Refresher course, topic RC-2. Cellular and molecular effects. Non-targeted biological effects of ionising radiation

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Abstract. The universality of the target theory of radiation-induced effects is challenged by observations on non-targeted effects such as bystander effects and genomic instability. Essential features of non-targeted effects are that they do not require direct nuclear exposure by radiation and they are particularly significant at low doses. This new evidence suggests a need for a new paradigm in radiation biology. The new paradigm would cover both the classical (targeted) and the non-targeted effects. New aspects include the role of cellular communication and tissue-level responses. A better understanding of non-targeted effects may have important consequences for health risk assessment and, consequently, on radiation protection. Non-targeted effects may contribute to the estimation of cancer risk from occupational, medical and environmental exposures. In particular, they may have implications for the applicability of the Linear-No-Threshold (LNT) model in extrapolating radiation risk data into the low-dose region. This also means that the adequacy of the concept of dose to estimate risk is challenged by these findings. Moreover, these effects may provide new mechanistic explanations for the development of non-cancer diseases. Further research is required to determine if these effects, typically measured in cell cultures, are applicable in tissue level, whole animals, and ultimately in humans.

KEYWORDS: non-targeted effects, bystander effect, genomic instability, adaptive response ionising radiation, radiation protection, LNT validity.

Non-targeted effects of ionising radiation

Cellular targets for radiation damage

The *target theory* of radiation induced effects [1, 2] postulates that cells contain at least one critical site or target that must be hit by radiation in order to kill a cell. Radiation damage outside of the target does not cause cell death. It is widely accepted that nuclear DNA is the critical target for radiation induced cell death. Early experiments demonstrated that damage to the DNA is more than 3000 times more effective than membrane damage in the killing of cells *in vitro* [3]. However, there is evidence suggesting that the cell membrane might also be a target of death in some instances [4, 5].

When a tissue absorbs ionising radiation, its energy results in the production of a fast recoil electron. This electron may then cause damage, either by direct interaction with the DNA, or indirectly through production of free radicals, particularly the hydroxyl radical (OH•), which can cause a break to the DNA helix. Charged particles with high linear energy transfer (LET) radiation such as $^3He^{2+}$ or α-particle would induce predominantly "direct" damage, whereas low LET radiation (γ and x-rays) predominantly cause "indirect" damage through the action of free radicals [6].

There are a few major types of DNA damage that can be produced by ionising radiation. *Single-strand breaks (SSBs)* occur due to the deposition of radiation 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 and finally DNA-DNA and DNA-protein crosslinks [7].

Radiation induced DNA damage can be repaired. There are three types of repair: *error-free repair* includes excision repair and generally does not result in mutations or lethality, *error-prone repair* may result in non-lethal or lethal mutations and *incomplete repair* does not result in the re-establishment of continuity in the DNA sequence and thus may be considered lethal [6].

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Non-repaired DNA breaks may lead to chromosomal aberrations. Many types of chromosomal aberrations are produced, some of them lethal (unstable aberrations like dicentrics, rings, fragments), and some non-lethal (stable aberrations i.e. reciprocal translocations). Non-lethal aberrations may lead to oncogenesis. Unstable aberrations may result in the formation of micronuclei, which are the consequences of separation of acentric fragments (or whole chromosome) from the mitotic spindle, and are clearly visible in cellular cytoplasm at the first post-irradiation mitosis [8]. These ultimately lead to loss of clonogenic survival.

In addition to 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 predetermine the later consequences of irradiation. Depending on the efficacy of the repair processes, damaged cells may undergo *necrosis*, *apoptosis*, *proliferative death*, *senescence (premature differentiation)* or ultimately survive and proliferate [6].

There is a range of *delayed effects*, which may occur in remote descendants of irradiated cells several generations after irradiation. If a cell survives and produces progeny then the initial biological response to the irradiation may influence cell differentiation, shorten life-span, induce *genomic instability* [9], or *carcinogenesis* [10].

Non-targeted effects. A new paradigm of Radiation Biology.

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 [11, 12]. 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"* [11] and include radiation-induced bystander effects [13], genomic instability [14, 15], adaptive response [16], low dose hyper-radiosensitivity (HRS) [17], delayed reproductive death [18] and induction of genes by radiation [19]. 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* [20] for radiation biology that challenges the universality of *target theory*.
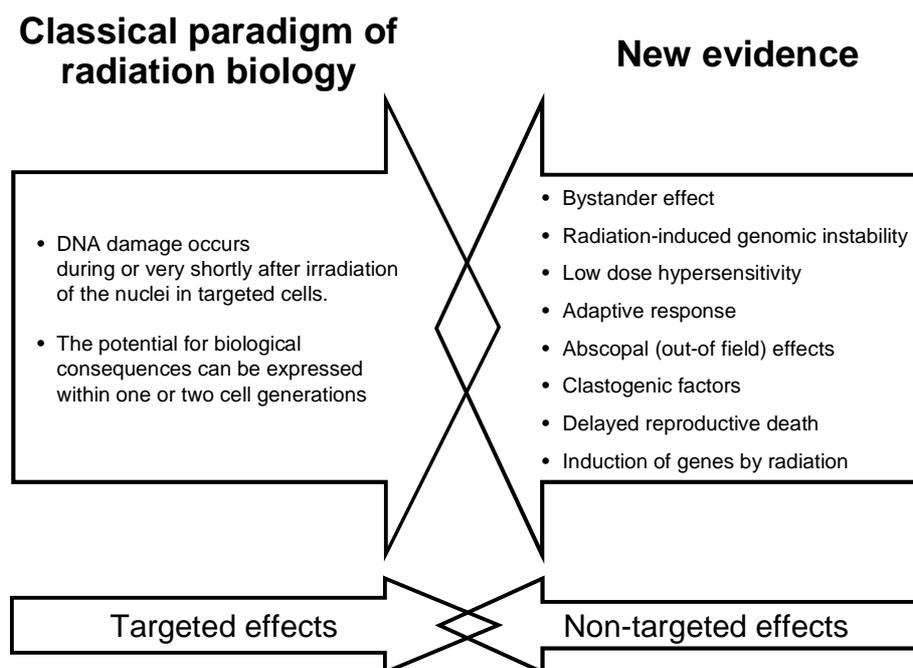

Classical paradigm of radiation biology

New evidence

- DNA damage occurs during or very shortly after irradiation of the nuclei in targeted cells.

- The potential for biological consequences can be expressed within one or two cell generations

- Bystander effect
- Radiation-induced genomic instability
- Low dose hypersensitivity
- Adaptive response
- Abscopal (out-of field) effects
- Clastogenic factors
- Delayed reproductive death
- Induction of genes by radiation

Targeted effects   Non-targeted effects

Figure 1. New paradigms for Low-Dose Radiation Response

Bystander effect and Genomic instability, definitions

This paper will discuss mainly the bystander effect and to a lesser extent, genomic instability.

The radiation-induced bystander effect is a phenomenon whereby cellular damage such as sister chromatid exchanges [21, 22], chromosome aberrations [23-25], apoptosis [23], micronucleation [26], transformation [27, 28], mutations [29-31] and changes of gene expression [32-35] is expressed in unirradiated neighbouring cells near to an irradiated cell or cells (Fig. 2).
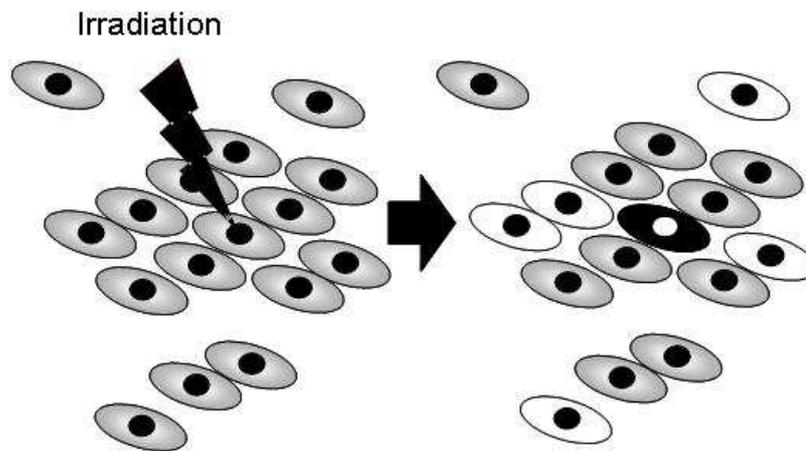

Figure 2. Scheme of the bystander effect. Directly damaged cell is marked black; bystander damaged cells are marked white.

Radiation-induced genomic instability is defined as a persistent elevation in the rate of *de novo* appearance of genetic changes (mutations, chromosome aberrations or micronuclei) within a clonal population [9, 14, 15], see Fig. 3.

Figure 3. Radiation induced genomic instability, damaged cells are marked white

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. There is as yet no evidence that the bystander effect persists for many generations. On the other hand, it was reported that persistent genomic instability can be induced via a bystander mechanism under *in vitro* [24] and *in vivo* [25] conditions. This evidence suggests that the initial cross-section for radiation damage is increased by the bystander effect, and cells that are affected by the bystander mechanism may remain at an increased risk of genetic change for many generations.

Evidence for bystander effects.

Interactions between hit and non-hit cells after exposure to ionising radiation have been known for many years in radiation biology. Much of the early data was obtained from studies of chromosome damage induced by plasma from radiotherapy patients [36, 37] and accidental exposures [38] in test cell cultures. These indirect effects were explained by the production of "clastogenic factors" [39]. These clastogenic factors were extensively studied in victims of the Chernobyl Accident [40-42]. It was hypothesised that they may be related to lipid peroxide products [43] ionisine nucleotides [44], cytokines [45] and reactive oxygen species (ROS) such as superoxide radicals [39].

Other evidence has come from abscopal or "out-of-field" effects, which are well known in radiotherapy [46-49]. These phenomena are defined as the effects of radiation on tissues of the same person or organism at some distance from the actual radiation site or target. A recent paper by [50] related radiation-induced out-of-field effects in lung of rodents with DNA damage. A strong correlation between lethality and DNA damage was found.

In the last few years, a large number of papers were published demonstrating evidence for the radiation induced bystander effect [13, 51]. Nagasawa and Little first published a paper, describing the bystander effect [22], measured as an increase of sister chromatid exchanges (SCE). They irradiated Chinese hamster ovary cells with low doses of α-particles from a conventional broad field source in a way that only a few cells within a population were actually traversed by a particle. A much higher level of SCEs were produced in cells than would be predicted on the basis of the number of cell nuclei targeted. The authors proposed a hypothesis that cell irradiation induces some indirect effects within neighboring cells via free radical cascades or signal transduction pathways.

Significant numbers of the recent publications with evidence for bystander effects have come from the studies with α-particle irradiation delivered with specially constructed conventional low doses broad-field sources [52]. In this case irradiation have been delivered to a population of cells in such a way that only a few cells within a population were actually traversed by α-particles. Hickman measured changes in the TP53 expression after rat lung epithelial cells were exposed to low doses of α-particles [35]. They found that a higher fraction of cells demonstrated an increased TP53 expression than were hit by α-particles.

A series of papers from the Los Alamos National Laboratory demonstrated that extracellular factors are involved in SCE formation following low dose α-particle exposure. Deshpande and co-workers [21] irradiated cell cultures of primary human fibroblasts with α-particles and observed a high level of sister chromatid exchanges. The percentage of cells showing SCEs was 9-fold higher than expected on the basis of the number of nuclei traversed. The authors provided convincing evidence for the production of extracellular factors, released into the cell culture medium [53]. Later, the same group [54] attributed the observed bystander effects to the action of TGF-*β1* and reactive oxygen species (ROS).

In a series of studies, Mothersill and Seymour demonstrated that medium from γ-ray irradiated cell cultures reduces the survival of unirradiated cells [23, 55-58]. Under this protocol supernatant from irradiated cells was transferred to test "reporter" cell cultures, which were analysed using the Puck and Marcus clonogenic assay [59] and for presence of micronucleated, apoptotic and cells with chromosome aberrations.

Another approach was utilized by Bishayee and co-workers [60, 61]. They detected a pronounced bystander effect in a V79 three-dimensional tissue culture model labelled with ^{3}H-thymidine when the isotope is localised in the cell nucleus and distributed non-uniformly among the cells. A related class of effects was demonstrated in thymocytes [62]. They demonstrated that interactions between different types of γ-irradiated cells lead to different degrees of radiation-induced apoptosis via the production of soluble autotoxic mediators. When irradiated cells were mixed with non-irradiated ones, less interphase-induced cell killing was observed than would be predicted on the basis of ratios of the cells mixed together. This protection effect is not observed when the medium from non-irradiated cells is added to the irradiated thymocytes.

Previous studies at the Gray Cancer Institute demonstrated that the target for chromosomal damage is larger than the nucleus on basis of calculations of the fraction of micronucleated Chinese hamster V79

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cells after α-particle irradiation [63]. It has been demonstrated a direct evidence of bystander effects in normal human AG01522B fibroblasts using the Gray Cancer Institute charged particle microbeam [64, 65]. Irradiation of a single fibroblast with a single $^3He^{2+}$ particle delivered by the microbeam through the nucleus would give a significant rise of bystander damaged cells measured as micronucleated and apoptotic cells. In general a 2-3 fold increase in the level of damaged cells was measured in comparison to controls.

Other groups have also utilised microbeam approaches to study bystander effects. Evidence for the existence of extra-nuclear target(s) for radiation-induced effects [66] was observed when the cytoplasm of human-hamster hybrid $A_{(L)}$ cells was irradiated avoiding traversal of the nucleus. Cytoplasmic irradiation led to considerable mutagenesis at the CD59 (S1) locus with minimal cytotoxicity. The mutations found were similar to those of spontaneous origin and are entirely different from those of nuclear irradiation. On other hand, it was demonstrated that cytoplasmic irradiation initiates the generation of reactive oxygen species. The final conclusion from the paper was that cytoplasmic irradiation might be more dangerous than nuclear irradiation, as mutagenicity is accomplished by little killing of the target cells.

Zhou and co-authors [30] demonstrated a bystander mutagenic effect after α-particle microbeam irradiation. They showed that cells, irradiated with a microbeam, could induce a bystander mutagenic response in neighbouring cells, which were not directly traversed by an α-particle. Intercellular communication plays a critical role in mediating the bystander phenomenon under these conditions. It was shown that irradiation of 20% of randomly selected human-hamster hybrid $A_{(L)}$ cells with 20 α-particles each, resulted in a mutant fraction that is 3-fold higher than expected, assuming no bystander effect. Analysis by multiplex PCR demonstrated that the types of mutations induced are significantly different from those of spontaneous origin.

Another study from the same group [31] showed that irradiation of even 10% of confluent human-hamster hybrid $A_{(L)}$ cells with a single α-particle per cell through the nucleus results in a mutant yield similar to that observed when all cells in the population are irradiated. This effect was significantly eliminated by an inhibitor of gap junction-mediated intercellular communication, or in cells carrying a dominant negative connexin 43 vector.

An important question is whether the bystander effect contributes to carcinogenesis. Lewis and co-authors [27] tested the response of non-irradiated cell cultures when these were exposed to medium from X-irradiated human CGL1 hybrid cells. They reported an increased radiation-induced bystander neoplastic transformation after treatment with medium from irradiated cells. Medium, exposed with 5 or 7 Gy of X-ray increased the frequency of neoplastic transformation significantly from $6.3x10^{-6}$ in control to $2.3x10^{-5}$ (~4-fold).

Sawant and co-authors [28] used the Columbia University microbeam system to delivered 0, 1, 2, 4 or 8 α-particles through the nuclei of all or 10% of C3H 10T1/2 cells. They demonstrated that when 10% of the cells are exposed to α-particles, the frequency of induced transformation is the same as that observed when every cell was exposed to the same number of α-particles.

Sigg [67] used β-particle emitting ^{90}Y wires (average energy 934 keV) to create an inhomogeneous radiation field in C3H 10T1/2 cell cultures. Total 24h doses ranging from 0 to 750 Gy across the exposure field were tested and at equal levels of toxicity a 10 fold enhancement of neoplastic transformation frequency was observed in the presence of heavily damaged cells. Homogeneous fields of low-dose-rate β-particle radiation produced neoplastic transformation frequencies typical for comparable photon exposures reported in the literature.

Radiation induced bystander effects may produce not only damage but other effect which can be interpreted as neutral or beneficial. For example, [54] reported that exposure of normal human lung fibroblasts to a low dose of α-particle stimulates their proliferation *in vitro*. On the other hand, this response also occurs when unirradiated cells were treated with media from α-particle irradiated cell cultures. The promitogenic response is attributed to superoxide dismutase and catalase-inhibitable increases in the concentrations of (TGF-*β1*) in cell supernatants and with intracellular increases in ROS, expression of TP53 and CDKN1A.

Matsumoto [68] found that the radiosensitivity of A-172 human glioblastoma cell lines to X-irradiation in the range of 0 to 10 Gy was increased in the case of treatment with pre-conditioned medium from irradiated cells in comparison to those irradiated in fresh medium. The key role in modification of the response is attributed to nitric oxide, which was emitted by irradiated cells and induced radioresistance in cells treated with supernatant.

Bystander effect can be induced by low and high LET irradiation

There is evidence that various types of radiation can induce the radiation bystander effect. The bystander effect induced by α-particles has already been discussed. β-particle irradiation is able to initiate a bystander response [60, 61]. Media transfer experiments showed that low LET γ-rays [23, 55] can also produce a significant effect. Unpublished data, which will be described in more detail later (part 5.3), demonstrated a bystander effect after targeted ultra-soft X-rays produced by the Gray Cancer Institute microprobe facility.

Characteristic features of radiation-induced bystander response

In comparison to direct, classical effect of irradiation the bystander effect has three characteristic features:

1. Bystander responses predominate in the low-dose region (< 0.5 Gy);

2. The bystander effect has a non-linear dose dependence, suggesting a switch-on ("all or nothing") mechanism for its activation;

3. The bystander effect is maximally induced by very low doses.

Nagasawa and Little first demonstrated evidence of the bystander effect induced by a very low dose of 0.16 mGy and saturating at 0.31 mGy without further statistically significant increases up to 4.9 mGy [22]. Hickman in his experiments with irradiation of rat lung epithelial cells, showed that the dose-effect for TP53 expression was different for α-particles in comparison to X-rays [35]. α-particles gave a no-threshold response whereas there was a low dose threshold observed with X-rays at around 0.1 Gy. Overall, the shape of the dose-effect curve for both types of irradiation had a tendency to flatten after exposure with 0.2-0.5 Gy and did not demonstrate a statistically significant increase with increasing dose. Deshpande and co-workers [21] did not observe a dose-dependence of the bystander effect above 0.02 Gy with saturation up to highest does tested, 13 Gy of α-particles. Zhou [30, 31] noted that a level of bystander mutagenesis effect after α-particle microbeam irradiation did not depend on the number of particles delivered. Lewis [27] also showed that the amount of cell death induced by bystander effects is not dependent on dose.

The bystander effect contributes to a significant proportion of the overall damage yield in the low-dose region by an apparently distinct mechanism from the "classical" radiation response. Recently obtained data [26, 64] demonstrated that the fraction of damaged (micronucleated and apoptotic) human fibroblasts was independent of the number of charged particles delivered to the targeted cell. One $^3He^{2+}$ ion, delivered to the nucleus of one cell among a few hundred non-irradiated neighbours induced the bystander effect to the maximum extent. Further increase of dose to the targeted cell does not change the dose response. Similarly, the effect was independent of the number of cells irradiated. The same level of damage was observed whether 1 or 4 cells were targeted within the dish. These data are considered in detail in [26, 64].

The general shape of the bystander effect dose response in comparison to direct radiation consequences is illustrated at Fig. 4. Most observations of bystander effects have shown a saturation of the response above the threshold dose (0.2 Gy is an estimation) and do not demonstrate a linear relationship to the dose, see review [69].

The model proposed here (Fig. 4) is supported by data, obtained with normal human fibroblast cell cultures published in [26, 64]. Experiments with primary urothelial explants similarly demonstrated the absence of a dose response,.
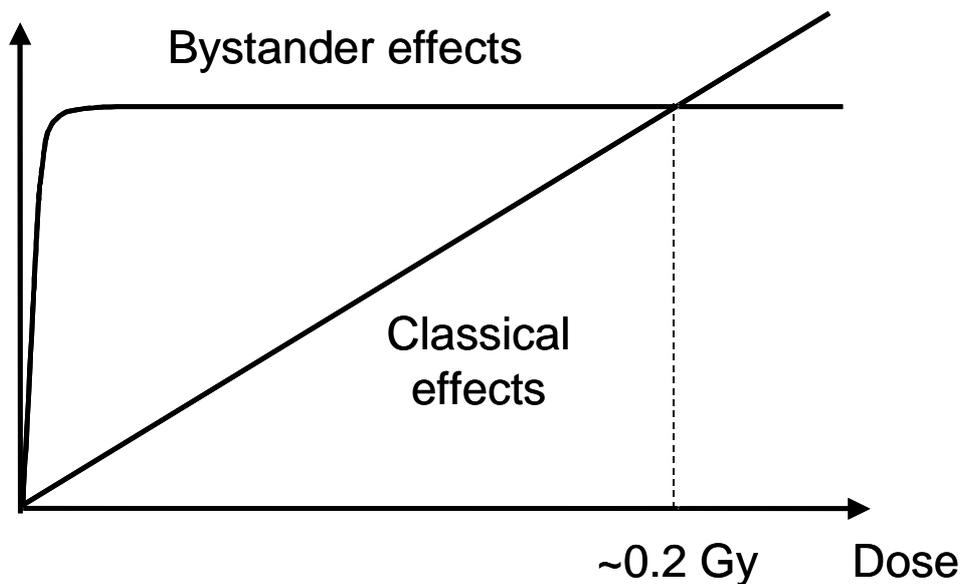
Figure 4. Comparison of "classical" and "bystander" types of response to ionising irradiation

The model proposed here is in marked contrast to that proposed by Brenner [70] as a quantitative model for the application of the bystander effect to carcinogenic risk. The BaD (Bystander and Direct) model of radiation response is supported by the data from the same group on *in vitro* oncogenic transformation after broad-field or microbeam α-particle irradiation [28]. BaD postulates that the bystander effect is a binary "all or nothing" phenomenon and might be expressed in a small sensitive sub-population of "interested neighbors" [71], which do not cover the entire cell population. The authors believe that there may be purely geometrical reasons for the existence of a subpopulation, susceptible to bystander effects. They assume that a hypothetical bystander factor has a limited penetration distance. However, in this case some clustering of damage should be observed around the irradiated cell. To date, no evidence of clustering has been reported and in contrast, data published in [64] suggest that cellular damage is uniformly distributed throughout the cell culture dish.

The BaD model also suggests that the bystander effect can only be observed at low doses (Fig. 5). At low doses, the bystander effect dominates the direct response. The authors point out that this may lead to an underestimation of low-dose risks extrapolated from high doses, where direct effects dominate. Similar to the model proposed here, BaD assume that the total response of a cellular system to ionising radiation has two components: direct and bystander damage. Direct damage has a linear dose-relationship, whereas bystander damage is induced to the maximum extent by very low doses (less than 1 cGy). In contrast to the proposed model, the authors believe that the bystander effect would decline with increasing dose because bystander signal-sensitive cells, whose nuclei are hit directly, cannot produce a bystander response Therefore, the total effect would be (as presented at Fig. 5) a result of summing bystander and direct effects at low dose region (up to 30 cGy). At the higher dose (from about 30 cGy) it would be predominantly influenced by direct effects. To date, however, there is not enough experimental data to assume that a direct hit would prevent a cell from releasing a bystander factor.

Therefore, a different model can be suggested (see Fig. 6), which has a more pronounced plateau in the low dose region and fits better to both the results, obtained during this project and other published experimental data. We assume that bystander signal-sensitive cells, whose nuclei are hit directly, can produce a bystander response. Finally, the model proposed here can be utilised to describe any dose-effect relationship for cellular damage whereas the BaD model is designed for the estimation of carcinogenic risk.
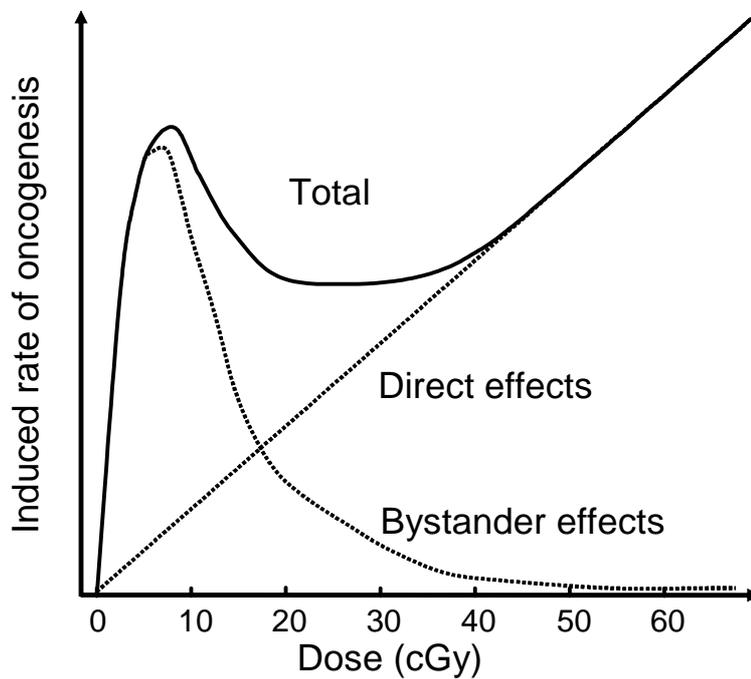
Figure 5. Contribution of bystander and direct component to the radiation induced rate of oncogenesis, a BaD model, reproduced from [70]
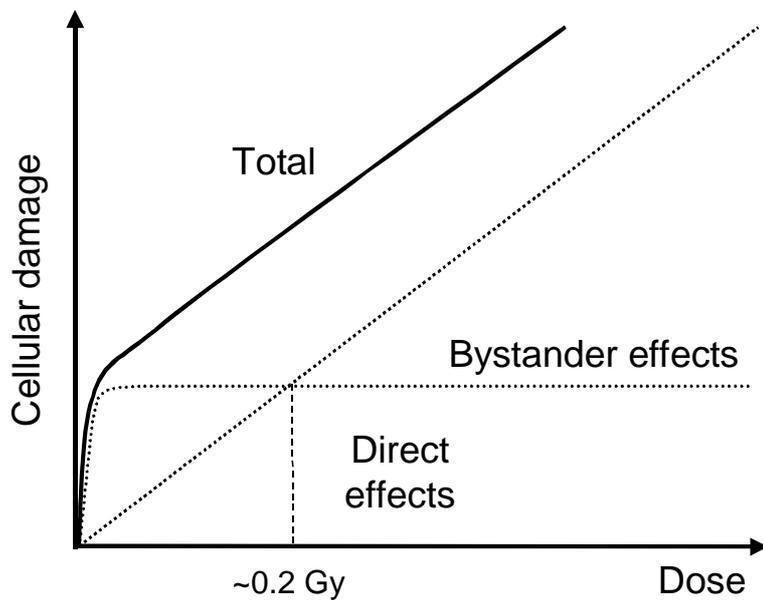

Figure 6. Contribution of bystander and direct component to the radiation induced damage, proposed model

Recently another novel stochastic model was proposed [72] A model of the radiation-induced bystander effect is developed that takes account of spatial location, cell killing and repopulation. The ionizing radiation dose- and time-responses of this model are explored, and it is shown to exhibit pronounced downward curvature in the high dose-rate region, similar to that observed discussed above. One significant advantage of this model is that this model is suitable for 3D modelling of bystander effect can be applied to the tissue data.

Bystander versus direct effects

For studies of cell killing, it is important to determine numerically the relative contribution of "classical" and "bystander" effects. Recently, Seymour and Mothersill [58] have presented a method of correcting the overall survival curve to enable analysis of the relative contributions of the bystander effect and the effects attributable to direct interaction of the radiation with the target cell. They used a standard Puck and Marcus assay [59] to obtain a clonogenic survival curve for HPV-G human keratinocytes. Two separate sets of cell culture flask were used. One set was irradiated with broad field of γ-rays with various doses, medium was harvested, filtered and added to a second set of flasks, which had not seen a direct radiation exposure. The survival results were converted to clonogenic death for both bystander and total effect and by subtraction, the percentage of cell death due to non-bystander induced death was determined. The data show that for this human epithelial cell line, doses within the range 0.01-0.5 Gy of γ-rays would induce clonogenic death only by the bystander effect (see Fig. 7).

It can be seen that there is a large bystander component at low doses but at doses of 0.5 Gy and above the direct effects of radiation begin to appear. The magnitude of the bystander effect is relatively constant and it appears to saturate at doses in the range of 0.03-0.5 Gy. After doses greater than 0.5 Gy, the clonogenic death curves are the result of a dose dependent non-bystander effect and a dose independent bystander effect.
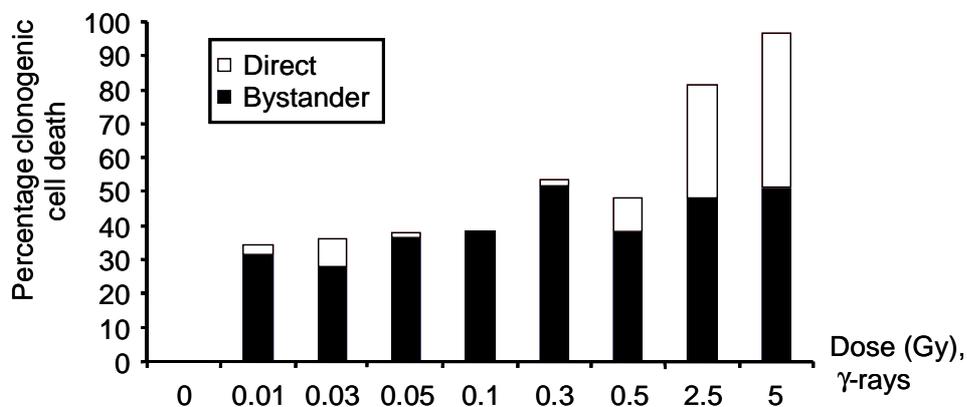

Figure 7. Clonogenic cell death measured in human keratinocytes. The total bar represents the total death detected after exposure of cells to the radiation dose. The death measured after exposure to medium from irradiated cell cultures (Bystander) is represented by the blue portion of the bar, and the remaining death determined by subtraction is represented by the red portion of the bar, giving a value (Direct) for death not attributable to bystander effects of radiation. Adapted from [58]

Mechanisms of the bystander effects

It is known that the bystander effect is cell type dependent [23], depends on cell proliferative state (discussed in [73]) and that energy/REDOX metabolism may be involved in the expression of a radiation induced bystander response [74]. 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 [51]. Bystander signalling is a complex and well-tuned system, which most likely involves more than one messenger and is connected with tissue microenvironment signalling [75, 76].

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 [77].

Azzam and co-workers [32] 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 [33]. Other workers have also shown that lindane treatment leads to inhibition of bystander-induced cell killing in hamster V79 cells [61]. The bystander effect was also significantly reduced in cells pretreated with 1 mM of octanol, which inhibits gap junction-mediated intercellular communication [31]. 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 [23, 58] 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 [53] 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 [53, 78], 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 [27] used a medium transfer protocol and observed delayed death and neoplastic transformation. And finally, Mothersill and Seymour [55] 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.

Hypothetical messenger(s)

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 [51].

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 [79, 80]. Among other candidates for GJIC mediated primary bystander messenger are antioxidants (thiols) [81], Ca^{2+} [82] Ip3 (storage form of intercellular Ca^{2+}) and cAMP [83], 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 [84], ceramide [5], death ligand (TNFSF6) produced from exfoliation [85]. Other evidence supports a role for cytokines as key signalling molecules in the transfer of bystander damage cytokines such as TNF-α [43, 86], TGF-β [54, 76] or IL-1 [43].

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 [54, 87]. Another group proposed that nitric oxide (NO) might play a central role in mediation of bystander effect [68, 88] 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 radiation induced bystander effect and genomic instability.

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 [24]. 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 [25].

The data published in [89] 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 $^3He^{2+}$ irradiation according to the results, published in [26, 64]. Also, the urothelial model, which demonstrates a pronounced bystander response [73, 90, 91] 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 [76]. This issue is discussed in greater detail in part 6.1 of this thesis. There is some indication that genomic instability may play a protective role as well. It was recently demonstrated [92] 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 [93, 94] and both have been observed *in vivo* [25, 95]. Finally there are suggestions that both the bystander effect [96] and genomic instability [97] are controlled through epigenetic mechanisms [98] such as DNA methylation [99].

Bystander effect in multicellular systems

The bystander effect 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 [100] 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 3-D structure, are essential for proper understanding of the bystander effect. The tissue microenvironment is also important for proper manifestation of the bystander effect [75]. Barcellos-Hoff and Brooks hypothesise that the radiation bystander effect and genomic instability are positive and negative manifestations of a tissue homeostatic process [76]. Extracellular signalling in normal tissues plays a crucial role in initiation and perpetuation of bystander effect.

Only a few papers have been published on bystander effects in multicellular systems. The radiosensitivity of HPV-G and HaCaT epithelial cells lines irradiated within microcolonies (>50 cells) was found to be lower than those irradiated as single cells [23, 101]. A series of papers by Bishayee and co-workers [60, 61] detected a pronounced bystander effect in a V79 three-dimensional tissue culture model labelled with ^{3}H-thymidine when the isotope is localised in the cell nucleus and distributed non-uniformly among the cells. Jen and co-workers [102] found that the radiosensitivity of mouse kidney cells that are irradiated under *in vivo* conditions *in situ* or *in vitro* as fragments was higher than those irradiated *in vitro* as single cells.

Our recent work [103] clarifies mechanisms of bystander responses in a 3D normal human-tissue system. Endpoints were induction of micronucleated and apoptotic cells. A charged-particle microbeam was used, allowing irradiation of cells in defined locations in the tissue yet guaranteeing that no cells located more than a few micrometers away receive any radiation exposure. Unirradiated cells up to 1 mm distant from irradiated cells showed a significant enhancement in effect over background, with an average increase in effect of 1.7-fold for micronuclei and 2.8-fold for apoptosis. The surprisingly long range of bystander signals in human tissue suggests that bystander responses may be important in extrapolating radiation risk estimates from epidemiologically accessible doses down to very low doses where non-hit bystander cells will predominate.

With the exception of abscopal effects and clastogenic factors in blood plasma of patient undergo radiation therapy, which were discussed above, little evidence of bystander effect under *in vivo* conditions is available. The one experimental paper, which deals with bystander effect under *in vivo* conditions is work by Watson and co-authors [25]. They utilised a bone marrow transplantation protocol to demonstrate that genomic instability could be induced in bystander cells. Mixture of irradiated and non-irradiated cells distinguished by a cytogenetic marker, was transplanted into CBA/H mice. Genomic instability was demonstrated in the progeny of non-irradiated cells. Another recent paper [104] demonstrated oncogenic bystander radiation effects in mouse cerebellum. Authors reported bystander (in fact "abscopal") tumour induction in cerebellum of radiosensitive Patched-1 (Ptch1) heterozygous mice after x-ray exposure of the other parts of the body. They also provided evidence supporting the role of gap-junction intercellular communication (GJIC) in transmission of bystander signals in the central nervous system.

Rationale for the current interest in non-targeted responses

The current interest in non-targeted effects such as bystander responses is particularly timely. Firstly there is currently a tremendous shift of emphasis from high-dose effects towards low and ultra-low doses, of relevance to environmental and occupational exposures both in terms of research needs and public interest. This has coincided with tremendous advances in the technical possibilities for precise low dose irradiation such as development of microbeams [105, 106], imaging and computerised automation. Apart from technical developments, low dose studies would not be possible without development of more specific and sensitive methods of cellular and molecular biology. Apoptosis assays, techniques to measure changes in cell cycle regulation, protein expression, advanced methods of cytogentic analysis has enabled radiation biology to start to probe low frequency changes in individual cells. This allows the systematic studies of processes (i.e. apoptosis, genomic instability or bystander effect) now considered to be important and are ultimately challenging the existing fundamentals of the understanding of the action of radiation on biological systems.

Hypothesis: bystander effect is a protective mechanism of tissue damage control.

The discovery of a bystander effect is important for understanding the dose-response mechanisms relevant to low-dose irradiation *in vivo*. One important question is whether the bystander effect is 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 function of the bystander effect is 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. The existence of a potentially sensitive group of cells, susceptible to the bystander response has also been proposed by [70]. However, not every cell will respond to the hypothetical bystander factor, which is released by targeted cells. Only 1-3% of the total number of cells in the system would express damage [26, 73] and approximately 10-15% would go on to bystander induced differentiation [91, 107]. Lehnert and co-workers believe that differences in the gene expression profiles and 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 [108]. The data obtained during this project are consistent with every cell being able to initiate the bystander effect. 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. Apoptosis is not playing a significant role in the urothelial explant system [26, 73]. Another way to isolate damage is to prompt affected cells into irreversible differentiation. Results [91, 107], which support this mechanism, have been obtained. Underlying this theory is that a normal 3-dimensional tissue microarchitecture is essential for the manifestation of the bystander effect [103, 109].Therefore, the bystander effect 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 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 [75]. Bystander induced differentiation seems to play a central role in this process. It is known that cellular senescence is a powerful tumour suppressor mechanism [110].

A general scheme explaining the proposed theory is illustrated in Fig. 8. Tissue, exposed to sparse natural irradiation, would respond as a single unit (1). The damaged cells would produce some bystander signal or signals. Some sensitive sub-population of potentially damaged cells would respond to the bystander messenger (2). The tissue response to sparse irradiation would affect just a fraction of cells within the tissue (estimated at 10-15%). A minor fraction of the cells will be eliminated (probably by apoptosis - estimated as < 1%). The majority of the cells would be removed from proliferating pool by being prompted into differentiation (3). Such a significant response of tissue might be explained by the great danger of even one transformation event induced by natural background radiation. Removing from the proliferating pool all the potentially damaged cells would significantly reduce the risk of transformation for any one cell.
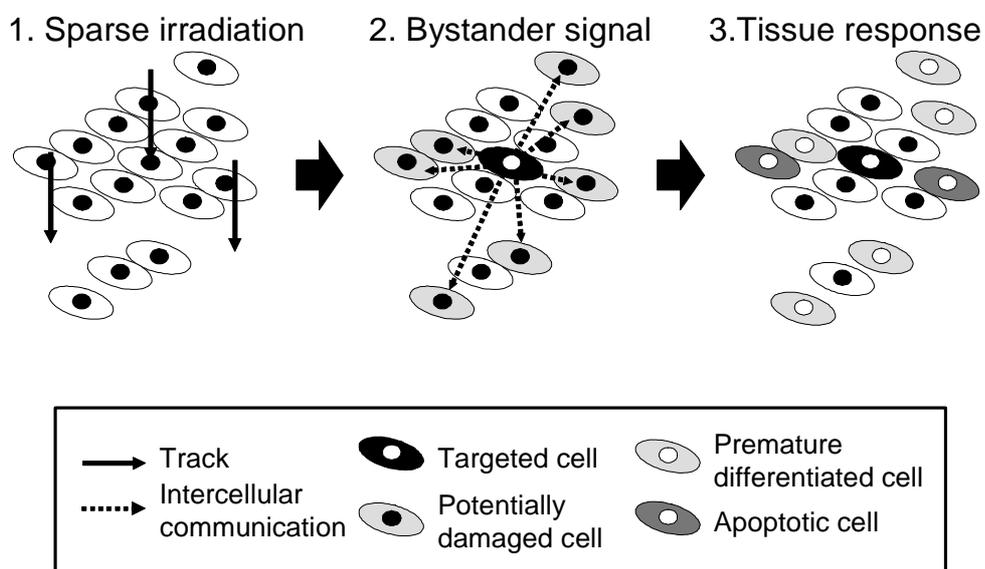

Figure 8. A general scheme of radiation induced bystander effect in tissue systems

Recently, two theories were proposed concerning the possible meaning of the bystander effect. One of them hypothesises that the radiation-induced bystander effect is a manifestation of a tissue homeostatic process [76]. Cell growth, differentiation and death are directed significantly by extracellular signaling through the interactions of cells with other cells and with the extracellular matrix and the tissue microenvironment. According to the authors' theory the bystander effect eliminates abnormal cells in order to inhibit neoplastic behavior and preserve tissue integrity. Genomic instability is interpreted by the authors as results of absence the bystander effect. They write: "radiation-induced bystander effects and genomic instability, are, respectively, positive and negative *cellular* manifestation of *multicellular* programs of damage response" [76]. Therefore, the bystander effect is hypothesised to be an important mechanism of tissue integrity maintenance.

Another theory concerning a possible role of the bystander effect for the genome as a whole was recently proposed by Baverstock [111]. The author proposed that the radiation induced bystander effect (as well as genomic instability) can be understood in the terms of the dynamic genome concept proposed in this paper. These phenomena are interpreted not just as the result of loss of stability from specific modifications of the genome sequence, but, as a response of the genome in order to preserve the integrity of the genomic sequence.

The relationship between the bystander effect and genomic instability

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 and bystander effect can both be induced *in vitro* and *in vivo* [25, 95]. The data published in [64, 65, 89] suggest that the same cell line (primary human fibroblasts) can express radiation induced genomic instability and bystander response, although a direct relationship between the two endpoints has not been tested implicitly. On the other hand, the experiments with irradiation of ureter tissue fragments [107, 112] demonstrate that genomic instability (i.e. *de novo* appearance of cellular damage) and the bystander effect could be closely linked. With the damaged or differentiated cells that are expressed 7 days later in the explant outgrowth, many must be several generations removed from the initially targeted cells and those which initially express the bystander phenotype. It is likely that a cascade mechanism of bystander cell damage probably dominates the initial phase of the targeted exposures. However a significant contribution of genomic instability (probably, bystander-induced) on the later stages cannot be ruled out.

Where the bystander effects might be important?

Bystander effect could be important in a few areas related to radiation. The bystander effect might contribute to the estimation of cancer risk from domestic radon exposure [113]; the effects of HZE particles during space mission to the Mars, see discussion on cosmic radiation at [114]; health effects of air crew personnel, exposed to radiation during i.e. inter-continental flight [115]; high energy radiotherapy outcome.

I would like to concentrate on two issues, where the bystander effects might contribute significantly: cancer radiotherapy and radiation protection.

Significance of the bystander effects for radiotherapy

The bystander effect is a low dose (up to 200 mGy) phenomenon. Therefore, at the first look, it cannot play any considerable role in radiotherapy, which operates with doses of tenth of Grays and more. However, the spectrum of secondary malignancies in radiotherapy patients may suggest some contribution of the bystander effect [116]. On the other hand, Trott [117] points out that the future experiments are needed to prove the potential therapeutic value of the bystander effect in radiotherapy and nuclear medicine.

The theory concerning a protective role of the bystander effect may be supported by the recent data of microbeam radiation therapy [118]. It was demonstrated that arrays of parallel X-ray microbeams could be efficiently used for treatment of central nervous system tumours because of minimal damage to normal tissues. Another group of publications [119-121] deals with microbeam radiation therapy of brain tumours. They have demonstrated an unusually high resistance of normal tissues irradiated with array microbeams of energetic synchrotron-generated X-rays and that this method can be successfully
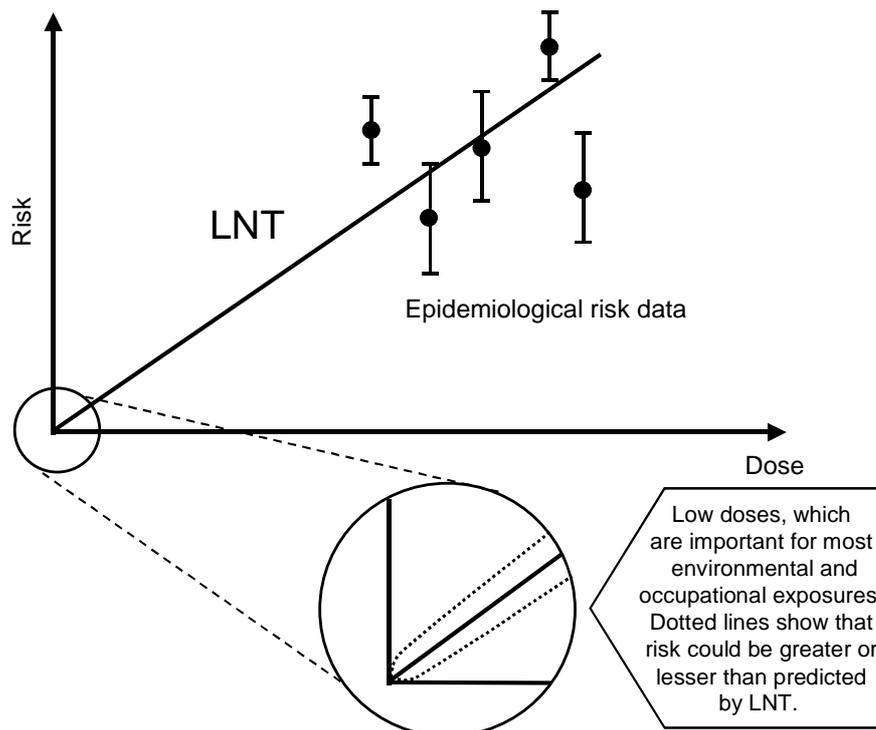
Figure 9. LNT and uncertainties in extrapolation of radiation risk

used for either curative or palliative treatment of brain tumours. It may point to that fact that the bystander effect, induced by microbeams would remove all potential targets in normal tissues, making them more radioresistant. On the other hand, the bystander effect as a phenomenon, which requires normal tissue microarchitecture and microenvironment would not act in the same way in tumours being either switched-off or damaging.

Finally, the finding of a significant bystander induced differentiation after microbeam irradiation would suggest a potential value of the bystander effect for differentiation therapy of cancer treatment; see review of [122].

Applicability to radiation protection and contribution to LNT discussion

According to the Linear-Non-Threshold (LNT) model, which currently dominates in radiation protection, cancer risk for low dose low LET exposures is derived from high-dose epidemiological data, mainly obtained from A-bomb survivors cohort [123]. The average dose of the A-bomb survivors was about 0.3 Gy, which corresponds to about 300 electron tracks at the cellular level (ignoring the very small neutron component) and which were delivered in a short time. Low-dose environmental exposures correspond to around 1 mGy per year of low LET radiation, which is roughly equivalent to 1 electron track per cell per year. The risk at low doses might be different than predicted by a linear extrapolation of the high dose epidemiological data. There is not any reliable epidemiological information in this dose region (Fig. 9).

The bystander effect does not demonstrate a linear relationship to dose. It is maximally induced by very low doses, suggesting a switch on mechanism for its activation. The general form of the bystander dose response curve may have implications for the applicability of the linear no-threshold (LNT) model in extrapolating radiation risk data into the low-dose region. How bystander effect might contribute to the risk estimation? The key question here: is whether the bystander effect is a *protective* mechanism or *non-specific damage* from irradiation.

There are findings, which point out that the bystander effect might be harmful. Several independent groups demonstrated evidence for bystander-induced mutagenesis [29-31]. Bystander-induced

transformation has also been demonstrated [27, 28]. It was proven that chromosomal damage is produced in bystander cells after low doses of radiation [24]. Considering this evidence, the bystander effect would increase the risk of carcinogenesis in the low dose region (Fig. 10).
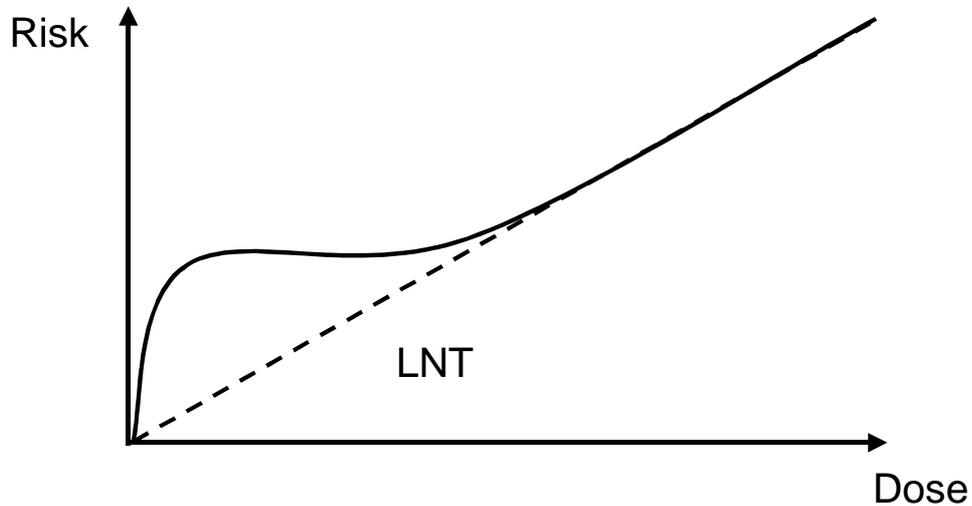

Figure 10. The risk at low doses might be *greater* than predicted by LNT

However, most of the data concerning the harmful character of the bystander effect was obtained from *in vitro* experiments with normally, immortalised, transformed or artificially constructed cell lines. This makes it difficult to apply these data to estimation of the carcinogenesis risk in the human population. There is however evidence for a protective nature of the bystander effect. A gross bystander induced differentiation has been demonstrated in the urothelial explant outgrowth versus a low level of cellular damage after microbeam irradiation. Matsumoto [68, 88] found that survival is increased after treatment with medium from irradiated cells. Similar data of a proliferation increase was reported by Iyer [54], although authors interpreted it as a step towards carcinogenesis. And finally, Barcellos-Hoff [76] published data and proposed a theory suggesting that the bystander effect is a mechanism of tissue integrity maintenance. This evidence suggests that bystander effects might
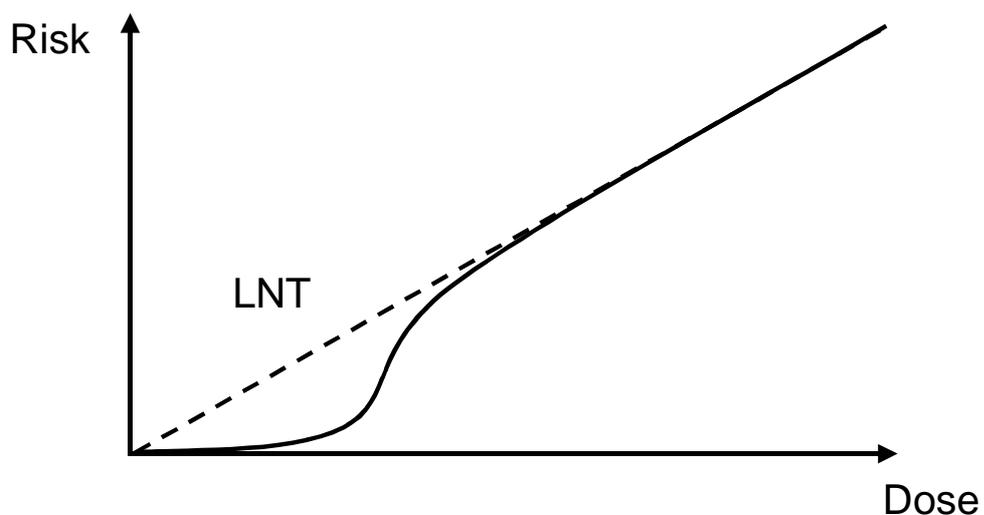

Figure 11. The risk at low doses might be *less* than predicted by LNT

decrease risk of carcinogenesis in low dose region (Fig. 11).

Regrettably, the current state of understanding of the underlying mechanistic basis of radiation induced bystander effect *in vivo* does not allow a firm conclusion to be expressed one way or the other on the validity of a association with a reduction or increase of cancer risk in human populations. The observation of the bystander phenomenon is preliminary in nature, and the applicability of any conclusion derived from *in vitro* studies to *in vivo* situation is still uncertain. The risk at low doses might be *greater* or *less* than predicted by a linear extrapolation of the high dose depending on consideration of data for *in vitro* or *in vivo* like systems. However, bystander effect will clearly result in an overall risk, which is a *non-linear* function of dose. It would be highly premature to consider revising current risk calculations on the basis of current *in vitro* and *in vivo* like studies of bystander phenomena. On other hand, the LNT model is important for radiation protection as a simple method to optimise procedures and regulations. However, it should not be mistaken as a scientific model directly derived from the present state of knowledge of the processes involved in radiation carcinogenesis [124].

Acknowledgements

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