Radiation-induced Bystander and Adaptive Responses in Cell and Tissue Models

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Abstract-*The use of microbeam approaches has been a major advance in probing the relevance of bystander and adaptive responses in cell and tissue models. Our own studies at the Gray Cancer Institute have used both a charged particle microbeam, producing protons and helium ions and a soft X-ray microprobe, delivering focused carbon-K, aluminium-K and titanium-K soft X-rays. Using these techniques we have been able to build up a comprehensive picture of the underlying differences between bystander responses and direct effects in cell and tissue-like models. What is now clear is that bystander dose-response relationships, the underlying mechanisms of action and the targets involved are not the same as those observed for direct irradiation of DNA in the nucleus. Our recent studies have shown bystander responses even when radiation is deposited away from the nucleus in cytoplasmic targets. Also the interaction between bystander and adaptive responses may be a complex one related to dose, number of cells targeted and time interval.*

I. BACKGROUND

Ionising radiation is commonly thought of as a two-edged sword. On the one hand harmful, in terms of risks to health from accidental exposure and its role as a carcinogen, but on the other, beneficial with its use in radiotherapy for cancer treatment and diagnostic procedures. For physicists and biologists, these roles have been studied for many years with a dogmatic appreciation of the underlying pathways, from individual radiation tracks interacting with cells and tissues, to biological response. The accepted paradigm has been based on the fact that direct damage to cellular DNA from the energy deposited by the radiation tracks is the triggering event leading to biological effects (1). In recent years however, this model has been questioned and a plethora of responses of cells to radiation in the absence of direct DNA damage, classified as non-targeted effects have been reported. These have included, genomic instability, low dose hypersensitivity, adaptive responses, inverse dose-rate effects and gene expression. Importantly, most of these responses appear to be major pathways of radiation effects at low doses and therefore they are of relevance to gaining a better understanding of the risks associated with radiation exposure and of the use and development of low dose therapy approaches such as fractionated radiotherapy.

A major challenge for radiation biologists has been the revelation that a cell does not even have to suffer direct exposure for an effect to be measured. This response, where neighbouring non-exposed cells next to an exposed cell respond is termed a "bystander effect". Its discovery highlights the recent advances in radiation and molecular technologies which are allowing the biological responses of radiation exposure to be followed at relevant doses (2, 3).

Ionising radiations consists of streams of photons or charged particles which interact with biological molecules via depositing energy by ionisation and/or excitation. Cellular DNA is very sensitive to radiation exposure with the DNA helix being easily broken by a few 10s of electron volts deposited in it. It is known that a whole range of different types of damages are produced in the DNA and that a double-strand break (dsb), where both strands of the helix and broken close to each other is a "toxic" lesion (4). Dsb are difficult for cells to repair

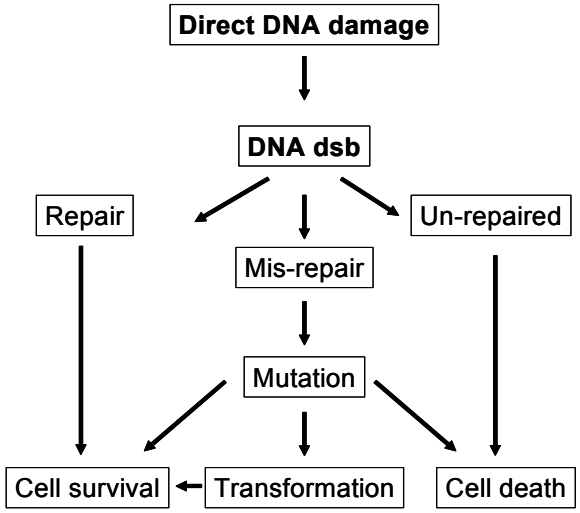

Fig 1. Standard model for radiation effects with a central role for direct DNA damage.

correctly and left unrejoined, they lead to loss of chromosomal material at cell division and cell death. When misrepaired they may lead to mutations and carcinogenesis (Fig. 1). Many less toxic lesions such as damaged bases and single-strand breaks may also lead to mutations ultimately leading to carcinogenesis.

This model has formed the basis of research into radiation effects since DNA was reported as the "sensitive target" back in the 1970s. For example, the current model of radon action in the lung assumes direct interaction of radon derived α-particles with the target bronchial epithelial cells (5). Two advances have challenged this model. Firstly, the advancing experimental evidence for non-targeted responses of radiation exposure and secondly the development of microbeam technologies which allow individual cells to be exposed to radiation and the central tenets of the direct DNA damage based models to be robustly tested. Coupled with molecular assays of cellular response on an individual cell basis, a complex pattern of response of cells to low dose irradiation is now emerging.

II. EVIDENCE FOR BYSTANDER RESPONSES IN CELLULAR SYSTEMS

Recent experimental studies showing evidence for bystander responses can be traced back to a seminal work published by Jack Little's group in 1992. They used an α-particle source to deliver low fluences of particles such that less than 1% of the CHO cells were exposed. They then measured the production of sister chromatid exchanges under these conditions. Surprisingly, they observed around 30% of the cells had produced sister chromatid exchanges (SCE). The yield of SCE increased up to a dose of 2.5 mGy, equivalent to less than 1% of the cells being traversed by an α-particle, and then saturated at higher doses. To obtain the equivalent yield of SCE with X-rays the cells had to be exposed to a dose of 2 Gy. They also monitored the role of reactive oxygen species (ROS) under these conditions (6). Similar studies in rat lung epithelial cells showed much more cells expressing the key damage sensor, TP53, than was predicted on the basis of the number of cells exposed (7). Importantly, they also showed a significant difference in the dose response for X-rays relative to α-particles. At 6 mGy of α-particles, there was a significant elevation in the numbers of cells expressing TP53, but none at the equivalent dose of X-rays. Several studies from the Los Alamos group, using a similar approach presented evidence for increased SCE in bystander cells. In common with the work of Nagasawa and Little, they observed little dose-effect in that saturation of the response occurred. A careful study was made of likely signals involved with evidence presented for a role of TNF-α and possibly interleukin-8 (IL-8) (8, 9). A common theme of the above approaches was that cells were irradiated as confluent monolayers suggesting that direct cell-to-cell communication could be involved.

The percentages of cells showing SCEs were 9-fold higher than expected on the basis of the number of nuclei traversed by one or more α-particles with no dose-dependence above 20 mGy (8). The authors showed that extracellular factors were involved (10). A short-lived factor could be generated in α-particle irradiated serum-containing medium in the absence of cells. A more persistent factor could be produced by fibroblasts after α-particle irradiation. This was heat-labile, could survive freeze-thawing and could be inhibited by superoxide dismutase. The authors considered that the short-lived factor could be involved in the formation of superoxide radicals, possibly as products of lipid peroxidation. The long-lived cell-dependent factor was postulated to be a cytokine such as TNF-α because of its known SCE inducing activity. Further studies by the group confirmed the involvement of ROS such as hydrogen peroxide and superoxide anions. The plasma membrane-bound NADPH-oxidase appeared to be primarily involved and the factors involved did not require direct nuclear or cellular hits to be produced (11). More recently they observed induction of the cytokine, IL-8, in parallel to increased production of ROS. They suggested that IL-8 may be involved in the inflammatory response observed in the respiratory tract and act as a promitogen in the response to inhaled radon (9).

The involvement of extracellular factors in the bystander effect has also been observed with low LET radiations. Mothersill and Seymour (12) found that medium from γ-irradiated epithelial cells could reduce the clonogenic survival of unirradiated cells. This effect was not observed when medium from fibroblasts was used. The effect was dependent on the numbers of cells present at the time of irradiation, but independent of dose between 0.5 and 5 Gy and was manifested in these cells by the production of high numbers of apoptotic cells. Further studies showed that delayed expression of lethal mutations and genomic instability was induced in the survivors of the bystander-killing environment (13). Cell-to-cell contact was not required for the bystander effects in irradiated keratinocytes. Treatment of cells with the tumour promoter phorbol myristate acid which closes gap junctions, involved in gap junctional intercellular communication (GJIC), led to increased cell killing by the bystander effect (14).

Evidence has also been found in fibroblast systems of the involvement of GJIC in bystander responses. Azzam *et al.* (15) followed the expression of TP53, CDKN1A (p21) and CDC2 in confluent primary human fibroblasts irradiated with low doses of α-particles. At levels of exposure where only 2% of the cells were traversed, induction of CDKN1A was observed in more cells than would be predicted. Importantly, they also observed clustering of expression in neighbouring cells. Treatment

of the population with lindane, which inhibits GJIC, led to a marked reduction in the α-particle induced increase in the levels of TP53 and CDKN1A. These effects were observed in 5 different primary human fibroblast strains. Underpinning this effect appears to be a role for membrane signalling pathways. When cells were incubated with the drug filipin which disrupts lipid rafts present in the cell membrane, bystander-induced mutations and SCEs can be prevented (16). Other studies have shown that lindane treatment leads to inhibition of bystander-induced cell killing (17). Little is known however regarding the signals which may be transferred via GJIC. The connexin proteins, which form the gap junctions, allow ions, second messengers and small metabolites to pass between cells and modification of these proteins 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 along with the role of membrane signalling events. The changes in TP53 levels reported in this study are in contrast to decreased levels of TP53 and increased proliferation reported by other workers (18) which also appears to be a media transferable effect.

Some studies have reported a close relationship between bystander and adaptive responses. Adaptive responses are where cells respond differently to subsequent irradiation after they have received a priming dose of radiation. A recent study by Iyer and Lehnert irradiated normal human fibroblasts with 10 mGy of γ-rays and then transferred the medium onto cells which were subsequently irradiated with 2 or 4 Gy of γ-rays. An increased clonogenic survival was observed, preceded by early decreases in TP53, increases in intracellular ROS and an increase in the redox and DNA repair protein AP-endonuclease (19). A similar response was observed when medium was transferred from cells pretreated with 10 mGy of α-particles onto cells which were then irradiated with 100 or 190 mGy of α-particles (20).

III. STUDIES WITH MICROBEAMS

An important contribution to the continuing study of bystander responses and other non-targeted responses has been the development of microbeam approaches. Although first developed many years ago (see (21) for a review), recent developments in imaging, software and hardware advances have allowed sophisticated microbeams to be constructed which can deliver targeted irradiation with high reproducibility. Our own laboratory has been fortunate to develop two microbeams. One based on the use of charged particles and the second based on ultrasoft X-rays. A typical configuration for a particle microbeam is shown in Fig. 2. Generally for charged-particle microbeams the radiation from an accelerator is either collimated, using an aperture or capillary, down to micron dimensions or focussed using electrostatic lenses. Particle detection can be done either before the cell position, using scintillation plastic coupled to photomultiplier tube detection or after the cell position using gas proportional counters. Computerised control of stage movement coupled to sophisticated imaging systems, based on intensified CCD cameras, allow automated cell detection and alignment. The current generation of particle microbeams utilise this general principle of collimation coupled with particle counting (see Fig. 2) (22-25). Using this approach, current systems can achieve 100% efficiency of particle detection, resolutions approaching 1 μm (23, 24) and a cell throughput approaching 3,000 cells per hour (26). For our focused soft X-ray microbeam, specialist diffraction lens used in soft X-ray microscopy, known as zone plates, focus characteristic X-rays (Carbon-K or aluminium-K at present) down to < 250 nm spot sizes. This is coupled to a similar microscope stage and imaging station to that used for the particle microbeam (27). The use of soft X-rays allows the terminal track electrons of conventional low LET radiations (X-rays, γ-rays) to be studied

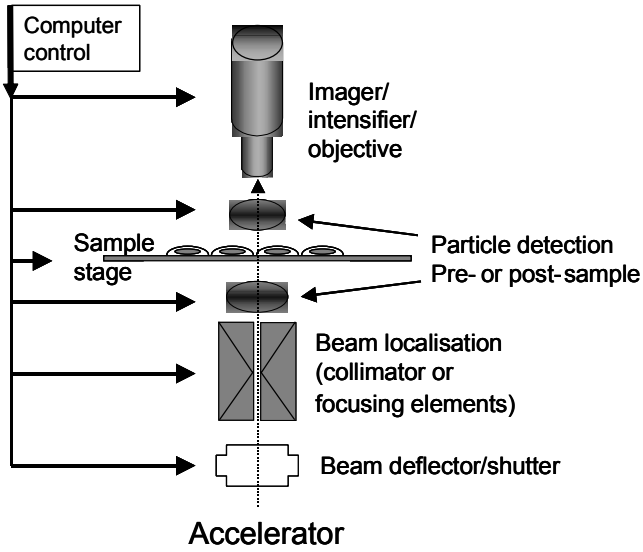

Fig 2. Outline of the essential elements of a charged particle microbeam

mechanistically. Several groups are also developing electron microbeams which will also utilise focussing systems (28, 29).

Our preliminary studies using this technology have tested for a bystander effect in primary human fibroblasts (30). G1-phase fibroblasts were seeded into specially constructed polypropylene-based dishes and allowed to attach. In an area of ~1cm^2 around 600-800 cells were normally present. One cell was located within this population and targeted with a known number of helium-3 ions (~100keV/μm) using our charged particle microbeam. The dish was revisited 3 days later which is

the peak expression time for chromosomal damage formation. Increased numbers of damaged cells, measured as micronucleated or apoptotic cells are observed even after a single particle traversal (31). In general a 2-3 fold increase in the level of damaged cells present is measured in comparison to controls. This typically results in an increase in the numbers of damaged cells in the population from ~ 40 to ~120 despite the fact that only a single cell was initially irradiated. No bystander effect is observed when a particle is targeted beside a cell. Similar changes are observed when up to 4 cells within the population are targeted. Fig. 3 shows the increase in the fraction of damaged cells under conditions where only 1 cell within the population was targeted.

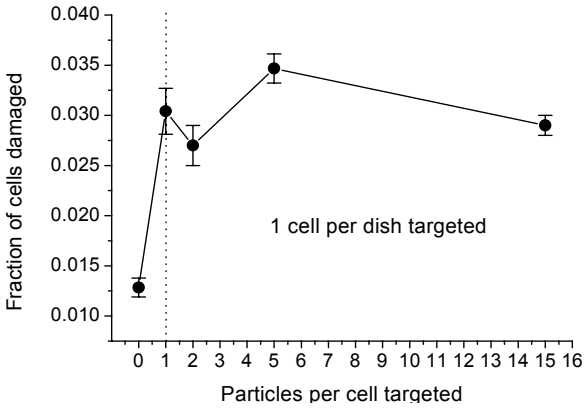

Fig 3. Fraction of damaged cells observed after a single cell within a population is exposed to individually counted particles

Importantly, a single helium ion delivered to a single cell is capable of switching on the effect and this saturates at higher numbers of particles. Also, the degree of bystander-mediated micronucleated cells produced did not vary with increasing numbers of cells targeted. The damaged cells were distributed throughout the dish (30). The dose response curves obtained for the bystander response in this model have key features which have been observed by many of the other reported studies. Firstly, the effect predominates at low doses. The dose delivered to a single cell by a single helium ion under these conditions is ~100 mGy. Secondly, the response appears to be fully switched on at this dose, with no additional effect when up to 15 helium ions are targeted to an individual cell.

We have also determined the effectiveness of targeted protons and focused soft X-rays at inducing bystander-mediated cell killing. Individual V79 hamster fibroblasts have been targeted with 1.0 or 3.2 MeV protons and clonogenic survival measured using a single cell revisiting protocol. A significant bystander-mediated cell killing is observed. Similarly to that observed with micronuclei, the effect saturates at low dose, when only a single cell is targeted. For 1.0 MeV protons, a single proton equivalent to a dose to the nucleus of 50 mGy was capable of inducing a bystander response. Typically between 100 and 200 cells are normally present on the dish at the start of an experiment. When only a single cell is targeted, a maximal bystander effect of ~10% reduction in viability is found equivalent to approximately 10 – 20 cells responding to the bystander signal. Importantly, the bystander-mediated cell killing is observed when only a single cell is targeted. This confirms, that in both this model and the fibroblasts shown earlier (see fig 3.) that every cell within a population is capable of releasing a bystander signal. Also, it is clear that, even although only a single cell is targeted within the centre of the dish, damaged cells are observed throughout the area of the dish which is analysed. There is an equal probability of finding cell killing anywhere over the 25mm^2 area of the dish. Similar results have been observed using focussed carbon-characteristic soft X-rays when these are targeted to individual cells.

Other studies with the microbeam system based at Columbia University have shown important evidence for the production of bystander mediated mutation and transformation events. In experiments where 20% of A_L cells were exposed, with particles delivered through the nucleus, an approximately 3-fold increase in mutation frequency was observed above that predicted from the number of cells targeted. The addition of the free radical scavenger DMSO had no effect although treatment with lindane (32), which inhibits GJIC, reduced the yield of bystander-mediated mutations. Similar studies in C3H10T1/2 cells detected bystander-induced transformation when only 10% of the cells were exposed to a single α-particle (33). These studies suggest that multiple endpoints can be induced under bystander conditions of relevance to radiation risk.

IV. STUDIES IN MULTICELLULAR SYSTEMS

Recently, there has been a considerable interest in the relative importance of bystander responses for *in vivo* systems. Little information is available for the role of bystander effects. Some studies have been done in multicellular models. Bishayee and colleagues have shown that in clusters of V79 cells, exposed to ^{3}H thymidine, additional cell killing is observed, based on the number of cells prelabeled. This additional cell killing effect could be modified by the addition of the OH scavenger DMSO or lindane which inhibits GJIC (17, 34). Other studies have shown that bystander and instability responses are related. When haemopoeitic stem cells were irradiated under conditions where only 50% of the cells were exposed, a significant level of bystander-induced genomic instability was observed (35). Further studies, where these cells were transplanted back into mice showed that the effects could be observed *in* vivo (36). Underlying the response was macrophage activation

which appears as an inflammatory response to the production of apoptotic cells (37). Instability is known to be a key step in the development of tumours so radiation-induced genomic instability has been postulated to play a role in radiation carcinogenesis. Whether bystander events also play a role needs to be determined.

Significant evidence has also existed for many years on the production of clastogenic factors from irradiated samples from humans. Examples include, early studies by Hollowell and Littlefield (38), where plasma from radiotherapy patients was able to induce chromosomal damage in normal unirradiated lymphocytes when these were cultured short-term. These were classified as indirect effects of radiation and thought to involve the production of clastogenic factors (see (39) for a review). These clastogenic factors have been postulated to be between 1,000 and 10,000 in size and include lipid peroxide products, ionisine nucleotides and cytokines such as TNF-α , but underlying their actions is the involvement of reactive oxygen species (ROS) such as superoxide radicals. Several reports in animals and patients of abscopal (i.e. out of field) effects after partial irradiation have been reported and it is interesting to speculate as to the underlying mechanisms and whether bystander effects are involved. Khan *et al*. (40), found that when partial irradiation of rat lung (i.e. the base) was performed, DNA damage, measured as micronucleus formation, was also observed in other areas of the lung principally the lung apex. Some of this response may involve increased production of TGF-β from partial irradiation of the liver. If these responses are proven in humans, they may require the incorporation of directional and geometrical information into calculations of normal tissue complication probabilities for lung, which are currently not considered in conventional dose-volume histograms (41). Other examples of abscopal events have also been observed in patients, such as bilateral pneumonitis after unilateral irradiation, (42) and these may also involve inflammatory responses.

Studies with internally deposited radioactive materials have also reported evidence for bystander effect *in vivo*. When hamsters were injected with the α-particle emitters $^{239}PuO_2$ or ^{230}Pu citrate, which concentrate in the liver, the induction of chromosome aberrations was independent of large changes in the local dose homogeneity when this was altered by injecting a range of particle sizes, but maintaining a constant total dose to the liver (43). A similar response was observed when the induction of liver tumours was observed (44). Thus the authors suggested that the liver was responding to the total energy and total dose to the liver, not to the numbers of cells traversed by an α-particle or the local dose distribution (45).

Our own studies of bystander responses in a multicellular model have used a urothelial model based on section of human or porcine ureter. Using this model we have irradiated cells within the explant outgrowth or targeted regions of the original tissue fragment. The ureter is highly organised with 4-5 layers of urothelium, extending from the fully differentiated uroepithelial cells at the lumen to the basel cells adjacent to the lamina propria or supporting tissue. Sections of ureter have been isolated and placed on microbeam dishes with the urothelium nearest to the dish surface. Using our charged particle microbeam, it is possible to locally irradiate a small section of ureter such that only 4 – 8 urothelial cells are targeted (see Fig. 4.). The tissue is then cultured to allow an explant outgrowth of urothelial cells to form.

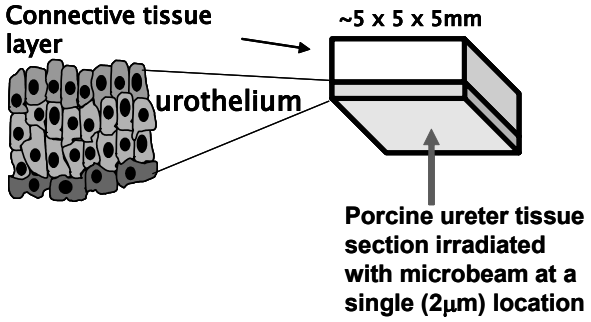

Fig 4. Diagram of the physical set-up for targeted irradiation of a localised (2μm) region of ureter. After irradiation, urothelial cells are selectively cultured to form an explant outgrowth which is analysed 7 days later.

When we have scored damaged cells (micronucleated or apoptotic) in this outgrowth, we typically find 3000 – 6000 damaged cells present. This is a much higher level of bystander effect that we have observed in the isolated cell studies on the basis of the number of damaged cells scored, but as a fraction of the total cells present this represents less than 1%. Importantly, the degree of this bystander-induced cell damage is independent of the number of cells targeted or the dose delivered to the tissue fragment. Also, we observe a significant elevation in the number or terminally differentiated urothelial cells. Overall, this involves a much greater fraction of cells than those which are expressing damage. Typically in the explant outgrowth 50 – 60% of the cells are normally differentiated, but this increases by 10 – 20 % when a localised region of the original tissue fragment in irradiated with the microbeam. This leads to an additionally 5 x 10^4 differentiated cells in the explant outgrowth (46). Therefore, in this model, the major response of the tissue is a protective one, namely switching off cell division.

We believe this is an important observation and suggests that in intact tissues, bystander responses may by entirely governed by the complex homeostatic mechanisms which control and maintain tissue integrity. These findings also add to continuing debate regarding the relevance of isolated cells culture systems to the

multicellular tissue environment we have *in vivo*. The role of cell to cell communication either directly via GJIC or indirectly via autocrine factors may be highly tissue specific and unlikely to be exactly mimicked in an *in vitro* test system. In a recent review, Barcellos-Hoff and Brooks (45) postulated that extracellular signalling pathways were an important integrator of multicellular damage responses which normally prevent cancer development through the removal of damaged cells and inhibition of neoplastic transformation. They predicted that bystander effects after low dose exposure were extracellular signalling pathways which modulate cellular repair and death programmes.

V. BYSTANDER EFFECTS – RISK VERSUS THERAPEUTIC GAIN?

Ultimately, the importance of non-targeted responses such as the bystander effect, is related to whether they are of relevance to the extent of risk associated with radiation exposure from environmental or occupational sources or whether they are an experimental phenomenon of little relevance to the *in vivo* situation.

Given the convincing evidence for radiation-induced bystander effects and genomic instability in cellular systems, it is important to consider their role *in vivo* and their relevance to radiation risk. For radiation risk, current models are based on direct damage to nuclear DNA being an initiating event in the carcinogenic process. For a given type of radiation, DNA damage is induced in proportion to dose, which implies a linear relationship between cancer induction and dose in the low-dose region. If, however, bystander effects contribute to the carcinogenic process, they may influence the shape of the dose-effect relationship. Most observations of bystander effects have shown a saturating response above a threshold dose (see Fig. 2). In our studies, even a single ion track through a single cell triggers a level of response throughout the population, which does not increase when further irradiation is given to the same or to other cells. Such behaviour could lead to various forms of non-linearity in the low-dose region, depending on whether the bystander effect leads to an increase in the number of cells affected or to a decrease in the number of cells at risk due to propagation of lethal effects. At higher dose levels where most bystander effects appear to saturate, other factors must switch on to give the normal acute responses observed beyond the range where most bystander effects appear to saturate. One potential mechanism is low dose hypersensitivity or induced radioresistance (47), another non-targeted response. With this effect a hypersensitive region is observed, for cell killing, at low doses which then switches to a more radioresistant response at higher doses. One could postulate that the hypersensitive region is due to bystander effects, which are then offset at higher doses by, for example, induced repair mechanisms.

Another aspect of bystander responses is that they may have consequences for the current role of radiotherapy and future novel therapeutic approaches. Firstly, they may be of importance in cancer risk at low doses, particularly for secondary cancer induction, the rates of which are increasing with improved primary tumour cure rates (48). Secondly, if the mechanisms can be elucidated, novel approaches to enhancing existing targeted radiotherapy approaches and/or reduction of secondary cancer risk could be employed. For example, bystander pathways could be inhibited to protect normal tissues close to tumours or they could be enhanced to improve cell killing within tumours. Bystander responses are of considerable importance in gene therapy regimens where not all tumour cells are targeted and indirect cell killing to untargeted cells is required to ensure maximal tumour cell kill (49). For example cells transfected by the herpes simplex virus thymidine kinase gene are killed by addition of ganciclovir along with neighbouring cells which have not been transfected (50). Understanding radiation-induced bystander responses may therefore provide useful insights into potential new therapeutic approaches which invoke mechanisms related to cell-cell communication of damage sensing signals.

In summary, the accepted model of radiation effects in cellular systems has been challenged with a range of studies showing effects in the absence of direct DNA damage due to energy deposition. The development of novel microbeam technologies has opened up the possibility of carefully mapping the mechanisms underlying the bystander responses observed in cell and tissue models and quantifying their role in both radiation risk and therapeutic regimens.

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REFERENCES

1. E.J. Hall, *Radiobiology for the radiologist*. 5th ed. 2000, Philadelphia: Lippincott Williams & Wilkins.
2. W.F. Morgan, Non-targeted and Delayed Effects of Exposure to Ionizing Radiation: II. Radiation-Induced Genomic Instability and Bystander Effects In Vivo, Clastogenic Factors and Transgenerational Effects. *Radiat Res.* **159,** 581-596 (2003).
3. W.F. Morgan, Non-targeted and Delayed Effects of Exposure to Ionizing Radiation: I. Radiation-Induced Genomic Instability and Bystander Effects In Vitro. *Radiat Res.* **159,** 567-580 (2003).

4. J.F. Ward, The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: A review. *Int. J. Radiat. Biol.* **57,** 1141-1150 (1990).
5. ICRP, *(International Commission on Radiological Protection)Human respiratory tract model for radiological protection. A report of the Task Group of the International Commission on Radiological Protection*. ICRP Publication 66. 1994, New York: Pergamon Press.
6. H. Nagasawa and J.B. Little, induction of sister chromatid exchanges by extremely low doses of α-particles. *Cancer Res.* **52,** 6394-6396 (1992).
7. A.W. Hickman, R.J. Jaramillo, J.F. Lechner and N.F. Johnson, α-particle-induced p53 expression in a rat lung epithelial cell strain. *Cancer Res.* **54,** 5797-5800 (1994).
8. A. Deshpande, E.H. Goodwin, S.M. Bailey, B.L. Marrone and B.E. Lehnert, Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: Evidence for an extranuclear target. *Radiat. Res.* **145,** 260-267 (1996).
9. P.K. Narayanan, K.E. LaRue, E.H. Goodwin and B.E. Lehnert, Alpha particles induce the production of interleukin-8 by human cells. *Radiat Res.* **152,** 57-63 (1999).
10. B.E. Lehnert and E.H. Goodwin, Extracellular factor(s) following exposure to α-particles can cause sister chromatid exchanges in normal human cells. *Cancer Res.* **57,** 2164-2171 (1997).
11. P.K. Narayanan, E.H. Goodwin and B.E. Lehnert, α-particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Res.* **57,** 3963-3971 (1997).
12. C. Mothersill and C. Seymour, Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of irradiated cells. *Int. J. Radiat. Biol.* **71,** 421-427 (1997).
13. C.B. Seymour and C. Mothersill, Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander-killing environment. *Radiat. Oncol. Investig.* **5,** 106-110 (1997).
14. C. Mothersill and C.B. Seymour, Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiat Res.* **149,** 256-262 (1998).
15. E.I. Azzam, S.M. de Toledo, T. Gooding and J.B. Little, Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat Res.* **150,** 497-504 (1998).
16. H. Nagasawa, A. Cremesti, R. Kolesnick, Z. Fuks and J.B. Little, Involvement of membrane signaling in the bystander effect in irradiated cells. *Cancer Res.* **62,** 2531-2534 (2002).
17. A. Bishayee, D.V. Rao and R.W. Howell, Evidence for pronounced bystander effects caused by nonuniform distributions of radioactivity using a novel three-dimensional tissue culture model. *Radiat Res.* **152,** 88-97 (1999).
18. R. Iyer and B.E. Lehnert, Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Res.* **60,** 1290-1298 (2000).
19. R. Iyer and B.E. Lehnert, Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. *Mutat. Res.* **503,** 1-9 (2002).
20. R. Iyer and B.E. Lehnert, Alpha-particle-induced increases in the radioresistance of normal human bystander cells. *Radiat Res.* **157,** 3-7 (2002).
21. K.M. Prise, M. Folkard and B.D. Michael, *The use of microbeams in radiation biology: An overview*, in *Radiation Research*, M. Moriarty, et al., Editors. 2000, Allen Press: Lawrence, KS. p. 174-177.
22. L.A. Braby, Microbeam studies of the sensitivity of structures within living cells. *Scanning Microsc.* **6,** 164-174 (1992).
23. M. Folkard, B. Vojnovic, K.J. Hollis, A.G. Bowey, S.J. Watts, G. Schettino, K.M. Prise and B.D. Michael, A charged particle microbeam: II A single-particle micro-collimation and detection system. *Int. J. Radiat. Biol.* **72,** 387-395 (1997).
24. M. Folkard, B. Vojnovic, K.M. Prise, A.G. Bowey, R.J. Locke, G. Schettino and B.D. Michael, A charged-particle microbeam I. Development of an experimental system for targeting cells individually with counted particles. *Int. J. Radiat. Biol.* **72,** 375-385 (1997).
25. G. Randers-Pehrson, C.R. Geard, G. Johnson, C.D. Elliston and D.J. Brenner, The Columbia University single-ion microbeam. *Radiat Res.* **156,** 210-214 (2001).
26. R.C. Miller, G. Randers-Pehrson, C.R. Geard, E.J. Hall and D.J. Brenner, The oncogenic transforming potential of the passage of single alpha particles through mammalian cell nuclei. *Proc Natl Acad Sci U S A.* **96,** 19-22 (1999).
27. M. Folkard, G. Schettino, B. Vojnovic, S. Gilchrist, A.G. Michette, S.J. Pfauntsch, K.M. Prise and B.D. Michael, A focused ultrasoft x-ray microbeam for targeting cells individually with submicrometer accuracy. *Radiat Res.* **156,** 796-804 (2001).
28. W.E. Wilson, D.J. Lynch, K. Wei and L.A. Braby, Microdosimetry of a 25 keV Electron Microbeam. *Radiat Res.* **155,** 89-94 (2001).
29. J.H. Miller, M. Sowa Resat, N.F. Metting, K. Wei, D.J. Lynch and W.E. Wilson, Monte Carlo simulation of single-cell irradiation by an electron microbeam. *Radiat. Environ. Biophys.* **39,** 173-177 (2000).

30. K.M. Prise, O.V. Belyakov, M. Folkard and B.D. Michael, Studies of bystander effects in human fibroblasts using a charged particle microbeam. *Int. J. Radiat. Biol.* **74,** 793-798 (1998).
31. O.V. Belyakov, A.M. Malcolmson, M. Folkard, K.M. Prise and B.D. Michael, Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. *Br. J. Cancer.* **84,** 674-679. (2001).
32. H. Zhou, G. Randers-Pehrson, C.A. Waldren, D. Vannais, E.J. Hall and T.K. Hei, Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci U S A.* **97,** 2099-2104 (2000).
33. S.G. Sawant, G. Randers-Pehrson, C.R. Geard, D.J. Brenner and E.J. Hall, The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res.* **155,** 397-401 (2001).
34. A. Bishayee, H.Z. Hill, d. stein, D.V. Rao and R.W. Howell, Free radical-initiated and gap junction-mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. *Radiat. Res.* **155,** 1-10 (2000).
35. S.A. Lorimore, M.A. Kadhim, D.A. Pocock, D. Papworth, D.L. Stevens, D.T. Goodhead and E.G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. *Proceedings of the National Academy of Sciences.* **95,** 5730-5733 (1998).
36. G.E. Watson, S.A. Lorimore, D.A. Macdonald and E.G. Wright, Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionizing radiation. *Cancer Res.* **60,** 5608-5611 (2000).
37. S.A. Lorimore, P.J. Coates, G.E. Scobie, G. Milne and E.G. Wright, Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? *Oncogene.* **20,** 7085-7095 (2001).
38. J.G. Hollowell and G. Littlefield, Chromosome Damage Induced by Plasma of X-Rayed Patients: An indirect Effect of X-Ray. *Proc. Soc. Exp. Biol. Med.* **129,** 240-244 (1968).
39. I. Emerit, Reactive oxygen species, chromosome mutation, and cancer: possible role of clastogenic factors in carcinogenesis. *Free Radic. Biol. Med.* **16,** 99-109 (1994).
40. M.A. Khan, R.P. Hill and J. Van Dyk, Partial volume rat lung irradiation: an evaluation of early DNA damage. *Int. J. Radiat. Oncol. Biol. Phys.* **40,** 467-476 (1998).
41. V.V. Moiseenko, J.J. Battista, R.P. Hill, E.L. Travis and J. Van Dyk, In-field and out-of-field effects in partial volume lung irradiation in rodents: possible correlation between early dna damage and functional endpoints. *Int. J. Radiat. Oncol. Biol. Phys.* **48,** 1539-1548 (2000).
42. G.W. Morgan and S.N. Breit, Radiation and the lung: a reevaluation of the mechanisms mediating pulmonary injury. *Int J Radiat Oncol Biol Phys.* **31,** 361-369 (1995).
43. A.L. Brooks, J.C. Retherford and R.O. McClellan, Effect of 239PuO2 particle number and size on the frequency and distribution of chromosome aberrations in the liver of the Chinese hamster. *Radiat Res.* **59,** 693-709 (1974).
44. A.L. Brooks, S.A. Benjamin, F.F. Hahn, D.G. Brownstein, W.C. Griffith and R.O. McClellan, The induction of liver tumors by 239Pu citrate or 239PuO2 particles in the Chinese hamster. *Radiat Res.* **96,** 135-151 (1983).
45. M.H. Barcellos-Hoff and A.L. Brooks, Extracellular signaling through the microenvironment: a hypothesis relating carcinogenesis, bystander effects, and genomic instability. *Radiat Res.* **156,** 618-627 (2001).
46. O.V. Belyakov, M. Folkard, C. Mothersill, K.M. Prise and B.D. Michael, Bystander-induced apoptosis and premature differentiation in primary urothelial explants after charged particle microbeam irradiation. *Radiat. Prot. Dosimetry.* **99,** 249-251 (2002).
47. B. Marples and J. M.C., The Response of Chinese Hamster V79 Cells to Low Radiation Doses: Evidence of Enhanced Sensitivity of the Whole Cell Population. *Radiat. Res.* **133,** 41-51 (1993).
48. D.J. Brenner, R.E. Curtis, E.J. Hall and E. Ron, Second malignancies in prostate carcinoma patients after radiotherapy compared with surgery. *Cancer.* **88,** 398-406 (2000).
49. R. Ramesh, A.J. Marrogi, A. Munshi, C.N. Abboud and S.M. Freeman, In vivo analysis of the 'bystander effect': a cytokine cascade. *Exp. Hematol.* **24,** 829-838 (1996).
50. M. Mesnil, C. Piccoli, G. Tiraby, K. Willecke and H. Yamasaki, Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proceedings of the National Acadamy of Science (USA).* **93,** 1831-1835 (1996).