Radiation Effects

Studies of Non-Targeted Effects of Ionising Radiation

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Introduction

According to the *target theory* of radiation induced effects, which forms a central core of radiation biology, DNA damage occurs during or very shortly after irradiation of the nuclei in targeted cells and the potential for biological consequences can be expressed within one or two cell generations (Ward, 1999; Ward, 2002). A range of evidence has now emerged that challenges the classical effects resulting from targeted damage to DNA (Fig. 1). These effects have also been termed *"non-(DNA)-targeted"* (Ward, 1999) and include radiation-induced bystander effects (Morgan, 2003a; Morgan, 2003b), 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). An essential feature of "non-targeted" effects is that they do not require a direct nuclear exposure by irradiation to be expressed and they are particularly significant at low doses.

This new evidence suggests a new *paradigm* for radiation biology that challenges the universality of *target theory*.

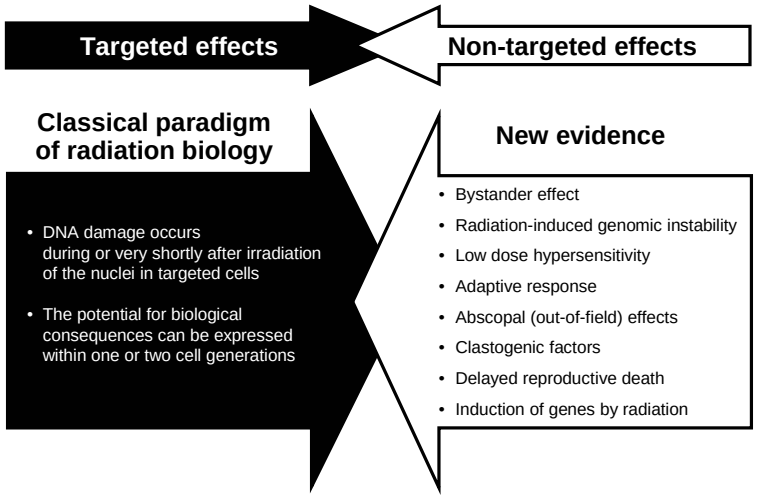

Figure 1. New paradigms for low-dose radiation response

The radiation-induced bystander effect is a phenomenon whereby cellular effects such as sister chromatid exchanges (Nagasawa and Little, 1992), chromosome aberrations (Watson*, et al.*, 2000), apoptosis, micronucleation (Belyakov*, et al.*, 2001; Belyakov*, et al.*, 2002), transformation (Sawant*, et al.*, 2001), mutations (Zhou*, et al.*, 2001) and changes of gene expression (Azzam*, et al.*, 2001) are expressed in *unirradiated neighbouring* cells near to an irradiated cell or cells. Radiation-induced genomic instability is defined as a persistent elevation in the rate of *de novo* appearance of genetic changes such as mutations, chromosome aberrations or micronuclei (Belyakov*, et al.*, 1999) within a clonal population (Wright, 2000). Genomic instability and the bystander effect are both non-targeted effects of irradiation. They have a cross-section much larger than the nucleus. The bystander effect and genomic instability might be related phenomena. It was reported that persistent genomic instability can be induced via a bystander mechanism under *in vitro* (Lorimore*, et al.*, 1998) and *in vivo* (Watson*, et al.*, 2000) conditions. This evidence suggests that the cells that are affected by the bystander mechanism may remain at an increased risk of genetic alterations for many generations. Other studies have suggested a common relationship between genomic instability and the bystander response. Some evidence of protective function of bystander effect is available (Barcellos-Hoff and Brooks, 2001). There is some indication that genomic instability may play a protective role as well. It was recently demonstrated (Limoli*, et al.*, 2001) that chromosome insta-

bility can lead to the development of cell variants that are more resistant to radiation. Bystander effect and genomic instability might be parts of a comprehensive system of oxidative damage control, which aims to reduce the risk of carcinogenesis (Pollycove, 1998). Finally there are suggestions that both the bystander effect and genomic instability are controlled through epigenetic mechanisms such as DNA methylation (Trosko, 1998).

Materials and Methods

Our work relates to experimentation with infinity telomerase-immortalized cell lines (human fibroblasts hTERT-BJ1 and human retinal pigment epithelial cells hTERT-RPE1), which allow investigation of bystander effect and genomic instability in wide time frame. However, the non-targeted effects cannot be comprehensively explained on the basis of a single cell reaction. It is well known that an organism is composed of different cell types that interact as functional units in a way to maintain normal tissue function. Radiation effects at the tissue level under normal conditions prove that individual cells cannot be considered as an isolated functional unit within most tissues of a multicellular organism. Therefore the radiation response is not simply the sum of cellular responses as assumed in classical radiobiology, predominantly from studies using cell cultures. Experimental models, which maintain tissue-like intercellular cell signalling and three-dimensional (3D) structure, are essential for proper understanding of bystander effect and genomic instability. The tissue microenvironment is also important for proper manifestation of non-targeted effects. Extracellular signalling in normal tissues plays a crucial role in initiation and perpetuation of both bystander effect and genomic instability. In order to fulfil these requirements we studied radiation-induced bystander effects and genomic instability in novel 3D artificial human skin tissue systems available from MatTek corporation (Boston, USA). Air-liquid interface culture technique is used to grow artificial tissues, which allow to model conditions present *in vivo*.

To treat cell culture samples, we were using "medium transfer technique" developed by (Mothersill and Seymour, 1997). "Donor" cultures were irradiated with broad field X-rays then medium from these flasks was filtered and added to the "recipient" samples according to a standard protocol. The Gray Cancer Institute (Northwood, UK) charged particle microbeam was used to irradiate tissue samples in a known pattern with a known number of $^{3}He^{2+}$ particles or protons. After irradiation, the tissues models were incubated for 3 days, fixed in 10% NBF, paraffin embedded and then sliced into 5 μm histological sections located at varying distances from the plane of the irradiated cells. In both cases of cell cultures and tissues we studied cell survival, micronucleation, *in situ* apoptosis and markers of differentiation.

Results

Briefly, we have demonstrated a clear bystander decrease in clonogenic survival, increase in micronucleation and apoptosis for both cell and tissue systems, studied the role of differentiation versus damage induction processes. Evidence of interaction between bystander response and genomic instability was shown in telomerase-immortalized cell lines.

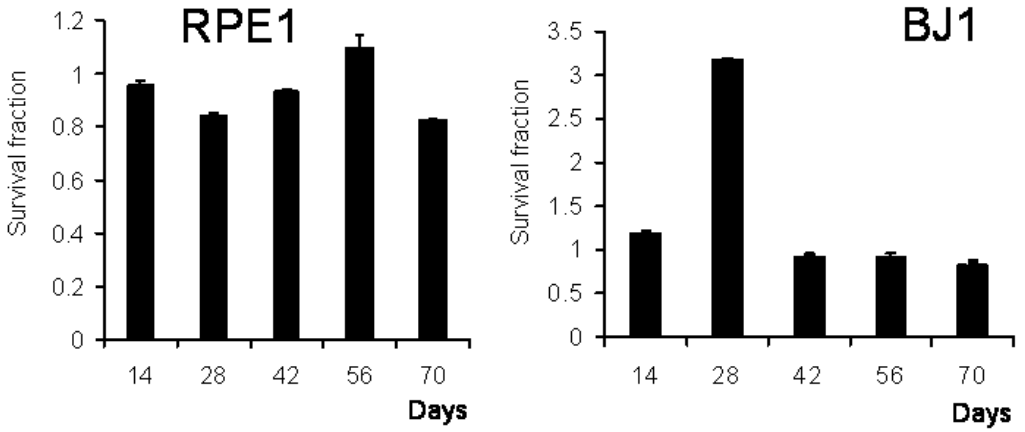

Figure 2. Cell survival, 10% of cells were microbeam irradiated at day 0 with 10 α-particles each.

We observed 5-10% reduction of cell survival (Fig. 2), 6-10 % increase of micronucleation (Fig. 3) and 1-3 % increase of apoptosis in infinity telomerase-immortalised cell lines after treatment with medium from irradiated cells at different timepoints (even tens of generation after the initial treatment), see Fig. 4.

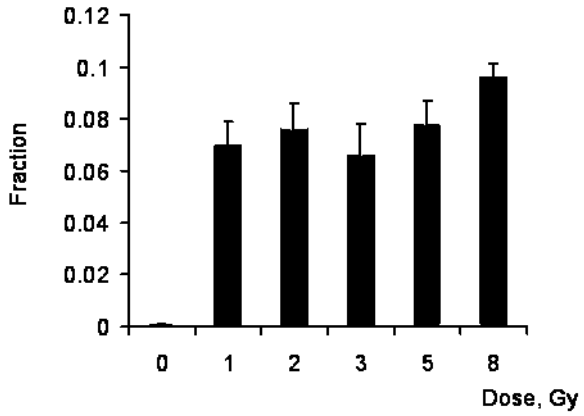

Figure 3. Fraction of micronucleated cells after medium transfer experiments (BJ1→RPE1), estimated in RPE1 cells

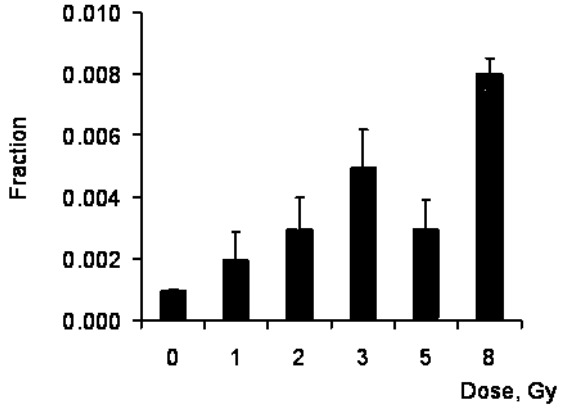

Figure 4. 3'-OH DNA end-labeled apoptosis, medium transfer experiments (BJ1→RPE1), estimated in RPE1 cells

Significantly elevated bystander induced apoptosis was observed with 3'-OH DNA end-labelling based technique in 3D artificial tissue skin systems. Pilot data on the bystander effect after microbeam irradiation (measured as increased fraction of damaged cells and bystander induced differentiation) were obtained (Fig. 5).

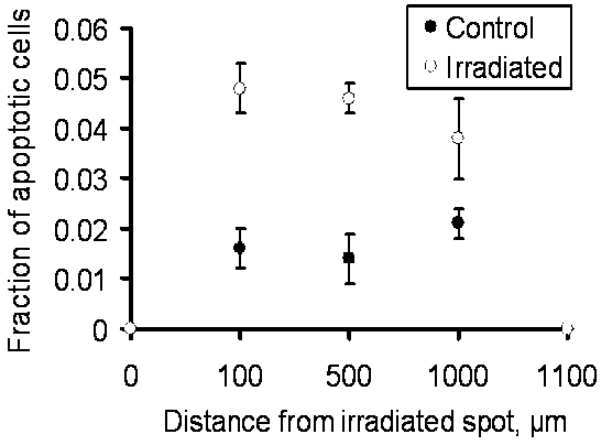

Figure 5. Bystander apoptosis in EPI-200 artificial human skin after spot microbeam irradiation

Our results suggested an importance of proliferation and differentiation status for bystander effect induction. A single 2 μm location on tissue section was pre-irradiated with 1-10 $^{3}He^{2+}$ particles (5 MeV; LET 75 keV/μm) using microbeam system. Studies of bystander-induced differentiation under *in situ* were performed using morphological measurements in underdeveloped EPI-201 model. Even although only a single region of the tissue section was targeted, thousands of additional cells were found to undergo bystander induced differentiation. This resulted in an overall increase in the fraction of differentiated cells for approximately 10-15%, which are much greater than that observed for the induction of damage (not more than 1-2% of apoptotic cells), Fig. 6.

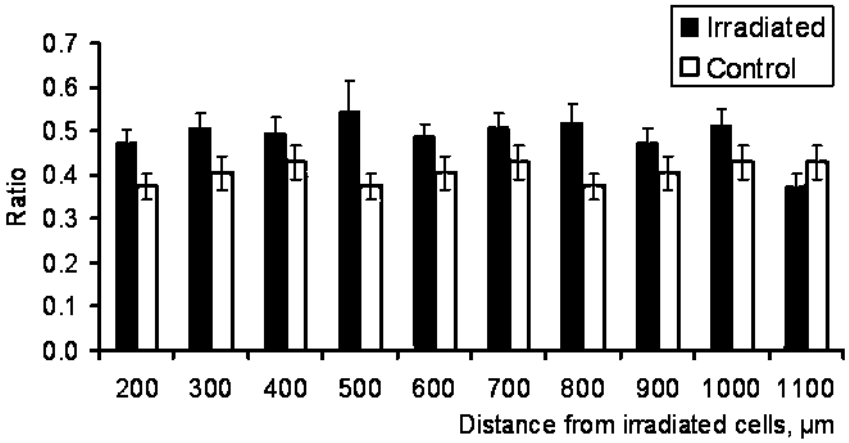

Figure 6. Differentiation is considerably increased in microbeam irradiated samples in comparison with controls up to a distance of approximately 1 mm from irradiated cells. This graph represents a ratio of thickness of cornified (differentiated) layer to the total thickness of artificial human skin.

Discussion

It is known that the bystander effect is cell type dependent (Mothersill and Seymour, 1997), depends on cell proliferative state (Belyakov*, et al.*, 2003) and that energy/REDOX metabolism may be involved in the expression of a radiation induced bystander response (Mothersill*, et al.*, 2000). The exact mechanisms of the bystander effect are not yet known. However, it is clear that bystander signal production and cellular response may involve different pathways (Grosovsky, 1999). Bystander signalling is a complex and well-tuned system, which most likely involves more than one messenger and is connected with tissue microenvironment signalling (Barcellos-Hoff, 1998; Barcellos-Hoff and Brooks, 2001).

There is experimental evidence that the bystander effect may have at least two separate pathways for the transfer of damage from irradiated cells to unirradiated neighbours: by gap junction intercellular communication (GJIC) or cell culture mediated factors. A junction between cells, which consists of many pores, mediates GJIC. Each pore is formed by a hexagonal array (connexon) of six transmembrane proteins (connexins) in each plasma membrane: when joined together the pores open, allowing communication and the interchange of metabolites between cells (Trosko and Chang, 2001).

Azzam and co-workers (Azzam*, et al.*, 1998) have demonstrated that the bystander effect is dependent on gap junction intercellular communication in confluent cultures of 5 different primary human diploid fibroblast lines exposed to low fluences of α-particles. They showed that TP53 and CDKN1A expression are activated in bystander cells after low dose α-particle irradiation. Importantly, they also observed clustering of expression in neighbouring cells. Treatment of the culture with lindane, which inhibits GJIC, led to a marked reduction in the increase in the levels of TP53 and CDKN1A. A recent paper from the same authors suggested direct evidence for the participation of GJIC in the transmission of damage signals from irradiated to non-irradiated cells (Azzam*, et al.*, 2001). Other workers have also shown that lindane treatment leads to inhibition of bystander-

induced cell killing in hamster V79 cells (Bishayee, *et al.*, 1999). The bystander effect was also significantly reduced in cells pretreated with 1 mM of octanol, which inhibits gap junction-mediated intercellular communication (Zhou, *et al.*, 2001). The same paper also reports that the bystander effect was suppressed in cells carrying a dominant negative connexin 43 vector, which is a part of the connexon complex.

Little is known concerning the signals, which may be transferred via GJIC. The connexin proteins, which form the gap junctions, allow ions, secondary messengers and small molecules to pass between cells and modification of these proteins, by phosphorylation, can open or close the pores. Whether specific signal molecules are transmitted between cells or the junctions are specifically opened, as part of a bystander response needs to be addressed.

The second proposed mechanism of the bystander effect is mediation by secretion of factors into the culture medium. Medium transfer experiments (Mothersill and Seymour, 1997; Seymour and Mothersill, 2000) suggest the existence of a relatively long-lived bystander effect mediator, which cannot be eliminated by media filtering. A series of studies 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. Lehnert and co-workers (Lehnert and Goodwin, 1997a) demonstrated that the culture medium harvested from cells irradiated with low fluences of α-particles could induce an increase in sister chromatid exchanges when incubated with unirradiated test cells. According to results (Lehnert and Goodwin, 1997a; Lehnert and Goodwin, 1997b), α-particle irradiated cells secrete into the serum containing medium some short-lived factor(s). It was found that the activity of this factor(s) could be inhibited by superoxide dismutase, can survive freeze and thawing but not heating. A recent paper by Lewis and co-authors (Lewis, *et al.*, 2001) used a medium transfer protocol and observed delayed death and neoplastic transformation. And finally, Mothersill and Seymour (Mothersill and Seymour, 1998) reported data which suggest that the bystander effect does not depend on communication through gap junctions formed between cells in contact but is due solely to media release factors, in contrast to that predicted from other studies.

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 excreted into the cell culture medium from the irradiated cells or be directly transmitted by GJIC - gap junction intercellular communication between hit and non-hit cells (Grosovsky, 1999).

Based on this distinction it can be speculated that at least two types of the bystander messenger might exist. Primary messenger is emitted by targeted cell. It is short lived, not very stable, travels through gap junctions, should be water soluble and most likely not a protein. One suitable candidate here could be long-lived organic radicals capable of transferring through gap junctions. Such radicals could have lifetimes of up to 20 hours (Watanabe, *et al.*, 1990; Koyama, *et al.*, 1998). Among other candidates for GJIC mediated primary bystander messenger are antioxidants (thiols) (Prise, 2002), Ca^{2+} (Lyng, *et al.*, 2001) Ip3 (storage form of intercellular Ca^{2+}) and cAMP (Lehnert, 1975), which is an important secondary messenger involved in Ca^{2+} metabolism.

Secondary bystander messenger should be long-lived, more stable, most likely emitted by activated, not directly traversed, cells. It might be a media borne factor and most likely a protein. 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 as key signalling molecules in the transfer of bystander damage 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).

There is a range of possible candidates for bystander effect mediation, which are medium borne and could be either primary or secondary messengers. Reactive oxygen species (H_2O_2/O^{-2}) have been proposed as possible signals involved in bystander responses (Narayanan, *et al.*, 1997; Iyer and

Lehnert, 2000). Another group proposed that nitric oxide (NO) might play a central role in mediation of bystander effect (Matsumoto*, et al.*, 2000; Matsumoto*, et al.*, 2001) potentially having a protective value.

In conclusion, it is most likely that there is no single mechanism underlying the bystander effect and both media borne and GJIC factors are involved in its induction and perpetuation. The mechanisms involved are probably cell type specific which may reflect a lot of the current uncertainty in the literature as to the processes involved.

The relationship between the bystander effect and genomic instability is not clear. It was reported that persistent genomic instability could be induced *in vitro* via a bystander mechanism. Chromosomal instability was demonstrated in the clonal descendants of haemopoietic stem cells after irradiating murine bone marrow with α-particles (Lorimore*, et al.*, 1998). The authors studied the effects of interposing a grid between the cells and the α-particle source so that 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 haemopoietic stem cells under *in vivo* conditions (Watson*, et al.*, 2000).

The data published in (Belyakov*, et al.*, 1999) suggest that the same AG01522B normal human fibroblast cell line is susceptible to radiation induced genomic instability (after both α-particle and X-ray irradiations), and bystander response after microbeam $^{3}He^{2+}$ irradiation according to the results, published in (Prise*, et al.*, 1998; Belyakov*, et al.*, 2001). Also, the urothelial model, which demonstrates a pronounced bystander response (Belyakov*, et al.*, 2002; Belyakov*, et al.*, 2003; Belyakov*, et al.*, 2005) may express genomic instability as a part of the response.

Other studies have suggested a common relationship between genomic instability and the bystander response. Some evidence of protective function of bystander effect is available (Barcellos-Hoff and Brooks, 2001). There is some indication that genomic instability may play a protective role as well. It was recently demonstrated (Limoli*, et al.*, 2001) that chromosome instability in GM10115 cells can lead to the development of cell variants that are more resistant to radiation. Bystander effect and genomic instability might be parts of a comprehensive system of oxidative damage control, which aims to reduce the risk of carcinogenesis (Pollycove, 1998; Pollycove and Feinendegen, 1999) and both have been observed *in vivo* (Watson*, et al.*, 2000; Watson*, et al.*, 2001). Finally there are suggestions that both the bystander effect (Neriishi*, et al.*, 2001) and genomic instability (Limoli*, et al.*, 2000) are controlled through epigenetic mechanisms (Trosko, 1998) such as DNA methylation (Bird, 1996).

The discovery of ionising radiation induced non-targeted effects is important for understanding the dose-response mechanisms relevant to low-dose irradiation *in vivo*. One important question is whether the non-targeted effects relates to a protective mechanism or whether, conversely, it amplifies the number of cells damaged by the isolated radiation tracks of low-dose exposures leading to an increased risk of carcinogenesis. One theory, supported by the experimental data obtained during this project is that the main functions of the non-targeted effects are to decrease the risk of transformation in a multicellular organism exposed to radiation. It can be speculated that individual cells within a tissue may not have the ability to detect irradiation such that an individual cell response is not expressed. An integrated multicellular system may be able to detect damage from irradiation and respond to it by removing a functional group of cells, which could be potentially damaged. However, not every cell will respond to the hypothetical non-targeted factor(s), which are released by targeted (or instable) cells. Only 1-3% of the total number of cells in the system would express lethal response (micronucleation or apoptosis) and approximately 10-15% would go on to premature induced differentiation. Differences in the gene expression profiles, temporal and spatial patterns of key proteins expressed in directly irradiated and bystander cells may determine how the

cells ultimately respond to low doses of radiation. Such a mechanism of co-operative response would make the tissue system much more robust. It would work only for low doses of charged particle irradiation (below~0.1-0.2 Gy, depending on system and type of radiation) because only in this case is the damage localised within a small fraction of the cell population. In some systems, the most convenient way to remove potentially damaged cells is via apoptosis. In particular, apoptosis allows the removal of affected cells without a negative impact on other cells via inflammatory responses. However many apoptotic pathways are controlled by cellular signals, which would also enable the selective removal of certain functional groups of cells. Another way to isolate damage is to prompt affected cells into irreversible differentiation. Underlying this theory is that normal 3D tissue microarchitecture is essential for the manifestation of the effect. Therefore, protective function of non-targeted effects might be a tissue-specific epigenetic phenomenon, which can be observed in full scale when there is presence of natural cellular stratification with differentiated and dividing cells present and an intact tissue microenvironment. However, the data suggest that initial nuclear damage seems to be essential for initiation of this system. Perpetuation of the bystander effect genomic instability might involve cascade-like epigenetic mechanisms. Tissues remove all potentially damaged cells from the system to avoid the risk of carcinogensis following sparse low dose irradiation or any other local oxidative damage. Bystander induced differentiation seems to play a central role in this process, see also (Belyakov*, et al.*, 2002; Belyakov*, et al.*, 2005).

Acknowledgments

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