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PROSPECTS

Radiation-Induced Genomic Instability: A Role for Secreted Soluble Factors in Communicating the Radiation Response to Non-Irradiated Cells

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Abstract Radiation induced genomic instability can be described as the increased rate of genomic alterations occurring in the progeny of an irradiated cell. Its manifestations are the dynamic ongoing production of chromosomal rearrangements, mutations, gene amplifications, transformation, microsatellite instability, and/or cell killing. In this prospectus, we present the hypothesis that cellular exposure to ionizing radiation can result in the secretion of soluble factors by irradiated cells and/or their progeny, and that these factors can elicit responses in other cells thereby initiating and perpetuating ongoing genomic instability. J. Cell. Biochem. 92: 1013–1019, 2004. © 2004 Wiley-Liss, Inc.

Key words: ionizing radiation; genomic instability; secreted factors

We live in an environment where radiation is ubiquitous. It is widely used clinically in diagnostic radiology, disease diagnosis, and treatment. In fact, the majority of cancer patients are treated with radiation therapy, either alone or in combination with chemotherapy. However, while radiation is widely used to diagnose, treat, and cure cancer, the public has major concerns about its carcinogenic potential and about the health effects of radiation contaminated environments, nuclear disasters, and potential radiological/nuclear terrorism. To address potential health risks associated with radiation exposure, we need to understand the long-term biological consequences of targeted and non-targeted effects in exposed cells and their progeny.

One of the hallmarks of the cancer cell is its inherent genomic instability. While there is compelling evidence that radiation can induce many of the genomic changes associated with carcinogenesis, a direct link between radiation induced genomic instability and radiation induced carcinogenesis has not yet been established [Goldberg, 2003; Sigurdson and Jones, 2003]. We will review the evidence for radiation induced genomic instability and present a new hypothesis to explain the observed effects. We propose that radiation induced DNA damage and the subsequent mutagenic and/or clastogenic effects are not the only explanation for the long-term delayed effects due to radiation exposure. In addition, we suggest that some factor or factors secreted by irradiated cells can elicit effects in non-irradiated cells, or in the progeny of irradiated cells, that can destabilize the genome resulting in phenotypes characterized by genomic instability.

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GENOMIC INSTABILITY AFTER IONIZING RADIATION EXPOSURE

There is abundant evidence both in vivo and in vitro, that exposure to ionizing radiation can lead to a host of effects that can be transmitted to the progeny of the irradiated cell. These effects include the dynamic ongoing production of chromosomal rearrangements, mutations, gene amplifications, transformation, microsatellite instability, and/or cell killing (reviewed in [Morgan, 2003a,b]). However, it is clear that the cell does not actually have to be hit by the radiation to elicit such an effect. Using low fluences of alpha particles, Kadhim et al. [1992] showed chromosomal instability in a significantly greater number of clonogenic survivors than could possibly have been hit by of radiation. Extending these initial observations, Lorimore et al. [1998] described chromosomal instability in cells that were protected from radiation exposure by a metal grid, while cells around them had been lethally irradiated. These observations suggest that an irradiated cell could communicate with a non-irradiated cell to pass on the legacy of radiation, that can then be observed in the progeny of those non-irradiated cells. Both the targeted, i.e., the hit cells and the non-targeted, i.e., the non-hit cells in the radiation environment can manifest evidence of delayed effects occurring in the clonally expanded progeny. This concept is presented in Figure 1.

GENOMIC INSTABILITY AFTER TRANSFER OF MEDIA FROM IRRADIATED CULTURES

A recent study by Seymour and Mothersill [1997] reported that irradiated, human epithelial cells produce a factor (or signal) in the culture medium that is capable of reducing the clonogenic survival of unirradiated cells cultured in this medium. The mechanism of this so called "bystander effect" is unknown, as is the nature of the signal or secreted factor (reviewed in [Morgan, 2003a]). Seymour and Mothersill [1997] went on to show that medium from irradiated cells was able to induce delayed effects in the progeny of some cell types that survived in this medium. The effects were cell line dependent [Mothersill and Seymour, 1997; Mothersill et al., 2001], but the data nevertheless indicated that signal(s)/factor(s) produced in medium by irradiated cells can induce genomic instability-type effects in distant progeny.

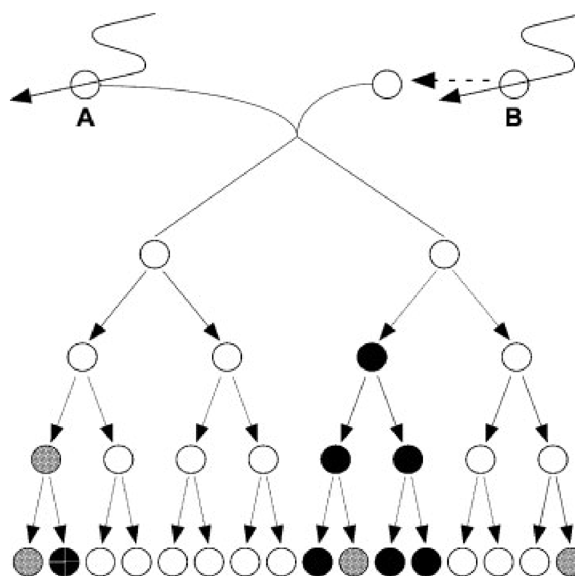


Fig. 1. Radiation-induced genomic instability. **A:** A cell survives irradiation and during clonal expansion instability develops in the progeny. **B:** An irradiated cell produces a soluble signal that affects other non-irradiated cells within the radiation environment. The progeny of that cell then develop instability during clonal expansion. The "normal" cells are represented by open circles, and the different shaded circles are different genomic rearranged cells that form "subpopulations" within the clone.

EVIDENCE FOR RADIATION INDUCED SOLUBLE FACTORS IN VIVO

The in vivo literature is replete with references to radiation induced secreted factors modulating effects outside the radiation field. These can be divided into in vivo bystander effects, abscopal effects, and clastogenic factors. In vivo bystander effects have been described after injecting mice with either neutron irradiated cells [Watson et al., 2000] or radioactively labeled cells [Xue et al., 2002], mixing these with non-irradiated cells and observing subsequent effects in the non-irradiated cells. These studies provided evidence supporting the numerous in vitro studies and demonstrated biological effects communicated in vivo. Nevertheless, these are still relatively artificial systems, and more convincing demonstrations of communicable effects between irradiated cells and non-irradiated cells have been the sporadic reports of abscopal effects (reviewed in [Morgan, 2003b]).

Usually derived from the clinical situation, abscopal effects describe those effects occurring outside the radiation field. For example, Ohba

et al. [1998] describe the case of a 76-year-old Japanese man presenting with back pain. Thoracic and abdominal computed tomograph (CT) scans revealed a significant thoracic vertebral bone metastasis and a number of defined hepatocellular carcinomas. The patient was treated with a conventional 36 Gy total dose to the vertebral bone metastasis for relief of the back pain. Subsequent CT scans 1 month and 10 months after treatment revealed the disappearance of the vertebral lesion and significant regression of the hepatic lesions outside the radiation field. Interestingly, the investigators performed retrospective analysis of a panel of cytokines from archived peripheral blood samples and found significantly elevated levels of tumor necrosis factor-alpha.

In a recent laboratory study, Camphausen et al. [2003] implanted Lewis Lung Carcinoma cells into the midline dorsum of p53 wild type mice and then used a fractionated radiation therapy-like schedule (5×10 Gy fractions or 12×2 Gy fractions) to irradiate the non-tumor bearing legs at a site distant from the implanted cells. They observed a significant delay in implanted tumor cell growth, but only in p53 wild type mice, not in p53 null mice or wild type mice treated with the p53 blocker pifithrin-alpha. These data implicate p53 as a key mediator of the radiation-induced abscopal effect and suggest that pathways downstream of p53 are important in eliciting the response.

Perhaps the most convincing demonstration of a role for secreted factors is the reports of clastogenic (chromosome breaking) factors in blood plasma from accidentally, environmentally, or therapeutically irradiated individuals (reviewed in [Huang et al., 2003; Morgan, 2003b]). When blood plasma is isolated from these individuals and co-cultured with peripheral blood lymphocytes from non-irradiated individuals, cytogenetic damage is observed in metaphase chromosomes from the non-irradiated donor. Similar results have been reported when plasma from non-irradiated individuals with the chromosome fragility disorders Ataxia telangiectasia [Shaham et al., 1980], Bloom syndrome [Emerit et al., 1982], or Fanconi anemia [Emerit et al., 1995]. These observations suggest that irradiated individuals as well as patients showing inherited chromosomal instability contain soluble factors in their blood plasma capable of damaging the genetic material of non-irradiated individuals.

IS RADIATION-INDUCED GENOMIC INSTABILITY THE RESULT OF SECRETED FACTORS?

There is evidence in the literature that stress induced secreted proteins can lead to decreased replication fidelity and subsequent genomic instability in cells irradiated with non-ionizing ultra violet radiation [Boesen et al., 1992] and that induced extracellular factors can communicate the ultra violet light response to non-irradiated cells [Schorpp et al., 1984]. This emphasizes the point that soluble factors may have a generic role in the long-term consequences of cellular exposure to DNA damaging agents.

The observations discussed above demonstrate the role of secreted factors in the induction of both in vitro and in vivo genetic instability and indicate that cells do not have to be traversed (hit) by radiation, or even in a radiation environment to elicit detrimental effects usually associated with direct radiation exposure. Furthermore, they suggest that some secreted factor, or factors, induced by radiation or by the progeny of an irradiated cell, is sufficient to stimulate and most likely perpetuate the instability phenotype.

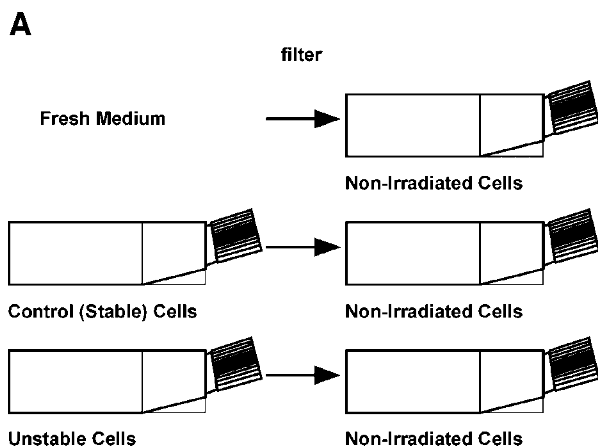
To explain these observations, we hypothesized that cellular exposure to ionizing radiation can result in the secretion of soluble factors by irradiated cells and/or their progeny. These factors can communicate the radiation exposure and subsequent response(s) to other cells initiating and perpetuating ongoing genomic instability. To test this hypothesis, we selected three clones isolated from our human/hamster hybrid GM10115 line that showed marked radiation induced chromosomal instability as manifest by multiple subpopulations of cytogenetically unique cells [Marder and Morgan, 1993; Limoli et al., 1998]. We then asked the question: “did medium from these chromosomally unstable cell clones contain soluble factors that could generate an unstable phenotype in cells that had never been irradiated?”

To answer this question, these clones were grown to confluence and the medium was replaced with fresh medium for 48 h. After this time, the medium was decanted from the unstable clones, filtered, and added to culture vessels containing 100 parental non-irradiated cells that had been plated 4 h previously. Flasks

of irradiated, but chromosomally stable, and non-irradiated parental cells served as controls. The goal was to clonally expand surviving colonies and determine if the medium from the unstable clones contained factors that could induce chromosomal instability in the non-irradiated parental clones. As expected, no effect on clonogenic survival as measured by colony forming ability, or induced genomic instability as measured cytogenetically, was observed in cells cultured with medium from parental cells or the progeny of an irradiated chromosomally stable clone. However, much to our surprize, we found that the medium from two of the three unstable clones was almost completely cytotoxic to parental non-irradiated cells ([Nagar et al., 2003a], Fig. 2).

The cytotoxic effect associated with this transfer of filtered medium from unstable clones to parental cells was termed the death inducing effect (DIE) [Nagar et al., 2003a]. Clearly, medium from some unstable cells contained factor(s) that could kill parental cells but the unstable clone itself was refractory to these cytotoxic effects (Fig. 2). We proposed that the unstable cells had adapted to this medium, but the factors secreted were instrumental in driving the chromosomal instability that characterized that clone. Thus, rather than killing the cell outright, the factor(s) produced the chromosomal rearrangements that made this clone unstable, and this in turn could account for the delayed reproductive cell death or increased lethal mutations that characterize chromosomally unstable clones [Marder and Morgan, 1993; Limoli et al., 1998]. Interestingly, not all unstable clones exhibited DIE [Nagar et al., 2003a], and the medium from one unstable clone will reduce but not completely eliminate clonogenic survival in another clone suggesting that more than one DIE factor is involved in the cytotoxic effect observed.

Nagar et al. [2003b] have performed a detailed analysis of how DIE kills non-irradiated parental cells. Within 30 min of transfer of unstable medium there was a significant increase in γH2AX foci in the recipient parental cells. GammaH2AX foci formation is thought to be associated with the induction of DNA double strand breaks [Rogakou et al., 1998], suggesting that the DIE factor can cause DNA cleavage. This would lend credence to the hypothesis that the DIE factor is responsible for perpetuating chromosomal instability in the unstable clone. DNA double strand breaks have been shown to be the primary lesion leading to chromosomal rearrangements [Morgan et al., 1988]. By extrapolation, this implicates the DIE factor in driving the dynamic production of chromosomal rearrangements that characterize the ongoing instability in the progeny of the irradiated cell. Over time the induction of γH2AX foci diminishes and the formation of micronuclei followed by cell fragmentation are observed. These are likely the consequences of DIE induced DNA double strand breaks [Nagar et al., 2003b]. Ultimately the cell dies a mitotic linked cell death or apoptosis (Fig. 3).



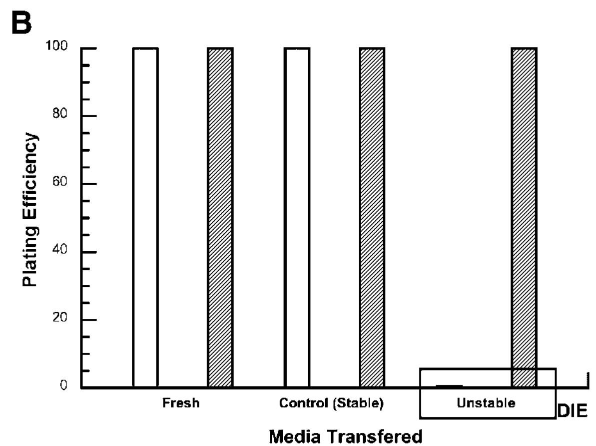


Fig. 2. Strategy for identification of the death inducing effect (DIE). **A**: Fresh media, media from non-irradiated cells or irradiated but chromosomally stable cells, or media from unstable cells is filtered and transferred to non-irradiated parental cells. **B**: The results of cell survival in this transferred medium as measured by colony forming ability. Effectively all cells survived in fresh medium or media from non-irradiated cells or irradiated but chromosomally stable cells. However, because of DIE (boxed) no surviving colonies were seen in the flasks receiving media from two of three chromosomally unstable clones.

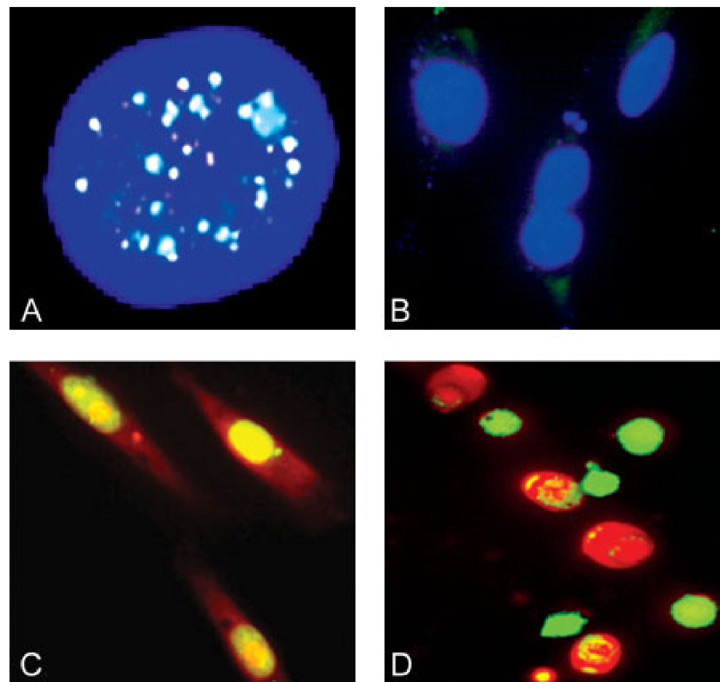


Fig. 3. The proposed sequence by which the DIE kills cells. **A**: Within 30 min of transferring unstable media a significant number of γH2AX foci are seen in recipient cells indicative of induced DNA double strand breaks. **B**: After 24 h of growth in unstable media many cells show induced micronuclei. **C**: Evidence of apoptosis by Annexin V staining, note the micronuclei. **D**: Evidence of apoptosis by TUNEL assay.

CHROMOSOMAL INSTABILITY INDUCED BY MEDIUM FROM RADIATION-INDUCED CHROMOSOMALLY UNSTABLE CELLS

Occasionally, single cell clones do survive culture in medium from chromosomally unstable cell clones. Interestingly, this is independent of the original number of cells plated and this is characteristic of other non-targeted, epigenetic effects of exposure to ionizing radiation [Kennedy et al., 1980]. These surviving colonies derived from single cells have been clonally expanded and while they will not grow in 100% medium from unstable cells, they will proliferate, albeit slowly, in a 50:50% mix of unstable medium:fresh medium. This mixture has previously been demonstrated to elicit DIE in non-irradiated cells [Nagar et al., 2003a]. Twenty surviving clones from the two clones that initially showed DIE have been analyzed cytogenetically for chromosomal instability. One clone that survived in medium from one unstable cell clone and three clones from another exhibited marked chromosomal instability [Nagar and Morgan, 2004]. While this is not such a clear cut result as one might desire, the results nevertheless do indicate that factors in medium from radiation induced chromosomally unstable clones can induce chromosomal instability in non irradiated cells. Furthermore, as the time in culture with unstable medium increases, the observed instability increases [Nagar and Morgan, 2004].

THE DIE IS NOT A CLASSICAL BYSTANDER EFFECT

It should be stressed that the non-targeted effect of exposure to ionizing radiation reported here is different from the bystander effect described following transfer of irradiated medium to non-irradiated recipient cells [Mothersill and Seymour, 1998]. No cytotoxic effect is observed following a medium transfer type of experiment. If we irradiate an exponentially growing flask of cells as a function of time and dose and then transfer the irradiated medium to non-irradiated parental cells, we find increased proliferation such that the number of colonies does not change but they are bigger and more robust. We attribute this to a "conditioned media" effect [Nagar et al., 2003a]. Thus, our

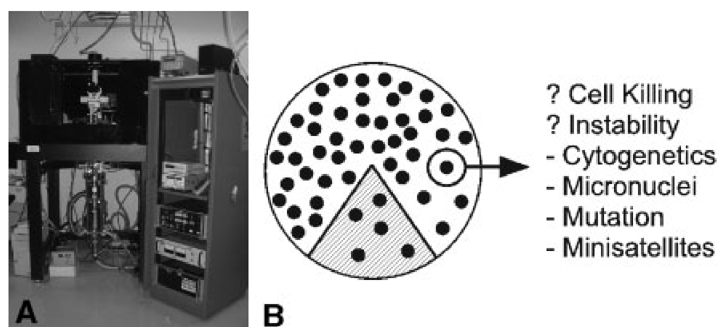


Fig. 4. Experimental strategy for investigating whether irradiated cells can communicate with non-irradiated cells. **A**: The electron microbeam at the Pacific Northwest National Laboratory. **B**: In total, 10% of the total cell number are irradiated using the microbeam. Potential cytotoxicity and/or induced genomic instability can then be investigated in the clonally expanded progeny of non-hit cells from the same culture environment.

human/hamster hybrid GM10115 cell line does not exhibit a classic radiation induced bystander effect [Mothersill and Seymour, 2004].

We speculate that there are two primary reasons for this. Firstly, our cells either do not produce a cytotoxic bystander factor or are not receptive to bystander like signals. Secondly, our cells have to be in a "radiation environment" to demonstrate an effect because irradiated cells communicate directly with non-irradiated cells in the culture environment. One way of testing this second alternative is to specifically irradiate a percentage of the cells in a population using a charged particle microbeam and investigate biological effects in those non-irradiated cells from the same culture environment.

Such a charged particle microbeam is available at the Pacific Northwest National Laboratory in Richland, Washington [Sowa Resat et al., 2004]. This variable-energy electron microbeam uses energetic electrons to mimic radiation damage from gamma- and X-ray sources while depositing energy in a preselected subset of cells for which the unirradiated neighbors can be easily identified [Sowa Resat and Morgan, 2004; Sowa Resat et al., 2004]. Using the microbeam, specific numbers of cells can be targeted with a known number of electrons with a given initial kinetic energy. This permits the selective irradiation of one or more cells within the culture environment (Fig. 4). As the region of direct irradiation is well defined, the biological effects observed due to direct irradiation versus effects due to a diffusible factor can be easily assessed for survival or induced instability.

CONCLUSIONS

There is compelling evidence from in vitro tissue culture studies, in vivo animal models, irradiated human subjects, and radiotherapy patients for a variety of effects occurring in either the progeny of an irradiated cell or outside the radiation field. Many of these non-targeted effects of exposure to ionizing radiation can be explained by the production of induced soluble factors either directly by the irradiated cell or in the progeny of an irradiated cell. We propose that these soluble factors can directly or indirectly lead to many of the phenotypes associated with radiation exposure. Clearly, one challenge ahead of investigators is to identify those soluble secreted factors responsible for these effects and test whether or not exposure to them can elicit the same phenotypes in the absence of radiation exposure.

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