NON-TARGETED EFFECTS OF RADIATION: APPLICATIONS FOR RADIATION PROTECTION AND CONTRIBUTION TO LNT DISCUSSION

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A NEW PARADIGM OF RADIATION BIOLOGY

According to the *target theory* of radiation induced effects (Lea, 1946), 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. A range of evidence has now emerged that challenges the classical effects resulting from targeted damage to DNA. These effects have also been termed *"non-(DNA)-targeted"* (Ward, 1999) and include radiation-induced bystander effects (Iyer and Lehnert, 2000a), genomic instability (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 (Hickman*, et al.*, 1994). 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*. In this paper we will concentrate on the radiation-induced bystander effects because of its particular importance for radiation protection.

BYSTANDER EFFECT, DEFINITION AND EVIDENCE

The radiation-induced bystander effect is a phenomenon whereby cellular damage such as sister chromatid exchanges (Nagasawa and Little, 1992; Deshpande*, et al.*, 1996), chromosome aberrations (Lorimore*, et al.*, 1998; Watson*, et al.*, 2000), apoptosis (Mothersill and Seymour, 1997), micronucleation (Prise*, et al.*, 1998; Belyakov*, et al.*, 2001; Belyakov*, et al.*, 2002), transformation (Lewis*, et al.*, 2001; Sawant*, et al.*, 2001), mutations (Nagasawa and Little, 1999; Zhou*, et al.*, 2000; Zhou*, et al.*, 2001) and changes of gene expression (Hickman*, et al.*, 1994; Azzam*, et al.*, 2001) is expressed in unirradiated neighbouring cells near to an irradiated cell or cells.

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 (Hollowell and Littlefield, 1968) and accidental exposures (Goh and Sumner, 1968) in test cell cultures. These indirect effects were explained by the production of “clastogenic factors” (Emerit, 1994). Other evidence has come from abscopal or “out-of-field” effects, which are well known in radiotherapy (Nobler, 1969). 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 (Moiseenko, *et al.*, 2000) related radiation-induced out-of-field effects in lung of rodents with DNA damage.

In the last few years, a large number of papers were published demonstrating evidence for the radiation induced bystander effect (Grosovsky, 1999; Iyer and Lehnert, 2000a). Nagasawa and Little first published a paper, describing the bystander effect (Nagasawa and Little, 1992), 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. 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 (Hickman, *et al.*, 1994). 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 (Deshpande, *et al.*, 1996) 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 (Lehnert and Goodwin, 1997). Later, the same group (Iyer and Lehnert, 2000b) 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 (Mothersill and Seymour, 1997; Seymour and Mothersill, 2000). Under this protocol supernatant from irradiated cells was transferred to test "reporter" cell cultures, which were analysed using clonogenic assay and for presence of micronucleated, apoptotic and cells with chromosome aberrations. Another approach was utilized by Bishayee and co-workers (Bishayee, *et al.*, 1999). 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.

Recently we demonstrated direct evidence of bystander effects in normal human AG01522B fibroblasts using the Gray Cancer Institute charged particle microbeam (Prise, *et al.*, 1998; Belyakov, *et al.*, 2001). Irradiation of a single fibroblast with a single $^{3}He^{2+}$ 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. Zhou and co-authors (Zhou*, et al.*, 2000) 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 (Zhou*, et al.*, 2001) 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 (Lewis*, et al.*, 2001) 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 6.3×10^{-6} in control to 2.3×10^{-5} (~4-fold). Sawant and co-authors (Sawant*, et al.*, 2001) 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.

Radiation induced bystander effects may produce not only damage but other effect which can be interpreted as neutral or beneficial. For example, (Iyer and Lehnert, 2000b) 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 (Matsumoto*, et al.*, 2001) 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.

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; (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 (Nagasawa and Little, 1992). 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 (Hickman, *et al.*, 1994). 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 (Deshpande, *et al.*, 1996) 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 (Zhou, *et al.*, 2000; Zhou, *et al.*, 2001) noted that a level of bystander mutagenesis effect after α-particle microbeam irradiation did not depend on the number of particles delivered. Lewis (Lewis, *et al.*, 2001) 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. Our recently obtained data (Prise, *et al.*, 1998; Belyakov, *et al.*, 2001) 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 $^{3}He^{2+}$ 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.

The general shape of the bystander effect dose response in comparison to direct radiation consequences is illustrated at Fig. 1. 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 (Michael, *et al.*, 2000).

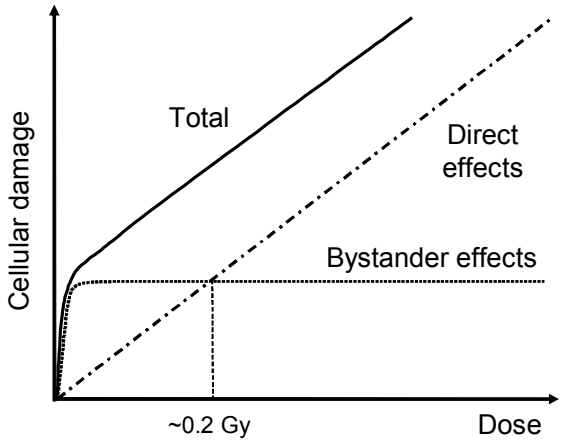

Figure 1. Contribution of bystander and direct component to the radiation induced damage

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 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. In a recent paper Barcellos-Hoff and Brooks hypothesise that the radiation bystander effect and genomic instability are positive and negative manifestations of a tissue homeostatic process (Barcellos-Hoff and Brooks, 2001). 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 (Mothersill and Seymour, 1997; Cummins*, et al.*, 1999). Bishayee and co-workers (Bishayee*, et al.*, 1999) 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 (Jen*, et al.*, 1991) 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.

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 only experimental paper, which deals with bystander effect under *in vivo* conditions is work by Watson and co-authors (Watson*, et al.*, 2000). 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.

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.

We propose a theory, supported by our experimental data, that the main function of the bystander effect is to decrease the risk of transformation in a multicellular organism exposed to radiation (Belyakov*, et al.*, 2002). 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 (Brenner*, et al.*, 2001). 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 (Prise*, et al.*, 1998; Belyakov*, et al.*, 2001; Belyakov*, et al.*, 2002) and approximately 10-15% would go on to bystander induced differentiation (Belyakov*, et al.*, 2002). Our data are consistent with every cell being able to initiate the bystander effect (Prise*, et al.*, 1998; Belyakov*, et al.*, 2001; Belyakov*, et al.*, 2002). 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 does not play a significant role in the systems studied (Prise*, et al.*, 1998; Belyakov*, et al.*, 2001; Belyakov*, et al.*, 2002). Another way to isolate damage is to prompt affected cells into irreversible differentiation. Results, which support this mechanism, have been obtained (Belyakov*, et al.*, 2002). Underlying this theory is that a normal 3-dimensional tissue microarchitecture is essential for the manifestation of the bystander effect. 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 (Barcellos-Hoff and Brooks, 2001). Bystander induced differentiation seems to play a central role in this process.

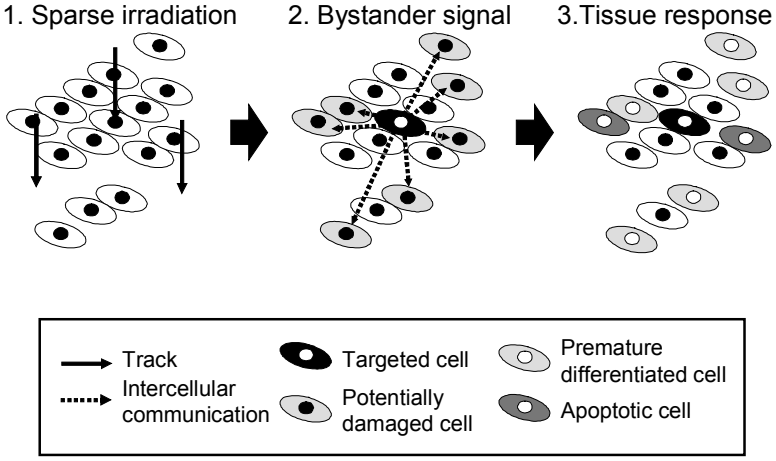

Figure 2. A general scheme of radiation induced bystander effect in tissue systems

A general scheme explaining the proposed theory is illustrated in Fig. 2. 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.

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 (Barcellos-Hoff and Brooks, 2001). 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" (Barcellos-Hoff and Brooks, 2001). 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 (Baverstock, 2000). 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.

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 (Kellerer, 2000). 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. 3).

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: 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 (Nagasawa and Little, 1999; Zhou, *et al.*, 2000; Zhou, *et al.*, 2001). Bystander-induced transformation has also been demonstrated (Lewis, *et al.*, 2001; Sawant, *et al.*, 2001). It was proven that chromosomal damage is produced in bystander cells after low doses of radiation (Lorimore, *et al.*, 1998). Considering this evidence, the bystander effect would increase the risk of carcinogenesis in the low dose region (Fig. 4).

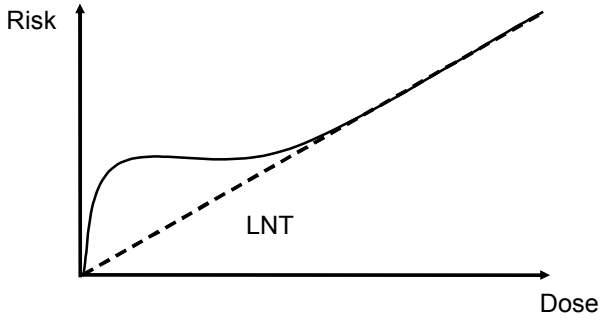

Figure 4. The risk at low doses might be *greater* than predicted by LNT

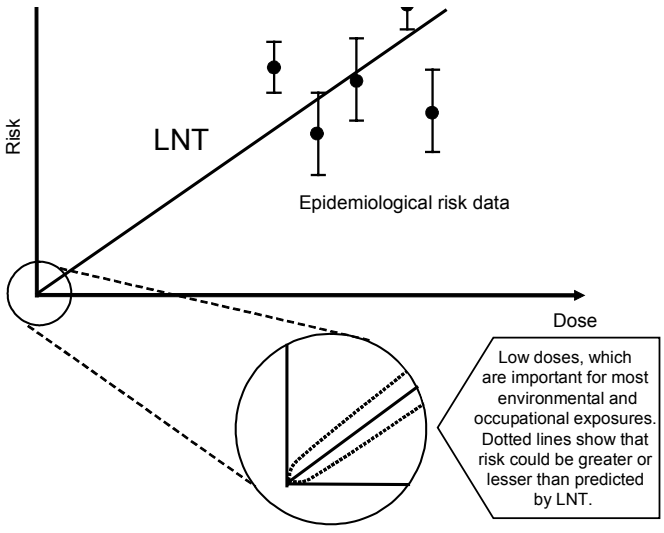

Figure 3. LNT and uncertainties in extrapolation of radiation risk

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 (Belyakov, *et al.*, 2002). Matsumoto (Matsumoto, *et al.*, 2001) found that survival is increased after treatment with medium from irradiated cells. Similar data of a proliferation increase was reported by Iyer (Iyer and Lehnert, 2000b), although authors interpreted it as a step towards carcinogenesis. And finally, Barcellos-Hoff (Barcellos-Hoff and Brooks, 2001) 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 decrease risk of carcinogenesis in low dose region (Fig. 5).

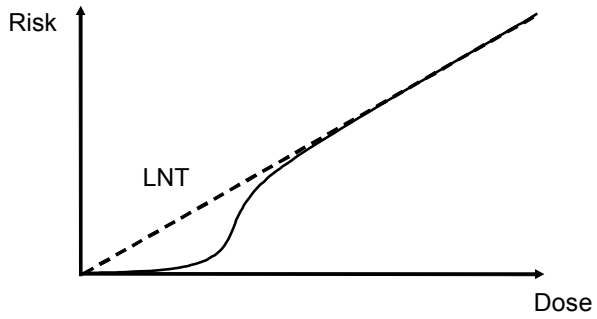

Figure 5. The risk at low doses might be *less* than predicted by LNT

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 (Trott and Rosemann, 2000).

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