at low doses. There was a slight further increase in micronuclei formation with increasing dose to the irradiated donor cells. When the bystander cells were cultured with non-irradiated medium no increase in micronuclei formation was detected. There was no difference between VH10 and VH10-hTERT cells.

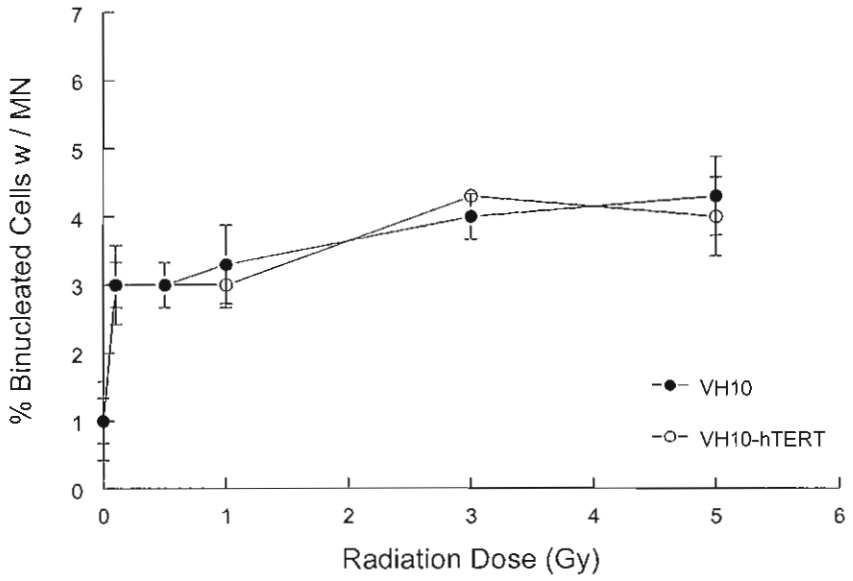


Figure 3.10: Dose dependence of micronucleus (MN) induction: % BNC+MN in bystander VH10 human fibroblasts (filled symbols) and bystander VH10-hTERT human immortalised fibroblasts (opened symbols) after irradiation with 200 keV X-rays. Data points represent the means from three experiments ± standard errors of the means.

3.2.3. γ-H2AX Foci Formation in Directly Irradiated Cells and Bystander Cells. Kinetics of Repair

One of the earliest steps in the cellular response to double strand breaks (DSBs) is the phosphorylation of serine 139 of H2AX, a subclass of eukaryotic histone proteins that are part of the nucleoprotein structure called chromatin (Rogakou et al, 1998)
Initial studies had observed a close correlation between the number of γ-H2AX foci and the number of expected DSBs after irradiation (Rogakou et al, 1999).
Using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX), discrete nuclear foci can be visualized at sites of DSBs.

Here γ-H2AX foci formation in direct irradiated cells and in bystander cells have been examined. Figure 3.11 shows that compared to directly irradiated cycling cells, non-irradiated bystander cells had fewer foci. The percentage of cells with γ-H2AX foci in bystander cells is less than twofold higher than of control cells, while in directly irradiated cells the percentage of cells increased rapidly with dose.

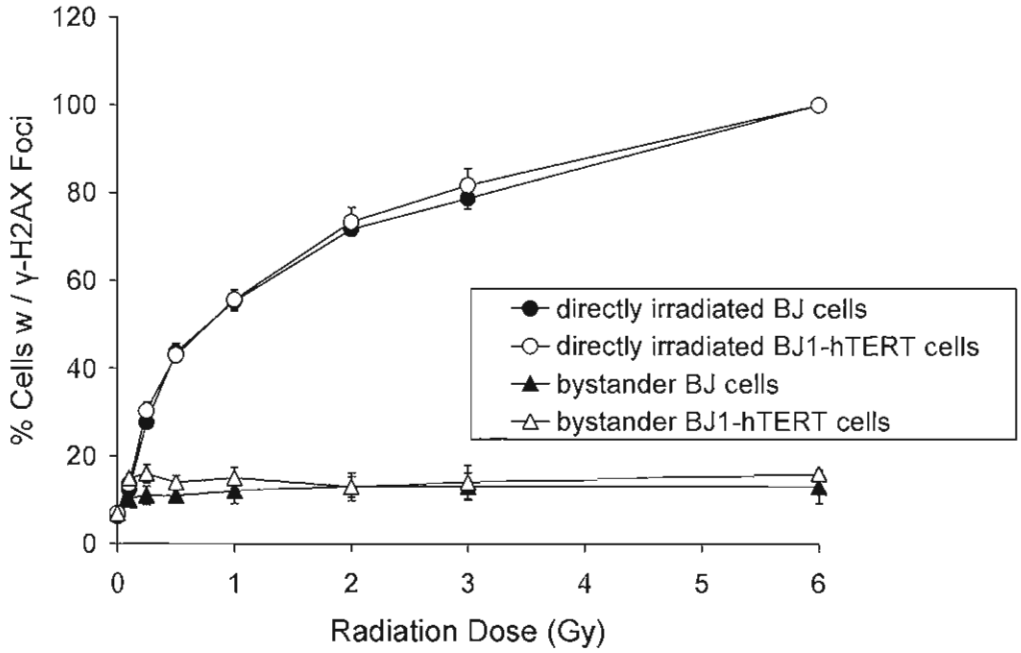


Figure 3.11: Dose response for induction of γ-H2AX foci in directly irradiated and bystander BJ and BJ1-hTERT proliferating cells 2h post irradiation. Results are the means of three experiments ± standard errors.

By examining γ-H2AX foci formation, the induction and repair kinetics of X-ray-induced double strand breaks in normal (BJ and VH10) and immortalised (BJ1-hTERT and VH10-hTERT) human fibroblasts in confluent cells were investigated. After ionising radiation, small foci are visible 10-30 min after irradiation and become more distinct after longer incubation times (Figure 3.12 and Figure 3.13). Distributions of cells with a given number of foci are shown in Figure 3.14; the mean values after 10 min were between 15 and 20 foci per cell for 0.5Gy, for an average of about 28-30 foci per cell per Gy. The number of foci in each cell line for each dose and fixation time was tested for

distributions to look for heterogeneity. No evidence for this was found. The distributions of foci 1 hour after irradiation were normal (Poisson with mean >> 1), yet they look broader for the hTERT cells, while 24 hours after irradiation there is no clear evidence for a Poisson distribution for all doses.

In Figures 3.15, 3.16, 3.17 and 3.18 the response of direct irradiated confluent cells to radiation in respect to γ-H2AX foci formation is shown.

Tables 3.3, 3.4, 3.5 and 3.6 give the distributions of γ-H2AX foci in non irradiated bystander BJ, BJ1-hTERT, VH10 and VH10-hTERT cells.

Figure 3.19 shows γ-H2AX foci in bystander cell populations.
In all bystander experiments performed in confluent cells, the medium from the irradiated donor cells was transferred to the recipient bystander cells 2 hours after irradiation. Media harvested from BJ or BJ1-hTERT cells irradiated with 0.25; 0.5 or 1Gy and conditioned for 2 hours did not produce any effect at 1 hour, 6 hours, 18 hours and 30 hours after transferring the medium when compared to the control. Thus, assuming that the effect would be evident at lower doses, the bystander experiment for the VH10 and VH10-hTERT cell lines was performed also for the dose of 0.1Gy. Again, no difference in the percentage of cells with γ-H2AX foci was observed between the control and the bystander cells 1h, 6h and 18h after transferring the irradiated medium to the non-irradiated bystander cells.

Initially 3 way ANOVA was performed in order to see if there is any difference between the direct irradiated wild type and immortalised cell lines. This model was not considered

further because the problems in a high factorial ANOVA with unequal N (number of cells counted) and interactions (dose, time and dose-time interactions) with the calculation of simple effects and comparisons are very complex. Instead, a one way ANOVA has been used followed by independent sample t tests for the relevant comparisons.

Individual Chi-square test was used to analyze the difference between control and bystander groups of cells.

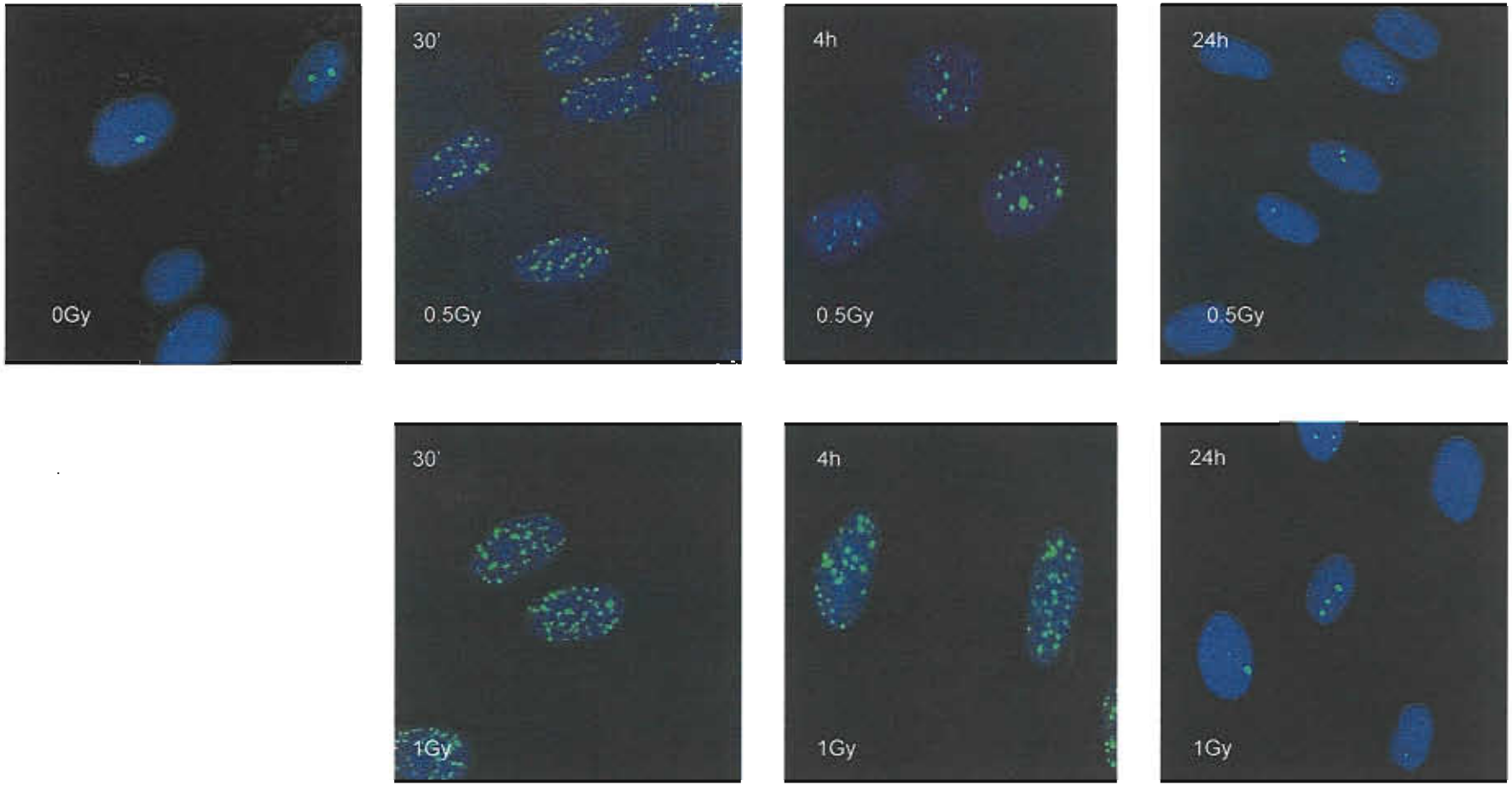


Figure 3.12: DSB repair in G1-phase BJ cells, as measured by γ-H2AX focus formation. γ-H2AX foci (green) in non irradiated and irradiated cells; nuclei were stained with 4,6-diamidino-2-phenylindole (blue).

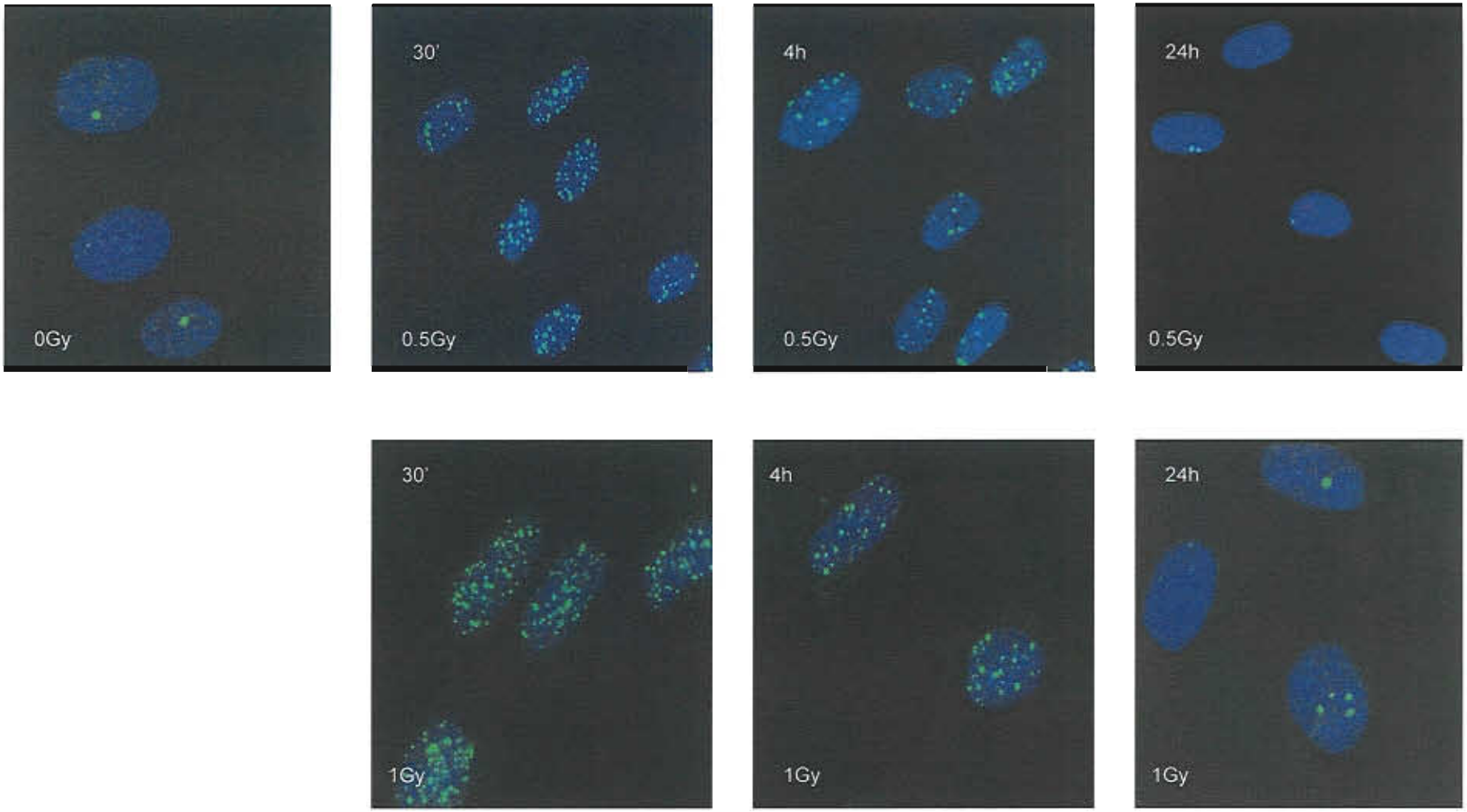


Figure 3.13: DSB repair in G1-phase BJ1-hTERT cells, as measured by γ-H2AX focus formation. γ-H2AX foci (green) in non irradiated and irradiated cells; nuclei were stained with 4,6-diamidino-2-phenylindole (blue).

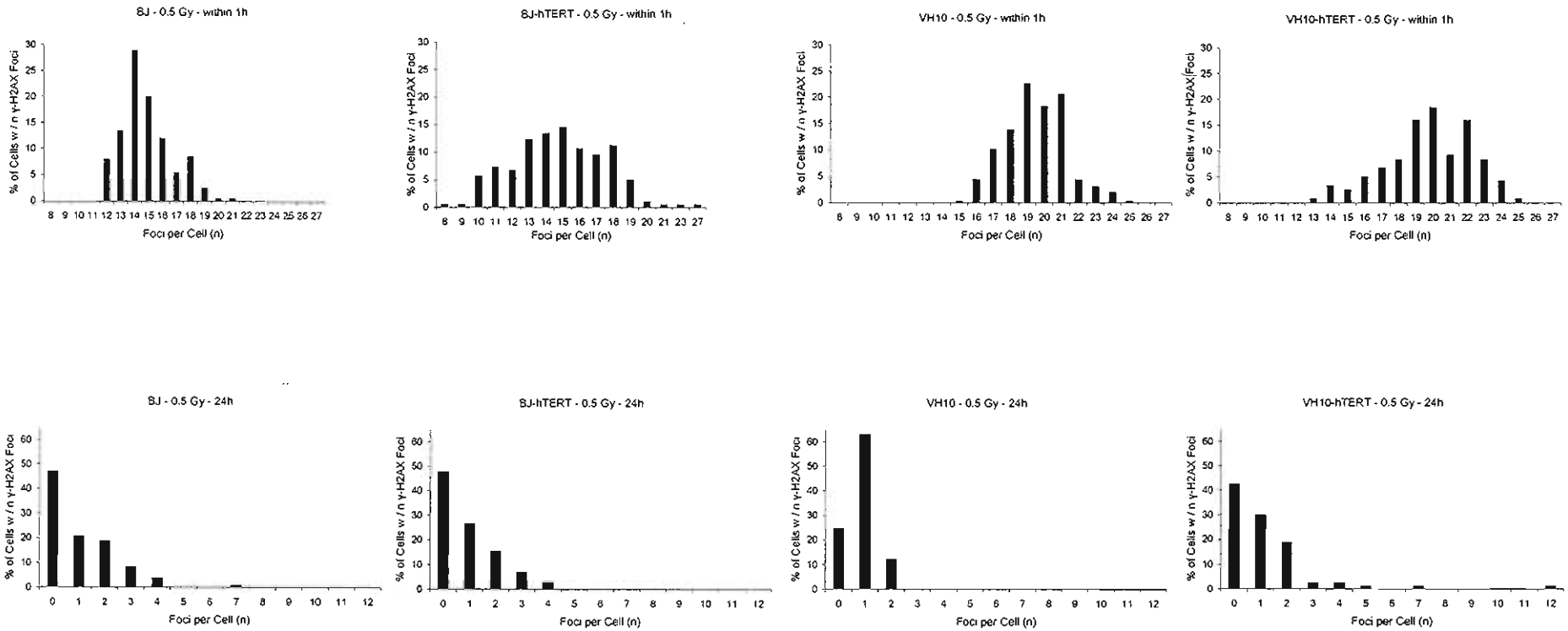
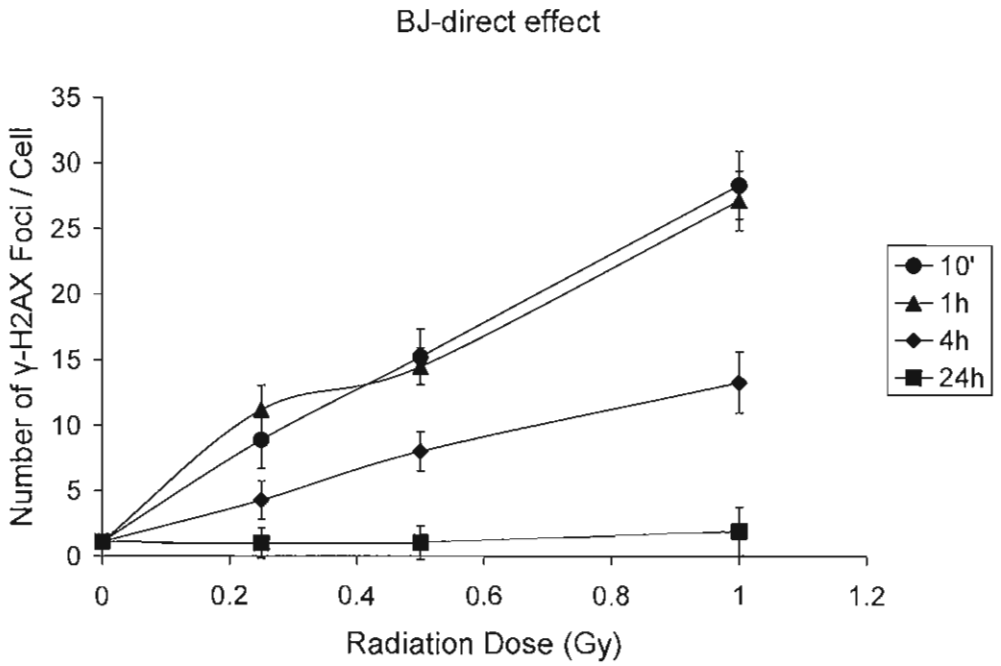


Figure 3.14: Distribution of BJ, BJ1-hTERT, VH10 and VH10-hTERT cells with n γ-H2AX foci either within 1h or 24 h after 0.5 Gy irradiation



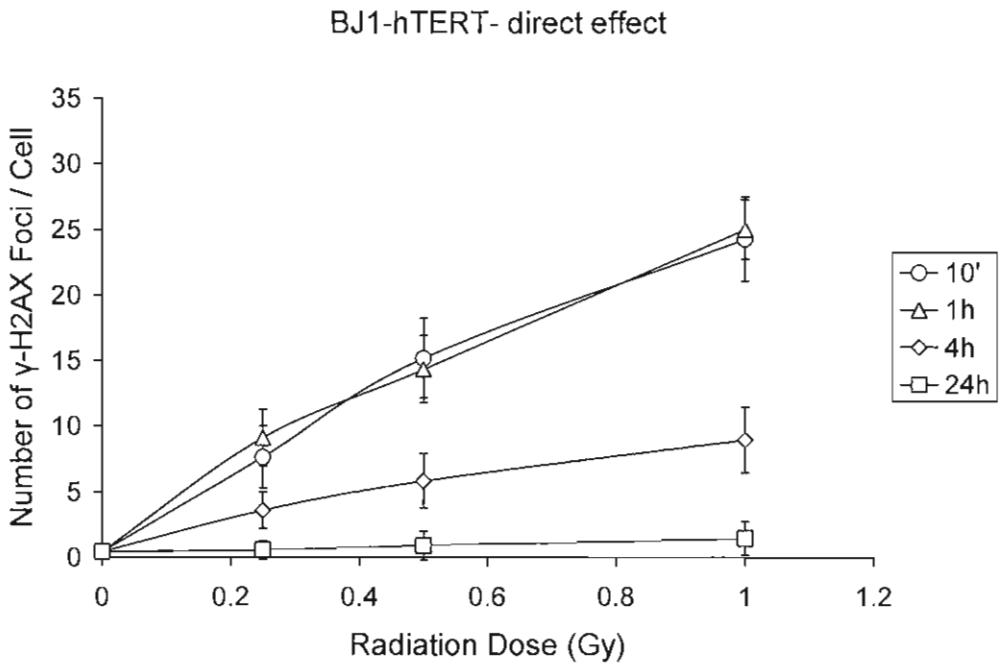


Figure 3.15: Mean number of γ-H2AX foci per cell for various repair times in irradiated confluent BJ (filled symbols) and BJ1-hTERT (opened symbols) cells.

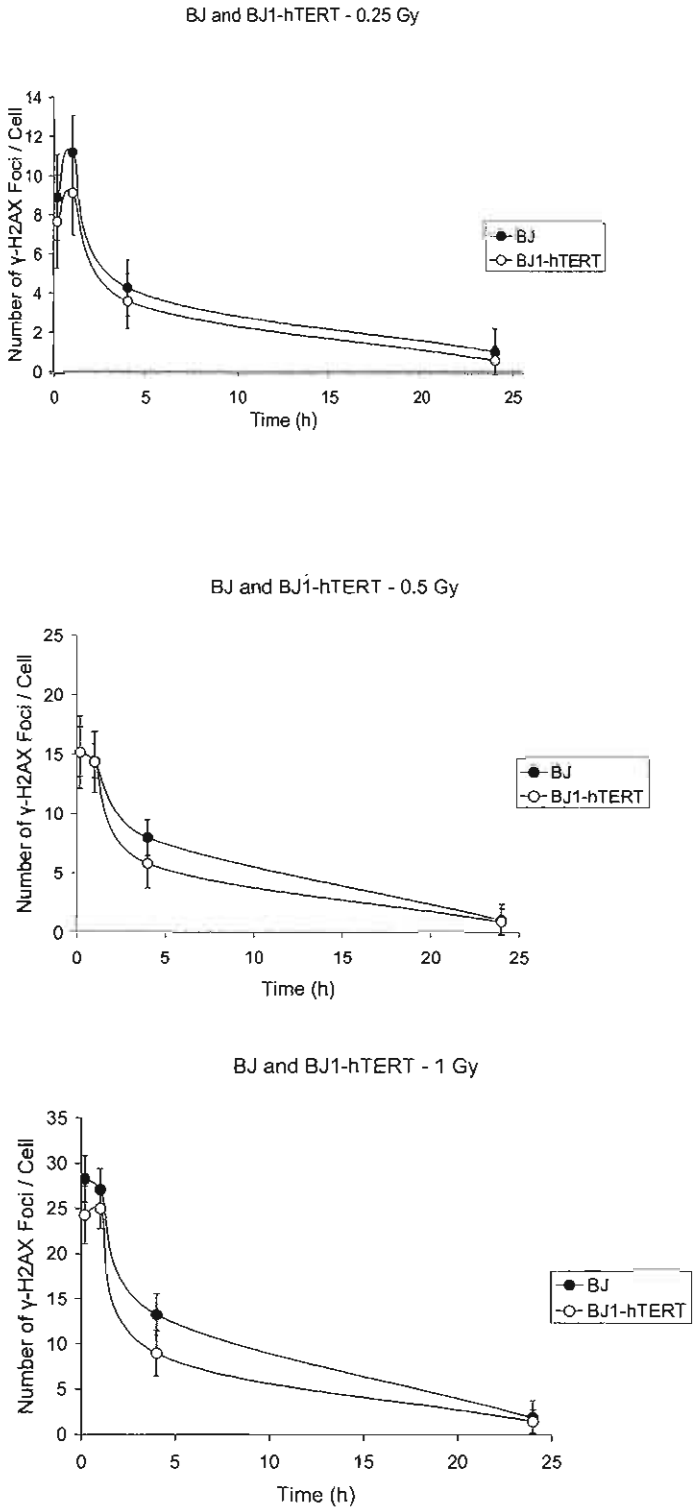
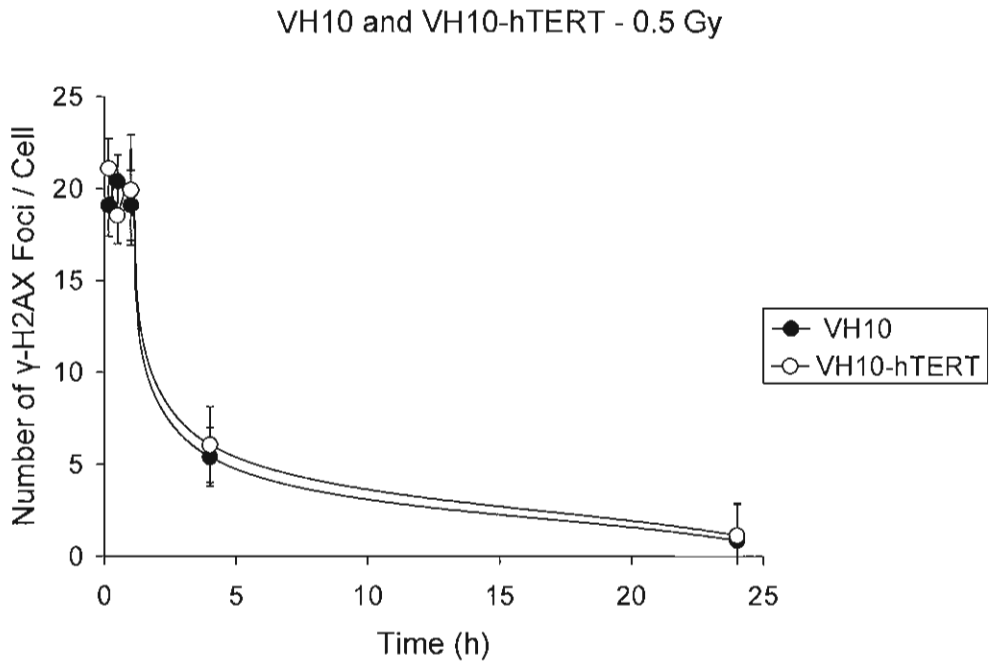


Figure 3.17: Time course for the repair of DSBs in BJ (filled symbols) and BJ1-hTERT (opened symbols) cells after different radiation doses. The mean number of γ-H2AX foci per cell for various repair times is shown.



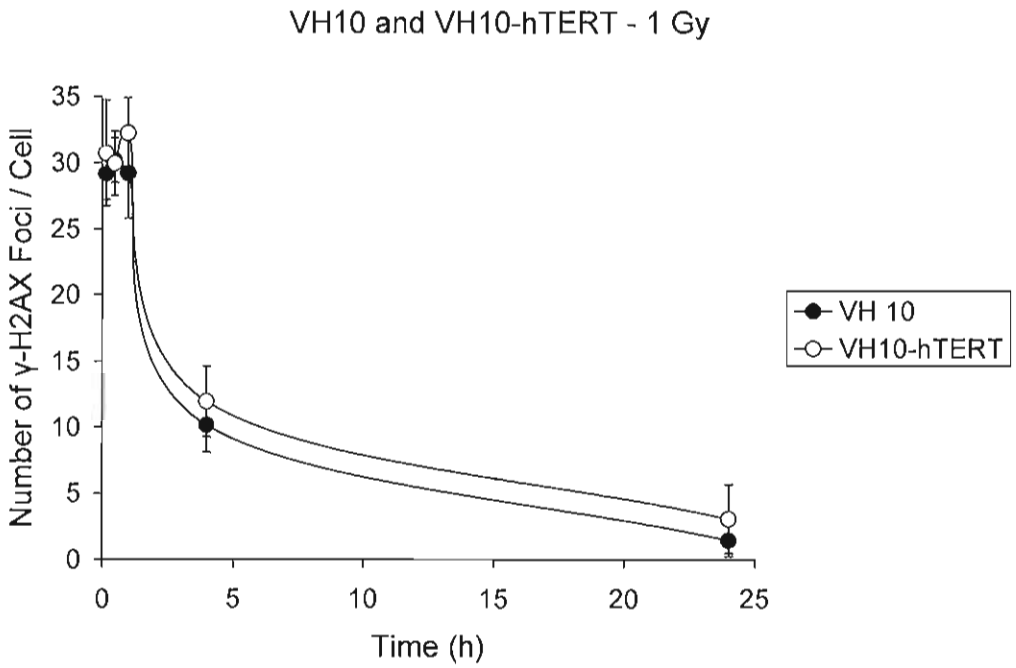


Figure 3.18: Time course for the repair of DSBs in VH10 (filled symbols) and VH10-hTERT (opened symbols) cells after different radiation doses. The mean number of γ-H2AX foci per cell for various repair times is shown.

No of Cells with n Foci		0	1	2	3	4	5	6-9	Total
Dose	Fixation time								
0 Gy	1h	53	38	29	9	3	3	1	136
	6h	48	39	33	9	4	5	2	140
	18h	57	43	32	12	5	3	2	154
0.25 Gy	1h	45	13	13	16	4	4	5	100
	6h	31	16	16	13	10	5	6	97
	18h	28	24	12	20	4	4	2	94
0.5 Gy	1h	23	27	15	19	5	6	2	97
	6h	30	37	24	13	6	4	1	115
	18h	32	27	20	10	2	4	2	97
1 Gy	1h	22	21	11	4	4	3	0	65
	6h	28	26	6	6	6	4	0	76
	18h	27	24	18	7	5	1	0	82

Table 3.3: Distribution of number of γ-H2AX foci in non-irradiated bystander **BJ** cells after different radiation doses. Media were conditioned on irradiated cells for 2hours and transferred to bystander cultures for 1h, 6h or 18h.

No of Cells with n Foci		0	1	2	3	4	5	6-9	10-15	>15	Total
Dose	Fixation Time										
0 Gy	1h	193	14	1	2	0	1	0	1	1	213
	6h	182	13	2	1	0	0	1	0	1	200
	18h	190	14	1	1	1	0	1	1	2	211
	30h	188	15	1	2	0	1	1	1	1	210
0.25 Gy	1h	180	13	3	3	0	0	1	5	0	205
	6h	250	16	1	0	1	1	0	0	3	272
	18h	230	16	2	2	0	0	2	0	2	254
	30h	434	47	5	3	3	2	3	0	3	500
0.5 Gy	1h	216	10	3	1	0	2	5	2	0	239
	6h	235	6	2	2	0	1	3	4	0	253
	18h	222	17	1	0	0	0	1	2	1	244
	30h	244	14	3	3	0	0	2	2	1	269
1 Gy	1h	170	13	2	3	0	0	0	1	1	190
	6h	159	12	1	3	0	1	1	2	0	179
	18h	211	10	1	1	0	0	1	0	2	226
	30h	228	23	6	0	0	0	3	1	2	263

Table 3.4: Distribution of number of γ-H2AX foci in non-irradiated bystander **BJ1-hTERT** cells after different radiation doses. Media were conditioned on irradiated cells for 2hours and transferred to bystander cultures for 1h, 6h, 18h or 30h.

No of Cells with n Foci		0	1	2	3	4	5	6-9	10-15	>15	Total
Dose	Fixation Time										
0 Gy	1h	80	21	7	1	0	1	1	0	0	111
	6h	70	20	12	0	0	0	0	0	0	102
	18h	87	22	13	1	1	1	1	1	0	127
0.1 Gy	1h	100	9	4	2	1	2	3	1	0	122
	6h	94	8	4	2	3	1	2	0	1	115
	18h	75	15	7	5	0	0	2	2	0	106
0.25 Gy	1h	130	15	5	4	1	1	1	1	1	159
	6h	111	18	4	6	0	1	2	2	0	144
	18h	106	16	5	3	3	2	4	3	0	142
0.5 Gy	1h	114	16	5	4	0	0	3	1	1	144
	6h	115	7	2	5	2	1	6	2	0	140
	18h	117	10	4	5	0	2	6	0	1	145

Table 3.5: Distribution of number of γ-H2AX foci in non-irradiated bystander **VH10** cells after different radiation doses. Media were conditioned on irradiated cells for 2hours and transferred to bystander cultures for 1h, 6h or 18h.

No of Cells with n Foci		0	1	2	3	4	5	6-9	10-15	>15	Total
Dose	Fixation Time										
0 Gy	1h	32	31	19	6	1	0	0	0	0	89
	6h	70	20	22	8	2	1	0	1	1	125
	18h	42	23	19	12	3	1	0	0	0	100
0.1 Gy	1h	75	25	14	11	3	1	6	2	0	137
	6h	71	11	14	10	9	2	6	1	0	124
	18h	33	30	14	9	6	5	3	1	0	101
0.25 Gy	1h	71	16	11	7	5	9	8	1	0	128
	6h	60	12	7	5	6	5	4	2	0	101
	18h	57	7	12	4	4	4	6	2	0	96
0.5 Gy	1h	41	35	40	20	8	2	0	0	0	146
	6h	70	32	20	14	6	6	8	4	0	160
	18h	46	47	11	7	3	5	9	0	0	128

Table 3.6: Distribution of number of γ-H2AX foci in non-irradiated bystander **VH10-hTERT** cells after different radiation doses. Media were conditioned on irradiated cells for 2hours and transferred to bystander cultures for 1h, 6h or 18h.

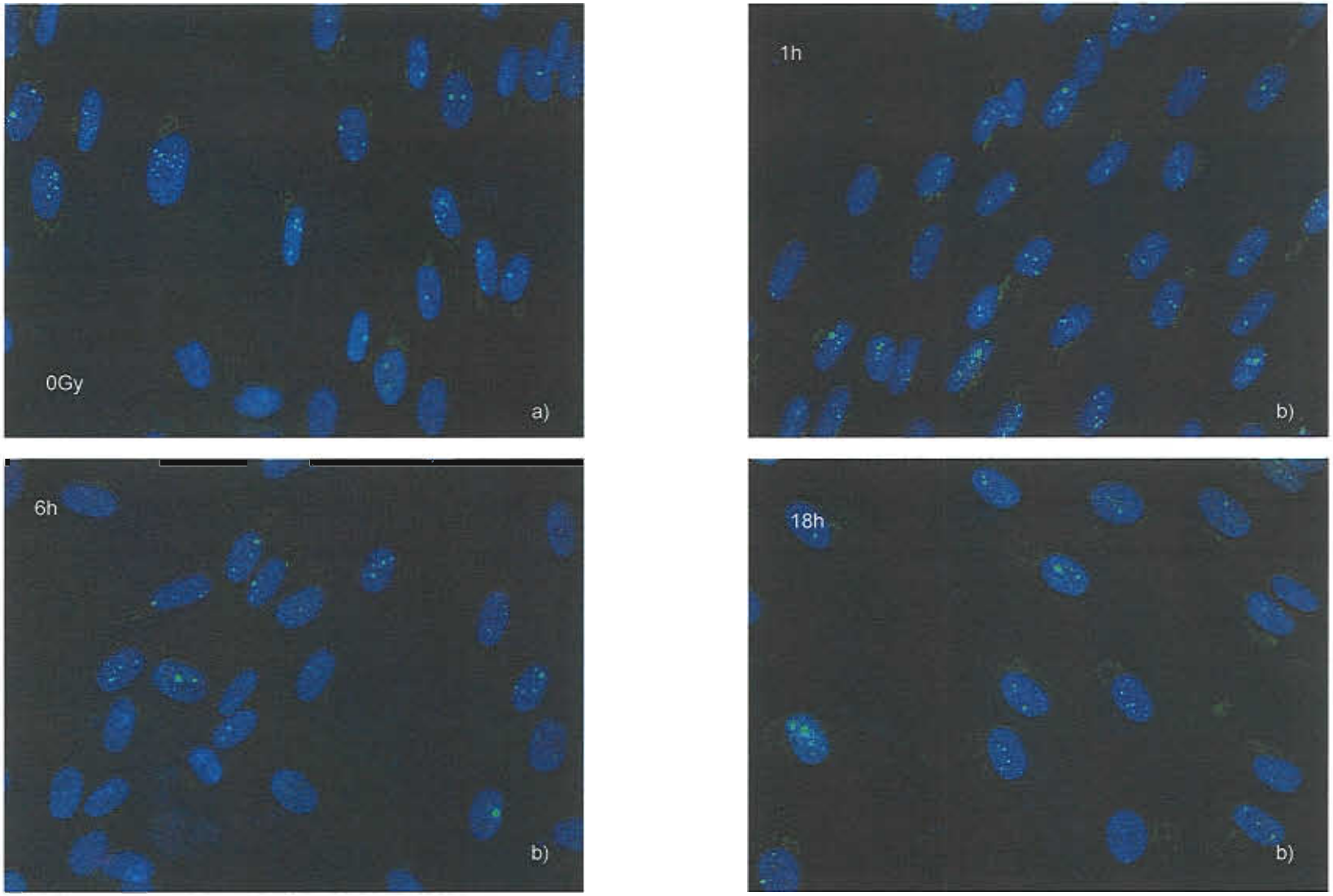


Figure 3.19: Presence of γ-H2AX foci in bystander cell populations: a) confluent unirradiated cells; b) confluent bystander cells after either 1h, 6h or 18h incubation with conditioned medium from 0.5Gy irradiated cells.

3.3. Premature Chromosome Condensation Assay

In this study, a premature chromosome condensation (PCC) method was used which involved an inhibitor of types 1 and 2A protein phosphatases such as Calyculin A. Table 3.7 presents the PCC index [(number of PCCs)/(number of interphases + number of PCCs) X 100] of the exponentially growing normal human fibroblast cell lines, in relation to Calyculin A concentration. The highest yield of PCC was obtained at 80 nM and after 45 min treatment.

Cell Type	40nM			80nM			120nM		
	30'	45'	1h	30'	**45'**	1h	30'	45'	1h
BJ	18	22	28	25	**55**	50	21	50	38
BJ1-hTERT	18	21	27	26	**56**	50	21	51	38

* PCC index = [(number of PCCs)/(number of interphases+ number of PCCs)X 100]

Table 3.7: Influence of concentration and treatment time of Calyculin A on PCC index (%) in normal human fibroblasts (BJ) and immortalised human fibroblasts (BJ1-hTERT)

The morphological characteristics of PCC allow judging the stage of the cells in the cell cycle at the time of induction of PCC (Figure 3.20). Chromosomes condensed in the G_1 phase are univalent, i.e. they exhibit a single chromatid (Figure A). As a consequence of the many nicks in the DNA, chromosomes in S phase are highly fragmented and look pulverized (Figure B, C). G_2 chromosomes are bivalent with two chromatids after PCC (Figure D) and look like metaphase chromosomes obtained after mitotic arrest by use of Colcemid.

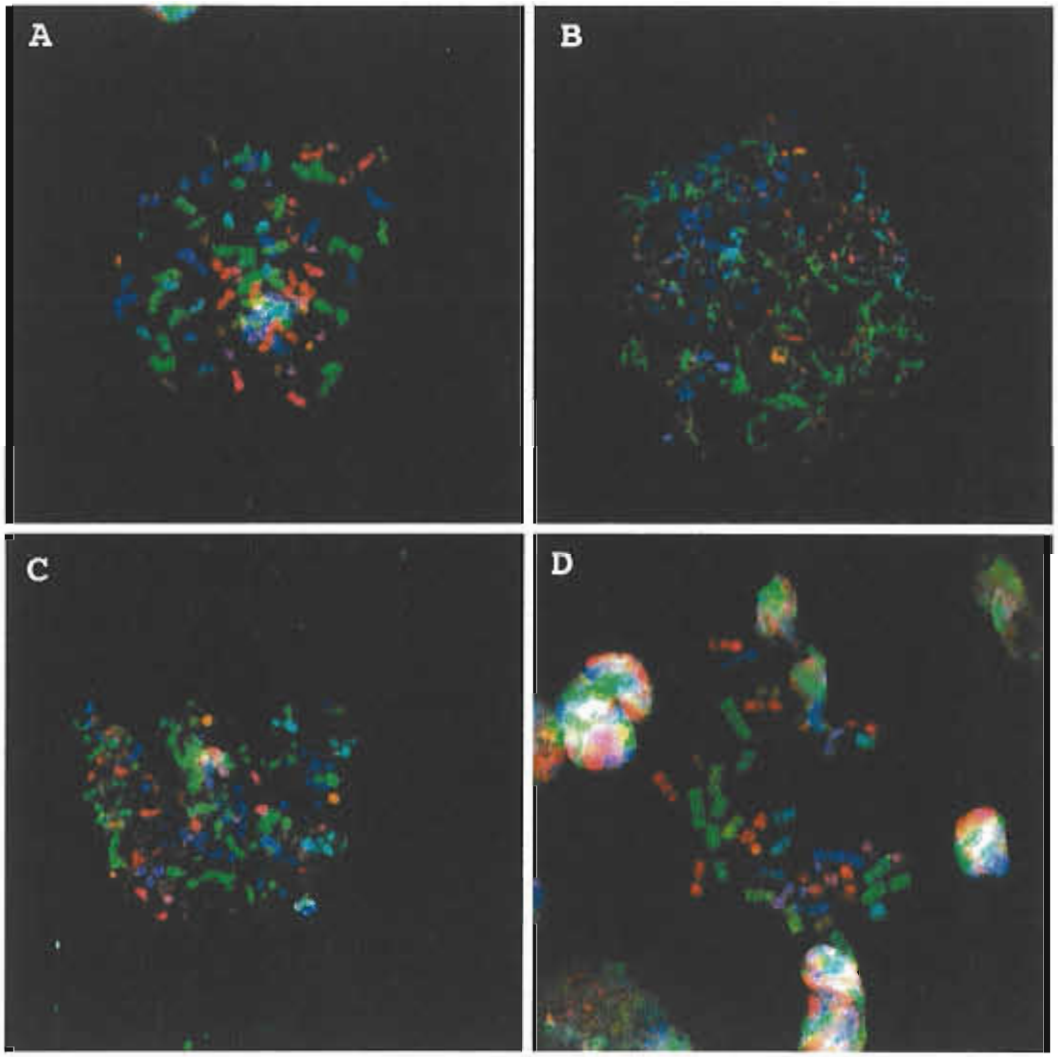


Figure 3.20: Premature chromosome condensation spreads with distinct morphological characteristics analysed by COBRA-FISH. G_1-PCC with univalent chromosomes morphology analysed by COBRA-FISH (A). B and C present S-PCC where chromosomes show univalent and fragmented morphology. D illustrates G_2-PCC, where chromosomes are bivalent.

We first observed the number of G2-type chromosomal alterations immediately after X-irradiation. Immediately after exposure chromatid exchanges and chromatid breaks were observed. It is clear that the irradiated sample shows significant numbers of chromosomal alterations when compared to the PCC spreads with no irradiation. Figure 3.21 summarises the dose response curves for chromatid-type breaks and chromatid-type exchanges after exposure to X-irradiation.

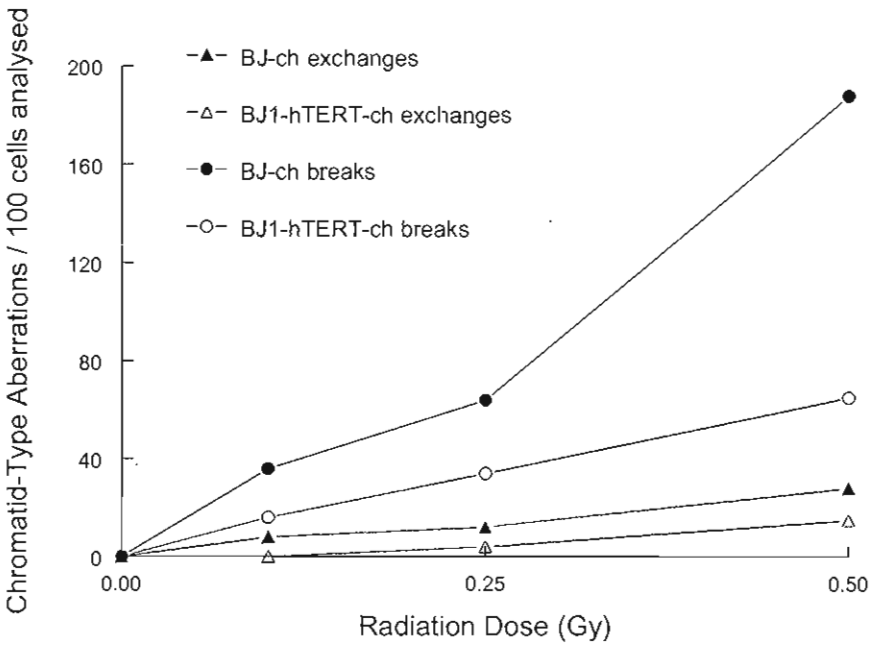


Figure 3.21: Initial numbers of G2-type chromosomal alterations as a function of radiation dose in proliferating BJ and BJ1-hTERT cells. These results are from two independent experiments. Triangles indicate the data for chromatid exchanges, and circles indicate the data for chromatid breaks for both cell lines.

The background frequency of chromatid breaks was approximately 0/cell for both BJ and BJ1-hTERT cell lines.

The curves for the BJ cell line show a significantly steeper dose response (188 breaks at 0.5Gy and 28 exchanges at 0.5Gy) than the curves for the immortalised cell line that give 65 breaks at 0.5Gy and 15 exchanges at 0.5Gy.

A linear increase in chromatid breaks and chromatid exchanges as a function of X-ray dose was observed; more than 75% of the initial alterations are breaks. The differences in patterns may be attributed to the structure of G_2 chromosomes.

3.4. Chromosomal Alterations Following Irradiation of Cells in G2-Phase of the Cell Cycle

The cytogenetic analysis demonstrates that there is a difference between the BJ and BJ1-hTERT cell lines due to chromatid type aberrations. Following 1Gy irradiation of cells in G2 phase of the cell cycle there is a higher incidence of breaks, exchanges and multi aberrant cells in BJ cells compared to BJ1-hTERT cells as shown in Table 3.8.

Cell line	Dose (Gy)	Abnormal cells (%)	Frequency of chromatid-type aberrations (%)		
			Breaks	Exchanges	Multi-Aberrant cells
BJ					
	0	2	2	0	0
	1	44	52	16	8
BJ-hTERT					
	0	2	2	0	0
	1	32	44	4	0

Table 3.8: Induction of chromosomal alterations in BJ and BJ-hTERT cell lines following irradiation of cells in G2-phase of the cell cycle with 0 or 1Gy. 50 cells were analysed per dose and cell line.

3.5. Rad51 Formation

The central homologous recombination molecule, Rad51, forms nucleoprotein filaments on DNA and promotes exchange between homologous sequences (Ristic et al, 2005). Furthermore, Rad51 displays a dynamic redistribution into nuclear foci after treatment with DNA damaging agents (Li et al, 1997). These foci are formed at the site of DNA damage and most probably represent centres for the repair of such lesions (Raderschall et al, 1999).

Several additional proteins involved in homologous recombination such as Rad51 paralogs, Rad52, Rad54, replication protein (RP)-A, BRCAI and BRCA2 are also involved in Rad51 foci formation, either directly or indirectly.

Rad51 foci were formed in all four cell lines.

In untreated cells between 1.33 % and 5.29 % of the cells displayed more than five nuclear Rad51 foci, most probably representing S-phase cells. This variation in Rad51 foci formation in untreated cells is most probably related to the cell cycle distribution since differences in growth were observed between the cell lines. After treatment with X-rays 51% of the BJ cells and 38 % of the BJ1-hTERT cells formed more than five Rad51 foci (Figure 3.22). Moreover, a clear decrease of Rad51 foci positive cells was observed 24 hours after irradiation (Table 3.9) for both cell lines. Yet, a difference in the percentage of cells with Rad51 foci between the proficient and deficient cell lines was seen for both recovery times (Table 3.9). Table 3.10 shows the number of VH10 and VH10-hTERT cells with Rad51 foci.

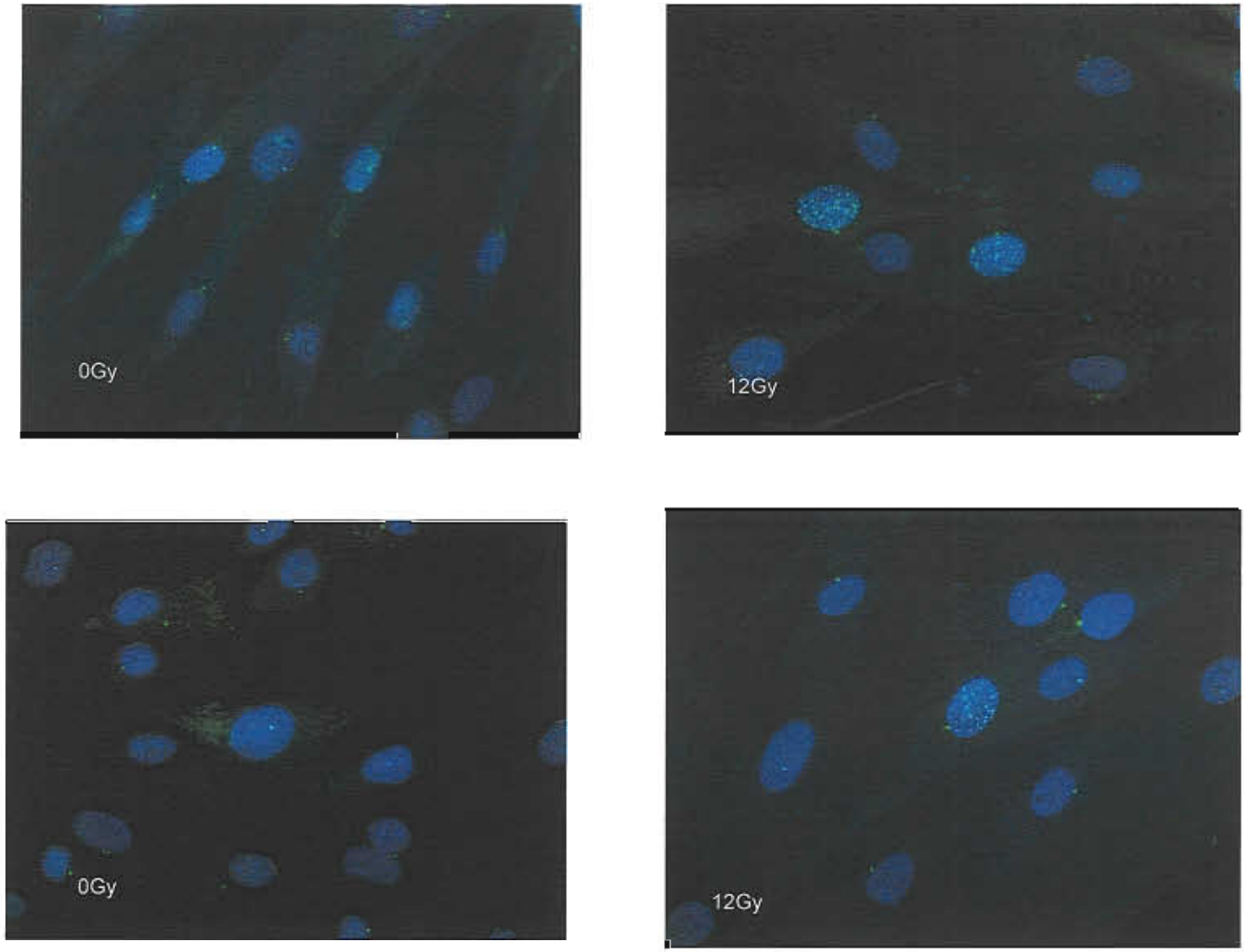


Figure 3.22: Formation of Rad51 foci (green) in irradiated and non irradiated BJ (upper panels) and BJ1-hTERT (lower panels) cells. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue).

		BJ cells		BJ1-hTERT cells	
Dose	Fixation Time	< 5 Foci(%)	≥ 5 Foci(%)	< 5 Foci(%)	≥ 5 Foci(%)
0 Gy	8 h	162 (95.85)	7 (4.14)	149 (98.67)	2 (1.33)
	24 h	286 (94.70)	16 (5.29)	314 (95.44)	15 (4.56)
12 Gy	8 h	148 (49)	154 (50.9)	190 (62.09)	116 (37.90)
	24 h	238 (75.3)	78 (24.75)	276 (85.98)	45 (14.01)

Table 3.9: Number and percentage of BJ and BJ1-hTERT cells with Rad51 foci. The data are means from three independent experiments.

		VH10 cells		VH10-hTERT cells	
Dose	Fixation Time	< 5 Foci(%)	≥ 5 Foci(%)	< 5 Foci(%)	≥ 5 Foci(%)
0 Gy	8 h	300 (96.77)	10 (3.22)	291 (95.41)	14 (4.59)
	24 h	284 (94.35)	17 (5.64)	278 (92.97)	21 (7.03)
12 Gy	8 h	153 (47.66)	168 (52.34)	177 (58.60)	125 (41.40)
	24 h	233 (75.16)	77 (24.84)	253 (83.77)	49 (16.23)

Table 3.10: Number and percentage of VH10 and VH10-hTERT cells with Rad51 foci. The data are means from three independent experiments.

3.6. Potential role of Reactive Oxygen Species (ROS)

To investigate whether oxidative stress was involved in the bystander effects, 2', 7'- dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a probe with fluorescence microscopy to examine the generation of radical oxygen species (ROS) in fibroblast by X-irradiation. This was a pilot experiment and was only performed in BJ cells.

The data in Table 3.11 show that compared to the controls there is no significant increase in ROS production in irradiated and non-irradiated bystander cells 8hours after irradiation, an approximately fourfold increase in irradiated cells and twofold increase in bystander cells 32 hours after irradiation and fourfold increase in irradiated cells and no increase in non- irradiated bystander cells 64 hours after irradiation.

Percentage of cells with DCF fluorescence			
8h post irradiation	32h post irradiation	64h post irradiation	
7.2 ± 0.8	4.0 ± 0.5	3.8 ± 0.3	Direct 0Gy
11.3 ± 2.2	16.8 ± 0.8	15.8 ± 0.8	Direct 6Gy
5.7 ± 0.5	7.2 ± 0.6	5.7 ± 0.6	Bystander 0Gy
6.4 ± 0.2	14.3 ± 1.4	4.9 ± 0.9	Bystander 6Gy

Table 3.11: Generation of radical oxygen species in directly irradiated and non-irradiated bystander BJ cells. Results are the means of two independent experiments ± standard errors.

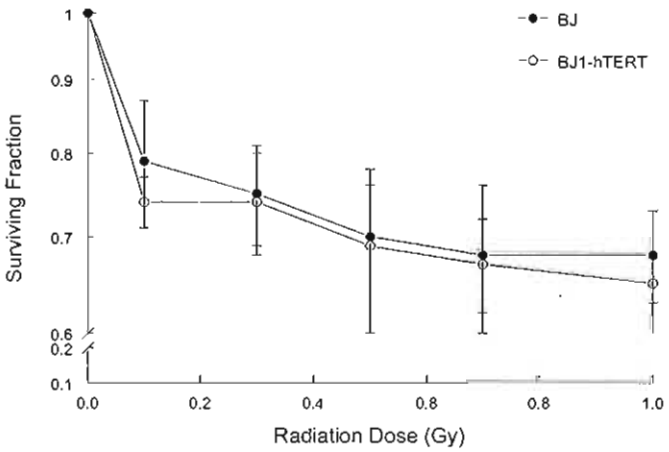
3.7. Low-Dose Hypersensitivity and Induced Radio-resistance

Recently, the response of cells to very low doses of ionizing radiation has generated considerable interest. A number of laboratories have now identified and reported a region of high sensitivity in the radiation survival response of cell systems at doses below 1 Gy. This phenomenon, which has been termed low dose hyper-radiosensitivity (HRR) is followed by increased radio-resistance (IRR). Results to date from studies using accurate measurements of clonogenic surviving fraction at very low levels of cell kill show that the linear-quadratic equation does not describe correctly the cell survival curve in the low-dose region (below 1Gy) (Lambin et al 1993, 1994, Joiner 1994).

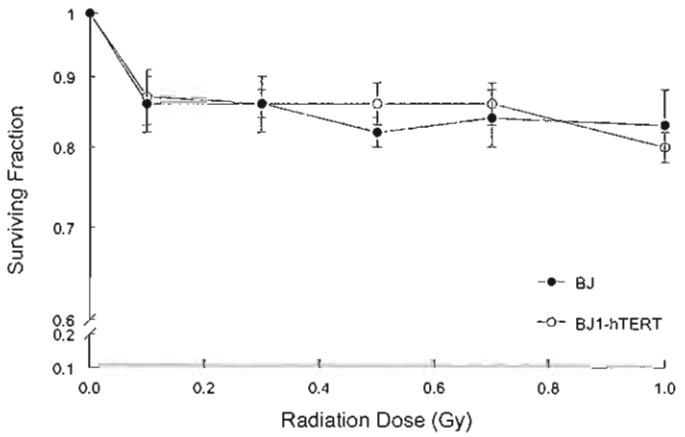
The data presented here (Figure 3.23 A) indicate that both BJ and BJ1-hTERT cell lines demonstrated HRR/IRR phenomenon at doses below 1Gy. There is a slight difference between the proficient and deficient cell lines. Moreover, the fraction of cells showing the low dose hypersensitivity phenomenon seems to be similar to that responding to the bystander effect (Figure 3.23 B).

These findings raise further questions about the type of damage which cause the bystander effect and low dose hyper-sensitivity at doses < 1Gy.

Figure 3.23 A shows that, between 0.2 and 1Gy there is approximately 25% reduction in the clonogenic survival of both cell lines. For the bystander effect (Figure 3.23 B), in the same dose range the survival fraction decreases to about 15%. This would support the interpretation that low-dose hypersensitivity in this cell lines is to a large extent or completely caused by a bystander mechanism.



A



B

Figure 3.23: A) Low-dose clonogenic cell survival of BJ (black filled symbols) and BJ1-hTERT (red opened symbols) human foreskin fibroblasts irradiated with cobalt-60 γ-rays. Each point represents the mean of three individual experiments (± SD).

B) Low-dose clonogenic cell survival of bystander BJ (black filled symbols) and BJ1-hTERT (red opened symbols) human foreskin fibroblasts which received medium from BJ and BJ1-hTERT cell cultures, respectively irradiated with cobalt-60 γ- rays. Each point represents the mean of three individual experiments (± SD).

Repeats of the clonogenic assay experiments for doses below 2Gy (Figure 3.24) and for doses below 0.1 Gy (Figure 3.25) were performed for both BJ and BJ-hTERT cell lines at GCI using 240 keV X-rays.

The results show that at very low doses of X-rays there is a significant difference between the immortalised and the wild type cell lines which was not seen after γ- rays. It cannot be ruled out that this is a real effect of the differences in radiation quality. Rather, it is suggested that this is due to uncontrolled different experimental conditions in different laboratories. A similar difference in the low-dose region between the cell lines was also seen in Dublin but was less pronounced (Figure 3.23 A). The reason why this difference in Dublin was not significant may be related to the large confidence limits of the results obtained here. Therefore, it is concluded that there may be an influence of the telomerase system on clonogenic survival at very low doses which is unrelated to the bystander effect.

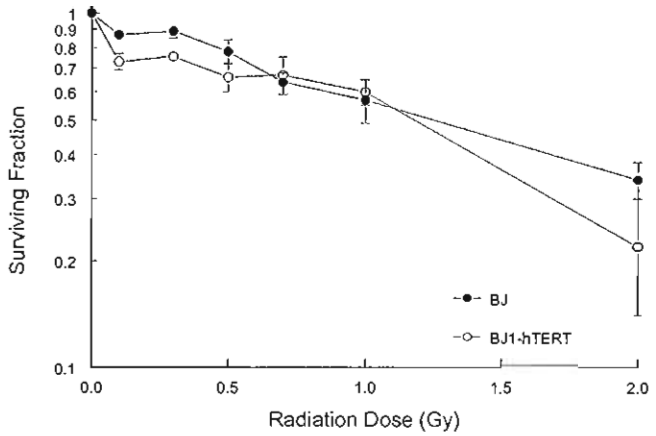


Figure 3.24: Low-dose clonogenic cell survival of BJ and BJ1-hTERT human foreskin fibroblasts irradiated with 240 keV X-rays. The points are the means of three independent experiments. The error bars show the standard deviation

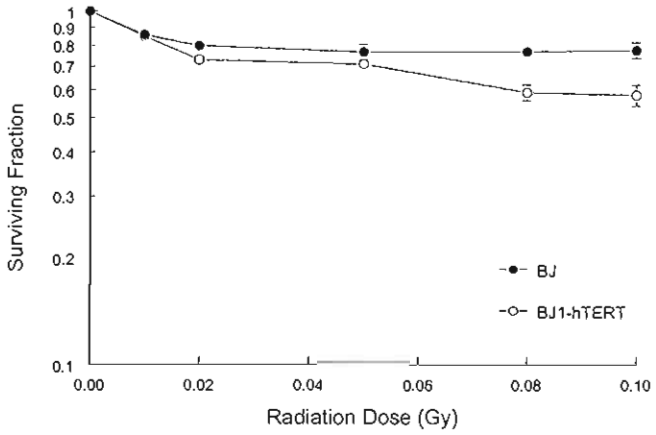


Figure 3.25: Comparison between the radiation dose dependence of the surviving fraction of BJ and BJ1-hTERT cell lines. The points are the means of three independent experiments. The error bars show the standard deviation.

The following figures show the individual data for the clonogenic survival of BJ and BJ1-hTERT cells lines of three repeats on direct and bystander effect. The data points are connected by straight lines. The curve in red shows the data of the direct effect fitted by the LQ equation.

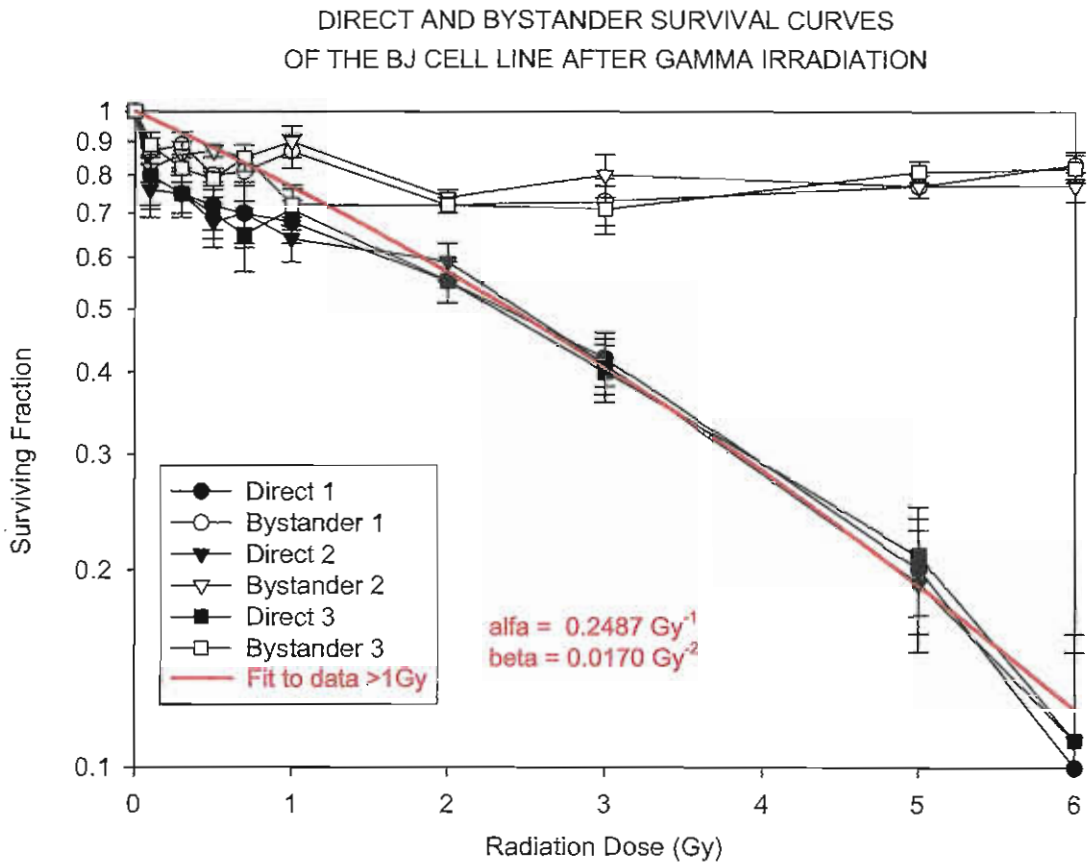


Figure 3.26: Radiation dose dependence of the survival fraction of BJ cells fitted by the linear quadratic equation (red curve).

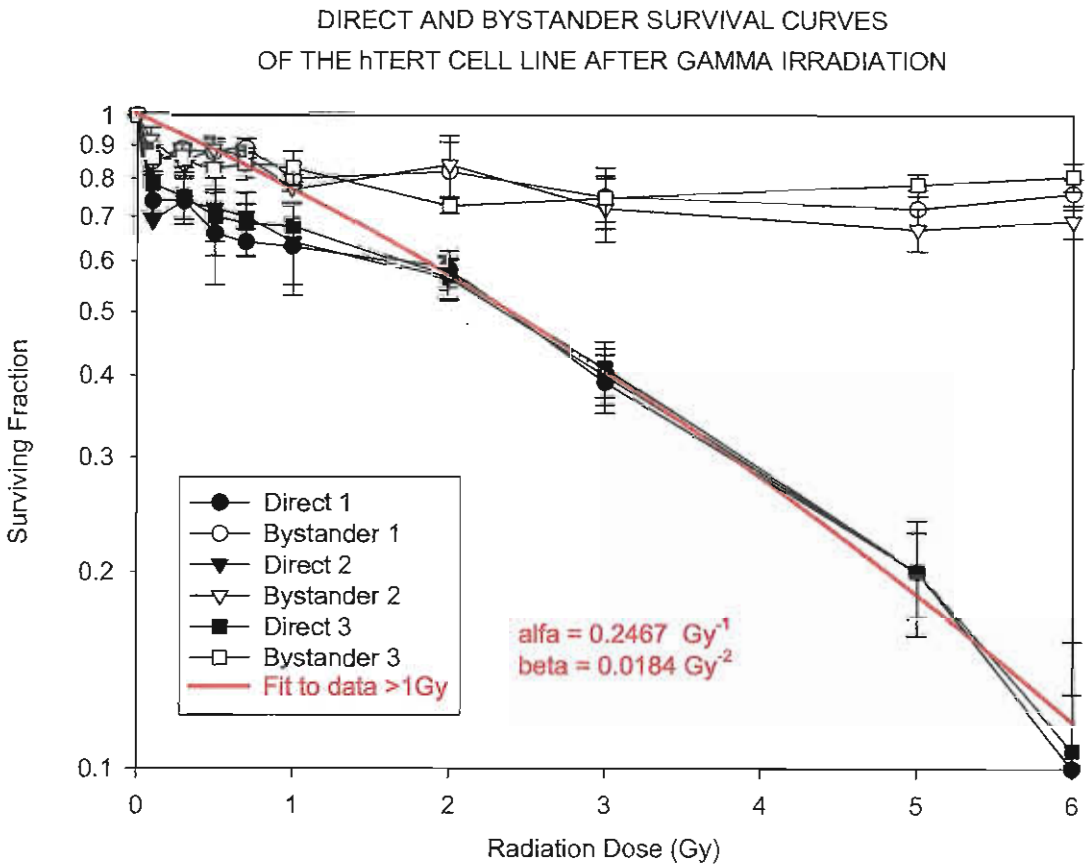


Figure 3.27: Radiation dose dependence of the survival fraction of BJ1-hTERT cells fitted by the linear quadratic equation (red curve).

Discussion

4.1. Mechanisms of Bystander Effects in Relation with the Telomere/ Telomerase System

Initial experiments aimed at characterising radiation responses of fibroblast cell lines and understanding if the telomere/ telomerase system affects the bystander effect. This study was initiated with wild type BJ and immortalised BJ1-hTERT cells and later extended to normal human VH10 and immortalised VH10-hTERT fibroblasts.

Increased clonogenic inactivation and chromosomal damage occurred not only in cells directly hit by ionising radiation, but also in unirradiated cells exposed to media from irradiated cells proving the evidence of bystander effects in all four cell lines.
In contrast, no significant bystander effect was observed in our experimental protocol for γ-H2AX formation (i.e. DSBs induction). Using these three criteria for studying the bystander effect there was no difference between the response of telomerase positive and negative cells.

Numerous reports have been published (Nagasawa and Little, 1992; Mothersill at al, 1997; Lorimore at al, 1998; Watson et al, 2000; Belyakov at al, 2002; Prise et al, 2002; Morgan, 2003; Little, 2003; Prise et al, 2003; Hall and Hei, 2003; Azzam et al, 2004;

Zhou et al, 2005) which suggest the existence of radiation-induced bystander effects in a number of cell types. The literature is contradictory on whether fibroblasts show any bystander responses, especially after low LET radiation such as X- or γ-rays and whether the same signal transmission pathways are involved. So far, much of the experimental evidence for bystander effects induced by low LET irradiation has been provided by studies where the medium from irradiated cells was transferred to unirradiated cells. However, the Gray Cancer Institute developed an X-ray microbeam to target individual cells within a sparse population of cells and demonstrated bystander cell killing (Prise et al 2003).

Especially influential were the studies by Mothersill and co-workers (Seymour and Mothersill 1997, 1999; Mothersill and Seymour 1997; 1998; 2003; 2004; 2005), who demonstrated that medium from irradiated cells, reduces the clonogenic survival of unirradiated cells, but there were differences between cell lines regarding the extent of the bystander effect. Human fibroblasts such as MSU-1 and human prostate PC3 cell lines showed no bystander effects after 0.5 Gy (cloning efficiencies around 100%), while SW 48 (human colon) and HaCaT (human keratinocyte) cell lines showed very pronounced bystander effects (cloning efficiencies about 11% and about 47% respectively) (Mothersill et al, 2002).

The data presented in this study demonstrate that conventional X-irradiation can induce medium mediated bystander responses in fibroblasts. This is similar to the results of Yang et al (2005) who showed that survival of bystander AGO1522 normal human fibroblasts cells after 0.5 Gy X-irradiation to the donor cells decreases to approximately 80% and stays at this level at higher doses. Mothersill and Seymour (1997) did not

observe a bystander response in clonogenic survival of unirradiated MSU-1 human fibroblasts that received medium transferred from γ-irradiated fibroblasts. Moreover, no bystander effect was observed when irradiated medium from MSU-1 human fibroblast cells was transferred to non-irradiated HaCaT human keratinocytes which usually show a very pronounced bystander effect if the transferred medium is from HaCaT cells. Surprisingly, if medium from irradiated HaCaT cells was transferred to non-irradiated human fibroblasts it killed nearly all cells, however, the significance of these results has not been further discussed (Mothersill and Seymour, 1997).

An explanation for the discrepancy of the results of different studies could be that different types of fibroblasts were used for the experiments. Another explanation is that the time for communication between the irradiated and non-irradiated cells was different in the different studies. This is also consistent with work done on human-hamster hybrid cells showing that bystander cells had lower or higher surviving fraction depending on the period (48h or 1h) the irradiated and non-irradiated cells were sharing the medium (Zhou et al 2002).

In order to look at relationships between different end points, independent of radiation dose, the fraction of inactivated cells was plotted as a function of the fraction of binucleated cells with one or more micronuclei (Figure 4.1) and as a function of the number of γ-H2AX foci formed after 1hour (Figure 4.2).

It is well recognised that the development of micronuclei in irradiated cells implies significant chromosome damage and rearrangements (Prise et al, 2003). Micronuclei have been suggested to arise predominantly from non rejoined DNA DSBs (Fimognari et al,

1997). From Figure 4.1 it appears that there is a non linear dose relationship between chromosome damage and inactivated cells. Within 20% binucleated cells with micronuclei nearly 40% are sterilised which is a similar ratio as described by other studies (Stap and Aten, 1990; Jamali and Trott, 1996). This suggests that the cellular mechanisms which cause the reduced survival of irradiated cells are partly responsible for the formation of micronuclei in irradiated cells (Littlefield et al, 1989; Jamali and Trott, 1996). The mechanism by which the micronuclei are formed after irradiation is related to the ability of radiation to cause chromosome damage. This study demonstrates that direct clonogenic cell inactivation by X-irradiation is associated with an increased micronucleus frequency.

On the other hand, in the micronuclei/ inactivation effect curve shown in Figure 4.1, the main surprise is the very rapid increase in the number of inactivated cells with very little chromosomal damage at very low radiation doses: with less than 5% binucleated cells with micronuclei approximately 30% of cells die. This means that five in six cells which do not have chromosome damage will die suggesting a different mechanism for inactivation of clonogenic cells in the low dose region. Since this effect is the same after direct and after indirect (bystander) exposure, this suggests an involvement of the bystander effect on cell inactivation at low radiation doses.

Between 0.5 Gy and 3Gy, the percentage of MN increase by a factor of 4, but there is only little increase in cell inactivation by a factor of 2. In other words, the lack of further cell inactivation at doses between 0.5 and 3 Gy is not caused by increased repair of chromosomal damage. This suggests that there are different mechanisms of loss of

reproductive integrity which occur at different doses. Moreover, no apoptosis was observed in bystander cells. So far this observation cannot be explained by established radiobiological theories and hypothesis.

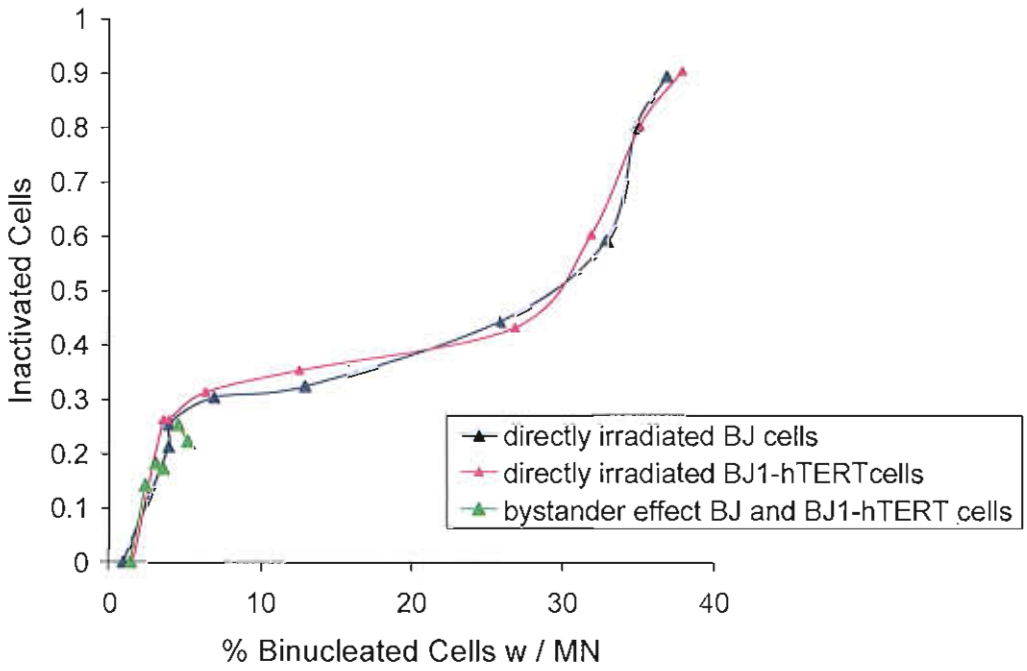


Figure 4.1: Fraction of inactivated cells as a function of fraction of binucleated cells with one or more MN. Since no difference in survival and MN formation was found between the BJ and BJ1-hTERT cell lines, the data for the bystander effect were pulled together.

Although γ-H2AX foci and micronuclei were observed in exponentially growing bystander cells, it is not proven that X-irradiation leads to DSBs in bystander cells. The data in this study show that for 20% bystander cell inactivation we would expect about 10 γ-H2AX foci (Figure 4.2). Instead, less than 5 foci are formed in the bystander cells which received medium from irradiated cells suggesting that lesions other than DNA double strand breaks are involved in the bystander effect and that different mechanisms are responsible for the production of γ-H2AX foci in direct irradiated and bystander cells.

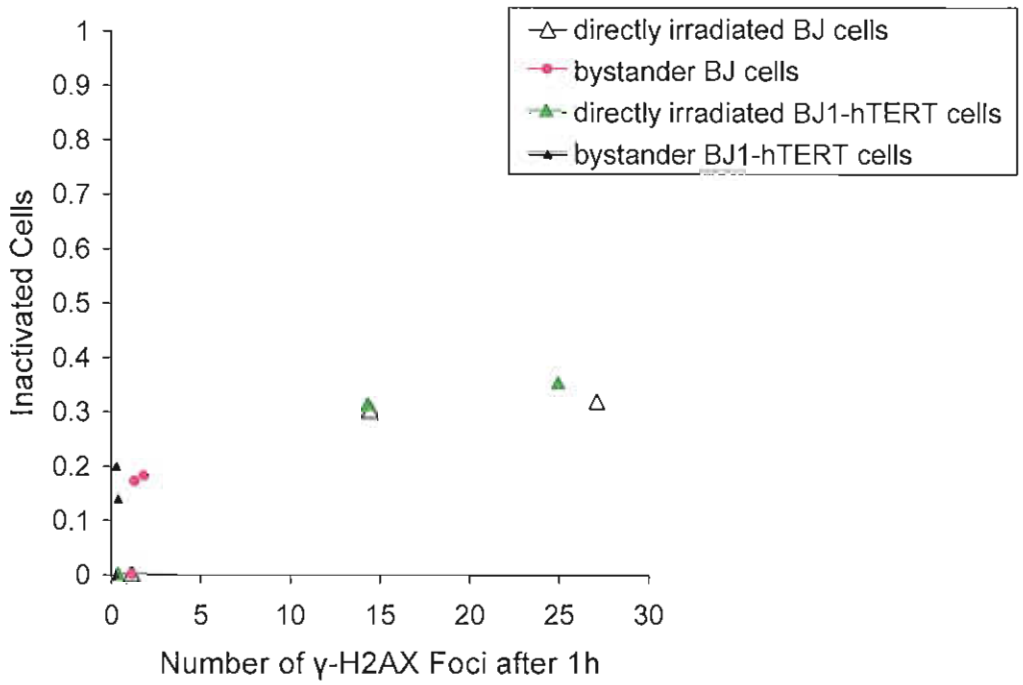


Figure 4.2: Fraction of inactivated cells as a function of number of γ-H2AX foci formed in BJ and BJ1-hTERT cells after 1h.

This appears to be in contrast to two recent publications from Columbia University (Sokolov et al, 2005) and Harvard Medical School (Yang et al, 2005).
Formation of γ- H2AX and micronuclei has been observed in H_2O_2 treated fibroblast cells (Yang et al, 2005) and it has been reported that H_2O_2 causes predominantly single strand breaks (Olive and Johnston, 1997). This suggests that formation of micronuclei and γ- H2AX is not caused exclusively by double strand breaks.

The bystander effect is different in cycling cells and confluent cells. In cycling cells a very small bystander effect was observed only for low doses, and only a small percentage of cells have γ-H2AX foci (Figure 3.11). 0.1 Gy exposure of the target cells resulted in greater γ-H2AX foci formation in bystander cells than for 1Gy, from approximately 7%to approximately 15% for the immortal cell line and from about 7% to 10% for the wild type cells.
Yang et al (2005) reported an increase in the frequency of cells with micronuclei and of cells with γ-H2AX foci in bystander cells from approximately 5% to approximately 10% (no numbers given, data extracted from graphs). They showed that the increased number of cells with γ-H2AX foci was independent of dose in the range of 0.1-10 Gy and that an increase was apparent for the dose of 0.1Gy at 2 hours post irradiation.
Sokolov et al (2005) did not observe any significant increase in γ-H2AX foci formation when donor cells were irradiated with 0.2 Gy, media was transferred to unirradiated cultures 1 hour later and foci were analysed in bystander cultures 4 hours post irradiation. If medium from cells irradiated with 0.2 Gy was transferred 1 hour later for 18 hours, the

proportion of cells with γ-H2AX foci increased from about 3% to about 10% (data extracted from graphs).

This discrepancy between results may be explained by the fact that different cell lines and/or different media transfer protocols were used.

In order to investigate medium borne bystander effects after X-rays, Yang et al (2005) used a novel transwell system in which, after irradiation the irradiated and non-irradiated cells shared the same medium, but did not touch each other until the time they were analysed for different end points. By using this system they only focused on mechanisms underlying medium mediated bystander effect, this way avoiding any involvement of intercellular communications. For the experiments, AGO1522 human diploid skin fibroblasts were plated from confluent cultures at a density that yielded proliferating cells at the time of irradiation. 24 hours after plating, the medium was changed and the cells irradiated using conventional broad field 250 kVp X-ray machine at a dose rate of 2.08 Gy / min. Immediately after the irradiation, the non-irradiated inserts were placed into the irradiated wells and they were co-cultured for 2 hours when they were fixed and stained for γ-H2AX following a standard immunostaining procedure. It is not mentioned how many cells were analysed for γ-H2AX foci, but the results are means of three experiments ± standard errors. In comparison with direct irradiated cells, bystander cells had fewer foci and the percentage of cells with foci in bystander cultures was about twofold higher than that of control cells, independent of dose between 0.1 and 10 Gy. Sokolov et al (2005) used different protocols for studying the bystander effect after irradiation. For the medium transfer protocol W138 normal human lung fibroblasts cells

were seeded onto two-well glass slides and irradiated with 0.2; 0.6 and 2 Gy at a dose rate of 0.6 Gy / min from a ^{137}Cs source. At different times (30 min, 1h, 2h, 4h, 8h) after irradiation the medium was removed from the irradiated cultures ; filtered and added to the non irradiated cells. The recipient cultures with the conditioned media were incubated for different times after the transfer of the media (30 min, 4h, 18h, 48h).Then they were fixed and stained for γ-H2AX.

In both papers (Yang et al, 2005; Sokolov et al, 2005), exponentially growing cells were used as donor and recipients, while in the present study, plateau phase cells were used. Initially, exponentially growing cells were used to study the bystander effect, but the assessment of γ-H2AX foci in cells which were in the S-phase of the cell cycle was difficult to follow. Therefore, it was decided to use confluent cells because it is possible that cells in the S-phase of the cell cycle are more sensitive to the development of γ-H2AX foci in relation to the normal DNA replication mechanism. Moreover, it was hoped to study differences in the cell cycle with regard to the bystander effect since Marples at el (2003) showed that the bystander effect is limited to the G_2 cell population.

In confluent cells there is no bystander effect as showed in Tables 3.3, 3.4, 3.5, 3.6, and if there were any γ- H2AX focus formation in bystander populations, their formation was limited to a subset of cells. Yet, such a subpopulation was also present in the control cells. These results are somehow in agreement with the results from the Columbia group (Sokolov et al, 2005). However, the percentage of bystander cells with foci in this study was lower than the percentage of cells with foci they observed.

Another source of difference in the results is the way foci are quantified. In this study foci per cell were recorded, while Sokolov et al (2005) had different criteria.

They used a threshold of ≥ 4 foci/ cell as being optimal for determination of % of affected bystander cells. This is a very questionable argument for the selection of a criterion.

In our study, statistical analysis did not show any significant difference when the threshold of ≥ 5 or no threshold was imposed for determination of γ-H2AX foci in bystander cells (Table 4.1).

It has also been found that the duration of media conditioning necessary to give the largest bystander effect depended on the radiation dose and was 1, 2 and 4 hours for 0.2; 0.6 and 2Gy, respectively (Sokolov et al, 2005). Besides this, the % of cells with ≥ 4 foci/ cell in the bystander population was maximum at 18 hours, but reduced at 48 hours post irradiation. Thus the bystander effect is manifested slowly and persists for longer time than the disappearance of γ-H2AX foci (Sokolov et al, 2005).

The results of this study, and in particular in relation to recently published work open more questions than they answer.

The molecular mechanisms of bystander cell inactivation in particular in relation to DNA damage and chromosome aberrations are still unclear. However, it is obvious that we succeeded in answering the first aim of our study and can confidently rule out any major influence of the telomerase in the bystander effect.

			foci cat	
			8 >15 foci	Total
dose radiation dose	1 controle	Count	4	624
		% within dose radiation dose	.6%	100.0%
	2 0.25 Gy	Count	2	254
		% within dose radiation dose	.8%	100.0%
Total		Count	6	878
		% within dose radiation dose	.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.682[a]	8	.953
Likelihood Ratio	3.700	8	.883
Linear-by-Linear Association	.012	1	.914
N of Valid Cases	878		

			focicat					
			0 0 foci	1 1 focus	2 2 foci	3 3 foci	4 4 foci	5 5 or more foci	Total
dose radiation dose	1 controle	Count	565	41	4	4	1	9	624
		% within dose radiation dose	90.5%	6.6%	.6%	.6%	.2%	1.4%	100.0%
	2 0.25 Gy	Count	230	16	2	2	0	4	254
		% within dose radiation dose	90.6%	6.3%	.8%	.8%	.0%	1.6%	100.0%
Total		Count	795	57	6	6	1	13	878
		% within dose radiation dose	90.5%	6.5%	.7%	.7%	.1%	1.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.562[a]	5	.990
Likelihood Ratio	.835	5	.975
Linear-by-Linear Association	.008	1	.928
N of Valid Cases	878		

Table 4.1: Results of the Chi-square tests. The statistical analysis did not show any significant difference when the threshold of ≥ 5 or no threshold was imposed for determination of γ-H2AX foci in bystander cells.

4.2. The Impact of the Bystander Effect on the Low-Dose Hypersensitivity Phenomenon

In Figure 4.3 and Figure 4.4, low-dose hypersensitivity up to about 0.5 Gy is closely related to the bystander effect in the induction of chromosomal damage. For higher doses there is no additional chromosomal damage from the bystander effect and no additional damage to clonogenicity.

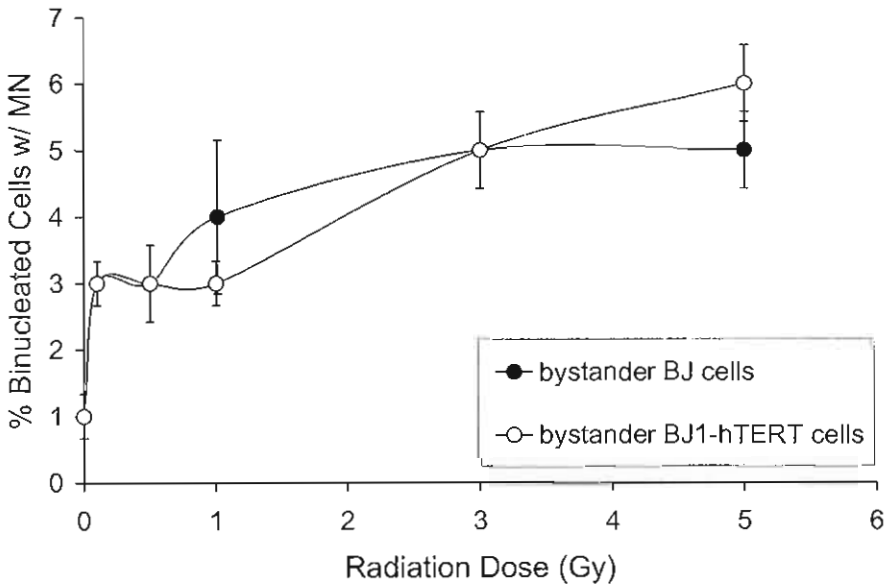
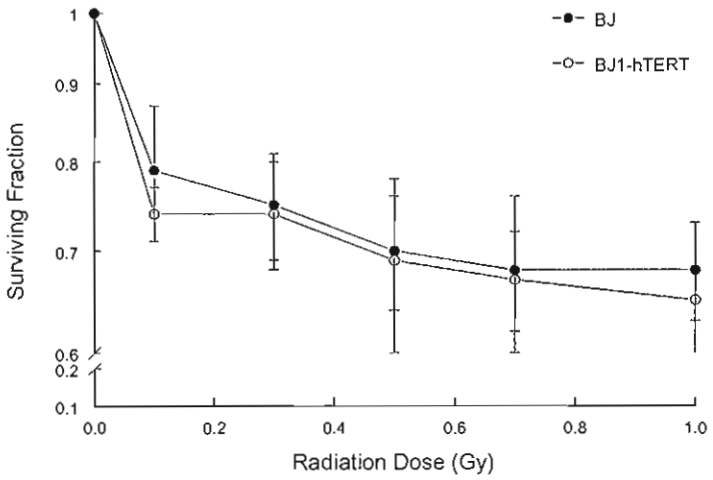
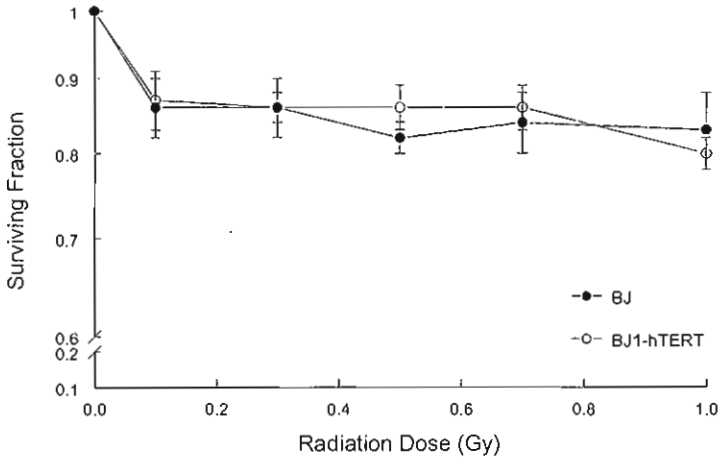


Figure 4.3: Dose dependence of micronucleus (MN) induction: % BNC+MN in bystander BJ human fibroblasts (filled symbols) and bystander BJ1-hTERT human immortalised fibroblasts (opened symbols) after irradiation with 200 keV X-rays. Data points represent the means from three experiments ± standard errors of the means.



A



B

Figure 4.4: A) Low-dose clonogenic cell survival of BJ (black filled symbols) and BJ1-hTERT (red opened symbols) human foreskin fibroblasts irradiated with cobalt-60 γ-rays. Each point represents the mean of three individual experiments (± SD).

B) Low-dose clonogenic cell survival of bystander BJ (black filled symbols) and BJ1-hTERT (red opened symbols) human foreskin fibroblasts which received medium from BJ and BJ1-hTERT cell cultures, respectively irradiated with cobalt-60 γ- rays. Each point represents the mean of three individual experiments (± SD).

There is a relationship supported by the graphs (Figures 4.1 and 4.2) that there is a close relation between the bystander effect and the survival fraction at low doses only, both for γ-H2AX foci formation (Figure 4.5) and chromosomal damage (Figure 4.3). One may suggest that, in our study at least, the hyper-radiosensitivity might be due to bystander factor induced cell inactivation in the low dose region.

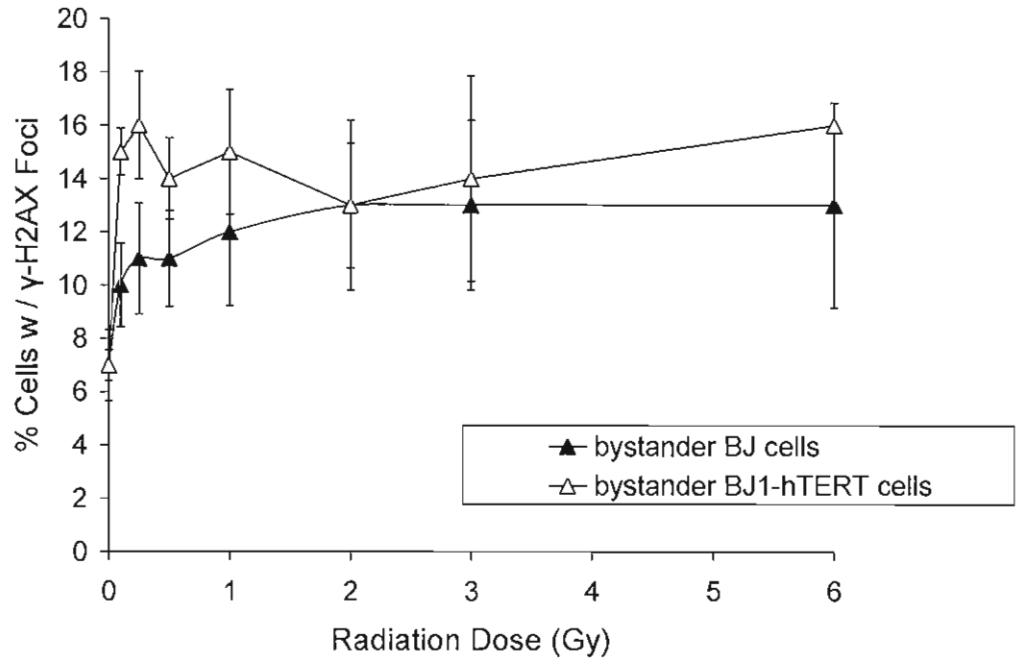


Figure 4.5: Dose response for induction of γ-H2AX foci in bystander BJ and BJ1-hTERT proliferating cells 2h post irradiation. Results are the means of three experiments ± standard errors.

The dose response curves (Figure 4.3 and Figure 4.5) obtained for the bystander response in this study have features which were observed in other reported studies: the bystander effect predominates at low doses and appears to be saturated with no significant additional effect at higher doses. Most observations on bystander effects show a saturating response above a threshold dose (Prise et al, 2003). Using microbeam irradiation, even a single ion track through a single cell triggered a level of response on the population of cells which did not increase when further irradiation was given.

In contrast, in an assessment of 13 different cell lines on response to low-LET radiation doses below 2Gy, two phenomena have been examined: the bystander effect and the hyper-radiosensitivity/ increased radioresistance (Mothersill et al, 2002). The results demonstrate that only 7 cell lines out of 13 show a bystander effect measured using clonogenicity as an end point. The cell death caused by the bystander effect in these cell lines varied between 6% and 90% after 0.5 or 2Gy irradiated cell conditioned medium. The conclusion was that there is a weak inverse correlation between the bystander effect and the hyper radiosensitivity/ increased radioresistance, as some cell lines which showed a large bystander effect were not sensitive to low doses of radiation (e.g. SW 48 cell line) or the opposite (e.g. HGL 21, RT 112 cell lines) (Mothersill et al, 2002).

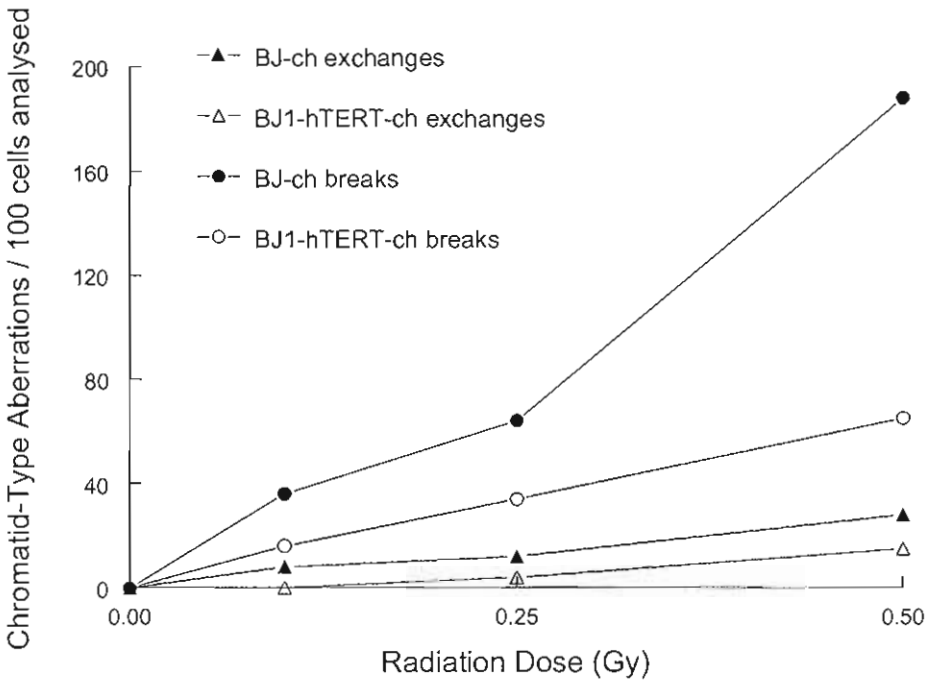
Thus, while the resulting increased mortality is similar in hyper radiosensitivity (HRS) and bystander effects, the underlying mechanisms may be different. HRS is centred on a hypersensitive subpopulation of targeted cells and bystander effect on factors transferred from targeted cells to non-targeted cells. The effects are independent in that cell lines may exhibit HRS, bystander effect, both or neither. For example, MSU-1 cultures exhibit a substantial HRS/ IRR response (Mothersill and Seymour, 1997). However, the media

from irradiated MSU-1 cells are non toxic and even somewhat beneficial to recipient MSU-1 cultures (Mothersill and Seymour, 1997).

Unfortunately, as yet very few data are available concerning the existence of the bystander response for low doses of low LET radiation such as γ and X-rays, particularly at exposures below several mGy where fewer than 100% of the cells will be traversed by a photon. Hopefully, such information will soon be available with the development of low LET microbeam sources. Based on studies with alpha radiation, this could be the region of the dose response curve where one might see non-linearity occurring based on a bystander effect.

4.3. Telomerase and Repair

The results suggest that there is a pronounced difference in repair kinetics between the cell lines. To address whether ionising radiation induces different initial levels of chromosomal damage in the proficient and deficient cell lines and/ or different efficiency of repair we used PCC to visualise directly chromosome events in interphase cells following irradiation. For the BJ cell line, 1h after 0.5 Gy irradiation the number of breaks is about 180. Assuming a linear relation between the radiation dose and the number of chromatid breaks, after a dose of 1Gy the number of breaks will be about 360. On the other hand, for the BJ1-hTERT cell line, the number of chromatid breaks/ Gy is approximately 120 (figure below).



G_2-type aberrations in both cell lines were also detected at metaphase and were predominantly the chromatid type. In Table 3.7, 3 hours after 1Gy irradiation we counted 52 breaks for the BJ cell line, while for the BJ1-hTERT cell line only 44 breaks were monitored. Furthermore, while such results confirm the difference in chromosomal aberrations between the cell lines and are consistent with the observations made for cells at interphase, a decrease by a factor of 7 (for the BJ cells) and 3 (for the BJ1-hTERT cells) in the kinetics of chromatid breaks is seen. This suggests the existence of a more efficient repair in the BJ cells. In a pilot experiment (data not shown), utilising the PCC technique, we found that there was a difference in the kinetics of repair between the cell lines, in agreement with the outcome of the analysis of chromosome abnormalities in the G_2 experiment.

These results suggest that telomerase is involved in the development of chromosomal damage and its repair in G_2 phase, although in the other cell cycle phases this does not appear to be the case as suggested from the results in exponentially growing cells where the overall effect is dominated by the cells in G_1 and S phase. The published literature on the role of telomerase in the development of radiation damage in cells does not provide any convincing explanation for our observation. Clearly, more experiments have to be performed on this topic, in particular also in different cell cycle phases in order to place our observations into perspective. Yet since these observations were only made in the last few weeks of this programme, it was not possible to include them in this thesis.

Whatever the result of these suggested additional experiments will be, it is clear that the telomerase may have besides its role in the maintenance of chromosome integrity during cell proliferation other physiological functions associated with the protection of

chromosomes from breakage. Yet the fact that breaks induced in the absence of telomerase were more easily repaired suggests that different types of breaks may be affected differently by telomerase.

Thus one may speculate that telomerase may play a role in the control of DSB repair by suppressing non homologous end joining (NHEJ), and facilitating or activating recruitment of error-free homologous recombination (HR) machinery during the S and G_2 phases of the cell cycle. Moreover, it needs to be considered whether hTERT could interfere directly with DNA repair or even participate in this process. So far, without additional data, any conclusion has to be rather speculative.

Both homologous recombination and NHEJ pathways are utilised to repair DSBs in mammalian cells. The exact mechanism that determines which pathway will be used is still unclear, but mammalian cells use homologous recombination as a mechanism for DSB repair less frequently than NHEJ (Karran, 2000). It has recently been shown in yeast and in mammalian cells that the choice of DSB repair pathway depends on cell cycle stage (Ferreira and Cooper, 2004; Kruger et al, 2004; Rothkamm et al, 2003; Saleh-Gohari and Helleday, 2004). Homologous recombination is a more dominant mechanism for DSB repair during the G_2 phase of the cell cycle, while the NHEJ pathway predominates particularly in G_0 and G_1 stages of the cell cycle (Karan, 2000; Johnson and Jasin, 2000).

A key feature of homologous recombination repair is DNA strand invasion which is catalysed by the Rad51 protein coating single stranded DNA; however, the exact roles of the accessory proteins that facilitate this process are not known. One of these proteins, Rad51D has been shown to be involved in the maintenance of telomeres (Tarsounas et al,

2004). Rad51 can form distinct nuclear foci in response to ionising radiation exposure (Tarsounas et al, 2004) and these foci are thought to be sites where repair reactions take place (Tarsounas et al, 2004).

In our results, a difference in the percentage of cells with Rad51 foci between the proficient and deficient cell lines was seen for both recovery times (Table 3.8).

These data, taken together, reinforce the idea that hTERT may defend the chromosomes from breakage, but breaks which occur in cells which do not have a telomerase activity are more easily repaired.

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Induction and Detection of Bystander Effects in Human Fibroblasts after Combined Treatment with BrdU, Hoechst 33258 and Ultraviolet A light

Abstract

Aims: A combined treatment of cells with 5-bromo-2'–deoxyurine (BrdU), Hoechst 33258 and ultraviolet A (UVA) light was used to introduce DSBs into cellular DNA for the study of bystander effects in human fibroblast cells.

Materials and Methods: Cells grown in the presence of BrdU on quartz glass slides coated with a thin layer of an antireflective material were exposed to Hoechst 33258 and/ or UVA light. Within this layer a regular pattern of small pores (circles or lines) is produced. When the attached cells are exposed to UV-light through the bottom of the slide, only those regions of the nucleus above the pore are exposed. Confluent cells on both exposed and non exposed areas were analysed for γ-H2AX foci formation.

Results: Combined treatment of cells with BrdU, Hoechst 33258 and ultraviolet A (UVA) light induced reduced cell survival and increased double strand break damage, while treatment with Hoechst 33258 and/ or ultraviolet A (UVA) had no effect on cells.

Conclusions: The combined treatment of cells with BrdU, Hoechst 33258 and UVA light is a valid method for the study of bystander effects as it enables both induction of DNA damage and discrimination of targeted cells and bystander cells (by using of the quartz slides).