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Commentary

Radiation-induced mutations in unirradiated DNA

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Until recently, it has been axiomatic that genetic alterations attendant to radiation exposure are attributable to radiation-induced DNA damage. According to this dogma, DNA damage occurs during or very shortly after irradiation of the nuclei in target cells, and the potential for genetic consequences is fixed within one or two cell generations. Several lines of evidence have now emerged that challenge each of the essential assumptions of this paradigm, and that are rapidly broadening views regarding the mechanisms by which ionizing radiation can produce genetic alterations. In addition to fundamental mechanistic interest in these new pathways for radiation-induced genetic change, there may also be considerable ramifications for the development of models used to estimate the risks of low-dose radiation exposure.

A report in a recent issue of the *Proceedings* by Hei and coworkers (1) represents the latest and most direct challenge to presumptions that radiation-induced genetic alterations require damage within the nucleus. Their experiments demonstrate that cytoplasmic irradiation with very low fluences of α-particles induces mutations in a human–hamster hybrid cell line. This observation of mutagenesis after low-fluence α-irradiation of the cytoplasm (1) builds on previous evidence for a "bystander effect" (2–4), which refers to the induction of genetic alterations in cells that are not themselves irradiated but that are neighboring to cells actually traversed by an α-particle. The current report (1) confirms suggestions from bystander effect studies that the relevant cross-section for mutagenic hits is much larger than the nucleus and may be expected to further stimulate rapidly growing interest in elucidation of the underlying mechanisms.

The investigation by Wu *et al.* (1) was made possible by the availability of a microbeam irradiation facility able to deliver a specific number of α-particles to precise locations within a target cell (5). This device, together with the physical characteristics of α-particles, enables the cytoplasm of individual cells to be irradiated without concomitant exposure of the nucleus. Because of their relatively high mass, α-particles have a short track length and deposit all of their energy within a dense sphere of ionizations very close to the original site of impact. One previous report (6) used microbeam α-irradiation to demonstrate that the induction of micronucleated or apoptotic human fibroblasts exceeded the number of cells traversed by an α-particle. Other bystander effect studies have used an inferential approach to conclude that genetic effects could be induced without direct nuclear irradiation. For example, in the initial report of the bystander effect by Nagasawa and Little (2), cell cultures were exposed to a very low fluence of α-particles, providing for traversal of approximately 1% of the cells. These conditions resulted in an increase in sister chromatid exchanges in 30% to 50% of the cells in the culture, providing the initial basis for the conclusion that the cross-section for genetic damage by α-particles is much larger than the nucleus.

Using the microbeam α-irradiation facility, Wu *et al.* (1) report that the induced mutant fraction produced by low-fluence cytoplasmic α-irradiation is only 2- to 3-fold lower than for microbeam irradiation of the nucleus with the same number of α-particles (5). Mutagenesis by cytoplasmic irradiation was induced even by a single particle traversal but quickly reached a maximal plateau after cytoplasmic hits by four to eight particles. In contrast, nuclear irradiation-induced mutations increase linearly with dose over a wide range. The spectrum of recovered mutations also differs depending on whether irradiation occurred in the nucleus or cytoplasm (1). Nuclear irradiation mutants are predominated by large deletions (5), whereas mutants induced by cytoplasmic irradiation consist of localized changes, perhaps reflecting base damage by reactive oxygen species (1). Therefore, particle traversals of the cytoplasm contribute a significant proportion of overall mutant yield in the very low-dose region by an apparently distinct mechanism. Importantly, because of the differences in dose-response functions, the cytoplasmic pathway for mutagenesis may be negligible after high doses that are often used as a starting point for the extrapolation of low-dose risks.

The bystander effect, operationally defined as the induction of genetic alterations in unirradiated nuclei, may reflect the occurrence of at least two separate mechanisms for the promulgation of damage from irradiated cells to unirradiated neighbors. One line of evidence (7) indicates that the bystander effect is dependent on gap junction intercellular communication, which stimulates a p53-mediated damage-signaling pathway. A separate series of studies (8–10) suggests a second mechanism in which irradiated cells secrete cytokines or other factors that act to increase intracellular levels of reactive oxygen species in unirradiated cells.

Evidence for a p53-mediated signaling pathway in the bystander effect was first reported by Hickman *et al.* (3) in a study of low-dose α-irradiation of rat lung epithelial cells. Flow cytometric analysis of the fraction of cells with elevated levels of p53 protein detected increased expression in a higher proportion of cells than were hit by an α-particle. A role for gap junction-mediated communication in inducing this signaling pathway was then reported by Little and coworkers (7), who investigated the response of confluent cultures of primary human diploid fibroblasts to low fluences of α-irradiation. Although only 5% of nuclei were traversed by a particle, an overall 3- to 4-fold increase was observed in p53 and $p21^{waf1}$ protein levels in Western blot analyses. In contrast, the increased level of expression was eliminated by pretreatment with the gap junction intercellular communication inhibitor lindane. Modulation of expression of cell-cycle related genes, including $p34^{cdc2}$, cyclin B, and rad51 was also observed under similar conditions. An elegant *in situ* immunofluorescence was then used to observe the patterns of expression in cultures exposed to a low fluence of α-particles resulting in traversal of approximately 2% of the nuclei. Increased expression of $p21^{waf1}$ was observed in a clustered pattern; some groups of cells displayed elevated levels of $p21^{waf1}$, whereas other groups of cells in the same culture remained at background levels of

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expression. These results indicate that cell contact is critical in the promulgation of damage and do not suggest a role for a secreted difusible factor that would be expected to produce a more homogeneous increase in expression of $p21^{waf1}$.

The second bystander effect mechanism is mediated by secretion of factors into the culture medium (8–10). A recent report suggests that this mechanism does not depend on communication through gap junctions formed between cells in contact (11). Lehnert and coworkers originally demonstrated that culture medium harvested from cells irradiated with low fluences of α-particles can induce an increase in sister chromatid exchanges when used to incubate unirradiated test cells (8). Because the originally irradiated medium is removed in this experimental protocol, factors in the harvested medium that mediate the bystander effect could originate only by secretion from the irradiated cells. This protocol can be used to observe a bystander effect for periods of at least 24 hr after the radiation exposure, suggesting a continual production and secretion of factors until the return of cellular DNA damage response to basal levels. Factors in the harvested medium also induce an elevation in intracellular levels of reactive oxygen species, including superoxide and hydrogen peroxide, and these are postulated to be critical intermediates in the promulgation of damage (9, 10). Elimination of the bystander effect by heat treatment of the harvested medium or by treatment of irradiated cells with protein synthesis inhibitors indicates that the secreted factors are proteins (9, 10). The bystander effect does not demonstrate a linear relationship to dose (2, 4, 8–10). Rather, it is maximally induced by very low doses, suggesting a switch mechanism for the activation of a generalized cellular response after damage to a large nonnuclear cellular target.

The induction of nuclear mutations by cytoplasmic α-irradiation (1) is directly related to the bystander effect on a conceptual level, because both involve the induction of genetic change in unirradiated nuclei. Beyond this syllogism, the cytoplasmic pathway for mutagenesis resembles the secreted factor bystander effect mechanism in the quickly saturated dose-response relationship and in the implication of reactive oxygen species as mediators of the response. These similarities suggest a fundamental mechanistic relationship or identity; it is possible that the two processes are only operationally distinguished by experimental design. The resemblance of these nonnuclear processes for genetic change to cellular UV-induced damage response (12, 13) also warrants some consideration. The UV response is maximally induced by low fluences of UV light and recognizes damage at or near the plasma membrane rather than in the nucleus. Furthermore, it involves the activation of secreted intercellular signaling proteins, i.e., cytokines. Finally, the UV response also appears to be elicited by oxidative stress, because it can be inhibited by reactive oxygen species scavengers (12, 13).

The question remains how the DNA is ultimately damaged. Wu *et al.* (1) have suggested that long-lived free radicals that are generated by cytoplasmic irradiation could migrate to the nucleus and induce oxidative damage to the DNA. A similar hypothesis has also been proposed by Lehnert and Goodwin (8), who specifically suggested that the superoxide anion has sufficient stability to permit diffusion to the nucleus. A speculative alternative possibility may be found in the induction of the cellular damage response itself. It may be possible that reduced replication fidelity or increased recombinational activity occurs even on undamaged DNA when a p53-mediated DNA damage response pathway is activated.

A great deal of recent attention has also been focused on radiation-induced genomic instability, defined here as a persistent elevation in the rate of genetic change within a clonal population (14–18). There is no evidence that the bystander effect persists for many generations; the evidence suggests that it would persist only until the irradiated cells returned to the basal levels of DNA damage response. On the other hand, one recent report (20) has demonstrated that persistent genomic instability can be induced via a bystander mechanism. This suggests that the target for α-radiation-induced genetic alterations is increased in two ways, when compared with conventional understanding of DNA damage and mutagenesis. The initial cross-section for damage is increased by the bystander effect, and cells that are affected by the bystander mechanism may remain at an increased risk of genetic change for many generations.

Widespread interest in the bystander effect has also been specifically stimulated by the implications for α-radiation-induced lung cancer in human populations. Exposure to α-particles, primarily from radon gas in residential settings (20, 21), accounts for as much as 50% of background radiation dose (20) and may be the causative agent in up to 10% of all lung cancers and 30% of lung cancers in nonsmokers (22). As in bystander effect experiments, exposure to background levels of α-radiation in the respiratory epithelium results in a rare and stochastic distribution of nuclear traversals; most cells receive no direct irradiation. Many risk estimates for α-radiation-induced carcinogenesis incorporate the presumption that the number of cells at risk in a target tissue is defined by the fraction whose nucleus is traversed by a particle (20, 21). The findings of Wu *et al.* (1), together with the rapidly growing literature on the bystander effect, demonstrate that these presumptions about the target for genetic and carcinogenic damage may now need to be reconsidered.

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