

Genomic instability and bystander effects: a historical perspective

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Data have been emerging over the past two decades concerning two phenomena in which important biological effects of ionizing radiation arise in cells that in themselves receive no radiation exposure. In the first, radiation-induced genomic instability, biological effects occur in the progeny of the irradiated cell after many generations of cell division. In the second, radiation-induced bystander effects, they arise in cells that receive no radiation exposure as a consequence of damage signals transmitted from neighboring irradiated cells; transmission may be mediated either by direct intercellular communication through gap junctions, or by factors released into the surrounding medium. In both phenomena, the biological effects appear to be associated with an upregulation of oxidative metabolism. The present paper is designed to review the historical background leading to our current knowledge of these two phenomena, and to indicate some future directions for research that will allow us to assess better their importance in the health effects of exposure to ionizing radiation.

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Introduction

A central radiobiological paradigm for many years contended that the biological effects of exposure to ionizing radiation occur in the irradiated cell as a direct consequence of DNA damage, damage that has not been correctly restored by metabolic repair processes. Genetic changes such as mutations and chromosomal aberrations, which are thought to be important events in the development of cancer, would thus arise at the site of DNA damage in the irradiated cell as a consequence of processing during normal DNA replication or enzymatic repair.

A number of early experiments provided evidence for the nucleus as the sensitive target in the cell (Bacq and Alexander, 1961). Zirkle and Bloom (1953), for example, employed a focused microbeam of high linear energy transfer (LET) particles to irradiate specific cellular substructures in several types of cells. In these

studies, cell killing was found to depend upon irradiation of the nucleus; little cytotoxic effect occurred by cytoplasmic irradiation alone. Later on, interest was focused specifically on the DNA molecule with the discovery of enzymatic DNA repair processes, (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964). Bacterial cells carrying mutants for several different repair pathways proved to be highly sensitive to the cytotoxic effects of ultraviolet light irradiation. The seemingly direct relationship between the modulation of DNA repair and the cytotoxic effects of irradiation (Hanawalt, 1977) provided a convincing, although indirect argument that biological effects occurred in irradiated cells as a direct consequence of DNA damage.

Further evidence to support this hypothesis arose from the use of techniques that allowed radiation exposure to be localized specifically to DNA molecules. This was accomplished by incubating cells with radioactive ¹²⁵Iododeoxyuridine (¹²⁵I), which is incorporated into DNA in the place of thymidine. When ¹²⁵I decays, it releases a shower of very low-energy electrons; when incorporated into cellular DNA, this intense release of energy is confined to a very small region of the DNA molecule, with most of the damage occurring within a few base pairs of the site of decay (Martin and Haseltine, 1981). Such ¹²⁵I decays that produced damage only in DNA proved to be highly mutagenic and cytotoxic; a single decay within target gene had a high probability of producing a mutation (Liber *et al.*, 1983). On the other hand, ¹²⁵I localized elsewhere within the cell such as in the cytoplasm or the cell membrane had no cytotoxic or mutagenic effect. Such findings led to the development of models for radiation action based on the assumption that dose-dependent effects are related directly to unrepaired or misrepaired DNA damage in irradiated cells. In cell populations exposed to very low fluences of high LET particles, the dose-dependent risk was based on the fraction of cells traversed by a particle track.

Over the past two decades, however, data have been emerging that challenge this paradigm, indicating that important biological consequences of exposure to ionizing radiation may arise in cells that in themselves receive no radiation exposure. The present paper is designed to review the historical background leading to our current knowledge of two of these phenomena. In the first, *radiation-induced genomic instability*, biological effects arise in the progeny of the irradiated cell after many generations of cell division. In the second, the

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radiation-induced bystander effect, biological effects arise in cells that receive no radiation exposure as a consequence of damage signals transmitted from neighboring irradiated cells.

Genomic instability

The genome in mammalian cells is constantly challenged by destabilizing factors including normal DNA replication and cell division, as well as a number of intracellular and extracellular environmental stresses such as oxidative metabolism, exposure to genotoxic chemical agents and background radiation. As a result, cells have developed elaborate mechanisms for maintaining genomic integrity, notably those that assure the fidelity of DNA replication and the enzymatic repair of endogenous and exogenous DNA damage, as well as the control of progression through the cell cycle. Failure of these processes can lead to destabilization of the genome with a resultant enhancement of the rate with which deleterious mutations arise that can lead to alterations in cellular function including cell death.

Genomic instability is considered a hallmark of the process of carcinogenesis. Most human tumors show such instability as manifested by multiple unbalanced chromosomal aberrations (Dutrillaux, 1997). It has been hypothesized that as many as six to eight separate genetic events may be required to transform a normal cell into one with a fully malignant phenotype; in certain types of colon cancer, for example, a number of specific genetic events have been identified that are associated with increasing levels of malignant change (Vogelstein and Kinzler, 1993).

Most investigators agree that the accumulation of such genetic events, including the activation of oncogenes and the loss of tumor suppressor genes, represent an important characteristic of carcinogenesis. If each mutation is an independent event occurring with a probability of around 10^{-5} , however, the question arises as to how so many genetic events can develop in a single cell lineage within the lifetime of an individual. It has thus been tempting to speculate that the development of genomic instability in cells may be an important mechanism in the initiation and/or progression of cancer (Cheng and Loeb, 1993), this instability rendering the potential cancer cells more susceptible to the accumulation of these multiple genetic abnormalities. Indeed, the loss of stability of the genome is becoming widely accepted as one of the most important processes in the development of cancer (Loeb *et al.*, 2003).

However, there is no widespread agreement as to how and when this process develops. Many believe that it occurs early in the development of cancer. Nowell (1976) suggested that such instability provides the genomic plasticity needed to drive the stepwise process of genetic changes required for the development of the neoplastic phenotype. It has been hypothesized that incipient cancer cells develop a mutator phenotype rendering them more prone to accumulating the requisite number

of mutations at critical loci within the cells (Loeb, 2001). The defect in DNA mismatch repair characteristic of hereditary nonpolyposis colon cancer is an example of a mutator phenotype that fits this model (Vogelstein and Kinzler, 1993). However, most cancers are associated with large-scale genetic changes including gains and losses of whole chromosomes and rearrangements either within or between chromosomes, rather than the point mutations characteristic of a defect in mismatch repair.

On the other hand, some investigators believe that the development of instability may arise later in the process of neoplastic transformation and carcinogenesis, rather than being an early or initiating event. If a clonal population of potential cancer cells develops a selective growth advantage, it could accumulate mutations more rapidly owing to enhanced cell turnover without invoking instability as a necessary event. This would be consistent with the observations that loss of p53 function may occur late in the development of many tumors; loss of p53 function is thought to lead to genomic instability. On the other hand, experimental studies of radiation-induced mammary cancer and malignant transformation *in vitro* suggest that instability may be an early event that facilitates the occurrence of p53 mutations (Selvanayagam *et al.*, 1995; Syljuåsen *et al.*, 2001).

Additional clues as to the importance of instability in carcinogenesis arise from the study of certain rare genetic disorders characterized by chromosomal instability and a heritable predisposition to the development of cancer (Fearon, 1997; Sankaranarayanan and Chakraborty, 2001). The genes from these disorders have now been cloned and characterized, and their functions determined. They include: *ATM*, an important sensor of DNA damage; *NBS*, *BRCA1* and *BRCA2*, which code for proteins involved in DNA repair, specifically recombinational repair pathways; the seven cloned *FA* genes, which have been recently linked with *BRCA1*, *ATM* and *NBS1* in cell cycle checkpoint and DNA repair pathways (D'Andrea and Grompe, 2003); and the Bloom's syndrome (*BS*) gene, which codes for a helicase involved in DNA replication and repair. As is evident, the gene products associated with most of these disorders are involved in the signalling pathways for DNA replication and repair, as well as cell cycle control.

While these rare 'chromosomal breakage' syndromes affect only a small fraction of the population, they offer an important insight into the mechanisms for genomic instability and its association with cancer. The genes responsible for these genetic disorders are involved in pathways that ensure the fidelity of DNA replication and repair; in a number of cases, this involves the nonhomologous end joining (NHEJ) DNA repair pathway. Defects in this pathway in transgenic mice have been associated specifically with the induction of chromosomal instability (d'Adda di Fagagna *et al.*, 2001). Overall, the findings in these cancer-prone disorders offer a potential link between defects in DNA replication and repair, genomic instability and the development of cancer.

Genomic instability can be manifested by a variety of cellular changes. Most notable among these is chromosomal instability that includes two general types of chromosomal abnormalities. 'Unstable' aberrations are usually lethal to dividing cells and include changes such as dicentric, ring chromosomes and large deletions, whereas 'stable' aberrations can be transmitted through many generations of cell replication and include changes such as chromosomal loss and reduplication, reciprocal translocations and small deletions. Other manifestations of genomic instability may include an increased rate of spontaneous mutations, particularly in microsatellite sequences, and enhanced sensitivity to genotoxic agents. Interestingly, however, although most tumor cells show multiple chromosomal abnormalities characteristic of instability, they are not generally hypermutable.

Induction of genomic instability by radiation

While the general dogma held that genetic changes were a direct consequence of radiation exposure mediated by nonrepaired or misrepaired DNA damage in the irradiated cell, scattered early experiments suggested that the genetic consequences of irradiation might be delayed and appear in descendants of the irradiated cell after several generations of replication. For example, Demerec and Latarjet (1946) reported, by use of a bacterial mutation resulting in resistance to a bacteriophage as a marker, that two types of effects were observed as a consequence of exposure to either ionizing radiation or ultraviolet light. One was immediate, resulting in phenotypic changes in the treated cells, whereas the other was delayed leading to phenotypic changes appearing in the offspring of these cells after several rounds of cell division. Although they were unable to establish a mechanism for this effect, they hypothesized that radiation may induce two types of changes, one affecting the gene directly and the other affecting either the chromosomes or the cytoplasm in such a way as to increase the mutability of the gene system.

Early evidence for such an effect in mammalian cells was derived from studies of the malignant transformation *in vitro* of cell lines derived from mouse embryo fibroblasts. In the usual transformation experiments (Terzaghi and Little, 1976), irradiated cells were seeded at low density in multiple replicate Petri dishes. The dishes were returned to the incubator to allow the cells to proliferate to confluence, a process that required 20–25 generations of cell division. The confluent cultures were subsequently maintained in the incubator for an additional 4–6 weeks with regular medium changes to allow transformed foci to develop overlying the confluent monolayer. When the cells from these foci were isolated, expanded and injected subcutaneously into syngeneic animals, a high frequency of fibrosarcomas developed. The interpretation of these results followed classical radiobiological thinking; as a direct consequence of irradiation, potentially transformed cells

proliferated until the cultures reached confluence, after which the resultant clone developed morphological changes that resulted in a visible, transformed focus.

When the number of cells initially seeded and irradiated was varied, however, an unexpected result emerged. That is, the yield of transformed foci appeared to be independent of the number of cells seeded over a wide range of initial cell densities (Kennedy *et al.*, 1980). In fact, seeding of only a single irradiated cell under certain conditions yielded a high probability of giving rise to a transformed focus (Kennedy and Little, 1980). The classical theory that radiation transformed occasional cells as a direct consequence of unrepaired or misrepaired DNA damage would predict that if 1000 rather than 100 cells were seeded initially, 10 times as many transformed foci should arise. However, approximately the same number of foci arose under both conditions.

This phenomenon is perhaps best illustrated by experiments in which a large number of replicate dishes were seeded with only a single viable irradiated cell. This cell was allowed to proliferate until confluence was reached as in a standard transformation experiment and the cultures incubated for an additional 4–6 weeks. If transformation had been a direct consequence of damage to an occasional irradiated cell, one would have expected all the cells in that culture dish to be transformed. On the contrary, however, one or more transformed foci arose overlying a normal appearing monolayer in many of the dishes. These results led to the hypothesis that radiation had turned on a type of genetic instability in many of the irradiated cells (a high-frequency event), which enhanced the probability that one or more of their progeny would become transformed after many generations of cell replication. This second, transforming event was a rare one occurring in less than 10^{-6} cells at confluence. Detailed studies of the kinetics of transformation showed that this second transforming event had the characteristics of a mutation in that it arose with a constant frequency per cell per generation during the growth of cells to confluence (Kennedy *et al.*, 1984). Although the critical genetic event (transformation) was an indirect effect of the initial irradiation exposure, it did not occur in a radiation-damaged cell.

Other reports began to appear supporting the general hypothesis that radiation may induce a type of transmissible genetic instability facilitating the delayed appearance of genetic effects in the progeny of irradiated cells. For example, Fabre (1983) presented preliminary evidence for the transmission of enhanced recombinational activity over a number of generations in gamma-irradiated yeast, while Pampfer and Streffer (1989) reported that irradiation of the mouse embryo in the single cell blastocyst stage gave rise to an enhanced frequency of chromosomal aberrations in the somatic cells of the full grown fetus. Frank and Williams (1982) reported that X-irradiation of Chinese hamster (CHO) V-79 cells led to a persistent hypersensitivity of their descendants to the induction of mutations by PUVA

treatment (8-methoxy-psoralen plus long wavelength UV light) over many rounds of cell division.

Of particular interest was the observation that a persistent reduction in cloning efficiency occurred among the progeny of irradiated cells for many generations post irradiation (Seymour *et al.*, 1986). These authors proposed that this reduced cloning efficiency resulted from an enhanced frequency of 'lethal mutations' occurring in the progeny of irradiated cells. This phenomenon of delayed reproductive failure was soon confirmed by others (Gorgojo and Little, 1989; Mendonca *et al.*, 1989; Chang and Little, 1991). This finding is reminiscent of the earlier descriptions of heritable small colony formation in irradiated cells (Sinclair, 1964; Nias *et al.*, 1965), which likely reflect the same phenomenon. In some cellular systems, an increased rate of apoptotic cell death has been shown to accompany delayed reproductive failure (Jamali and Trott, 1996; Limoli *et al.*, 1998; Belyakov *et al.*, 1999). It has been proposed that oxidative stress perhaps consequent to enhanced, p53-independent apoptosis may contribute to the perpetuation of the instability phenotype in these populations (Limoli *et al.*, 1998; Redpath and Gutierrez, 2001).

About this time, Stamato *et al.* (1987) described the occurrence of delayed mutations in CHO cells following treatment with the alkylating agent ethylmethanesulfonate (EMS). Although many mutations occurred immediately after treatment, a residual elevation in the frequency with which new mutations arose was observed up to 10–12 cell generations later. The authors noted that EMS produces DNA lesions, which are only slowly removed from DNA. Thus, they surmised that the persistence of such DNA lesions might account for the delayed appearance of mutations. They further hypothesized that such long-lived DNA lesions, which have the potential of producing mutations many generations after their induction, could be a significant factor in the process of carcinogenesis. Shortly afterward, Little *et al.* (1990) reported a similar finding in CHO cells exposed to ionizing radiation. The critical DNA lesions produced by ionizing radiation are generally considered to be DNA double-strand breaks (DSBs); there has been no evidence to suggest that this type of damage may persist for 10–12 generations of cell replication.

The hypothesis that radiation may itself induce transmissible genomic instability over many cell generations has now been confirmed in a number of experiment systems for various genetic end points (Morgan *et al.*, 1996; Little, 1998; Baverstock, 2000; Romney *et al.*, 2001b). In terms of mutagenesis, approximately 10% of clonal populations derived from single cells surviving radiation exposure showed a significant elevation in the frequency of spontaneously arising mutations as compared with clonal populations derived from nonirradiated cells (Chang and Little, 1992; Little *et al.*, 1997). This increased mutation rate persisted for approximately 30 generations post irradiation, then gradually subsided. Interestingly, the molecular structural spectrum of these late-arising mutants resembles

those of spontaneous mutations in that the majority of them are point mutations (Grosovsky *et al.*, 1996; Little *et al.*, 1997), indicating that they arise by a different mechanism from that of direct X-ray-induced mutations that involve primarily deletions.

An enhancement of both minisatellite (Li *et al.*, 1992) and microsatellite (Romney *et al.*, 2001a) instability has also been observed in the progeny of irradiated cells selected for mutations at the *thymidine kinase* locus, further evidence that a subpopulation of genetically unstable cells arises in irradiated populations. It is of interest that instability as measured in minisatellite sequences of X-ray-transformed mouse 10T $\frac{1}{2}$ cells was markedly enhanced when the cells were grown *in vivo* as compared to prolonged cultivation *in vitro* (Paquette and Little, 1994). It has been reported recently that delayed reactivation of p53 may occur in the progeny of cells surviving radiation exposure (Suzuki *et al.*, 2003).

An enhanced frequency of nonclonal chromosomal aberrations was first reported in clonal descendants of mouse hematopoietic stem cells examined 12–14 generations after exposure to alpha radiation (Kadhim *et al.*, 1992). Persistent radiation-induced chromosomal instability has since been demonstrated in a number of other cellular systems (Sabatier *et al.*, 1992; Holmberg *et al.*, 1993; Marder and Morgan, 1993; Kadhim *et al.*, 1995; Little *et al.*, 1997; Ponnaiya *et al.*, 1997). Susceptibility to radiation-induced chromosomal instability differs significantly among cells from different strains of mice (Ponnaiya *et al.*, 1997; Watson *et al.*, 1997). It is now clear that genomic instability, both chromosomal and mutational, can be induced by high or low LET radiation (Little *et al.*, 1997; Belyakov *et al.*, 1999; Limoli *et al.*, 2000; Evans *et al.*, 2001) and in most, although not all, cell types (Dugan and Bedford, 2003). It has been shown recently that long-term instability can be induced by irradiation of cells with single α particles from a focused microbeam (Kadhim *et al.*, 2001), supporting earlier observations that the instability phenotype can be activated by low radiation doses, becoming saturated at higher doses (Kadhim *et al.*, 1995; Grosovsky *et al.*, 1996; Little *et al.*, 1997).

Of importance in terms of radiation protection is whether this phenomenon occurs *in vivo* and thus may be related to the induction of cancer. The transmission of chromosomal instability *in vivo* has been reported in several distinct experimental models (Pampfer and Streffer, 1989; Watson *et al.*, 1996; Ullrich and Davis, 1999; Watson *et al.*, 2001), although not in others (Bouffler *et al.*, 2001b). Evidence for transmissible instability in irradiated human populations is at present weak (Nakanishi *et al.*, 2001; Whitehouse and Tawn, 2001). While it has been suggested that instability induced in X-irradiated mouse hematopoietic stem cells may be related to the occurrence of the nonspecific genetic damage found in radiation-induced leukemias in these mice (MacDonald *et al.*, 2001), other work from this laboratory indicates that susceptibility to radiation-induced leukemia/lymphoma is generally separable from sensitivity to induced genomic instability (Boulton *et al.*, 2001).

One interesting model involves the induction of mouse mammary tumors by radiation. Sensitivity to tumor induction was found to be strain specific and to correlate with the induction of chromosomal instability in mammary epithelial cells irradiated *in vivo* (Ullrich and Davis, 1999). The induction of such instability was dose dependent. It was shown subsequently that the sensitive, cancer-prone mouse strain (BALB/c) was characterized by reduced expression of the DNA repair enzyme DNA-PKcs, leading to inefficient end joining of DNA DSBs induced by radiation (Okayasu *et al.*, 2000). This finding is of interest in relation to the evidence for the involvement of chromosome telomeres in radiation sensitivity and genomic instability (Bouffler *et al.*, 2001a). DNA-PKcs has been shown to play an essential role in telomere function and capping (Bailey *et al.*, 2001; Gilley *et al.*, 2001). A high frequency of telomere fusions occur in DNA-PKcs-deficient cells (Gilley *et al.*, 2001); the loss of telomeres has been associated with the development of chromosomal instability in cancer cells (Fouladi *et al.*, 2000). Transmissible instability might thus be a consequence of successive bridge-breakage fusion cycles resulting from telomere loss.

Bystander effects

The concept of a 'bystander effect' was elucidated by immunologists in the 1970s to denote the appearance of an immune response in cells in mixed populations that were not directly stimulated. In an early *in vitro* study (Kettman and Skarvall, 1974), spleen cells that were not stimulators of the allogeneic effect (CBA/H cells) could be stimulated to antibody synthesis when cultured with stimulator cells (CBA/J cells), which had been prevented from showing antibody responses by X-irradiation. There was a strong stimulation of the immune response in B cells of the CBA/H strain, even though these cells were 'bystanders' to the ensuing allogeneic response. These and other findings suggested that the effect was mediated by the release of a B-cell stimulatory activity that could stimulate a primary immune response (Kettman and Skarvall, 1974).

Previous studies *in vivo* had suggested that the allogeneic effect in mice was dependent upon direct cell to cell interaction (Rajewsky *et al.*, 1972). Johnson and Hersey (1976) produced a graft-versus-host reaction in a rat strain by the transfer of spleen cells from another strain; these recipients were subsequently injected with leukemic cells derived from the recipient rat strain. The authors observed a marked inhibition of the growth of this highly malignant leukemia, concluding that the occurrence of this 'bystander effect' suggests that nonspecific cytotoxic mechanisms may play a role in the inactivation of tumor cells *in vivo*.

There has been long-standing interest in the role of cell-to-cell interactions in biological processes, given impetus by the observations that structures termed 'gap junctions' develop between mammalian cells in contact

with each other. These gap junctions are membrane-associated protein channels, which allow the passive transfer of ions and small molecular weight molecules between cells. There has been particular interest in the role of gap junction-mediated intercellular communication (GJIC) in the process of carcinogenesis, as most cancer cells have lost the capacity for such communication (Yamasaki, 1990; Trosko and Ruch, 1998, 2001). GJIC is mediated by connexin proteins that are coded by a family of highly evolutionary-conserved genes called connexin genes (Yamasaki and Naus, 1996). The connexon, a hexameric unit of these proteins in one cell, couples with a corresponding connexon in an adjoining cell to join their cytoplasm and allow the passive transfer of small molecules between the cells. Thus, there are well-established mechanisms for crosstalk between and among cells in a population.

Evidence for a role of bystander effects in two important biological phenomena emerged in the early 1990s. In the first, it was observed that in a mixed population of irradiated and nonirradiated cells, genetic changes arose in those cells that had received no radiation exposure, suggesting that damage signals had been passed from the irradiated cells to the bystander cells in the population (Nagasawa and Little, 1992). The second phenomenon involved gene therapy experiments in which herpes simplex virus (HSV-1) was transduced into tumor cells rendering them sensitive to the cytotoxic effects of the antiviral drug ganciclovir (Freeman *et al.*, 1993). In this study, the population of cultured cells was found to contain only 10% HSV-positive cells; however, ganciclovir was also found to be lethal to the HSV-negative bystander cells.

Very shortly thereafter, a similar phenomenon was demonstrated in various tumor cell systems (Smythe *et al.*, 1994), including those derived from brain tumors (Kato *et al.*, 1994; Wu *et al.*, 1994). The effect appeared to require cell-to-cell contact (Wu *et al.*, 1994), rather than being mediated by the release of a soluble factor into the medium. It was later found that GJIC was directly involved in the phenomenon by the transfer of toxic metabolites of ganciclovir from the HSV-expressing cells to the surrounding bystander cells (Wygodka *et al.*, 1997; Mesnil and Yamasaki, 2000). This phenomenon has offered hope for targeted gene therapy of cancer, as it has become evident that the suicide gene would not have to enter each individual cell, a prospect that is not realistic, in order to become effective (Touraine *et al.*, 1998; Mesnil and Yamasaki, 2000).

Radiation-induced bystander effect

The first evidence for a bystander effect of ionizing radiation arose from a study of the induction of sister chromatid exchanges (SCEs) in monolayer cultures exposed to very low fluences of α particles, fluences whereby only a small fraction of the cells in the population received any radiation exposure (Nagasawa and Little, 1992). An enhanced frequency of SCE was

observed in 20–40% of the cells, whereas only about 0.1–1.0% of the cell nuclei were actually traversed by an α particle. This finding was later confirmed in studies with normal human lung fibroblasts (Deshpande *et al.*, 1996), and evidence presented to suggest that the phenomenon involved the secretion of cytokines or other factors by the irradiated cells leading to the upregulation of oxidative metabolism in the bystander cells (Narayanan *et al.*, 1997; Lehnert *et al.*, 1997).

It was shown subsequently that an enhanced frequency of specific gene mutations also occurs in bystander cells in cultures exposed to very low fluences of α particles (Nagasawa and Little, 1999). As a result, the induced mutation frequency per alpha particle track increased unexpectedly at low fluences, where bystander as well as directly irradiated cells are at risk for the induction of mutations. This leads to hyperlinearity of the dose–response curve in the low-dose region (Nagasawa and Little, 1999). Interestingly, the mutations arising in bystander cells were primarily point mutations, whereas those occurring in directly irradiated cells were largely partial and total gene deletions (Huo *et al.*, 2001). The high frequency of point mutations in bystander cells is reminiscent of the spectrum found in unstable progeny of irradiated cells where point mutations also predominated (Little *et al.*, 1997).

It was reported in earlier studies that α particle irradiation could induce the intracellular generation of reactive oxygen species (ROS), including the superoxide anion and hydrogen peroxide (Narayanan *et al.*, 1997). This ROS response did not require direct nuclear irradiation, as an ROS response was induced in nonirradiated cells incubated with conditioned medium from α -irradiated cultures. On the other hand, based on the lack of a suppressive effect of DMSO, it has been suggested that ROS are not involved in the mutagenic response of bystander cells in monolayer cultures of the human-hamster AL hybrid cell line following microbeam irradiation (Zhou *et al.*, 2000).

In recent experiments (Azzam *et al.*, 2002), the role of oxidative stress in modulating signal transduction and micronucleus formation in bystander cells was examined in confluent monolayer populations of human diploid cells exposed to low fluences of α particles. The results support the hypothesis that superoxide and hydrogen peroxide produced by flavin-containing oxidase enzymes mediate the activation of several stress-inducible signalling pathways as well as micronucleus formation in bystander cells (Azzam *et al.*, 2002). These include the p53 damage-response pathway as well as the MAP kinase family of signalling pathways. It has also been reported that nitric oxide may initiate intercellular signal transduction pathways influencing the bystander response to radiation (Matsumoto *et al.*, 2001; Shao *et al.*, 2002). This upregulation of oxidative stress in bystander cells is reminiscent of the enhanced oxidative stress associated with radiation-induced genomic instability (Limoli *et al.*, 2001; Redpath and Gutierrez, 2001); it is tempting to speculate that the preponderance of point mutations in both conditions is the result of oxidative damage.

Changes in gene expression, notably upregulation of the p53 damage-response pathway, have also been shown to occur in bystander cells in monolayer cultures exposed to very low fluences of alpha particles (Azzam *et al.*, 1998). As was observed in the gene targeting experiments, this radiation-induced bystander effect also appeared to be mediated by gap junction intracellular communication (Azzam *et al.*, 2001). The observation that p53 in bystander cells was phosphorylated on serine 15 (Azzam *et al.*, 2001) suggests that the upregulation of p53 in bystander cells is a consequence of DNA damage. The activation of the p53 damage-response pathways in bystander cells was confirmed by *in situ* immunofluorescence studies in which it was shown that upregulation of p21^{Waf1} occurred in clusters of cells in the monolayer population, whereas only 1–2% of the cell nuclei were traversed by an α particle. Evidence was also presented for the activation of MAP K proteins and their downstream effectors in bystander cells (Azzam *et al.*, 2002); this is of particular interest in terms of the observation that membrane signalling may be involved in the bystander effect in monolayer cultures (Nagasawa *et al.*, 2002).

A significant advance in the study of radiation-induced bystander effects has come from the availability of precision, charged-particle microbeams, which allow the irradiation of substructures of single cells in a monolayer population with one or more α particles. As well as manipulating the number of particles with which targets cells are irradiated, the fraction of cells in the population can also be varied. Consistent with the findings noted above for broad field irradiation, nuclear irradiation of targeted cells led to the occurrence of an enhanced frequency of mutations (Zhou *et al.*, 2000, 2001) as well as of malignant transformation (Sawant *et al.*, 2001) in bystander cells in monolayer populations. In addition, evidence has been presented for an enhanced frequency of micronucleus formation and apoptosis in bystander cells (Prise *et al.*, 1998; Belyakov *et al.*, 2001) as well as a reduction in clonogenic survival (Sawant *et al.*, 2002).

As noted earlier, DNA damage in bystander cells appears to differ from that occurring in directly irradiated cells; whereas the mutations induced in directly irradiated cells were primarily partial and total gene deletions, over 90% of those arising in bystander cells were point mutations (Huo *et al.*, 2001). This would be consistent with the evidence that oxidative metabolism is upregulated in bystander cells, and has led to the hypothesis that the point mutations are a result of oxidative base damage occurring in bystander cells (Huo *et al.*, 2001). A similar mechanism has been proposed for the observation that localized cytoplasmic exposure from a microbeam irradiator led to a significant increase in the frequency of point mutations, as it appeared to involve the generation of ROS (Wu *et al.*, 1999).

It has been reported recently that bystander cells defective in the NHEJ pathway including mouse knock-out cell lines for Ku80, Ku70 and DNA-PKcs are extremely sensitive to the induction of mutations and chromosomal aberrations (Little *et al.*, 2003; Nagasawa

et al., 2003). These studies also provided evidence that once a cell received a bystander signal, it was refractory to additional signals arising from other irradiated cells. Interestingly, the mutations in these repair-deficient bystander cells were primarily the result of partial and total gene deletions (Nagasawa *et al.*, 2003), in contrast to those occurring in wild-type bystander cells. The marked sensitization of repair-deficient bystander cells to the induction of mutations and chromosomal aberrations may be a consequence of unrejoined DNA DSB occurring as a result of clustered damage arising from opposed oxidative lesions and single-strand breaks, whereas mutations in wild-type cells in which DSB are efficiently repaired arise primarily from oxidative base damage.

Another technique for studying bystander effects involves mixing irradiated with nonirradiated cells, whereby the nonirradiated bystander cells can be identified and examined. When cells labelled with ^{125}I dUrd were mixed with unlabelled cells and multicellular clusters formed by centrifugation, a decrease in clonogenic survival occurred in the unlabelled cells that appeared to depend upon GJIC (Bishayee *et al.*, 1999; Howell and Bishayee, 2002). A similar effect was observed *in vivo* when radiolabelled tumor cells were injected into nude mice and tumor growth was employed as an end point (Xue *et al.*, 2002). In another series of experiments (Watson *et al.*, 2000), a mixture of irradiated and nonirradiated bone marrow cells was transplanted into mice. Chromosomal instability was observed in the progeny of the nonirradiated hematopoietic cells, providing a link between the bystander effect of ionizing radiation and the induction of genomic instability *in vivo*.

Distant 'bystander' effects: medium transfer experiments

The protocols described in most of the foregoing experiments involved either confluent or subconfluent monolayer cultures or mixed cell populations in which irradiated and nonirradiated cells were allowed to grow together either *in vitro* or *in vivo*. In both cases, the bystander and targeted cells were in physical contact. This physical contact allows ions and small molecules to be passed between cells through gap junctions. There are, however, provocative findings indicating that bystander effects may be mediated by damage signals released into the culture medium by irradiated cells. Thus, incubation of nonirradiated cells with conditioned medium from irradiated cultures may lead to biological effects in these 'bystander' cells. Such a mechanism would also explain the bystander effects observed in microbeam experiments when targeted cells are irradiated in sparse monolayer cultures (Prise *et al.*, 1998; Belyakov *et al.*, 2001).

There is a long history suggesting that irradiated individuals may release into their plasma clastogenic factors that will induce chromosomal damage when transferred to cultured cells from unirradiated donors

(Goh and Sumner, 1968; Hollowell and Littlefield, 1968; Scott, 1969). Such factors have since been reported to occur in various populations of exposed individuals (Emerit *et al.*, 1997), as well as to be associated with a variety of hereditary chromosomal breakage syndromes and pathological conditions where they are thought to be biomarkers of oxidative stress (Emerit, 1998).

Although evidence for these factors has been accumulating over the past three decades, their exact nature has proven elusive, as well as the mechanisms by which they may be continually produced for many months or years after radiation exposure. In an experimental study in rats, for example, clastogenic activity persisted in the circulating plasma of irradiated animals for the 10-week duration of the study, and was not abrogated by dilution with nonirradiated serum (Faguet *et al.*, 1984). Irradiation resulted in the rapid appearance of clastogenic activity in the plasma of the rats; this activity was not due to chemical-physical changes of normal plasma components, but resulted from circulating factors released by irradiated cells. Serum irradiated *in vitro* was not clastogenic.

These studies have been reviewed in detail by Mothersill and Seymour (2001) and by Morgan (2003). Mothersill and Seymour (1998) reported that the exposure of cells in cultures or explants of tissue to gamma radiation doses as low as 1 cGy can lead to the release of factors into the medium by the irradiated cells; when this conditioned medium was transferred to nonirradiated cells, their cloning efficiency was reduced associated with increased levels of apoptotic cell death. This phenomenon was associated with early changes in mitochondrial membrane permeability and the induction of ROS (Lyng *et al.*, 2001).

Lehnert and co-workers (Lehnert *et al.*, 1997; Narayanan *et al.*, 1997) also showed by medium transfer experiments that extracellular factors including ROS were released by alpha particle-irradiated cells, leading to the induction of SCEs in nonirradiated cells. Furthermore, they showed that incubation of nonirradiated cells with irradiated culture medium alone led to an enhancement in SCE and ROS in these 'bystander cells'. On the other hand, other workers have shown this to be a cell-mediated response finding no effect of irradiated medium alone (Belyakov *et al.*, 2001; Zhou *et al.*, 2002). Furthermore, Zhou *et al.* (2002) reported that while irradiated cells released cytotoxic factors into the culture medium that killed nonirradiated cells, such factors had little or no effect on mutation induction.

It has also been reported that conditioned medium from alpha particle-irradiated cells can stimulate cell proliferation in nonirradiated cells; this promitogenic response was attributed to an increase in TGF β_1 acting as a mediator of the increased intracellular ROS observed in bystander cells (Iyer *et al.*, 2000). An increase in protein levels of AP endonuclease, a redox and DNA base excision repair protein, was also measured in bystander cells but not in directly irradiated cells. The promitogenic response was associated with an increase in cloning efficiency (Iyer and Lehnert, 2002).

This finding is of interest as it suggests a possible beneficial bystander effect related to an increase in DNA repair capacity and clonogenic survival, and is thus reminiscent of the feeder layer effect for clonogenicity as well as the earlier finding that incubation with conditioned medium from plateau-phase cultures facilitated the repair of potentially lethal radiation damage (Little, 1971).

Another provocative finding (Nagar *et al.*, 2003) has arisen from preliminary studies in which conditioned medium was harvested from clonal cell populations, derived from single cells surviving radiation exposure, which showed persistent genomic instability. The conditioned medium from certain unstable clones was found to be highly cytotoxic to normal cells; the effect was lost by heating or freezing the medium, but not by diluting it with a fresh medium.

Overall, a clear picture has yet to emerge from the experience with medium transfer experiments. There is convincing evidence that factors are released into the medium by irradiated cells such that incubation with conditioned medium harvested from irradiated cultures can lead to changes in the viability of nonirradiated cells. The results from different laboratories, however, are not entirely consistent. Some workers report that incubation with such a conditioned medium leads to a reduction in cloning efficiency of the recipient cells (Lyng *et al.*, 2002; Sawant *et al.*, 2002), while others find that it is enhanced (Iyer and Lehnert, 2002) or dependent on cell type (Mothersill and Seymour, 1997). The effect of irradiated medium alone is particularly controversial. In terms of genetic effects, one laboratory describes a bystander effect for SCEs in conditioned medium transfer experiments (Lehnert *et al.*, 1997), whereas another finds little or no evidence for a bystander mutagenic effect under similar conditions (Zhou *et al.*, 2002). The effect appears likely to be mediated by cytokines or ROS, but the exact nature of the factor or factors responsible for the biological effects that arise in the nonirradiated, bystander cells remains to be elucidated.

Future directions

There is now strong evidence from studies in a number of different cell culture systems that these two phenomena are real. That is, genetic effects of radiation may arise in cells that in themselves receive no radiation exposure as a result of signals that are transmitted from irradiated cells. In the first case, these signals are transmitted to the progeny of the irradiated cell, whereas in the second they are passed from the irradiated cell to nonirradiated cells in the population. In the latter case, this may involve direct intercellular communication through gap junctions or be a consequence of factors released into the cellular medium.

While it is tempting to speculate that these phenomena may be of importance in the carcinogenic effects of radiation, considerable additional information is necessary before such nontargeted effects can be incorporated

into models of radiation-induced cancer. Although provocative preliminary data from mouse models suggest that both these phenomena may occur *in vivo*, these studies need to be extended to include other model systems to show both the generality of these responses and their relationship to radiation carcinogenesis. Also of importance is more precise knowledge of the dose–response relationships, particularly for sparsely ionizing (low LET) radiation such as most gamma- and X-rays. A corollary of this is the extent to which they occur as a consequence of very low radiation doses as are associated with environmental exposures.

Existing data suggest that radiation-induced genomic instability may result from fairly low doses, in the range of 0.1 Gy, and saturate at higher doses. However, this needs more rigorous investigation. On the other hand, there have been relatively few studies of the bystander effect induced by low LET radiation, and these have generally involved quite high doses (in the range of 1–10 Gy). Clearly, the bystander effect can be induced by very low fluences of high LET alpha particles, although it should be noted that each alpha particle traversal of a nucleus leads to a significant dose to the irradiated cell (0.1–0.2 Gy). Furthermore, we have no information as yet on the influence of dose rate for α particle-induced bystander effects; human exposures such as those arising from residential Radon involve very low dose rates.

Of greatest importance, however, will be a better understanding of the molecular mechanisms involved in these two phenomena. In neither case do we know the nature of the signal that is transmitted from the irradiated cell to its progeny or to bystander cells, and how these signals are generated. Second, only scattered information is available that allows us to formulate preliminary hypotheses as to how the instability phenotype may be maintained over many generations of cell replication. Finally, in neither case do we have firm data to indicate how long these phenotypes may persist.

One common observation in both phenomena has been evidence for the upregulation of oxidative metabolism in the cells at risk, be they unstable progeny cells or bystander cells. In both cases, molecular structural analyses of the induced mutations indicate that they are consistent with those one would expect from oxidative stress. The nature of the signal leading to this upregulation of oxidative metabolism, and how it may be maintained in the case of the instability phenotype, remains to be answered.

A clear understanding of the cellular and molecular mechanisms for these phenomena, along with clear evidence of their occurrence *in vivo* and as a consequence of low-dose radiation exposures, will allow us to assess their importance in the health effects of exposure to ionizing radiation.

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