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Substrate-Dependent Cell Cycle Distributions in Response to Ionizing Radiation

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Abstract

The amount of DNA damage that a cell incurs from ionizing radiation is thought to be minimized by molecular "checkpoint" mechanisms. Most research that has been done in this field has focused on events that occur inside the cell to control cell growth. However, important molecular signals are known to occur in the environment outside the cell. In this study, we show that the checkpoint response varies greatly depending on the type of extra-cellular matrix and that the cells differentiate on certain types of ECM. We also find that an inhibitory extra-cellular factor is produced by the irradiated cells. These results are unique because they demonstrate that the cell environment is critical to the checkpoint response and may be valuable in understanding and perhaps controlling cancer, radiation fibrosis, wound healing, and scleroderma.

Background and Research Objectives

Studying the effect of ionizing radiation on human cells is important because sufficient doses of radiation can cause cancer and/or tissue damage. This becomes a consideration in the case of accidental exposure of workers or in assessing the maximum dose of radiation that can be administered in cancer therapy. DNA damage is a potential, detrimental, outcome of radiation exposure, but, depending on the dose and conditions of radiation, most DNA damage is repaired by cellular molecular pathways. However, any damage that is not repaired before the cell divides will be inherited by the genes of daughter cells. Cancer results if radiation mutates genes that are necessary for the control of cell growth. Cell growth is described in terms of the cell cycle, which is the temporal series of molecular events that a cell must complete before it can divide and form two daughter cells. The cell cycle consists of four phases called G_1 (Gap 1), S (Synthesis of DNA), G_2 (Gap 2), and M (Mitosis). After irradiation, cells temporarily halt in either G_1 or G_2 phases at "checkpoints," presumably to give cells more time to repair damaged DNA (for review, see 1). Consequently, checkpoints would decrease the amount of DNA damage that is inherited by daughter cells and, therefore, decrease the chance of cancer. However, a certain cell type, the human fibroblast, does not temporarily delay at checkpoints after irradiation but, instead, arrests indefinitely (2-4). This observation caused us to question whether factors other than DNA damage affect the length of time that cells cease proliferation after irradiation.

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Most research on radiation checkpoints has centered around the molecular pathways within the cell that directly control growth and repair DNA damage. These studies have produced a wealth of information about proteins that can contribute to cancer, but have not considered that cells receive many signals from their extra-cellular environment. For example, cells in tissue and in cell culture adhere to surfaces by interacting with the extra-cellular matrix (ECM), which is a network or web of proteins such as collagen, fibronectin, and laminin. In short, it is thought that the adhesion of cells to the ECM allows the cell to sense molecular signals from the environment outside of the cell that can affect important cellular processes such as cell motility, morphogenesis, differentiation, and cell proliferation. Also, amounts of extra-cellular factors such as the growth factor, Transforming Growth Factor β 1 (TGF- β 1), have been shown to increase after irradiation. The production of such molecules could dramatically affect cell proliferation after irradiation. In fact, a major concern of radiation therapy of lung cancer is the potentially lethal side effect of radiation fibrosis, in which elevated amounts of TGF- β 1 and the ECM protein, collagen are produced, which results in changes in proliferation of fibroblasts but, more importantly, a decrease in lung elasticity. Very similar situations are observed in fibroblasts during the process of wound healing and in the disease scleroderma.

Because most radiation research has focused directly on checkpoint pathways, and because of the importance in understanding radiation fibrosis, our studies have focused on the role that the ECM and extra-cellular factors play in radiation-induced proliferation delay of human fibroblasts. Specifically, we: 1) measured the effect that two different plastics, polystyrene and Mylar, have on radiation arrest; 2) measured the effect that the ECM proteins, collagen IV, fibronectin, and laminin have on radiation arrest; and 3) determined if the production of extra-cellular factors are involved in radiation arrest.

Importance to LANL's Science and Technology Base and National R&D Needs

This research is important to the mission of OBER/DOE because of their interest in the health effects of radiation. This study could lead to the identification of molecular markers that respond to radiation, so that the danger of low dose radiation to the public can be determined. This research is important to national science efforts because, as explained in the previous section, little research has been done that investigates the effect of extra-cellular matrix and extra-cellular factors on radiation cell cycle arrest. This is pertinent to understanding, and perhaps controlling, radiation fibrosis, wound healing, and scleroderma. In fact, this research has led to a follow-on project funded by the NIH radiation research study section, and has spurred invitations to speak at the Annual Radiation Research Society Meeting, at the

Scientific Approach and Accomplishments

Several studies show that ionizing radiation causes normal human fibroblasts to accumulate in the G₁ phase of the cell cycle in an apparently permanent arrest (2,3). However, we show that the arrest is reversible in many cells if cell adhesion interactions with the extracellular matrix (ECM) are disrupted (4). Since cell adhesion influences radiation arrest in human fibroblasts, particular ECM proteins might lead to varied cell cycle (checkpoint) responses to radiation. In an initial attempt to test this hypothesis, we compared the radiation arrest we observe when cells attach and grow on tissue culture polystyrene to radiation arrest on Mylar. Mylar was tested because it is a commonly used culture substrate when irradiating cells with another type of radiation, α -particles. Also, the surface chemistry of the plastic polymer dictates the amounts and conformation of adsorbed ECM molecules. This means that, although it appears that we simply are testing two different kinds of plastic, we are really testing different kinds of ECM adsorbed to the plastic. The results are dramatic: only about 20% of the cells remained arrested 72 h after irradiation on Mylar compared to about 70% arrested cells on polystyrene (Figure 1 in reference 5).

We next determined the effect of three ECM proteins, collagen IV, fibronectin and laminin on radiation cell cycle arrest. As with the plastic substrates, large differences in the percentage of arrested cells were observed at 72 h post-irradiation: 60% of cells irradiated on collagen IV or fibronectin ceased proliferation, whereas 40% of cells that were irradiated on laminin ceased proliferation (Figure 2 in reference 5). Therefore, the trend that we observe for percentage of proliferating, irradiated fibroblasts on different substrata is Mylar > laminin > fibronectin $\geq$ collagen IV > polystyrene.

Radiation causes fibroblasts to differentiate to another cell type, myofibroblasts (6). Differentiation means that a cell of one type undergoes developmental changes that result in a different type of cell that has a more specialized function. The function of myofibroblasts in tissue is to produce molecular signals that control the growth patterns of neighboring epithelial cells. For example, radiation causes epithelial cells (the primary target of cancer) to die through a process called programmed cell death, or apoptosis, so that damaged cells are subtracted from the reproductive population. However, this is an indirect effect of radiation, because it is actually the myofibroblasts that result from radiation that produce the molecular death signals that epithelial cells respond to.

Myofibroblasts may also be involved in radiation fibrosis. As previously mentioned, fibrosis is a dose-limiting and debilitating side-effect of radiation cancer therapy in which

copious amounts of collagen and other ECM proteins are deposited in the irradiated tissue, resulting in aberrant ECM composition and loss of tissue elasticity. It is thought that the myofibroblasts support ECM remodeling in fibrotic tissue, and that changes in the differentiation pattern of fibroblasts reflect the risk of patients to develop fibrosis. Radiation fibrosis is often compared to wound healing. In both, there is a remodeling of ECM, an increase in TGF- β 1, and differentiation of fibroblasts to myofibroblasts. The difference is that these events are reversed when the wound heals but are permanent in fibrotic tissue.

Another potential function for myofibroblasts is in cell-cell signaling, as suggested in prostate tissue (7). In the prostate gland, the epithelial cell mass is organized into distinct regions of viable or apoptotic (dying) epithelial cells. The surrounding tissue is also spatially organized: fibroblasts neighbor the regions of viable epithelial cells, and myofibroblasts neighbor areas of apoptotic epithelial cells. Androgen ablation, which is sometimes used in conjunction with radiotherapy or chemotherapy of prostate cancer, causes an increase in TGF- β 1 and mass apoptosis of epithelial cells throughout the prostate gland. Concurrently, the number of fibroblasts in the surrounding tissue decreases, and are replaced with myofibroblasts that then surround the epithelial mass. It is proposed that the myofibroblasts are responsible for producing TGF- β 1 that leads to epithelial cell apoptosis.

Because of the importance of differentiation of fibroblasts to myofibroblasts in radiation therapy and wound healing, we decided to investigate whether the prolonged cell cycle arrest observed on some ECM substrates after irradiation is actually differentiation. A molecular marker characteristic of myofibroblasts is a protein called smooth muscle (SM) α -actin (8). Therefore, we measured the amount of SM α -actin in irradiated fibroblasts. To do this, we used a procedure called Western analysis. A protein extract is made from irradiated cells and then individual proteins are resolved or ordered on gels according to their size. Because the gels are fragile and difficult to work with, the resolved proteins are then transferred to a membrane, and an antibody that only binds to SM α -actin is used to detect SM α -actin. Therefore, the intensity of the bands corresponds to the amount of SM α -actin that is made by the cell at the given time after irradiation. We observed that the amount of SM α -actin increases during the cell cycle arrest on polystyrene, collagen IV, and fibronectin, but not when irradiated fibroblasts remain proliferating on laminin or Mylar (Figure 4 in reference 5). This is the first data that demonstrates that fibroblasts differentiate to myofibroblasts in an ECM-dependent manner.

It is interesting that irradiated fibroblasts do not arrest well or differentiate on laminin, because laminin is not an ECM protein that is commonly thought to be associated with fibroblasts in tissue. Rather, laminin interacts more with epithelial cells. However, some cancerous fibroblasts exhibit increased expression of the cell surface receptor that binds

receptor, and decreased expression of the receptor that binds fibronectin (9,10). Perhaps the irregular expression pattern of ECM receptors in transformed fibroblasts or an aberrant ECM environment around non-transformed fibroblasts would result in an abnormal cell cycle response to radiation or other stress insults. This could result in an abnormal response by normal epithelial cells because myofibroblasts would not be produced, and, therefore, there would be a lack of the molecular death signals produced that would typically kill epithelial cells.

Extra-cellular molecules that are synthesized by either fibroblasts or myofibroblasts are also important to the radiation checkpoint response. In fact, it is proposed that the production of an extra-cellular molecule, in particular TGF- β 1, is involved in myofibroblast differentiation because direct treatment of human and rat fibroblast cultures with activated TGF- β 1 causes differentiation to myofibroblasts (11-13). In fact, TGF- β 1 induction of specific ECM proteins might evoke myofibroblast differentiation. TGF- β 1 expression leads to a large increase in the amounts of a particular form of fibronectin, ED-A fibronectin, in cultured fibroblasts (14). The increase in ED-A fibronectin amounts is followed by increases in amounts of SM α -actin (15). Blocking the function of ED-A fibronectin stops the induction of SM α -actin by TGF- β 1. It has been postulated that the increase of TGF- β 1 in fibroblasts causes a cell cycle arrest that then predisposes the cells to differentiation (12). The basal amounts of TGF- β 1 produced by myofibroblasts are 10-15 times higher than those produced by fibroblasts; however, radiation induces TGF- β 1 only in fibroblasts (12). Thus, it was further suggested that the high basal amounts of TGF- β 1 in myofibroblasts maintain the differentiated phenotype. This scenario agrees with our data that shows that cell cycle arrest occurs early after irradiation, whereas differentiation is a later event.

We decided to test for the presence of growth inhibitory extra-cellular factors because of the potential importance of such molecules to the checkpoint response. Extra-cellular factors are molecules that are synthesized inside the cell but then are released into the culture medium that surrounds the cell. These molecules, whether growth inhibitory or activating, then function by binding to receptors on the cell surface. The concentration of the molecules must be sufficient in order to have an effect on the cell. Thus, replacing the culture medium on the irradiated cells with fresh medium would remove such a factor and allow the arrested cells to grow. We performed such experiments and found that this, indeed, was the case. Another situation that would occur if there is an inhibitory factor present is that increasing the amount of medium on a given number of cells would cause an increase in growth because the factor would be diluted. We found this situation also to be true. These experiments, plus other control experiments, suggest that the irradiated fibroblasts do release an extra-cellular factor that inhibits the growth of the irradiated cells.

In conclusion, this study shows that: 1) the extent of the radiation checkpoint response is influenced by the ECM; 2) irradiated fibroblasts differentiate to the myofibroblast cell type in an ECM-dependent manner; and 3) a growth inhibitory extra-cellular molecule is synthesized by the irradiated fibroblasts. These three observations may be interconnected because, in some cases, extra-cellular factors are released into the medium in an inactive form and are stored in only certain types of ECM. Then when the cells are irradiated, the inactive form of the factor can quickly be released and activated without taking time to synthesize the molecule. This is known to occur when TGF- β 1 is activated in situations other than radiation insult. It is the goal of the NIH proposal that was funded as a result of this LDRD project to determine if radiation activates TGF- β 1 in an ECM dependent manner. In any case, the general importance of this work is that it shows that, obviously, the extra-cellular environment of the cell is of extreme importance to radiation checkpoints. Studies that consider the effect of the extra-cellular environment more closely represent the situation that is present in the tissue. Therefore, these studies may provide new insight into the treatment of disease states such as radiation fibrosis, wound healing, and cancer.

Publications

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