

# Genomic instability and bystander effects induced by high-LET radiation

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An understanding of the radiobiological effects of high-linear energy transfer (LET) radiation is essential for radiation protection and human risk assessment. Ever since the discovery of X-rays was made by Röntgen more than a century ago, it has always been accepted that the deleterious effects of ionizing radiation, such as mutation and carcinogenesis, are due mainly to direct damage to DNA. With the availability of a precision single-particle microbeam, it is possible to demonstrate, unequivocally, the presence of a bystander effect with many biological end points. These studies provide clear evidence that irradiated cells can induce a bystander mutagenic response in neighboring cells not directly traversed by  $\alpha$ -particles, and that cell–cell communication processes play a critical role in mediating the bystander phenomenon. Following exposure to high-LET radiation, immortalized human bronchial (BEP2D) and breast (MCF-10F) cells have been shown to undergo malignant transformation through a series of successive steps, before becoming tumorigenic in nude mice. There is a progressive increase in genomic instability, determined either by gene amplification or allelic imbalance, with the highest incidence observed among established tumor cell lines, relative to transformed, nontumorigenic and control cell lines.

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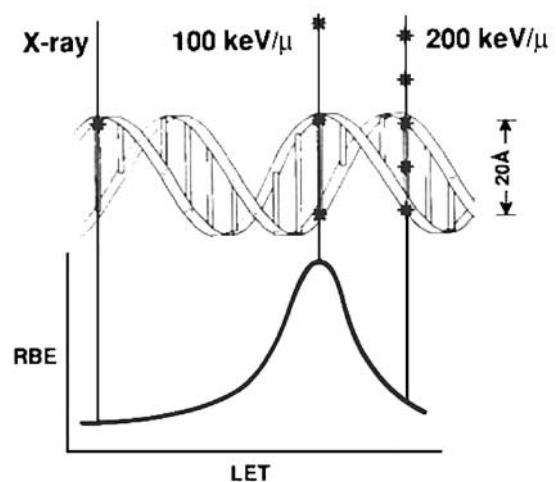
**Keywords:**  $\alpha$ -particles; microbeam; bystander effects; gene amplification; allelic imbalance

## Introduction

Many concepts in radiation biology are more clearcut and easier to understand when the source of radiation considered is of high linear energy transfer (LET) – specifically when it is an  $\alpha$ -particle. The traversal of a mammalian cell by a single  $\alpha$ -particle is sufficient to produce profound biological consequences that are readily detectable by currently available technology; that is not always the case for a single electron.

The relative biological effectiveness (RBE) of a given radiation is a function of the LET. As the LET increases, the RBE increases and reaches a maximum at about 100 keV/ $\mu$ m. Beyond this value for the LET, the RBE again falls to lower values. The LET at which

the RBE reaches a peak is much the same for a wide range of mammalian cells, from mouse to human, and is the same for mutation as an end point as for cell killing. It is of interest to ask why radiation with this LET is optimal in terms of producing a biologic effect. At this density of ionization, the average separation between ionizing events just about coincides with the diameter of the DNA double helix (20 Å or 2 nm). Radiation with this density of ionization has the highest probability of causing a double-strand break by the passage of a single-charged particle, and double-strand breaks are the basis of most biologic effects. This is illustrated in Figure 1. In the case of X-rays, which are more sparsely ionizing, the probability of a single track causing a double-strand break is low, and, in general, more than one track is required. As a consequence, X-rays have a low biologic effectiveness. At the other extreme, much more densely ionizing radiations (with an LET of 200 keV/ $\mu$ m, for example) readily produce double-strand breaks, but energy is ‘wasted’ because the ionizing events are too close together. Since RBE is the ratio of doses producing equal biologic effect, this more densely ionizing radiation has a lower RBE than the optimal LET radiation.



**Figure 1** Diagram illustrating why radiation with a LET of 100 keV/ $\mu$ m has the greatest RBE for cell killing, mutagenesis, or oncogenic transformation. For this LET, the average separation between ionizing events coincides with the diameter of the DNA double helix (i.e., about 20 or 2 nm). Radiation of this quality is most likely to produce a double-strand break from one track for a given absorbed dose

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The more densely ionizing radiation is just as effective *per track*, but less effective per unit dose. It is possible, therefore, to understand why RBE reaches a maximum value in terms of the production of double-strand breaks, because the interaction of two double-strand breaks to form an exchange-type aberration is the basis of most biologic effects. In short, the most biologically effective LET is one where there is a coincidence between the diameter of the DNA helix and the average separation of ionizing events. Radiations having this optimal LET include neutrons of a few hundred kiloelectron volts, as well as low-energy protons and  $\alpha$ -particles.

It is either a remarkable fact or a remarkable coincidence that as life evolved on earth, the most common radiations to which it was exposed have an LET that make them the most efficient at causing double-strand breaks and producing mutations.

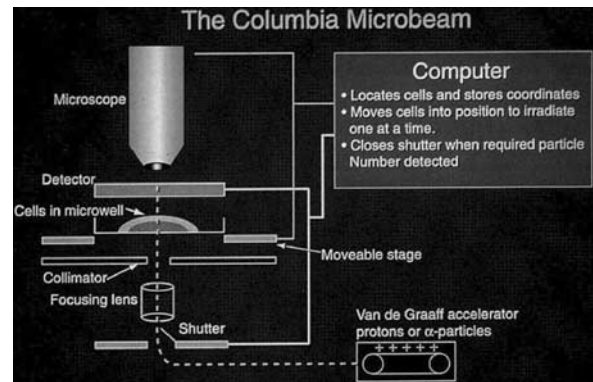
### The bystander effect

Generations of students in radiation biology have been taught that heritable biological effects require direct damage to DNA. In fact, evidence has been available for many years that this simple statement is not strictly true.

As early as the 1940s, there were reports that the inactivation of biological entities may be brought about equally by ionizations produced within the entity, or by the ionization of the surrounding medium (Dale, 1940, 1942, 1943; Lea *et al.*, 1944). Kotval and Gray (1947) had shown that  $\alpha$ -particles which pass close to the chromatid thread, as well as those which pass through it, have a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges.

The term used today to describe such phenomena is 'the bystander effect', a name borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (Cheng *et al.*, 1999, for example).

In the radiation field, it has come to be loosely defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are. Interest in this effect was sparked by the report of Nagasawa and Little (1992) that, following a low dose of  $\alpha$ -particles, a larger proportion of cells showed biological damage than were estimated to have been hit by an  $\alpha$ -particle; specifically 30% of the cells showed an increase in sister chromatid exchanges, even though less than 1% were calculated to have undergone a nuclear traversal. The number of cells hit was estimated by a calculation, based on the fluence of  $\alpha$ -particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature, since it was not possible to know on an individual basis which cells were hit and which were not.



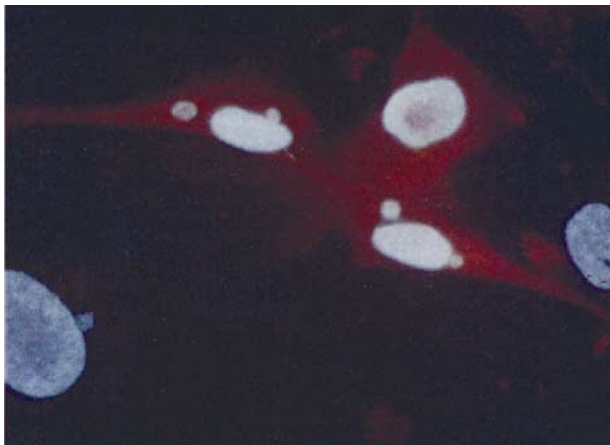
**Figure 2** Schematic drawing showing the overall layout of the Columbia University microbeam facility and the integrated control system

This situation has been remedied by the development of a single-particle microbeam, a device capable of putting a predefined exact number of  $\alpha$ -particles (including one) through a particular cell nucleus.

The layout of the Columbia University microbeam is illustrated in Figure 2. A detailed description has been described previously (Randers-Pehrson *et al.*, 2001). Briefly, each cell attached in a monolayer to a thin polypropylene base of a cell culture dish is identified and located by using an image analysis system, and its coordinates are stored in a computer. The cell dish is then moved under computer control such that the centroid of each cell nucleus (or a region of the cytoplasm remote from the nucleus, according to the plan of the particular experiment) is in turn positioned over a highly collimated shuttered beam of  $\alpha$ -particles generated by a Van de Graaff accelerator. Each cell is exposed to a predetermined exact number of  $\alpha$ -particles and a detector positioned above the cell signals, to close the accelerator shutter when the desired number of particles (e.g., one) is recorded, after which the next cell is moved over the beam. Continuous developments in hardware and software have increased the microbeam throughput so that individual cells can be irradiated, one at a time, in about 1 s; this permits sufficient cells to be exposed for mutation and oncogenic transformation studies. Earlier, microbeam systems were much slower, so that biologic studies were limited to chromosomal aberrations, which can be scored in a smaller number of cells. Using this device, the bystander effect for  $\alpha$ -particle irradiation has been demonstrated for a variety of biological end points.

### Micronuclei in normal human fibroblasts

Perhaps, the most direct and most dramatic demonstration of the bystander effect involves the observation of micronuclei in irradiated human fibroblasts. Cells of one population were lightly stained with cyto-orange, a cytoplasmic vital dye, while cells of another population were lightly stained blue with a nuclear vital dye. The

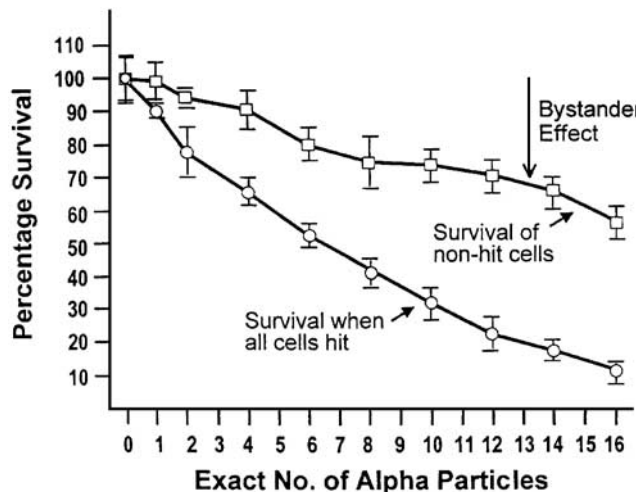


**Figure 3** The bystander effect with human fibroblasts. Cells of one population were stained with the vital nuclear dye Hoechst 3342 (blue fluorescence), and cells of another population were stained with the vital cytoplasmic dye cell tracker orange (orange fluorescence), and mixed at a ratio of 1:1. Only blue nuclei were microbeam irradiated with  $\alpha$ -particles; the orange cells were thus 'bystanders.' Cells were fixed and stained 44 h after exposure to radiation. A micronucleus is clearly visible in an orange (non hit) cell (courtesy of Dr Charles Geard)

two cell populations were mixed and allowed to attach to the culture dish, and the computer controlling the accelerator was programmed to irradiate only blue-stained cells with 10  $\alpha$ -particles directed at the centroid of the nucleus. The cells were fixed and stained 48 h later, at which time micronuclei and chromosome bridges were visible in a proportion of the nonhit (i.e. orange-stained) cells (Figure 3). This is an astonishing demonstration of the bystander effect, because the development of micronuclei implies significant chromosome damage and rearrangement, which is clearly visible in nonhit cells that have been fixed *in situ*.

### Cell lethality

Lines of hygromycin- and neomycin-resistant V79 cells were produced. Before exposure, the hygromycin-resistant cells were stained with a low concentration of a vital nuclear dye. They were then plated in microwells in the proportion, nine neomycin-resistant for every one hygromycin-resistant cell. The computer was programmed to irradiate only 10% of cells stained with a nuclear dye with various numbers of  $\alpha$ -particles from 1 to 16, aimed at the centroid of the nucleus. The cells were then removed and cultured for survival in the appropriate growth media, which made it possible to obtain survival curves for hit and nonhit cells (Sawant *et al.*, 2002). The data are shown in Figure 4. There is a considerable degree of cell killing in the nonhit cells, implying a substantial bystander effect. The magnitude of the bystander effect in these studies is much greater than that reported by The Gray Cancer Institute, where only 5–10% lethality is seen in nonhit cells, using

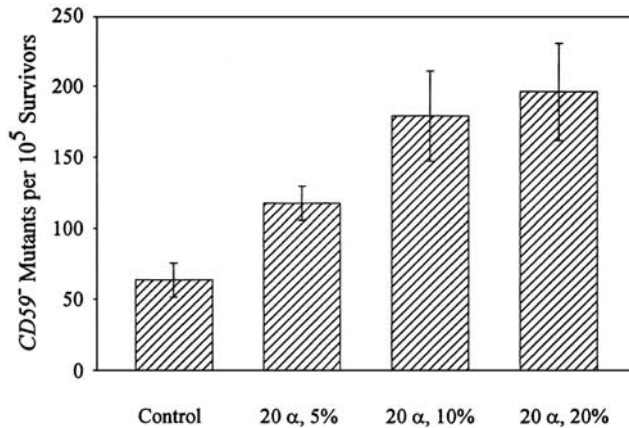


**Figure 4** The bystander effect for cell survival in V79 cells. Each data point (mean  $\pm$  s.d.) on the line with circles refers to the survival of cells when all cell nuclei on each dish were exposed to the same exact numbers of  $\alpha$ -particle traversals using the microbeam system. The squares show survival for various numbers of  $\alpha$ -particles, from 1 to 16, traversing 10% of the cell population. The extent to which this falls below the 100% survival for the nonhit is an indication of the magnitude of the bystander effect. Each data point represents the mean  $\pm$  s.d. of the clonogenic survivals from three culture plates (redrawn from Sawant *et al.*, 2001)

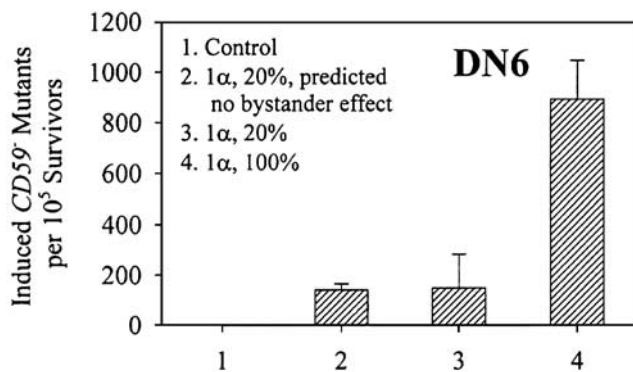
protons or soft X-rays in a microbeam (Prise *et al.*, 1998, 2002). The difference is probably accounted for by the cell density (Mitchell *et al.*, 2003). In The Gray Cancer Institute studies, only about 200 cells were seeded in an area of  $10 \times 10 \text{ mm}^2$ . The average distance between cells, therefore, was some hundreds of microns; so it is likely that communication via gap junctions did not contribute to the effect observed. By contrast, in the studies reported here, 1000–1200 cells were plated in a miniwell of 6.3 mm diameter so that 50–60% were in contact, allowing gap-junction communication that has been demonstrated to be of importance in mutation studies with the microbeam. Therefore, this study also supports the need for gap-junction communication as a mediator of bystander effects in relation to radiation-induced cell killing. It is likely that multiple mechanisms are involved in the bystander process depending on the cell types used, biological end points measured and types of radiation used.

### Mutagenic effects in human-hamster hybrid cells

Zhou *et al.* (2000) reported a study in which human-hamster hybrid ( $A_L$ ) cells were exposed to  $\alpha$ -particles by the use of the Columbia microbeam. After all cells on the dish were identified and located, the computer was programmed to expose 20% of the cells, randomly selected, to a near lethal dose 20  $\alpha$ -particles directed through the centroid of the nucleus. This irradiation allows less than 1% of the cells to survive, and yet when assayed for mutations in the human chromosome 11, the



**Figure 5** The bystander effect for mutations in the human-hamster hybrid ( $A_L$ ) cells when 20% of the cells receive 20 nuclear traversals by  $\alpha$ -particles. There is a substantial incidence of mutations over the background level, despite the fact that no irradiated cells survive (redrawn from the data of Zhou *et al.*, 2000)



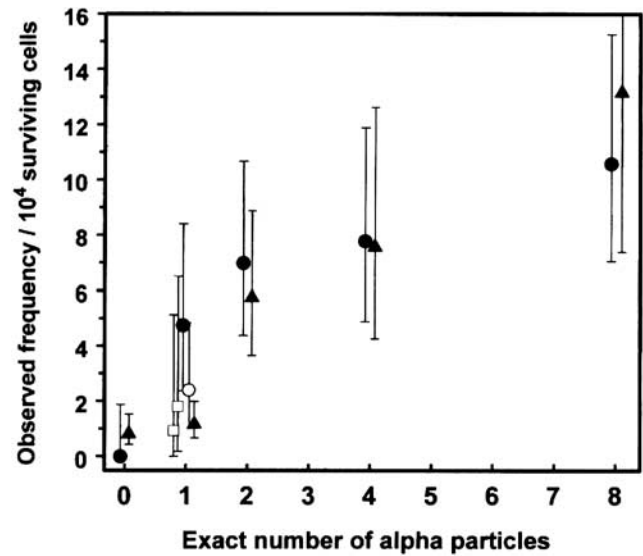
**Figure 6** Mutation fraction ( $M_F$ ) from population of  $A_L$ -AH1-9 cells transfected with a dominant-negative connexin 43 vector (DN6). Error bars represent mean  $\pm$  s.d. The population of AH1-9 cells used in these experiments have higher mutant induction as well as background mutant level than the parental  $A_L$  cells (redrawn from the data of Zhou *et al.*, 2001)

mutation yield was four times that of the background (Figure 5). These mutations must clearly arise from neighboring cells, not directly exposed, but in close proximity to irradiated cells.

A further series of experiments identified the importance of cell-cell communication via gap junctions as a mechanism of the bystander effect (Zhou *et al.*, 2001). When  $A_L$  cells were transfected with a dominant-negative connexin 43 vector (DN6), which eliminates gap-junction communication, the bystander effect essentially disappeared. This is illustrated in Figure 6.

### Oncogenic transformation in mouse fibroblasts

Mouse fibroblast (C3H 10T1/2) cells were plated in a monolayer, and the computer was programmed to

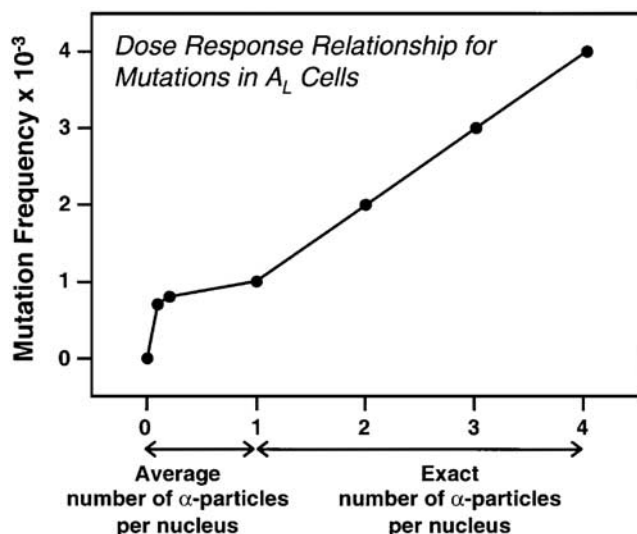


**Figure 7** Yield of oncogenically transformed cells per  $10^4$  surviving C3H 10T1/2 cells produced by nuclear traversals by 5.3 MeV  $\alpha$ -particles. Triangles represent exposure of all cell nuclei on each dish to exact numbers of  $\alpha$ -particles, using the microbeam system. Solid circles represent the exposure of one in 10 cell nuclei on each dish to exact numbers of  $\alpha$ -particles. Open squares represent subsequent repeats of the experiment in which one in 10 cell nuclei was exposed to exactly one  $\alpha$ -particle. Open circle represents combined data for all the experiments, in which one in 10 cell nuclei was exposed to one  $\alpha$  particle including these repeat experiments. Standard errors ( $\pm$  s.d.) were estimated, assuming an underlying Poisson-distributed number of transformed cells (redrawn from the data of Sawant *et al.*, 2001)

irradiate either every cell, or every tenth cell, selected at random with one to eight  $\alpha$ -particles directed at the centroid of the cell nucleus (Sawant *et al.*, 2001). The cells were subsequently removed by trypsinization, replated at low density, and transformed foci were identified 6 weeks later by their morphologic appearance. The results are shown in Figure 7, and illustrate that (a) more cells can be inactivated by  $\alpha$ -particles than were actually traversed by one. (b) When 10% of the cells on a dish are exposed to two or more  $\alpha$ -particles, the resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of  $\alpha$ -particles.

### Implications in risk assessment

It is important to note that the experimental results discussed in this paper involve laboratory model systems, since bystander experiments with *in vivo* systems, particularly in the human, are clearly not possible at the present time. However, if these results were applicable *in vivo*, they could have significant consequences in terms of extrapolation of radiation risks from high to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase



**Figure 8** Mutation frequency as a function of the number of  $\alpha$ -particles per nucleus (data for the average number of particle traversals were calculated from cell population in which defined proportion of cells were exposed to a single  $\alpha$ -particle). Due to the bystander effect, which is evident when only a proportion of the population is exposed, the risk at low doses is higher than predicted by a linear extrapolation from high doses (based on the data of Hei *et al.*, 1997; Zhou *et al.*, 2001)

more slowly, if at all, at intermediate doses. Thus, a simple linear extrapolation of radiation risk from intermediate (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity, at least at high LET.

This is illustrated in Figure 8, which combines the data of Zhou *et al.* (2001) where only a proportion of cells are irradiated with a single particle (allowing the bystander effect to be manifest), together with a previous compilation of data by Hei *et al.* (1997) where all cells were exposed to various numbers of particles from 1 to 4. Under these experimental conditions, it is evident that a linear extrapolation of risks from high to low doses (which average less than one particle per cell) would underestimate the risks at low doses. This applies, at this stage, strictly to  $\alpha$ -particles, and it is not known whether it would apply in an *in vivo* situation to, for example, radon exposure in homes and mines.

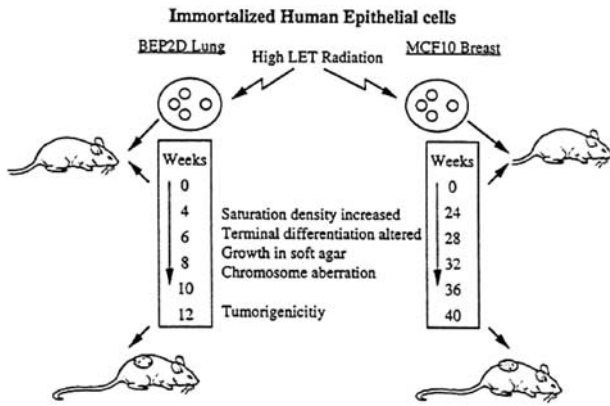
### Genomic instability

Carcinogenesis is generally considered to be a progressive process involving a number of mutational events, with each mutation conferring an additional growth advantage for the clonal expansion of genotypically altered cells (Fearon and Vogelstein, 1990). While ionizing radiations have been widely studied as human carcinogens, and are known to induce malignancies in a wide variety of tissues, the underlying mechanisms at the cellular and molecular levels are not known with any certainty. An attractive hypothesis is that radiation

induces 'genomic instability' in a tissue or population of cells, which is the source of the multiple mutational events that appear to be required in the transformation of a normal tissue into a metastasizing invasive tumor (Morgan, 2003 for review). The notion is that a mutation may occur in a gene responsible for the stability of the genome and the fidelity of replication, resulting in what has been referred to as mutator phenotype, that is, a single induced mutation followed by a cascade of further mutations. Support of this concept comes from the observation of microsatellite instability in a wide range of human tumors (Loeb *et al.*, 2003 for review). The discovery of mutations in one of the five mismatch repair genes in cases of hereditary nonpolyposis colorectal cancer also support the idea that an induced mutation can result in instability and a mutator phenotype (Loeb, 1991). It should be noted that a mutator phenotype can also be induced by non-DNA-damaging pathways such as aberrant gene expression and methylation status (Loeb *et al.*, 2003). The observations that (1) several cell cycle checkpoint genes such as cyclin B1 and RAD 51 have been shown to be overexpressed in radiation-induced bystander cells (Azzam *et al.*, 1998), and (2) that DNA repair-deficient cells have a higher bystander chromosomal aberration and mutagenic response (Nagasawa and Little, 2002; Nagasawa *et al.*, 2003) provide a possible link between mutator phenotype and bystander response, though direct evidence has yet to be demonstrated. Although there is no clearcut evidence that genomic instability actually occurs among radiation-induced cancer, the notion of global chromosomal instability arising from an initial event, possibly a mutation, makes it possible to understand conceptually how a single low dose of radiation can lead to a cancer many years after the initial exposure, and seemingly involves multiple steps. In this regard, a recently developed neoplastic transformation model based on human epithelial cells provides some useful clues of the process.

### Transformation models based on human epithelial cells

Compared with cells of rodent origin which are notoriously unstable, it is much more difficult to induce instability by radiation in cells of human origin. In this regard, it is worth noting that, based on the observed incidence of (for example) breast cancer in the Japanese A-bomb survivors, and a very crude estimate of the number of cells at risk, the probability per cell of oncogenic change is between  $10^{-12}$  and  $10^{-14}$  per Gray (Hei *et al.*, 1996b). Consequently, it is no surprise that no one has ever successfully transformed primary human cells by a single dose of radiation, since a sufficiently large experiment *in vitro* is not practical in a laboratory setting. Immortalized cell cultures, of human lung or breast epithelial cells, offer the next best alternatives as useful models to assess the phenotypic and genotypic changes leading to cancer after exposure to radiation. Although these cells are immortalized

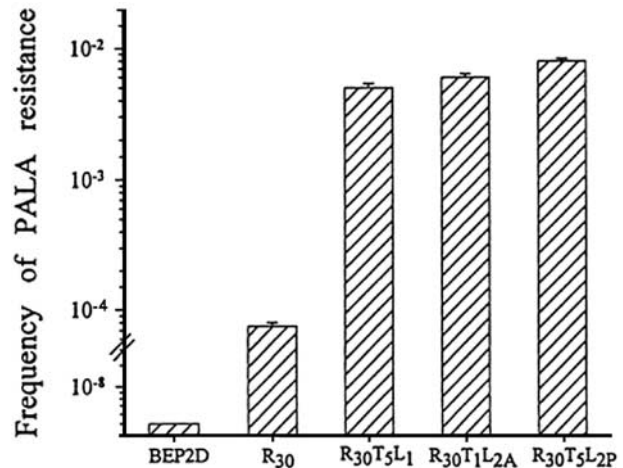


**Figure 9** Schematic diagram illustrating the multistep neoplastic transformation process in both immortalized human bronchial and breast epithelial cells irradiated with a single 60 Gy dose of  $\alpha$ -particles. Irradiated cells need to undergo successive passages with the concomitant accumulation of additional mutagenic changes, before tumorigenicity can be demonstrated. The time scale necessary for breast epithelial cells to undergo neoplastic changes is significantly longer than comparatively treated lung cells (from Hei *et al.*, 2001)

either spontaneously (MCF-10F human breast) or through viral transduction (BEP2D human bronchial), they are phenotypically normal, and do not express any transformed characteristics such as anchorage-independent growth and tumorigenicity in nude mice. After exposure to either 140 keV/ $\mu\text{m}$   $\alpha$ -particles or 1 GeV nucleon  $^{56}\text{Fe}$  ions, transformed cells arise through a series of sequential stages including altered growth pattern, resistance to serum-induced terminal differentiation, agar-positive growth, tumorigenicity and metastasis (Figure 9, Hei *et al.*, 1994; Piao *et al.*, 1999; Calaf and Hei, 2000). A single 30 or 60 cGy dose of these high-LET radiations can induce neoplastic transformation of the BEP2D cells in a stepwise fashion at a frequency of  $\sim 10^{-7}$  3–6 months after exposure (Hei *et al.*, 1996b).

### Genomic instability induced by high-LET radiation

It has long been recognized that genomic instability is an essential genotypic component of carcinogenesis, and that aneuploidy is a hallmark of malignancy. Although BEP2D cells are virally immortalized, the cell line is very stable genetically with only a small increase in the number of chromosomal markers from one to four in passage 17 and passage 35, respectively (Weaver *et al.*, 1997). In contrast, tumorigenic BEP2D cells have an increased incidence of chromosomal alterations including deletions and loss of heterozygosity in chromosomes 8 and 14, as determined using PCR amplification of short tandem repeats (Weaver *et al.*, 2000). One aspect of genomic instability is gene amplification, which is frequently observed in tumors and transformed cell lines. Amplification in the CAD gene which results in



**Figure 10** Frequency of PALA resistance among control, transformed (R30), and tumorigenic BEP2D cells induced by 150 keV/ $\mu\text{m}$   $^4\text{He}$  ions. R30T5L1 is derived from a primary tumor; R30T1L2A is derived from a secondary tumor of a different lineage, whereas R30T5L2P is from a tertiary tumor. The latter two lines are metastatic as well. Data are pooled from three experiments. Bars represent + s.e.m. (from Piao and Hei, 2001)

the acquired resistance to the chemotherapeutic agent PALA (*N*-phosphonacetyl-L-aspartate) has been demonstrated in rodent tumor cell lines previously (Tlsty *et al.*, 1989), but not in normal human fibroblasts (Wright *et al.*, 1990). CAD is a multifunctional protein that catalyses the first three steps in the *de novo* biosynthesis of uridine monophosphate. PALA is a competitive inhibitor of the enzyme aspartate transcarbamylase (ATCase). Figure 10 shows the frequencies of PALA resistance among control, transformed but not yet tumorigenic, and tumorigenic BEP2D cells derived from either a primary, secondary, or tertiary tumor. Transformed cells were induced by a single 60 Gy dose of 150 keV/ $\mu\text{m}$   $\alpha$ -particles (Hei *et al.*, 1994), and cultures were exposed to a  $9 \times \text{LD}_{50}$  concentration of PALA (180–200  $\mu\text{m}$ ) for 3 weeks, to assess the frequency of drug-resistant clones. The frequency of PALA resistance among the immortalized, control BEP2D cells was less than  $10^{-8}$ . In contrast, the frequency of gene amplification among the four transformed cell lines examined, including R30, ranged from  $7 \times 10^{-5}$  to  $9 \times 10^{-5}$ , whereas the PALA-resistant frequencies among the tumorigenic cell lines were 100-fold higher (Piao and Hei, 2001).

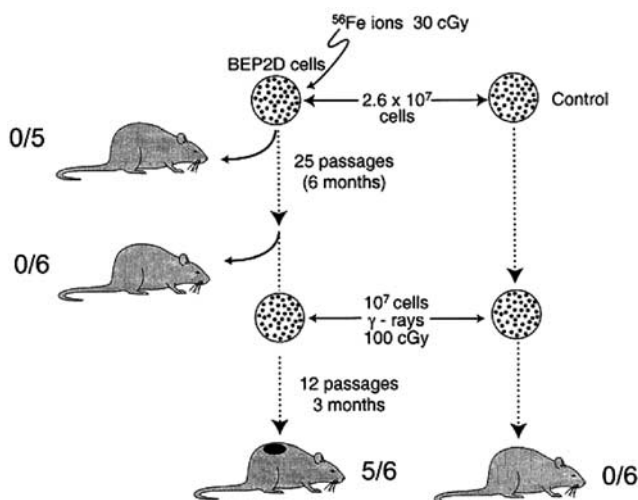
The results demonstrate that the stepwise neoplastic transformation process induced by  $\alpha$ -particles is clearly associated with a gradual increase in genomic instability as determined by CAD gene amplification. Although the initial molecular events leading to gene amplification are not known, there is evidence to suggest that chromosomal breakage followed by formation of acentric fragments that harbor the target gene may play a role (Windle and Wahl, 1992). The significant increase in PALA resistance among the tumorigenic compared to control BEP2D cells may be useful as a predictive assay for tumorigenicity in BEP2D cells transformed by high LETO radiation.

## Genomic instability in animal studies

Radiation-induced genomic instability can also be demonstrated *in vivo*, as shown in Figure 11. Exponentially growing BEP2D cells plated in T25 tissue culture flasks were irradiated with a single, nontransforming 30 cGy dose of 1 GeV/nucleon  $^{56}\text{Fe}$  ions, accelerated with the Alternating Gradient Synchrotron at the Brookhaven National Laboratory. After irradiation, cells were subcultured continuously for 30 passages. Control and irradiated cells were then irradiated with a single, nontransforming 1 Gy dose of  $\gamma$ -rays from a cesium irradiator, at a dose rate of 1.18 Gy/min. Cultures were then trypsinized, counted and replated for both survival and the expression of transformed phenotypes, as described previously (Hei *et al.*, 1994, 1996a). Although neither the 30 cGy dose of  $^{56}\text{Fe}$  ions nor the 1 Gy dose of  $\gamma$ -rays were effective in inducing neoplastic transformation by themselves, BEP2D cells irradiated with the combined protocol demonstrated progressive neoplastic changes and induced tumors in 5/6 animals, when subsequently inoculated into nude mice. This represents a model system where the high-LET radiation induces a genomic instability which takes many generations to produce the multiple genetic changes that lead, after exposure to low doses of X-rays, to a malignant change in cells of human origin.

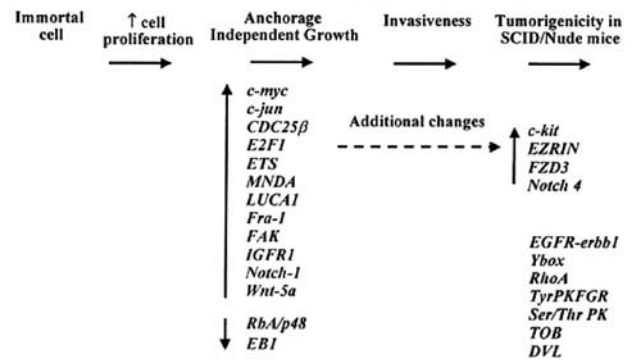
## Differential gene expression in radiation-induced tumorigenic breast cells

The human breast epithelial cell line MCF-10F, spontaneously immortalized and derived from the breast tissue of a 36-year-old female undergoing mastectomy for fibrocystic disease, is unique in the sense that it has the morphological characteristics of normal breast epithelial cells. These characteristics include dome



**Figure 11** Induction of genomic instability *in vivo* by a single 30 cGy dose of heavy ions in BEP2D cells

## Alterations in Gene Expression with Phenotypic Progression in MCF-10F Cells Induced by High LET Radiation



**Figure 12** Differential gene expression as a function of transformation stages in MCF-10F cells (from Hei *et al.*, 2001)

formation in confluent cultures, three-dimensional growth in collagen gel, and dependence upon hormonal and growth factors for optimal growth *in vitro* (Soule *et al.*, 1990). MCF-10F cells irradiated with 60 cGy or double doses of 60/60 cGy of  $\alpha$ -particles showed gradual phenotypic changes including altered morphology, increase in cell proliferation relative to control, anchorage-independent growth and invasive capability, but no tumorigenicity in nude mice (Calaf and Hei, 2000). However, MCF-10F cells irradiated with two doses of  $\alpha$ -particles in the presence of estrogen progress to the tumorigenic and metastatic stages as well.

Using cDNA expression arrays for oncogenes/suppressor genes, the differential expression patterns of these genes between the transformed cells in various stages of the neoplastic process, relative to control MCF-10F cells, were determined as shown in Figure 12 (Hei *et al.*, 2001). Autoradiographic analyses showed that of the 190 genes analysed, 49 genes showed a high level of altered expression (image quant value  $>1.60$ ), and 12 genes showed minor difference in expression levels. Among these 49 genes, 17 genes were altered at all stages of the transformation process, 21 were altered only at the early stage and the remaining 11 genes were changed only at the late tumorigenic stage. Among these 11 late-stage-associated genes, seven were found exclusively in the tumorigenic cell lines, and their expression levels were further confirmed by Northern blots (Roy *et al.*, 2001a).

## Frequent allelic imbalance in transformed human epithelial cells

Using a battery of polymorphic microsatellite markers located on chromosomes 6 and 17, the incidence of microsatellite instability and loss of heterozygosity in transformed MCF cells at various stages of the neoplastic process has been examined (Roy *et al.*, 2001b). These chromosomes are chosen since they are



frequently found to be altered in human breast cancers. A progressive degree of allelic imbalance, ranging from 21 to 50%, was detected at 6q, 17p and 17q. The array of genetic anomalies during neoplastic transformation increases the probability of random rearrangements, which not only favors chromosomal disintegration, which leads to loss of heterozygosity, but at the same time, also favors mitotic recombination, which lead to microsatellite instability. Thus, the detection of allele loss on both chromosome 6 and 17 in human breast epithelial cells induced by radiation and estrogen treatment *in vitro* seem to be similar to regions of imbalance found in primary breast cancer, which highlights the relevance and usefulness of this model. Identification and characterization of these altered loci are also helpful for the systematic studies to identify the cellular and molecular changes associated with radiation-induced breast carcinogenesis.

## Conclusions

Both genomic instability and the bystander effect are phenomena, discovered relatively recently, that result in a paradigm shift in our understanding of radiation biology. In the past, it seemed reasonable to assume that the direct deposition of energy, by a charged particle crossing a cell nucleus, led to the production of single- and double-strand breaks in DNA, which are the basis for most radiation effects. It turns out that biology is not as simple as this.

Genomic instability and the bystander effect have one thing in common, namely that both involve nontargeted effects in unirradiated cells, exhibiting responses typically associated with direct radiation exposure, but occurring in one case in the *progeny* of irradiated cells and in the other case in the *close neighbors* of irradiated cells. In addition to this similarity, there is some evidence that they share a common mechanism; for example, a recent review article (Lorimore and Wright, 2003) makes a compelling case that both result from an inflammatory-type response to radiation-induced stress and injury. In the case of genomic instability, gene mutations, chromosomal aberrations and/or delayed lethality show up in a population of cells many generations later, that is, events occur in the progeny of cells irradiated many generations earlier, with the intervening generations being apparently normal. In the case of the bystander effect, chromosomal aberrations, cell lethality, mutations or oncogenic transformation show up in cells that have not themselves been traversed by a charged particle, but are in close proximity to cells that have. Although there is indication that genomic instability may occur in the progeny of bystander cells, the evidence is not consistent (Nagar *et al.*, 2003).

*Genomic instability* provides a possible answer for one of the longstanding questions in radiation biology, namely, how can a single brief exposure to a low dose of radiation result in six or seven mutations at different

loci? This number of mutations may be consistent with the interpretation of Armitage and Doll (1954) that the incidence of cancer in adults increases with the sixth power of age, but that so many mutations could be caused by a single modest dose of radiation is, to say the least, improbable. Yet, there is hard evidence from the Japanese survivors of the A-bomb that a prompt exposure to radiation can induce a whole spectrum of malignancies including cancers of the digestive tract. The phenomenon of genomic instability, demonstrated in so many different biological systems, provides a ready answer. However, the mechanism that gives rise to genomic instability is far from clear: apart from the mutator phenotype mentioned above, there is indication that epigenetic modification may also be involved (Xu *et al.*, 1999). This is a fertile area for future research.

The *bystander effect* has major implications for the estimation of the risks of the deleterious effects of radiation at the low doses of concern in radiation protection, in the medical use of radiation for diagnostic purposes, and in the level to which sites used in the past for nuclear energy or nuclear weapons need to be cleaned up.

The 50-year study of the Japanese A-bomb survivors, which has cost the US tax payers in excess of half a billion dollars, not to mention the contribution from Japan, provides the best quantitative risk of radiation-induced cancer. For radiation doses up to about 2.5 Sv, and down to about 0.05 Sv (50 milliSv), there appears to be a linear relationship between excess cancer risk and radiation dose, which amounts to about 10% at 1 Sv. The study sample amounted to about 100 000 and the effect on individuals exposed to doses below about 50–100 mSv cannot be assessed. On the other hand, the average background radiation to which the US population is inevitably exposed from natural sources is about 3 mSv (Hall, 2000). Life on earth has been exposed to this, or higher levels, throughout evolutionary time. The question is, what is the risk of radiation exposures, above the level of natural background, but below the lowest dose, for which risks are known from the A-bomb survivors. This is a much debated issue, since it involves issues of major societal and economic concern. National and International standard setting bodies assume that a linear extrapolation of risks from high to low doses represents a conservative and prudent policy (ICRP, 1991; NCRP, 1993). A vocal minority consider that low doses have no deleterious effects, since life evolved in a continuous radiation environment (Feinendegen *et al.*, 1998; Cohen, 2002). The bystander effect contributes to this debate by implying that the biological effects of low doses, where not all cells are traversed by a charged particle, are amplified by the transfer of factors to unirradiated neighbors. If phenomena demonstrated *in vitro* are applicable *in vivo* (which is a big 'if'), then the bystander effect implies that a linear extrapolation of risks from high to low doses may underestimate rather than overestimate low-dose risks. This, too, is a fertile area for future research; the answers are not all in by any means.



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