Radiation-induced effects in unirradiated cells: A review and implications in cancer

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Abstract. A long-held central dogma of radiation biology has been that the carcinogenic effects of ionizing radiation (IR) are induced by the direct and radiolytic actions of IR on nuclear DNA. Numerous investigations, however, have revealed that several cancer relevant effects of IR can occur in cells that have received only cytoplasmic or plasmalemmal membrane exposure to IR. Further, mounting evidence now indicates that many effects that have been attributed to IR-induced damage to nuclear DNA or that occur following irradiation of the cytoplasmic compartment of cells can also occur in cells that have received no direct exposure to IR *per se*. These so-called 'bystander effects', i.e., radiation-induced effects in unirradiated cells, include cell killing, increases in intracellular reactive oxygen species, the induction of mutations, enhanced cell growth, the induction of apoptosis, the induction of genomic instability and neoplastic transformation. In this report, we summarize the evidence that demonstrates IR can cause this array of effects in non-irradiated cells, and we discuss recent findings concerning the potential mechanisms that may underlie IR-induced effects in unirradiated, or 'bystander' cells. Additionally, we discuss IR-induced bystander effects and their possible relationship to some *in vivo* observations, how bystander effects may pertain to carcinogenesis the treatment of tumors with radiotherapy, and how effects in bystander cells contribute to uncertainties in assessing cancer risks associated with exposure to IR.

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Contents

1. Introduction

A longstanding paradigm in radiation biology has been that many effects induced by ionizing radiation (IR), including its carcinogenic effects and ability to kill cancer cells, are the result of DNA damage arising from the actions of IR in cell nuclei, especially interactions of IR and its products with nuclear DNA (1,2). Consistent with this view, IR undoubtedly can damage DNA by directly ionizing DNA itself and by indirect processes in which DNA reacts with numerous radiolytic reactive products, e.g., $OH\bullet$, $H\bullet$, O_2^{-}, and H_2O_2, that are generated in aqueous fluid surrounding DNA. These processes unquestionably can result in a variety of types of DNA damage, including DNA single and double strand breaks, modifications of deoxyribose rings and bases, intra- and inter-strand DNA-DNA cross-links and DNA-protein cross-links (1,3,4). About a third of all DNA damage is caused by the direct effects of sparsely ionizing γ- and X-rays, with the remaining balance being attributable to the indirect actions of IR. With high-linear energy transfer (LET) radiations like more densely ionizing α particles that are emitted by radon and radon progeny, boron neutron capture tumor therapy, and the new α particle atomic nanogenerator approach for targeting tumor cells using actinium-225 coupled to internalizing antibodies (5), the direct actions of IR on DNA become more predominant and the nature of DNA modifications become much more complex. Whether caused by low- or high-LET IR, all of the above forms of DNA damage can lead to untoward effects in cells if unrepaired or misrepaired. With specific regard to carcinogenesis, genomic mutations caused by IR are widely thought to arise from DNA damage that is subsequently converted into a mutation as a result of processing by DNA repair mechanisms

or that is converted into a heritable mutation when DNA undergoes replication.

As recently reviewed elsewhere (6), many cancer-relevant effects of IR in directly irradiated cells, including cell killing and the induction of mutations, can occur in the absence of direct irradiation of cell nuclei. In this report, we summarize the mounting lines of evidence that indicate IR can also cause an array of important effects even in cells that have not experienced any direct exposure to IR *per se*, and we discuss evidence concerning the potential physical and biochemical mechanisms that may underlie IR-induced effects in unirradiated, or what are now called 'bystander' cells. Further, we discuss how IR-induced bystander effects may relate to several *in vivo* observations, how they may pertain to the development of cancer and radiotherapy, and how bystander effects contribute to uncertainties in assessing cancer risks associated with exposure to IR.

2. Earlier indications that ionizing radiation can affect unirradiated cells

Evidence that exposure to IR can cause effects in unirradiated mammalian cells both *in vivo* and *in vitro* began to emerge in the 1950s starting with a report of changes in the sternal bone marrow of children who received radiation to their spleens for the treatment of chronic granulocytic leukemia (7). These observations were followed by the demonstration of IR-induced clastogenic activity in the plasma from patients receiving high dose radiotherapy and after accidental total body irradiation (8,9). Other studies reported that the injection of plasma and plasma filtrates from irradiated rats or sheep into unirradiated rats resulted in a higher incidence of mammary tumors than when unirradiated rats were injected with plasma preparations from unirradiated animals (10).

Subsequently, Hollowell and Littlefield (11) showed that the addition of plasma from patients who received radiotherapy caused dicentrics, and chromatid and chromosome breaks in non-irradiated lymphocytes in culture. Further experiments performed *in vitro* shortly thereafter demonstrated that irradiating plasma alone can also yield clastogenic activity (12). That such effects may be manifested *in vivo* and not merely as an artifact of *in vitro* cell culture was suggested by a study (13) in which lymphocytes from children conceived months after their mothers received non-pelvic radiation exposure showed unstable aberrations, a finding that was duplicated in baby rabbits. Along the same line, Poncy *et al* (14) reported that inhalation exposure of rats to α particle-emitting radon resulted in both acute and delayed increases in sister chromatid exchanges (SCEs) in their bone marrow cells, a result consistent with the possibility that one or more blood borne SCE-inducing factor(s) may have translocated from the lung to the more peripherally located bone marrow where they exerted their effects. SCEs as an indicator of DNA damage (15,16) can be produced in response to DNA synthetic activity on a damaged template, and they involve the breakage and rejoining between the DNA duplexes on both sister chromatids after DNA synthesis (17) by way of a process that involves DNA double strand break repair via homologous recombination (18). More recently, clastogenic activity has also been observed in the plasma of atomic bomb survivors and Chernobyl residents (19,20). Remarkably, such clastogenic activity in the plasma of exposed subjects evidently can persist, often still present months, years, or even after over three decades following IR exposure (21-23).

A variety of chemical species were proposed in some of the above reports to function as clastogenic factors, including aldehydic breakdown products of lipid peroxidation, the cytokine tumor necrosis factor-α (TNF-α), and inosine nucleotides (19,24,25). Aside from their chemical nature, the fact that the effects of many of the IR-induced transmissible DNA damaging factors can be inhibited by the Cu-Zn metalloenzyme superoxide dismutase (SOD) (19), which catalyzes the conversion of superoxide anions into H_2O_2 and O_2, implies a role(s) for extracellular ROS in mediating their effects. Superoxide anions are sufficiently stable to allow diffusion within cells, and the generation of clastogenic hydroxyl radicals from these reactive species and/or hydrogen peroxide via Fenton-type reactions would not be unexpected. It is also conceivable that free radicals generated by radiolysis could result in the initiation of lipid peroxidation in fatty acids contained in blood or other extracellular fluids, with the subsequent outcome being the formation of lipid peroxidation products, e.g., 4-hydroxynonenol, that cause DNA damage when present even at low concentrations. Yet, how the production of ROS after IR exposure may be sustained in a manner that continues the production of clastogenic factors literally years later remains an unsolved mystery, if in fact ROS-related factors underlie the above IR-associated chromosome damaging effects in unirradiated cells. Even so, that soluble, extracellular factors can cause DNA damage has been known for some time. Conditioned media from cells grown from individuals with cancer-prone disorders such as Fanconi's anemia, Bloom syndrome, and ataxia-telangiectasia, for example, cause chromosomal aberrations and increases in SCEs in normal cells (19,26,27). Despite the above cited reports, most researchers in the radiation biology and medical communities continued to view the nucleus as the main target in IR-induced effects, and all other findings as mere curiosities until the early 1990s.

3. Recent developments in radiation-induced bystander effects

Bystander terminology (28) has been used to describe the transfer of transfected DNA into untransfected cells via gap junctions (29) or special cases in which tumor cells are transfected with a gene encoding for a non-mammalian enzyme that converts a non-toxic agent into a cytotoxic drug that kills or radiosensitizes neighboring unmodified tumor cells (30-32). Not to be confused with these phenomena, 'bystander effects' in the parlance of radiation biology refer to radiation-induced effects in non-targeted cells, and cells experiencing such effects are often called 'bystander cells'. As discussed below, several lines of evidence now indicate that such bystander effects: a) consist of a potentially broad spectrum of responses that may be cell type specific, b) can occur in a manner that may preclude predictive extrapolations from effects induced in directly irradiated cells, c) may have either or both benign and detrimental effects, and d) can be communicated by soluble transmissible factors, by direct

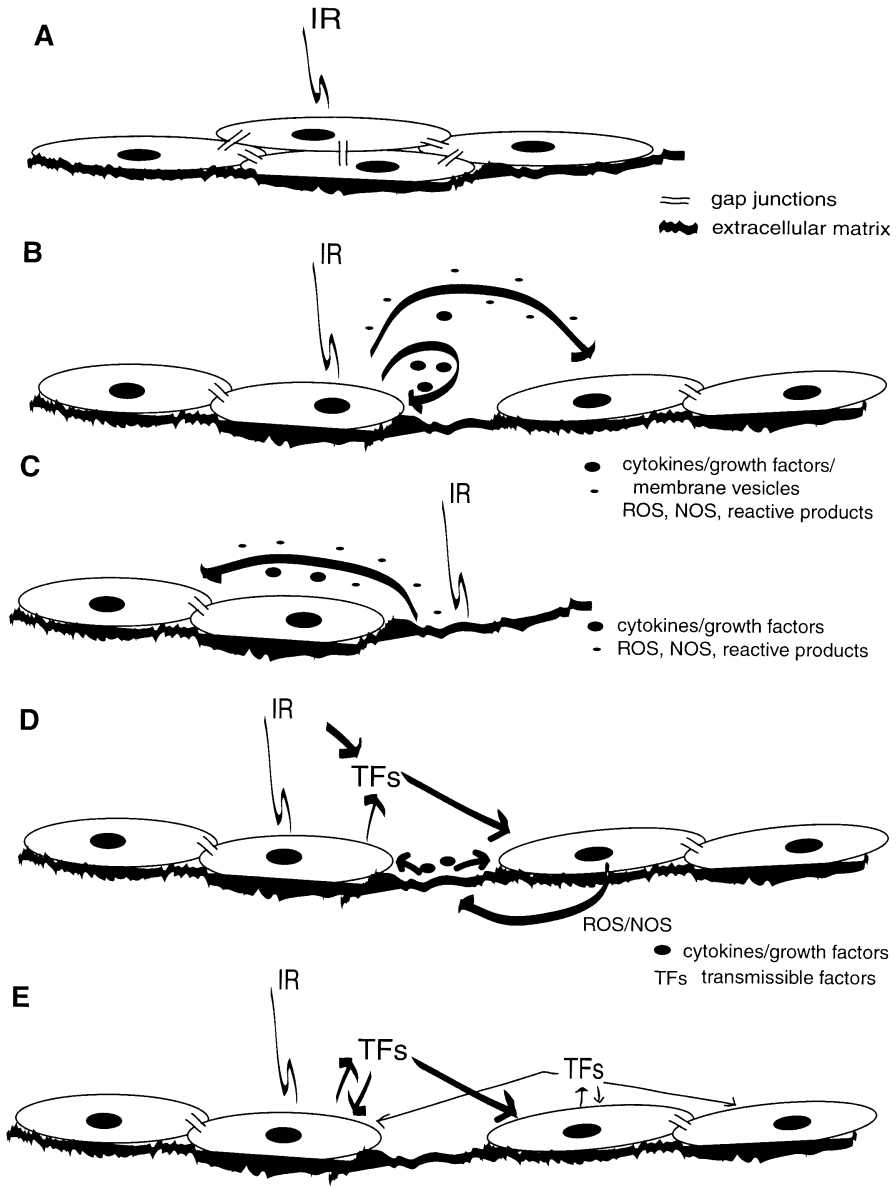

Figure 1. Demonstrated and potential mechanisms involved in mediating ionizing radiation (IR)-induced bystander effects. (A), Irradiated cells can induce responses in neighboring cells by transmitting signals to unirradiated cells via gap junctions. (B), Irradiated cells can produce soluble, signaling proteins, e.g., cytokines and growth factors. These, in turn, can induce responses in unirradiated cells that possess the necessary surface receptors that recognize the signaling molecules. It is possible that such signaling molecules additionally may induce paracrine effects in the directly irradiated cells that originally produced them, thereby altering the effects of their targeted exposure as well as responses to subsequent bouts of exposure to IR. Further, direct irradiation of cells can lead to the induction of supra-basal levels of reactive oxygen species and, perhaps, reactive oxides of nitrogen, which can affect both targeted and non-targeted cells. The production of membrane vesicles with biologically active factors may also be exfoliated from directly targeted cells, and, like with soluble factors, these may modify responses in directly irradiated cells and/or induce effects in unirradiated bystander cells. (C), Irradiation of the extracellular microenvironment, including extracellular fluid and the extracellular matrix, can result in the generation of fluid-phase reactive oxygens species and perhaps reactive oxides of nitrogen, as well as the release of otherwise matrix-bound soluble factors that in turn can affect unirradiated cells. (D), Direct irradiation of cells and extracellular fluids can result in the production of transmissible factors as illustrated in. Some of these can induce the production and release of reactive oxygen species by responsive bystander cells. Interactions between these oxidatively active species and the extracellular matrix, in turn, may cause the release of yet other factors that can affect both directly irradiated and bystander cells. (E), Direct irradiation of cells results in the extracellular availability of transmissible factors, as illustrated in (B). In response to these, bystander cells may produce yet other factors that can affect either or both directly irradiated cells and bystander cells.

cell-cell communications via gap junctions, and perhaps by exfoliated plasma membrane constituents. Demonstrated and potential mechanisms by which radiation-induced effects may be communicated to unirradiated cells are illustrated in Fig. 1.

An interest in bystander effects was stimulated by a study in 1992 in which Nagasawa and Little (33) observed increases in the frequency of SCEs in ~30% of immortalized Chinese hamster ovary cells that received low dose exposure to α particles, even though less than 1% of the cells' nuclei were estimated to actually received direct nuclear hits by an α particle. That a relatively low percentage of the cells experienced one or more direct 'hits' by the α particles, be they in the cytoplasm or in nuclei, suggested the possibility that some mechanism was conveying a radiation-associated

response to unirradiated cells. In a subsequent investigation, Lehnert and co-workers (34) at Los Alamos also obtained evidence that α particles cause increases in SCEs in normal human lung fibroblasts in the absence of direct nuclear traversals, e.g., ~23% of the cells showed excessive SCEs after exposure to a low dose of α particles, whereas the nuclei of less than 3% of the cells were actually traversed by an α particle. Interestingly, the elevations in SCEs reached a plateau level over a dose range of 1.8-12.9 cGy in a manner suggestive of an all or nothing 'on/off' mechanism for their induction. Using data from the study by Nagasawa and Little (1992), the Los Alamos group calculated that the potential target size for the SCE-inducing effects of α particles for the lowest dose that gave a positive response under those exposure conditions was a spatial distance equivalent to ~350 times the area of a typical CHO cell's nucleus. Further, the estimated potential target size for α particles to cause excessive SCE in the experiments using human fibroblasts was ~9 times the area of the cells' nuclei. Accordingly, the potential target size for α particles to cause the excessive SCE responses in both investigations was clearly much larger than the size of cell nuclei and even whole cells. That extracellular processes induced by IR may lead to DNA damage is consistent with the conclusion drawn from several studies examining genomic instability in which the target size for IR to mediate its destabilizing effects on DNA has been determined to be large (35,36).

Based on the above calculations, Lehnert's group investigated the possibility that the induction of excessive SCEs in normal human fibroblasts may be mediated at least in part by the generation of extracellular factors that communicate the response to unirradiated cells (37,38). These experiments consisted of irradiating culture medium with α particles and then transferring the medium onto fresh cells at various times thereafter, or transferring the supernatants from α-irradiated cells onto unirradiated cells. α particles indeed caused the generation of extracellular factors, which upon transfer to otherwise unexposed cells caused the induction of excessive SCEs in unirradiated cells to the same extent as that observed after cells were directly irradiated. A short-lived (<30 min) SCE-inducing factor(s) was generated in α-irradiated culture medium containing serum in the absence of cells, and a more persistent SCE-inducing factor(s), which could survive freeze-thawing but was heat labile, was present in the supernatants of α-irradiated cells. These results provided direct evidence that extracellular factors generated by α-irradiation could mediate DNA damage, as indexed by increases in SCEs. Pointing to the possibility that these SCE-inducing factors involve ROS, further analyses revealed that both the short-lived medium- and the more persistent cell-derived SCE-inducing activities could be inhibited by superoxide dismutase (SOD) (37,38).

In a follow-up series of experiments, the same group investigated the possibility that exposure to α particles can cause increases in extracellular and intracellular ROS (39). Direct exposure of fibroblasts to 0.4-19 cGy of α particles resulted in significant increases in intracellular $O_2^{\cdot}$ and H_2O_2, which were associated with NADPH oxidase activation, a nuclear translocation of the transcription factor NFκB, increased interleukin 8 (IL-8) gene expression, and increases in extracellular IL-8 as a potential bystander mediator (40). Like with the SCE response in bystander cells, the intracellular ROS response was induced to the same extent in unirradiated cells treated with α-irradiated medium or with the supernatants from α-irradiated cells as in directly irradiated cells. And, like the induction of SCEs in bystander cells, the induction of ROS as a bystander effect could be inhibited by SOD (39).

Evidence that IR-induced bystander effects can occur by other mechanisms in addition to transmissible extracellular factors first came from the work of Azzam and colleagues (41) at Harvard. They found that the exposure of confluent human fibroblasts to low doses of α particles causes unexpectedly high increases in the DNA damage-inducible, cell cycle regulating proteins TP53 and downstream CDKIN1A (formerly called $p21^{Waf\text{-}1,Cip\text{-}1}$) in cell populations in which only a low percentage of the cells actually experienced nuclear traversals. From a functional perspective, increases in these proteins expectedly would disfavor cell growth (42-46). Immunofluorescence studies of exposed monolayers of fibroblasts revealed that the increases in CDKN1A appeared in localized 'islands of cells' in a manner that suggested a conveyance of a signal(s) from irradiated cells to their nearby neighbors. Pre-exposure treatment of their cells with lindane, an inhibitor of gap junctions that normally serve to couple cell responses by providing an intercellular route for translocations of messenger molecules ≤1.2 kDa, eliminated the greater than expected α particle-induced increases in TP53 and CDKN1A in cells neighboring presumably directly irradiated cells. The role of gap junctions in mediating this effect was subsequently confirmed by the Harvard group by more directly showing the involvement of connexin 43-mediated intercellular communication in the transmission of damage signals from irradiated to non-irradiated cells (47). Further support for a relationship between gap junctions and cell growth inhibitory bystander responses under at least some conditions comes from a study by Vance and Wiley (48) in which the role gap junctions may play in mediating a phenomenon called 'competitive cell proliferation disadvantage' was assessed. With aggregated chimeras consisting of γ-irradiated and non-irradiated cleavage stage mouse embryos in which gap junction formation was inhibited by 18 α-glycyrrhetinic acid, evidence was obtained that gap junctions were involved in mediating decreased cell proliferation rates in the irradiated components of the chimeras.

Iyer and Lehnert (6) investigated the possibility that the α particle-induced bystander-mediated increases in TP53 in a higher fraction of cells than can be explained by nuclear traversals alone may be caused, at least in part, by fluid-phase, soluble factors generated in response to the α particles. Unexpectedly, the transfer of supernatants from α-irradiated fibroblasts to unirradiated cells caused decreases in cellular levels of the TP53 and CDKN1A proteins in the latter cell populations, not increases like those that occur in directly irradiated cells. These decreases in bystander cells were accompanied by increases in proliferating cell nuclear antigen (PCNA) and CDC2 (49). 'All or nothing' prompt increases in transforming growth factor-ß1 (TGF-ß1) in the supernatants of α-irradiated cells were determined to be

involved in mediating the α particle-associated 'increased intracellular ROS bystander response' described above as well as the 'decreased TP53/CDKN1A bystander effect' (6). Importantly, the TGF-ß increases were inhibitable by SOD- and catalase, thereby suggesting that the extracellular ROS generated by interactions of α particles with medium constituents may play a role in initiating the bystander process.

Consistent with the bystander-associated changes in the cell cycle regulating proteins TP53, CDKN1A, PCNA and CDC2 and the well-documented association of increases in basal levels of ROS and cell proliferation (50-52), evidence was also obtained in the same study that the transfer of supernatants from cells irradiated with a low dose of α particles (1 cGy) onto non-irradiated cells causes greater than normal cell proliferation, a response that could be mimicked by low concentrations of recombinant TGF-ß1 commensurate with those found in the medium of α-irradiated cells (6). Hence, yet another bystander effect that can be mediated by transmissible, fluid phase factors generated in α-irradiated cell cultures is a stimulation of cell growth. This response would not be predicted from results obtained from cells that were directly irradiated with higher doses of IR, which induce cell cycle checkpoints and diminish cell growth (46). How the above promitogenic effect in bystander cells can be reconciled with the results by Azzam *et al* (41) in which low dose α particles can induce increases in TP53 and CDKN1A in a manner that expectedly would inhibit cell growth remains unclear. Conceivably, the extracellular factor(s)-mediated decreased TP53/CDKN1A and promitogenic bystander effects may serve to reduce the extent of the gap junction-communicated increased TP53/CDKN1A response in α particle exposed populations, but this possibility has yet to be evaluated experimentally.

Irradiation of cell nuclei can lead to nuclear damage and the death of unirradiated cells as another bystander effect. Investigators at the Gray Laboratory in the United Kingdom (53,54), for example, used a microbeam to irradiate the nuclei of a very low percentage of fibroblasts in subconfluent cultured populations and observed micronuclei formation and apoptosis in higher percentages of the cells than could be accounted for by directly targeted cells alone. Mothersill and Seymore (55) showed that cell killing as a bystander effect extends to low-LET IR as well in an investigation in which supernatants from immortalized HaCaT keratinocytes that were exposed to ^{60}Co γ-rays reduced the cloning efficiency of unirradiated cells and induced apoptosis. As an example how bystander effects may differ with different cell types, these effects did not occur when performed with human fibroblasts and their supernatants post-irradiation. Of note, immortalized keratinocytes such as the HaCaT cell line undergo apoptosis following γ-irradiation, whereas primary normal human keratinocytes do not, a difference that may be related to cell cycle checkpoint deficiencies in the former (56). In a follow-up investigation, Mothersill and Seymour (57) examined the possibility that gap junctions may be involved in mediating the distribution of the cell killing factor(s) generated in the supernatants of γ-irradiated keratinocytes by using phorbol myristate acid (PMA) to close their gap junctions prior to irradiation. Treatment of the cells with PMA did not diminish the ability of supernatants from the irradiated cells to reduce the clonogenic survival of unirradiated cells, but, instead, it actually increased cell killing by the bystander effect, as well as the killing of directly irradiated cells. These latter results are not necessarily surprising, given that PMA is an activator of protein kinase C and it causes elevations in intracellular ROS (58,59). Regardless, the finding that a factor that reduces cell survival can be produced by irradiated cells may offer some explanation for observations such as enhancements in radiosensitivity to very low dose IR exposure (60).

Perhaps more like the *in vivo* condition in which cells are arranged three-dimensionally, Bishayee and co-workers (61) used a multicellular cluster model consisting of mixtures of differing ratios of Chinese hamster V79 cells with or without tritiated thymidine incorporated into their DNA. The relatively short range of the ^{3}H ß particles presumably only caused irradiation of the cells with incorporated tritiated thymidine and not unlabeled cells included in the cell assemblies. These investigators observed greater than expected decreases in cell survival in the cell mixtures than could be accounted for by only the lethal effects of radiation in the radiolabeled cells, an effect that was inhibitable by gap junction disrupting lindane and DMSO (62). In conjunction with other previously described observations, it is now clear that cell killing as a bystander effect can be mediated by soluble extracellular factors and by direct cell-cell communications via gap junctions.

Changes in radiosensitivity can also be a radiation-induced effect in non-irradiated cells. Matsumoto and co-workers (63) reported reduced radiosensitivity in cells cultured in conditioned medium harvested from other cells that had been exposed to ≥2.5 Gy doses of X-rays. Such a finding suggests the possibility that at least relatively high dose, low-LET IR exposure may induce enhanced radioresistance in unirradiated cells via one or more soluble factors. Although this 'bystander-like' response was observed under special conditions that are too detailed to describe here and likely lack generalization to most other cell models, the investigation's focus on enhanced radioresistance in unirradiated cells deviated from prior investigations of radioadaptive responses *in vitro* in which adaptive responses have been studied in cell populations that received direct exposure to a low 'priming' dose of IR prior to being exposed to much higher dose IR (64-66). More recently, Iyer and Lehnert (67) extended upon the observation by Matsumoto *et al* (63) and found that a radioadaptive response can be induced in unirradiated cells by a transmissible factor(s) present in the medium of cells exposed to a low dose of γ-rays like that used in more typical radioadaptive response experiments. The potential implications of these radioadaptive responses in bystander cells from a human health perspective remains unclear. If radioadapted bystander cells are like some other cell types that become radioresistant in response to a low priming dose of IR, they may potentially manifest more complex types of DNA damage (68) and be more susceptible to transformation (69) after subsequent exposure to a higher dose of IR. It should be noted, however, that IR-associated bystander effects do not necessarily lead to increases in radioresistance. Increases in cell radiosensitivity, for example, can occur in the presence of dead and dying cells (70).

While details about the mechanism(s) by which the low dose (1 cGy), low-LET IR radioadaptive effect is mediated remain to be determined, the adaptive bystander response in normal human lung fibroblasts was found to be associated with decreases in basal levels of TP53 protein, increases in intracellular ROS, and increases in the redox and base excision repair protein AP-endonuclease. A similar radioadaptive response in bystander cells treated with supernatants from α-irradiated human fibroblasts has also been demonstrated recently by the same group (71). Conceivably, these radioadaptive bystander responses may be linked to the progression of cells to a later point in G_1 where DNA repair processes and radioresistance can be induced (72).

The induction of a process that leads to chromosomal instability in response to IR, which is often taken to reflect a condition of genomic instability, also can be mediated by a bystander mechanism. In the early 1990s, Kadhim and colleagues (35,36) reported that the phenomenon of chromosomal instability occurred at a higher level in clonal descendants from α-irradiated hematopoietic stem cells than simply could be accounted for by the expected proportion of cells that actually were traversed by α particles and survived. These investigators (73) later examined this unexpected outcome by using a grid configuration to shield some cells in the irradiated populations of murine bone marrow cells from direct traversals by α particles. The phenomena of chromosomal instability, which the investigators previously associated with increases in intracellular ROS, oxidative DNA base damage, and vulnerability to free radical-mediated membrane damage (74), indeed occurred in the descendants of unirradiated cells in irradiated cell cultures. Also consistent with the induction of genomic instability and the emergence of lethal mutations is the delayed killing of some unirradiated cell types that have been treated with supernatants from irradiated cells (57). Thus, it appears that interactions between irradiated and unirradiated cells and/or perhaps IR-reactants formed in culture medium, a physiologic fluid surrogate, in turn cause responses that may be important processes that underlie genomic instability as a carcinogenic mechanism. Importantly, chromosomal instability in bystander cells is not merely an *in vitro* phenomenon in terms of its transmission after cell exposures to IR *in vitro*. Watson and co-workers (75) recently used a bone marrow transplantation approach in which a mixture of irradiated and unirradiated cells that could be distinguished by a cytogenetic marker were transplanted into mice. With their system, chromosomal instability was observed in the progeny of the unirradiated stem cells.

Also germane to carcinogenesis is recent evidence that α particles can cause mutations in unirradiated cells neighboring irradiated cells. Nagasawa and Little (76), for example, observed a greater than expected induction of HPRT mutations in CHO cells by very low doses of α particles in a manner that was best explained by the occurrence of mutations in unirradiated cells via a bystander mechanism. That most of the HPRT mutations occurring among bystander cells were point mutations suggested the possibility that they may have been caused by ROS (77). Zhou *et al* (78,79) also found greater than expected mutations in human-hamster hybrid cell populations in which only some of the cells were directly irradiated with as few as one α particle delivered to cell nuclei by way of a charged particle microbeam. Pretreatment of the cells with the free radical scavenger DMSO prior to IR exposure did not attenuate the response, thereby suggesting oxidative stress or at least hydroxyl radicals were not a predominant cause of the mutations in bystander cells. The gap junction inhibitors lindane and octanol, on the other hand, were found to reduce the mutant yield. As well, the mutagenic effect was inhibited in cells carrying a dominant negative connexin 43 vector (79). Using the same human-hamster hybrid cells, the same group (80) showed that the mutagenic effect was not due to an extracellular factor in culture medium, but did find evidence for a cell-derived cytotoxic factor(s) that killed unirradiated cells. Thus, in the same model system one bystander effect can be mediated by gap junctions while another results from cellular responses to a soluble, extracellular factor(s).

Investigators have also obtained evidence that neoplastic or oncogenic transformation can result as an IR-associated bystander effect *in vitro*. To study this possibility, Sawant *et al* (81) used a microbeam to irradiate the nuclei of 10% of the cells in C3H 10T1/2 cell populations and assessed for morphologically transformed cells thereafter. The resulting frequency of transformation when only a fraction of the cells were irradiated was not less than that observed after every cell was exposed to the same number of α particles, a result that appear to be best explained by a bystander mechanism. Lewis and colleagues (82) have demonstrated that neoplastic transformation as a bystander effect is not restricted to high LET α particles or low dose exposure to IR. Using CGL1 cells, which are a HeLa-skin fibroblast cell line, these investigators observed that medium from cells that were irradiated with 5 or 7 Gy X-ray doses caused cell killing via a process that did not appear to involve apoptosis, while additionally causing a reduction in plating efficiency and a 4-fold increase in the neoplastic transformation of the cells. While the results from these studies are interesting and suggest a role for bystander effects in mediating neoplastic transformation, the cell models used in the study were already immortalized cells with an unknown number of pre-existing abnormal genomic changes. That normal human cells can be transformed by a bystander mechanism has yet to be demonstrated. Regardless, experimental results like those above may be related to other findings, such as the marked increases in neoplastic transformation frequencies with cells exposed to IR in the presence of heavily-irradiated cells (83).

Finally, some mention should be made about the role(s) plasma membrane-derived constituents may play in mediating IR-associated bystander effects. The plasma membrane has been recognized for some time as an IR-sensitive cell component (84,85), and recent evidence suggests that important membrane-bound constituents such as the 'death ligand' TNFSF6 (formerly called FasL or CD95) can be released on exfoliated vesicles at abnormally high levels following exposure to IR (86). Existing evidence also indicates that several important regulatory molecules, including TGF-ß1, can be released as bioactive components of shed, extracellular vesicles (87,88). Roles for these as well as cytokines, chemokines and growth factors produced by irradiated cells (56,89-91) in mediating bystander effects is

only now beginning to receive experimental attention. Along the same line, little is known about how IR changes in the extracellular matrix, including the release of growth factors and other cell signaling factors, may figure in the mediation of bystander effects (92).

4. Bystander effects and cancer

It is clear that the numerous bystander effects reported to date, which are summarized in Table I, could have important implications in cancer development as well as its treatment with radiotherapy. How such effects may be involved in bringing about undesirable clinical outcomes such as second cancers (93,94) remains to be determined, assuming bystander-mediated untoward effects do indeed occur in the *in vivo* condition as results from several studies now suggest. Further, it remains possible that some bystander effects may function as response modifiers to subsequent fractions of IR given in fractionated radiotherapy. For example, new evidence indicates that cells experiencing the previously described bystander-mediated 'decreased TP53/CDKN1A response' fail to normally increase their TP53 protein levels when subsequently irradiated with a low dose of IR (49), and bystander responses can take the form of increased or decreased radiosensitivity in bystander cells in a not yet predictable manner (63,67,70,71).

For the sake of balance, however, it should be pointed out that firm evidence that mechanisms such as IR-associated bystander effects are operational *in vivo* is currently limited. Nagarkatti and co-workers (95), for example, have reported that inhalation exposure of mice to radon can cause alterations in extra-pulmonary splenic, thymic and peripheral lymph nodal lymphocytic subpopulations. Such findings are consistent with the possibility that exposure to α particles can result in the generation of biologically active, diffusible products that may act at more distant, unirradiated body sites. Further, the previously cited findings of persistent clastogenic activity in the plasma of irradiated human subjects (11,19,26) seemingly is best explained as a mechanism that could cause chromosomal damage in cells *in vivo* at times well after exposure.

It is also unclear what the biological consequences may be of the ROS and promitogenic bystander effects discussed above. Clearly, increases in intracellular ROS are associated with the induction of excessive SCE, but aside from associations with mutagenicity and carcinogenicity (96), cause-and-effect relationships between SCEs and oxidative stress have not been unequivocally established. Yet, ROS can cause a spectrum of DNA lesions, they have been associated with genomic instability, they are regarded as having mutagenic and carcinogenic potential, and they probably play roles in tumor promotion (74,97). Further, the DNA-damaging effects of excessive ROS can activate cell cycle checkpoints, induce a senescence-like state, or cause cells to undergo apoptosis and necrosis *in vitro* (50,98). Based on these findings, one might expect that exposure to high levels of intracellular ROS, if any thing, would curtail cell proliferation. Paradoxically, however, cells along the conducting airways of the lower respiratory tract often undergo enhanced proliferation, i.e., hyperplasia, *in vivo* in a background of ROS-associated stimuli, including inhalation exposure to radon/radon progeny (99-101). In this regard, the promitogenic bystander effect provides a possible mechanistic explanation for such hyperproliferative responses, which could be interpreted as a protective response in terms of increasing the epithelial barrier in airways. On the other hand, and in the context of a potentially detrimental consequence of a bystander-mediated promitogenic response, excessive cell proliferation, especially in a concurrent background of DNA-damaging oxidative stress, or enhanced states of mutagenesis (78), or the induction of genomic instability in bystander cells, may very well contribute to carcinogenic processes (97,102-105). Decreases in the reproductive capacity and the induction of apoptosis in bystander cells (55) seemingly would provide some protection against the development of cancer from cells that may be experiencing yet other phenomena such as mutations and genomic instability (106).

Table I. Radiation-induced effects in unirradiated cells.

Sister chromatid exchanges
Micronucleus formation
Cell killing/delayed cell death
Increased intracellular ROS
Apoptosis
Gene and protein expression changes
Increased radioresistance/radioadaptation
Mutagenesis
Chromosomal instability
Neoplastic transformation

These issues obviously require further study, and definitive conclusions about the roles bystander effects may play *in vivo* are tempered by the fact that results obtained from radiation studies with cells *in vitro* can markedly depart from those observed *in vivo* (107). As discussed before, chromosomal instability following the exposure of hemopoietic cells to low dose exposure to α particles *in vitro* has been attributed to a large extent to this phenomenena occurring in unirradiated, bystander cells (73). The results from some studies, however, seriously question that the same type of effect is manifested *in vivo*. Whitehouse and Tawn (108), for example, found no evidence in a carefully performed investigation for *de novo* chromosomal aberrations as an index of persistent transmissible genomic instability in peripheral blood lymphocytes from individuals whose bone marrows were experiencing α particle irradiation from deposited plutonium. Similarly, Bouffler and colleagues (109) found no evidence for transmissible genomic instability in bone marrow samples harvested over a prolonged period after mice were exposed to bone-seeking radium-224 or after mice were exposed to whole body X-irradiation.

Information that provides some insight into the possibility that IR-associated bystander effects can cause cancer in humans comes from the radiation oncology literature where several investigations have noted the development of second

cancers at sites distant from irradiated fields. While potentially informative, such studies must be viewed with caution, given that even shielded or distant sites can receive some low dose level of exposure to IR during radiotherapy due to equipment 'leaks' (small amounts of ionizing radiation that is transmitted through the lead shielding) and internal scatter of secondary electrons within the patient. Furthermore, by convention, the radiation treatment field edge is defined at the 50% isodose line, indicating that there is a small, but non-zero, amount of radiation delivered centimeters beyond the identified field edge. Also complicating the analysis of post-radiation therapy secondary cancer development is the fact that patients administered radiation therapy for their tumors have often received chemotherapy as part of their anti-cancer treatment that can be directly carcinogenic and/or alter cellular responses to radiation. Further, cancer patients have close medical follow-up, leading to an increased rate of diagnosis of second malignancies, as well as often continuing exposure to carcinogens/known risk factors (e.g., continued cigarette smoking), which may increase the second malignancy rate above that of the untreated population. A factor that may be operational in the opposite direction, that is to decrease the reported frequency of second tumors, is that for a second malignancy to be recorded as such it must be of a different histologic type or significantly separate in time to distinguish it from a recurrence or metastasis from the index tumor. One further caveat to a review of this literature is the awareness that these patients do not represent a random selection from the general population, but a group of people who have developed one cancer, which therefore puts them at greater risk for developing a second cancer, be it from continued lifestyle/behavioral choices or underlying genetic suseptibility. Nevertheless, several studies do raise the possibility that bystander effects may be operational in humans.

5. Bystander effects in humans

There are well recognized effects in radiation therapy that may represent bystander effects if one considers that any effect beyond the radiation field is, by definition, evidence of a bystander effect. The almost universal fatigue response to IR is clearly a systemic effect that is believed to be mediated by central nervous system responses to factor(s), believed to be cytokines, released into the circulatory system from local treatment effects (110). Likewise, radiation of part of the lung for the treatment of primary lung cancer or as part of breast conserving radiation therapy has been associated with radiation pneumonitis that does not necessarily adhere to the field edges of lung that received direct irradiation (111-113), consistent with observations in animal models that indicate that cytokine release is critical in mediating this process. However, since the radiation portal field edge is defined by the 50% isodose line, a significant volume of lung beyond that line is exposed to radiation doses that may be sufficient under some physiologic conditions to cause pneumonitis. Internal scatter within the lung may also account for the reported cases of pneumonitis beyond field edge. Internal scatter in the lung has been difficult to accurately model in the past because of the lung/soft tissue interface as well as the low tissue density of the lung itself. These limitations can now be overcome with Monte Carlo based CT radiation treatment planning (i.e., Peregrine dose calculation) (114), but this approach is not yet in widespread clinical use, nor has it as yet been used to investigate 'bystander' pneumonitis.

Bystander effects can only be examined when less than the whole body has been irradiated and there is knowledge regarding doses and volumes exposed to radiation. Thus, studies of second malignancies from total body irradiation (TBI), atomic bomb exposures or accidental environmental exposures are uninformative in this regard. The best available data comes from second malignancy studies following radiation therapy for cancers of childhood or young adulthood where there is a high survival rate and long follow-up potential. The retinoblastoma experience was the first to raise the possibility of radiation causing a high rate of both in-field and out of field second cancers, mostly osteosarcomas. A more complete analysis of these data, once the underlying genetic link of hereditable mutations in the Rb gene was identified, demonstrated that homozygous deletions of the gene were responsible for both retinoblastoma and osteosarcoma. Patient data, segregated based on the underlying genetics, revealed that it was the genetic form of retinoblastoma, regardless of whether the child received radiation therapy that correlated with both in-field and distant sarcoma development (115,116). Indeed, Koten *et al* reported that in non-heritable retinoblastoma there was no development of second malignant tumors (117,118).

Hodgkin's disease survivors are a second group of patients where second malignant tumors are a well documented fact that may yield clues to the action of bystander effects in carcinogenesis *in vivo* in humans. The Late Effects Study Group (LESG) reported on follow-up of almost 1000 children treated for the disease between 1955-1979. The patients received combined chemoradiation and at 20 years post-treatment had a risk of leukemia or solid tumor development of 18%. All of the solid tumors developed within the radiation portals (119). Other groups have reported out of field second malignancies, but they did not provide details on the distance of the tumor from the radiation portal, thus making it impossible to judge whether the tissue could have been exposed to low dose IR in the area of radiation fall-off (120). Some of these patients received concurrent cytotoxic chemotherapy, while others had such a short period of time between IR and the second tumor (i.e., less than 2.5 years) that it seems biologically unlikely that the radiation exposure could have played a causative role in the carcinogenic process. Nevertheless, the possibility that bystander effects may have been active cannot be excluded. Leukemia development as a separate risk has been studied in Hodgkin's disease survivors, but all of these patients had irradiation of active bone marrow and the vast majority (138/149 cases) had chemotherapy (121).

In a more general examination of all second malignant tumors in survivors of childhood cancer, 69 of 102 second malignancies were deemed to be radiation associated (developed within the radiation portals), another 12 arose in children with a known genetic susceptibility, and a separate cohort of 12 never received radiation (122). Therefore there were 9 cases of cancer arising in irradiated patients beyond the treatment field edge. Of these 2 were clearly identified

as being within the range of scatter. All of the children were treated in the pre-linac, pre-computer planning era on 250 KV (low energy) machines which further complicates any analysis for evidence of bystander effects. All one can conclude is that bystander mechanisms may be operational, but other undocumented factors may better explain the findings, e.g., genetic susceptibilities that were not known at the time of the original report.

Epidemiologic data from the SEER database, amongst others, has been examined to evaluate second malignancy rates following radiation therapy vs. surgical treatment (123). Comparing two treatment options for a given malignancy, e.g., prostate or cervical, has been suggested as a way of avoiding the many biases inherent in comparing a population with a malignancy against the general population. These studies have documented a higher second malignancy rate in the radiation cohort, beyond the radiation field edges, specifically lung cancer, that raises the possibility of bystander effects being operational *in vivo*. However, since these studies were epidemiologic in nature, and lacked information about smoking history, other lung cancer risk factors, detailed information on radiation doses and treatment portals, and uniform follow-up, no firm conclusions can be drawn.

Overall, the clinical literature does not provide strong evidence for or against the existence of radiation bystander effects *in vivo*. Many studies can be interpreted to suggest a functional bystander response, but until prospective clinical trials are undertaken, where detailed field and dose information, as well as other cancer risk factors for each patient is documented, no firm conclusions can be made.

6. Bystander effects and human cancer risk assessment

Demonstrations of the effects of extranuclear irradiation and bystander effects have brought to the forefront new complexities that require rethinking about radiation risk assessment and as just discussed, information about the potential carcinogenic effects of IR-induced bystander effects in humans is lacking. Current risk assessments for IR-induced cancer is based largely on the cancer incidence data that have been obtained from the survivors of the atomic bombings of Hiroshima and Nagasaki nearly 60 years ago in conjunction with estimates of dose exposures to the survivors. While useful for assessing cancer risk to higher doses of IR, especially low-LET IR, cancer risk due to doses below 20 cGy remains an unsettled issue for lack of reliable dose-response information in this low dose region. To address this limitation, the US National Council on Radiation Protection and Measurements (124) and the International Commission on Radiation Protection (125) have recommended use of a linear no-threshold model for extrapolating low dose IR cancer risk from the more definitive high dose-cancer incidence data.

To be sure, new mechanistic possibilities must now be considered in interpreting the results of both *in vitro* and *in vivo* studies inasmuch as nuclear DNA alone no longer can be viewed as the only relevant target for the actions of IR, or even necessarily the most important target for eliciting at least some detrimental effects of IR under some exposure conditions. Instead, the new findings that many effects previously attributed to DNA damage induced at or near the time of exposure and its attendant effects, including reproductive incapacitation, apoptosis, mutation, genomic instability, and cell transformation can occur in cells that receive no direct exposure to IR at all are at odds with prior models of IR-associated cancer risk. For example, numerous risk estimate models (Committee on the Biological Effects of Ionizing Radiations, 1990; United Nations Scientific Committee on the Effects of Atomic Radiation, 1993, Human Respiratory Tract Model for Radiological Protection, 1994), have been developed for assessing radiation dose to sensitive cells for estimating cancer risk associated with the inhalation of α particle-emitting radon and radon progeny (126-129). An underlying assumption shared by these models is that traversals of α particles through the nuclei of target cells in the lower respiratory tract alone are of primary concern in terms of being cancer-causing. In this regard, considerable attention has been directed toward morphometrically determing distances to the nuclei of airway cells for use in calculating the probability density in specific energy delivered to their nuclei by α particles from the decay of radon/radon progeny. Even though the likelihood that α particles from inhaled radon/radon progeny directly traverse the nuclei of airway basal epithelial cells is low in average household settings (130), recent estimates suggest that exposure to indoor radon may be responsible for as many as 24,000 new cases of lung cancer yearly (131), or ~10% and 30% of all lung cancers and lung cancers in non-smokers, respectively (132). Such circumstantial evidence suggests the possibility that extranuclear mechanisms may contribute to radon-induced cancers, a possibility that gains support from several of the studies discussed earlier. In essence, much of the information that has been obtained from *in vitro* experiments involving low dose high- or low-LET IR appear to indicate that low dose exposure may be more detrimental than high dose exposure, i.e., responses in the low dose exposure region are disproportionately greater than those that occur at higher doses on a cGy-by-cGy basis.

Some initial efforts have been recently undertaken toward quantitatively unraveling the relative contributions of targeted and non-targeted effects in IR-exposed cell populations. Seymour and Mothersill (106), for example, reported that the clonogenic death of bystander cells predominated over a low-LET IR dose range of 0.01-0.5 Gy, with the effect remaining relatively constant over the lower doses. At doses higher than 0.5 Gy, clonogenic death appeared to be the result of a dose-dependent non-bystander effect coupled with a dose-independent bystander response. Brenner and co-workers (133,134) have also developed a new model termed 'BaD' (Bystander and Direct) that takes into account the role of bystander effects in the α particle-induced oncogenic transformation of C3H 10T1/2 cells *in vitro*. Similar to the aforementioned conclusion made by Seymour and Mothersill (106), bystander effects according to the BaD model are importantly operational at relatively low doses below 0.2 Gy where they may dominate the overall oncogenic response. While attempts to discriminate between the targeted and non-targeted effects of IR are at present rudimentary, the above studies both suggest bystander effects in exposed cell populations may enhance risk especially under conditions of

relatively low dose exposure. In this regard, risk estimates extrapolated from high dose data to low dose exposures may result in underestimations of risk. For the sake of balance, however, it should be pointed out that more recent results from an extension Brenner *et al*'s model coupled with additional experimental data sets have suggested that the oncogenic transformation type bystander effect seen with C3H 10T1/2 cells does not play a large role in radon-induced lung cancer in humans (135).

7. Summary

Many important IR-associated effects can occur in cells that have experienced no direct exposure to IR *per se*. While these bystander effects have been amply demonstrated *in vitro*, how they may pertain to the *in vivo* condition and the induction or perpetuation of pathologic responses remains uncertain. Some evidence, but certainly not all, implicates ROS as a central mechanism of bystander effects under at least some conditions. In addition to carcinogenesis, radiation bystander effects may play an important role in a range of other ROS-associated processes *in vivo*, including aging and wound healing. Until these matters are better understood, the effects of IR-associated bystander effects will continue to contribute to the uncertainties inherent to current risk assessment models. Aside from their mechanistic bases, even some very basic phenomenological features of bystander effects remain to be investigated. These include: i) the demonstration of their occurrence *in vivo*, ii) the total spectrum of bystander effects relative to those that occur in response to IR in directly targeted cells, iii) the relationships between radiation quality and quantity as inducers of bystander effects, iv) how bystander effects may differ with different cell phenotypes and stages of cell differentiation, v) the persistency of the bystander responses, vi) how bystander effects may be agonistic, antagonistic, or synergistic relative to the targeted effects of IR or how they otherwise affect one another and the overall responses of cell populations, vii) how IR dose-response relationships with cells experiencing bystander effects may deviate from those of normal cells, viii) relationships between genomic heterogeneity among individuals and the occurrence of IR-induced bystander effects, and, of course, ix) the biological consequences of the effects, both *in vitro* and *in vivo*, be they beneficial or untoward. In addition to these issues, a cogent understanding of the mechanistic underpinnings of bystander effects is lacking. Conceivably, new information about such mechanisms may provide insight into how bystander effects may some day be beneficially regulated, while also providing new information for advanced predictive assessments of the hazards of exposure to IR, including, perhaps, individualized risk assessments.

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References

1. Goodhead DT: Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. Int J Radiat Biol 65: 7-17, 1994.
2. Iliakis G: The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. Bioessays 13: 641-648, 1991.
3. Ward JF: DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. Prog Nucleic Acid Res Mol Biol 35: 95-125, 1988.
4. Ward JF: The complexity of DNA damage: relevance to biological consequences. Int J Radiat Biol 66: 427-432, 1994.
5. McDevitt MR, Ma D, Lai LT, Simon J, Borchardt P, Frank RK, *et al*: Tumor therapy with targeted atomic nanogenerators. Science 294: 1537-1540, 2001.
6. Iyer R and Lehnert BE: Effects of ionizing radiation in targeted and non-targeted cells. Arch Biochem Biophys 376: 14-25, 2000.
7. Parsons WB, Watkins CH, Pease GL and Childs DS: Changes in sternal bone marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukemia. Cancer 7: 179-189, 1954.
8. Goh KO and Sumner S: Breaks in normal human chromosomes: are they induced by a transferable substance in the plasma of irradiated persons exposed to total-body irradiation? Radiat Res 35: 171-174, 1968.
9. Hollowell JG and Littlefield LG: Chromosome aberrations induced by plasma from irradiated patients. A brief report. JSC Med Assoc 63: 437-442, 1967.
10. Souto J: Tumour development in the rat induced by the blood of irradiated animals. Nature 195: 1317-1318, 1962.
11. Hollowell JG and Littlefield LG: Chromosome damage induced by plasma of X-ray patients: an indirect effect of X-ray. Proc Soc Exp Biol Med 129: 240-243, 1968.
12. Scott D: The effect of irradiated plasma on normal human chromosomes and its relevance to the long-lived lymphocyte hypothesis. Cell Tissue Kinet 2: 295-305, 1969.
13. Goyanes-Villaescusa V: Chromosomal abnormalities in lymphocytes of children and baby rabbits born from mothers treated by x-radiation before pregnancy. A transplacentary plasmatic chromosome-damaged factor? Blut 22: 93-96, 1971.
14. Poncy JL, Walter C, Fritsch P, Masse R and La Fuma J: Delayed SCE frequency in rat bone-marrow cells after radon inhalation. Sanders CL, Cross FT, Dagle GE and Mahaffrey JA (eds). CONF-791002, Technical Information Center, US Department of Energy, 1980.
15. Wolff S, Bodycote J and Painter RB: Sister chromatid exchanges induced in Chinese hamster cells by UV irradiation of different stages of the cell cycle: the necessity for cells to pass through S. Mutat Res 25: 73-81, 1974.
16. Schvartzman JB and Goyanes V: A new method for the identification of SCE's per cell cycle in BrdUrd-substituted chromosomes. Cell Biol Int Rep 4: 415-423, 1980.
17. Kato H: Spontaneous and induced sister chromatid exchanges as revealed by the BUdR-labeling method. Int Rev Cytol 49: 55-97, 1977.
18. Sonoda E, Sasaki MS, Morrison C, Yamaguchi-Iwai Y, Takata M and Takeda S: Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. Mol Cell Biol 19: 5166-5169, 1999.
19. Emerit I, Levy A, Cernjavski L, Arutyunyan R, Oganesyan N, Pogosian A, *et al*: Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor. J Cancer Res Clin Oncol 120: 558-561, 1994.
20. Emerit I, Oganesian N, Sarkisian T, Arutyunyan R, Pogosian A, Asrian K, *et al*: Clastogenic factors in the plasma of Chernobyl accident recovery workers: anticlastogenic effect of Ginkgo biloba extract. Radiat Res 144: 198-205, 1995.

21. Emerit I: Radiation induced transferable clastogenic factors. In: Frontiers in Radiation Biology. Balaban, Frankfurt, pp679-684, 1990.
22. Pant GS and Kamada N: Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals. Hiroshima J Med Sci 26: 149-154, 1977.
23. Faguet GB, Reichard SM and Welter DA: Radiation-induced clastogenic plasma factors. Cancer Genet Cytogenet 12: 73-83, 1984.
24. Auclair C, Gouyette A, Levy A and Emerit I: Clastogenic inosine nucleotide as components of the chromosome breakage factor in scleroderma patients. Arch Biochem Biophys 278: 238-244, 1990.
25. Lazutka JR and Rudaitiene S: Modulation by novobiocin of sister-chromatid exchanges induced by tumor necrosis factor in human lymphocytes. Mutat Res 268: 217-221, 1992.
26. Emerit I and Cerutti P: Clastogenic activity from Bloom syndrome fibroblast cultures. Proc Natl Acad Sci USA 78: 1868-1872, 1981.
27. Shaham M, Becker Y and Cohen MM: A diffusable clastogenic factor in ataxia telangiectasia. Cytogenet Cell Genet 27: 155-161, 1980.
28. Djordjevic B: Bystander effects: a concept in need of clarification. Bioessays 22: 286-290, 2000.
29. Moolten FL: Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. Cancer Res 46: 5276-5281, 1986.
30. Faulds D and Heel RC: Ganciclovir: a review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs 39: 597-638, 1990.
31. Ramesh R, Marrogi AJ, Munshi A, Abboud CN and Freeman SM: *In vivo* analysis of the 'bystander effect': a cytokine cascade. Exp Hematol 24: 829-838, 1996.
32. Kievit E, Nyati MK, Ng E, Stegman LD, Parsels J, Ross BD, *et al*: Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. Cancer Res 60: 6649-6655, 2000.
33. Nagasawa H and Little JB: Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res 52: 6394-6396, 1992.
34. Deshpande A, Goodwin EH, Bailey SM, Marrone BL and Lehnert BE: Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. Radiat Res 145: 260-267, 1996.
35. Kadhim MA, MacDonald DA, Goodhead DT, Lorimore SA, Marsden SJ and Wright EG: Transmission of chromosomal instability after plutonium alpha-particle irradiation. Nature 355: 738-740, 1992.
36. Kadhim MA, Lorimore SA, Hepburn MD, Goodhead DT, Buckle VJ and Wright EG: Alpha-particle-induced chromosomal instability in human bone marrow cells. Lancet 344: 987-988, 1994.
37. Lehnert BE and Goodwin EH: A new mechanism for DNA alterations induced by alpha particles such as those emitted by radon and radon progeny. Environ Health Perspect 105 (Suppl. 5): 1095-1101, 1997.
38. Lehnert BE and Goodwin EH: Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. Cancer Res 57: 2164-2171, 1997.
39. Narayanan PK, Goodwin EH and Lehnert BE: Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Res 57: 3963-3971, 1997.
40. Narayanan PK, La Rue KE, Goodwin EH and Lehnert BE: Alpha particles induce the production of interleukin-8 by human cells. Radiat Res 152: 57-63, 1999.
41. Azzam EI, De Toledo SM, Gooding T and Little JB: 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.
42. Agarwal ML, Agarwal A, Taylor WR and Stark GR: p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci USA 92: 8493-8497, 1995.
43. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW: Participation of p53 protein in the cellular response to DNA damage. Cancer Res 51: 6304-6311, 1991.
44. Di Leonardo A, Linke SP, Clarkin K and Wahl GM: DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev 8: 2540-2551, 1994.
45. El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, *et al*: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 54: 1169-1174, 1994.
46. Gadbois DM, Crissman HA, Nastasi A, Habbersett R, Wang SK, Chen D, *et al*: Alterations in the progression of cells through the cell cycle after exposure to alpha particles or gamma rays. Radiat Res 146: 414-424, 1996.
47. Azzam EI, De Toledo SM and Little JB: Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to non-irradiated cells. Proc Natl Acad Sci USA 98: 473-478, 2001.
48. Vance MM and Wiley LM: Gap junction intercellular communication mediates the competitive cell proliferation disadvantage of irradiated mouse preimplantation embryos in aggregation chimeras. Radiat Res 152: 544-551, 1999.
49. Lehnert BE and Iyer R: Cell growth-related bystander responses to alpha particles and gamma-rays. Proceedings of the Eleventh International Congress of Radiation Research 2: 488-491, 1999.
50. Burdon RH, Alliangana D and Gill V: Hydrogen peroxide and the proliferation of BHK-21 cells. Free Radic Res 23: 471-486, 1995.
51. Burdon RH, Alliangana D and Gill V: Endogenously generated active oxygen species and cellular glutathione levels in relation to BHK-21 cell proliferation. Free Radic Res 21: 121-133, 1994.
52. Murrell GA, Francis MJ and Bromley L: Modulation of fibroblast proliferation by oxygen free radicals. Biochem J 265: 659-665, 1990.
53. Prise KM, Belyakov OV, Folkard M and Michael BD: Studies of bystander effects in human fibroblasts using a charged particle microbeam. Int J Radiat Biol 74: 793-798, 1998.
54. Belyakov OV, Malcolmson AM, Folkard M, Prise KM and Michael BD: Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. Br J Cancer 84: 674-679, 2001.
55. Mothersill C and Seymour C: Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int J Radiat Biol 71: 421-427, 1997.
56. Petit-Frere C, Capulas E, Lyon DA, Norbury CJ, Lowe JE, Clingen PH, *et al*: Apoptosis and cytokine release induced by ionizing or ultraviolet B radiation in primary and immortalized human keratinocytes. Carcinogenesis 21: 1087-1095, 2000.
57. Mothersill C and Seymour CB: 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.
58. Sakka E, Rae M, Aitken J and Bramley T: Protein kinase C- and Ca^{2+} ionophore-stimulated production of reactive oxygen species in mechanically dispersed isolated bovine luteal cells. Biol Reprod 57: 428-435, 1997.
59. Roy S, Sen CK, Kobuchi H and Packer L: Antioxidant regulation of phorbol ester-induced adhesion of human Jurkat T-cells to endothelial cells. Free Radic Biol Med 25: 229-241, 1998.
60. Marples B and Joiner MC: 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, 1996.
61. Bishayee A, Rao DV and Howell RW: Evidence for pronounced bystander effects caused by non-uniform distributions of radioactivity using a novel three-dimensional tissue culture model. Radiat Res 152: 88-97, 1999.
62. Rao D and Howell R: Free radical-initiated and gap junction-mediated bystander effect due to non-uniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. Radiat Res 155: 335-344, 2001.
63. Matsumoto H, Hayashi S, Hatashita M, Ohnishi K, Shioura H, Ohtsubo T, *et al*: Induction of radioresistance by a nitric oxide-mediated bystander effect. Radiat Res 155: 387-396, 2001.
64. Wolff S: Aspects of the adaptive response to very low doses of radiation and other agents. Mutat Res 358: 135-142, 1996.
65. Shadley JD, Afzal V and Wolff S: Characterization of the adaptive response to ionizing radiation induced by low doses of X rays to human lymphocytes. Radiat Res 111: 511-517, 1987.
66. Marples B and Skov KA: Small doses of high-linear energy transfer radiation increase the radioresistance of Chinese hamster V79 cells to subsequent X irradiation. Radiat Res 146: 382-387, 1996.

67. Iyer R and Lehnert BE: Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. Mutat Res (In press).
68. Ueno AM, Vannais DB, Gustafson DL, Wong JC and Waldren CA: A low, adaptive dose of gamma-rays reduced the number and altered the spectrum of S1-mutants in human-hamster hybrid AL cells. Mutat Res 358: 161-169, 1996.
69. Sasaki MS: On the reaction kinetics of the radioadaptive response in cultured mouse cells. Int J Radiat Biol 68: 281-291, 1995.
70. Djordjevic B, Lange CS, Rotman MZ, Torres C and Zheng Z: Increasing radiosensitivity in the course of fractionated X irradiation: the effect of contact with dead and dying cells. Radiat Res 150: 275-282, 1998.
71. Iyer R and Lehnert BE: Alpha-particle-induced increases in the radioresistance of normal human bystander cells. Radiat Res 157: 3-7, 2002.
72. Boothman DA, Meyers M, Odegaard E and Wang M: Altered G1 checkpoint control determines adaptive survival responses to ionizing radiation. Mutat Res 358: 143-153, 1996.
73. Lorimore SA, Kadhim MA, Pocock DA, Papworth D, Stevens DL, Goodhead DT, *et al*: Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. Proc Natl Acad Sci USA 95: 5730-5733, 1998.
74. Clutton SM, Townsend KM, Walker C, Ansell JD and Wright EG: Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. Carcinogenesis 17: 1633-1639, 1996.
75. Watson GE, Lorimore SA, MacDonald DA and Wright EG: Chromosomal instability in unirradiated cells induced *in vivo* by a bystander effect of ionizing radiation. Cancer Res 60: 5608-5611, 2000.
76. Nagasawa H and Little JB: Unexpected sensitivity to the induction of mutations by very low doses of alpha-particle radiation: evidence for a bystander effect. Radiat Res 152: 552-557, 1999.
77. Huo L, Nagasawa H and Little JB: HPRT mutants induced in bystander cells by very low fluences of alpha particles result primarily from point mutations. Radiat Res 156: 521-525, 2001.
78. Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ and Hei TK: Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc Natl Acad Sci USA 97: 2099-2104, 2000.
79. Zhou H, Suzuki M, Randers-Pehrson G, Vannais D, Chen G, Trosko JE, *et al*: Radiation risk to low fluences of alpha particles may be greater than we thought. Proc Natl Acad Sci USA 98: 14410-14415, 2001.
80. Zhou H, Suzuki M, Geard CR and Hei TK: Effects of irradiated medium with or without cells on bystander cell responses. Mutat Res 499: 135-141, 2002.
81. Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ and Hall EJ: 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.
82. Lewis DA, Mayhugh BM, Qin Y, Trott K and Mendonca MS: Production of delayed death and neoplastic transformation in CGL1 cells by radiation-induced bystander effects. Radiat Res 156: 251-258, 2001.
83. Sigg M, Crompton NE and Burkart W: Enhanced neoplastic transformation in an inhomogeneous radiation field: an effect of the presence of heavily damaged cells. Radiat Res 148: 543-547, 1997.
84. Stefani S, Chandra S and Tonaki H: Ultrastructural events in the cytoplasmic death of lethally-irradiated human lymphocytes. Int J Radiat Biol Relat Stud Phys Chem Med 31: 215-225, 1977.
85. Chandra S and Stefani S: Plasma membrane as a sensitive target in radiation-induced cell injury and death: an ultrastructural study. Int J Radiat Biol Relat Stud Phys Chem Med 40: 305-311, 1981.
86. Albanese J and Dainiak N: Ionizing radiation alters Fas antigen ligand at the cell surface and on exfoliated plasma membrane-derived vesicles: implications for apoptosis and intercellular signaling. Radiat Res 153: 49-61, 2000.
87. Dainiak N and Sorba S: Intracellular regulation of the production and release of human erythroid-directed lymphokines. J Clin Invest 87: 213-220, 1991.
88. Dolo V, Pizzurro P, Ginestra A and Vittorelli ML: Inhibitory effects of vesicles shed by human breast carcinoma cells on lymphocyte ^{3}H-thymidine incorporation, are neutralised by anti TGF-beta antibodies. J Submicrosc Cytol Pathol 27: 535-541, 1995.
89. Huber MA, Kraut N, Addicks T and Peter RU: Cell-type-dependent induction of eotaxin and CCR3 by ionizing radiation. Biochem Biophys Res Commun 269: 546-552, 2000.
90. Kyrkanides S, Olschowka JA, Williams JP, Hansen JT and O'Banion MK: TNF alpha and IL-1beta mediate intercellular adhesion molecule-1 induction via microglia-astrocyte interaction in CNS radiation injury. J Neuroimmunol 95: 95-106, 1999.
91. Barcellos-Hoff MH: How do tissues respond to damage at the cellular level? The role of cytokines in irradiated tissues. Radiat Res 150 (Suppl. 5): S109-S120, 1998.
92. Barcellos-Hoff MH and Brooks AL: Extracellular signaling through the microenvironment: a hypothesis relating carcinogenesis, bystander effects, and genomic instability. Radiat Res 156: 618-627, 2001.
93. Wolden SL, Lamborn KR, Cleary SF, Tate DJ and Donaldson SS: Second cancers following pediatric Hodgkin's disease. J Clin Oncol 16: 536-544, 1998.
94. Rich DC, Corpron CA, Smith MB, Black CT, Lally KP and Andrassy RJ: Second malignant neoplasms in children after treatment of soft tissue sarcoma. J Pediatr Surg 32: 369-372, 1997.
95. Nagarkatti M, Nagarkatti PS and Brooks A: Effect of radon on the immune system: alterations in the cellularity and functions of T cells in lymphoid organs of mouse. J Toxicol Environ Health 47: 535-552, 1996.
96. Abe S and Sasaki M: SCE as an index of mutagenesis and/or carcinogenesis. In: Sister Chromatid Exchange. Sandburg AA (ed). Alan R. Liss, New York, pp461-514, 1982.
97. Ames BN, Shigenaga MK and Gold LS: DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. Environ Health Perspect 101 (Suppl. 5): 35-44, 1993.
98. Chen Q and Ames BN: Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. Proc Natl Acad Sci USA 91: 4130-4134, 1994.
99. Taya A, Morgan A, Baker ST, Humphreys JA, Bisson M and Collier CG: Changes in the rat lung after exposure to radon and its progeny: effects on incorporation of bromodeoxyuridine in epithelial cells and on the incidence of nuclear aberrations in alveolar macrophages. Radiat Res 139: 170-177, 1994.
100. Auerbach O, Stout AP and Hammond EC: Changes in bronchial epithelium in relation to cigarette smoking and in response to lung cancer. N Engl J Med 265: 253-257, 1961.
101. Mossman BT, Marsh JP, Dantona R, Bergeron M and Senior A: Involvement of active oxygen species (AOS) in injury, repair, and proliferation of tracheobronchial epithelial cells after exposure to asbestos fibers. In: Biology, Toxicology, and Carcinogenesis of Respiratory Epithelium. Nettesheim DTAP (ed). Hemisphere Publishing Corp., Washington, DC, pp145-154, 1990.
102. Cayama E, Tsuda H, Sarma DS and Farber E: Initiation of chemical carcinogenesis requires cell proliferation. Nature 275: 60-62, 1978.
103. Cohen SM and Ellwein LB: Cell proliferation in carcinogenesis. Science 249: 1007-1011, 1990.
104. Shi X, Castranova V, Halliwell B and Vallyathan V: Reactive oxygen species and silica-induced carcinogenesis. J Toxicol Environ Health B Crit Rev 1: 181-197, 1998.
105. Preston-Martin S, Pike MC, Ross RK, Jones PA and Henderson BE: Increased cell division as a cause of human cancer. Cancer Res 50: 7415-7421, 1990.
106. Seymour CB and Mothersill C: Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiat Res 153: 508-511, 2000.
107. Terzaghi-Howe M and Ford J: Effects of radiation on rat respiratory epithelial cells: critical target cell populations and the importance of cell-cell interactions. Adv Space Res 14: 565-572, 1994.
108. Whitehouse CA and Tawn EJ: No evidence for chromosomal instability in radiation workers with *in vivo* exposure to plutonium. Radiat Res 156: 467-475, 2001.
109. Bouffler SD, Haines JW, Edwards AA, Harrison JD and Cox R: Lack of detectable transmissible chromosomal instability after *in vivo* or *in vitro* exposure of mouse bone marrow cells to 224Ra alpha particles. Radiat Res 155: 345-352, 2001.
110. Loprinzi L, Goldberg RM and Peethambaram PP: Cancer anorexia/cachexia. In: Principles and Practise of Supportive Oncology. Berger A, Portenoy RK and Weissman DE (eds). Lippincott-Raven, Philadelphia, p133, 1998.

111. Arbetter KR, Prakash UB, Tazelaar HD and Douglas WW: Radiation-induced pneumonitis in the 'non-irradiated' lung. Mayo Clin Proc 74: 27-36, 1999.
112. Matsuura K, Hirokawa Y, Matsuura A, Akagi Y and Ito K: Migratory pneumonitis similar to bronchiolitis obliterans organizing pneumonia after conservative treatment of breast cancer: a case report. Nippon Igaku Hoshasen Gakkai Zasshi 59: 412-414, 1999.
113. Martin C, Romero S, Sanchez-Paya J, Massuti B, Arriero JM and Hernandez L: Bilateral lymphocytic alveolitis: a common reaction after unilateral thoracic irradiation. Eur Respir J 13: 727-732, 1999.
114. Hartmann Siantar CL, Walling RS, Daly TP, Faddegon B, Albright N, Bergstrom P, *et al*: Description and dosimetric verification of the Peregrine Monte Carlo dose calculation system for photon beams incident on a water phantom. Med Phys 7: 1322-1337, 2001.
115. Smith LM, Donaldson SS, Egbert PR, Link MP and Bagshaw MA: Aggressive management of second primary tumors in survivors of hereditary retinoblastoma. Int J Radiat Oncol Biol Phys 17: 499-505, 1989.
116. Abramson DH, Ellsworth RM, Kitchin FD and Tung G: Second nonocular tumors in retinoblastoma survivors. Are they radiation-induced? Ophthalmology 91: 1351-1355, 1984.
117. DerKinderen DJ, Koten JW, Nagelkerke NJ, Tan KE, Beemer FA and Den Otter W: Non-ocular cancer in patients with hereditary retinoblastoma and their relatives. Int J Cancer 41: 499-504, 1988.
118. Koten JW, Der Kindren DJ and Otter WD: Editorial reply. N Engl J Med 318: 581-582, 1988.
119. Meadows AT, Baum E, Fossati-Bellani F, Green D, Jenkin RD, Marsden B, *et al*: Second malignant neoplasms in children: an update from the Late Effects Study Group. J Clin Oncol 3: 532-538, 1985.
120. Nelson DF, Cooper S, Weston MG and Rubin P: Second malignant neoplasms in patients treated for Hodgkin's disease with radiotherapy or radiotherapy and chemotherapy. Cancer 48: 2386-2393, 1981.
121. Kaldor JM, Day NE, Clarke EA, van Leeuwen FE, Henry-Amar M, Fiorentino MV, *et al*: Leukemia following Hodgkin's disease. N Engl J Med 322: 7-13, 1990.
122. Meadows AT, D'Angio GJ, Mike V, Banfi A, Harris C, Jenkin RD, *et al*: Patterns of second malignant neoplasms in children. Cancer 40 (Suppl. 4): 1903-1911, 1977.
123. Brenner DJ, Curtis RE, Hall EJ and Ron E: Second malignancies in prostate carcinoma patients after radiotherapy compared with surgery. Cancer 88: 398-406, 2000.
124. Measurements USNCoRPa. Report 116. Bethesda, 1993.
125. Protection ICoR. Recommendations. Report No. 60. New York, 1991.
126. James AC: Lung dosimetry. In: Radon and its Decay Products in Indoor Air. Nero AV (ed). John Wiley, New York, pp259-309, 1988.
127. Hui TE, Poston JW and Fisher DR: The microdosimetry of radon decay products in the respiratory tract. Radiat Protect Dosimet 31: 405-411, 1990.
128. Fisher DR, Hui TE and James AC: Model for assessing radiation dose to epithelial cells of the human respiratory tract from radon progeny. Radiat Protect Dosimet 38: 73-80, 1991.
129. Hofman W, Crawford-Brown DJ, Menaches MG and Martonen TB: Radon progeny effects at bronchial bifurcations. Radiat Protect Dosimet 38: 91-97, 1991.
130. VI) NRCCoHRoEtRB. National Academy of Sciences, Washington, DC, 1994.
131. Puskin JS and Boice JD: EPA's perspective on risks from residential radon exposure. J Air Pollut Control Assoc 39: 915-920, 1989.
132. Lubin JH, Boice JD Jr, Edling C, Hornung RW, Howe GR, Kunz E, *et al*: Lung cancer in radon-exposed miners and estimation of risk from indoor exposure. J Natl Cancer Inst 87: 817-827, 1995.
133. Brenner DJ and Elliston CD: The potential impact of bystander effects on radiation risks in a Mars mission. Radiat Res 156: 612-617, 2001.
134. Brenner DJ, Little JB and Sachs RK: The bystander effect in radiation oncogenesis: II. A quantitative model. Radiat Res 155: 402-408, 2001.
135. Little MP and Wakeford R: The bystander effect in C3H 10T cells and radon-induced lung cancer. Radiat Res 156: 695-699, 2001.