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THE EFFECTS OF IONIZING RADIATION ON INTEGRIN-MEDIATED ADHESION OF BREAST CANCER CELLS

A Thesis

Presented to

The Honors Tutorial College

Ohio University

In Partial Fulfillment

of the Requirements for Graduation

from the Honors Tutorial College

with the degree of

Bachelor of Science in Chemistry

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by

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June 2011

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Acknowledgements

I would like to thank several people for their contributions to this thesis: Dr. Shiyong Wu, for his continuous support in my personal, academic, and professional life; Dr. ShinHee Lee, for her help with experimental design and execution, especially of the adhesion assays, and for being flexible with my schedule; Huiwen Cheng, for her technical assistance with experiments and for helping ShinHee in my absence; Megan Vogel, for voluntarily scanning dozens of Western films; and Brett Blevins, for his emotional support.

I would also like to recognize my sources of funding, the Provost's Undergraduate Research Fund and Dr. Wu's research grant, without which this research would have been impossible.

Abstract

There were over 207,000 new cases of invasive breast cancer and nearly 40,000 deaths from the disease in the U.S. in 2010. It is, therefore, critical to develop a better understanding of the disease process. Invasive cancer is marked by the loss of integrity of the basement membrane of a tissue, a specialized form of extracellular matrix (ECM) that normally separates a tissue from its surroundings; however, a complex, dynamic interaction involving many overlapping signaling pathways exists between breast tissue and its neighboring ECM. Invasion is initiated when the tumor cells have increased adhesion to the ECM, which is mainly modulated by interactions with the integrin family of adhesion proteins on the surface of the tumor cells. Each integrin heterodimer has a different adhesive affinity for each of the ECM proteins, namely fibronectin, vitronectin, collagen I, and collagen IV. This project examined the relative expression of integrins following exposure to ionizing radiation, which is commonly used for cancer therapy. Ionizing radiation forms free radicals and reactive oxygen species, which then cause damage within the cell. Although the exact mechanism is unknown, the hypothesis of this study was that exposure to high dose ionizing radiation would change the expression of the integrins and change integrin-mediated adhesion to the ECM proteins. Results showed that in the MDA-MB-231 cell line, ionizing radiation induces significant changes in both integrin expression and integrin-mediated adhesion to fibronectin, laminin, and collagens I and IV. After gathering further evidence from this and other cell lines, this information could potentially be used to optimize treatment for patients with invasive cancers.

Introduction

Significance and Overview

Breast cancer is the second leading type of cancer—behind skin cancer—and the second leading cause of cancer death—behind lung cancer— for women in the U.S.[1]. The American Cancer Society estimates that in 2010 there were over 207,000 new cases of invasive breast cancer in women, and there were nearly 40,000 deaths from the disease[1]. The five-year survival rate of non-invasive breast cancer is 93%, while the rate for breast cancer that has invaded the lymphatic system ranges from 41% to 88%, depending on tumor size and location[1]. For breast cancer than has spread to other organs, or metastasized, the five-year survival rate drops to only 15%[1]. While both local invasion and distant organ metastasis become problematic when they compress, destroy, and/or prevent normal tissue function, metastasis usually results in significantly worse prognosis for the patient because the disease changes from localized and potentially curable to generalized and likely incurable[2]. Therefore, developing a better understanding of the metastatic process is critical.

Breast cancers are usually carcinomas, which arise from the epithelial cells of the breast and typically metastasize to the bones, lungs, liver, or brain[3]. The metastatic potential of a tumor is dependent on both the genetic changes involved and the influences of the tumor microenvironment, which consists of sister tumor cells, stroma connective tissue of neighboring cells, and local inflammatory and vascular activity, all of which are reciprocally modulated[2]. This yields a regulatory system that is tumor- and environment-specific.

This complex, dynamic regulation is what enables cancerous cells to invade local tissue, including the normally impenetrable extracellular matrix and basement membrane of the stroma[4]. Invasion is initiated when the tumor cells have increased adhesion to the extracellular matrix proteins, which is mainly modulated by the integrin family of proteins on the tumor cell surface[4]. Integrins are highly involved in invasion and metastasis because of both their direct adhesion to the extracellular matrix and their indirect signaling pathways that send signals to and receive signals from the tumor microenvironment[5]. Integrin-linked signaling pathways regulate tumor cell proliferation, survival, gene expression, cell shape, cytoskeletal organization, contractile force generation, and cell adhesion, each of which plays a different role at different points in the metastatic cascade[5,6].

Integrins form alpha-beta heterodimers, of which there are over twenty combinations between the different alpha and beta subunits[5]. Each heterodimer has a different adhesive affinity for the different extracellular matrix proteins, namely fibronectin, vitronectin, collagens, and proteoglycans[7]. Therefore, the relative expression of the different integrin heterodimers on the surface of the cell affects its adhesion to the extracellular matrix as well as activation of downstream pathways that affect tumor stability. This project examined the relative expression of different integrins following exposure to ionizing radiation, which is commonly used for cancer therapy. Ionizing radiation forms free radicals and reactive oxygen species, which then cause damage within the cell. Although the exact mechanism is unknown, the hypothesis of this study is that exposure to high dose ionizing radiation will change

the expression of the integrins and change integrin-mediated adhesion to the extracellular matrix proteins.

Cancer

A tumor consists of a heterogeneous population of cells that differ by their relative states of differentiation[8]. The outside of a tumor mass contains fully differentiated cells that are susceptible to radiation and chemotherapy because of their close vicinity to the non-tumorigenic microenvironment as well as the sufficient blood flow due to induced angiogenesis, or blood vessel growth[8]. The region closer to the center of the tumor contains progenitor cells, which can undergo a limited number of mitotic cycles to form several daughter cells[9]. These daughter cells can then differentiate into select types of cells based on their relative microenvironments[9]. Within the heart of the tumor lies the cancer stem cells (CSCs), which are both structurally and functionally distinct from the other cells within a tumor mass[10].

Cancer is the proliferation and accumulation of abnormal cells—cells that defy structural and functional properties necessary for tissue integrity[11]. Cancerous, or malignant tumor cells, differ from non-cancerous, or benign, tumor cells because of the former's ability to invade adjacent tissue[11]. This invasive property is governed by an abnormal genetic code[11]. Genes that are mutated, lost, translocated, or amplified in the DNA are transcribed into RNA messages that are qualitatively or quantitatively aberrant[11]. These RNA messages are then translated in abnormal proteins, which carry out the functional properties of cancerous cells[11].

A recent theory suggests there are six hallmarks of cancer[12]. These hallmarks are enabled by underlying genomic instability and mutation, tumor-promoting inflammation, reprogrammed energy metabolism, and evasion of immune destruction[12]. The first hallmark, the ability to sustain proliferative signaling, is a result of dysregulated cell cycle progression and cell growth, which can be caused by activation of proliferative pathways or disruption of pathways that normally attenuate proliferation[12]. The second hallmark, evasion of growth suppressors such as the TP53 and RB proteins, results in a loss of contact inhibition; this allows the cancer cells to exhibit growth that is independent of cell density and architectural support[12]. The third hallmark, the ability to resist cell death or apoptosis, can be due to increased survival signaling or decreased apoptotic signaling[12]. The fourth hallmark, enabling replicative immortality, allows the cancer cells to undergo unlimited replication, whereas normal cells enter senescence, a form of differentiation, or cell death after limited replication cycles[12]. The fifth hallmark, inducing angiogenesis or blood vessel growth, supplies the tumor with the nutrients and oxygen needed for rapid growth as well as the removal of toxic metabolic waste and carbon dioxide[12]. The sixth hallmark, activating invasion and metastasis, is what distinguishes malignant tumors from their benign counterparts[12].

During embryogenesis, a series of transcription factors regulate migration of germ cell layers by changing the morphology, protease expression, motility properties, and apoptotic signaling of the cells[12]. These transcription factors and subsequent processes are also activated in invasive cells, representing their transition from a

differentiated epithelial cell to an undifferentiated mesenchymal stem cell; hence, the process is called the epithelial-mesenchymal transition (EMT) [12]. While the exact mechanisms of EMT are unknown, its transcription factors appear to be involved in the entire metastatic cascade, except the final colonization[12]. The EMT can be transient or stable, and it can be expressed in different degrees, depending on the step of metastasis[12].

Invasion and Metastasis

Invasive cancer is marked by the loss of integrity of the basement membrane of a tissue[4]. The basement membrane is a specialized form of extracellular matrix (ECM) that normally separates a tissue from its surroundings. While the extracellular matrix consists of fibronectin, vitronectin, collagens, and proteoglycans, the basement membrane is mainly composed of type IV collagen and laminin[7]. Invasive cells typically express proteases active against collagen IV[5]. The exact composition of the ECM is tissue specific, and under normal conditions this helps control tissue organization; however, this mechanism is dysregulated in metastatic cells[5].

The invasion of neighboring ECM is an early yet critical step in metastasis—the movement of cells from a primary tumor to a distant location where a secondary tumor is formed. Metastasis consists of a cascade of events[13]. The primary tumor must first develop its own blood supply via angiogenesis[13]. Cells from the primary tumor then de-adhere, invade the surrounding epithelial ECM and basement membrane, and invade the endothelial BM of neighboring blood or lymphatic

vessels[13]. Following this intravasation, the cells circulate or disseminate to distant sites where they must then adhere to the capillary endothelium, invade the endothelial BM, and extravasate into the new organ tissues, where they develop into a secondary tumor with its own blood supply [13]. Complete metastasis requires the coordination of several integrin-linked signaling pathways for tumor cell proliferation, modification of the surrounding environment, invasion and migration into different tissues, and differentiation[5].

Integrins

Tissue organization and polarity is normally regulated by cell-cell interactions and cell-stroma interactions, via the cadherin and integrin family of adhesion proteins, respectively. Depending on the extracellular ligand bound to these adhesion proteins, different intracellular signals are initiated[2]. The integrin family consists of transmembrane glycoprotein heterodimers, each consisting of one alpha and one beta subunit that are non-covalently linked[14]. Combinations of the different subunits create at least 25 different dimers[5], each with different affinities for different ligands[15]. Integrin expression has been specifically linked to cancerous transformations; malignant activation of the same pre-neoplastic tumor resulted in different expression and distribution of integrins[16], and malignant activation in breast epithelial tissues specifically is associated with reduced expression of integrins α1, α6, β1, or β4[16].

Each integrin subunit has an extracellular domain, a single membrane spanning domain, and a short, non-catalytic cytoplasmic tail[5]. The extracellular domain ligates

different proteins, including the ECM proteins[5]. The affinity of the extracellular domain for ECM proteins can be altered, in part, by conformational changes in the quaternary structure of the integrin initiated by binding of another protein, due to intracellular signaling, to the cytoplasmic tail[5]. Integrin avidity, or the strength of adhesion, is affected by the formation of the focal adhesion sites where several heterodimers diffuse to form a multimeric complex[5]. This increased density of integrins strengthens adhesion by allowing binding of the ECM protein ligand to multiple integrins, called chelation, and by allowing rapid re-binding of the ECM to another integrin if contact with one is lost [17].

Formation of the focal adhesion sites, a result of ECM ligand binding, initiates significant intracellular activity, including migration. Focal adhesion sites contain many actin-associated proteins, which physically link the integrins to the cytoskeleton[18]. Migration involves polarization of the cell with chemotactic receptors[19], integrin focal adhesion sites[20] and associated cytoskeletal proteins[21] aggregating at leading edge, where actin polymerization and decreased membrane tension allows the projection of the lamellipodia[22]. In order for the cell to advance forward, there must be decreased adhesion at the trailing edge of the cell, which is mediated via decreased integrin affinity or enzyme-mediated integrin dissociation[22,23,24,25]. Then actin-myosin contractile forces project the cell forward[26]. Therefore, there must be a balance between adhesion and anti-adhesion of integrins at the leading and trailing edges of the migrating cell, respectively. Maximum migratory

rate requires intermediate adhesiveness; at low levels there is insufficient traction, but at high levels the cells cannot break contact and are immobile[27,28].

Similarly, there must be a balance between degrading the ECM to allow migration and maintaining enough matrix for appropriate traction, and integrins regulate the proteases that degrade and remodel the ECM[5]. Integrins act as part of a proteolytic complex to activate and localize matrix metalloproteinases (MMPs) that are secreted in an inactive form[29]. In addition, negative feedback prevents excessive degradation[5]. In this fashion, integrins activate MMPs specific to local ECM as well as protease inhibitors to prevent excessive ECM degradation[5].

Integrin Signaling

Besides their adhesive function, integrins also have an important role in invasion and metastasis because they act as signaling molecules in tumor cell proliferation, survival, gene expression, cell shape, cytoskeletal organization, contractile force generation, and cell adhesion, each of which plays a different role at different points in the metastatic cascade[5,6]. Integrin focal adhesion sites also co-localize with growth factor receptors[30] and cross-talk with other transmembrane receptors, such as the receptor tyrosine kinases [31,32,33,34,35] to affect their signaling pathways. Both intracellular and extracellular signals modulate the dynamic interaction between the tumor cells and the ECM that is necessary for invasion and migration[5]. Integrins send signals in based on extracellular interactions and they also respond to intracellular signals that change their extracellular interactions[5].

Ligand-activated integrins form focal adhesion sites, which act as signaling complexes for such intracellular pathways as protein phosphorylation, calcium mobilization, and GTP exchange[5]. Intracellular focal adhesion kinase (FAK) is co-localized to the cytoplasmic tails of the integrins at the focal adhesion sites, where it is activated upon formation of the multimeric complex[36]. Interestingly, FAK expression is higher in invasive tumors than pre-malignant counterparts[37,38]. Activated FAK then recruits several different adaptor proteins, which activate small GTPases and other downstream effectors[5].

For example, integrin clustering and formation of focal adhesion sites leads to autophosphorylation of FAK at tyrosine 397, which then recruits SRC kinases, leading to cell motility in unknown mechanism[5]. Ligation of certain integrins, namely α1β1, α6β4, α5β1, or αvβ3, leads to subsequent phosphorylation of FAK and recruitment of SHC; this then results in cell cycle progression, migration, anti-apoptotic signaling, and ERK activation[39,40]. Phosphorylated FAK (P-FAK) also recruits adaptor proteins such as RAS, RAC, and CDC42 to activate ERK, which regulates cell proliferation and gene transcription, promotes survival, generates actin/myosin contractile force, and causes integrin contact release at the trailing end of the migrating cell[5].

Further downstream effectors also play a role in integrin signaling. In mammary epithelial cells, for example, active RAC and CDC42 expression disrupts cell polarization through integrin localization, which induces a PI3K-dependent invasive, motile phenotype[41] that is dependent on subsequent lamellae formation in breast carcinoma[42]. In addition, PI3K is required for RAC activated pathways in

mammary epithelia[41], and it is required for EGF-mediated adhesion enhancement of integrin β1 in breast carcinoma[43]. However, in normal epithelial cells PI3K activation decreases adhesion via integrin α3β1[44], suggesting other pathways regulating tumor cell-ECM interactions may be involved.

Some proteins act as effectors that signals out from the inside of the cell. For example, RRAS increases integrin affinity for the ECM while HRAS decreases integrin affinity for the ECM in some cell lines[5]. In addition, activation of RAC, CDC42, and PKC induces formation of focal adhesion sites[5]. PKC, a serine/threonine kinase, is actually required for focal adhesion formation[45], phosphorylation of FAK[46], cell spreading [46], SHC-dependent ERK activation [47], and migration [48]. PKC also regulates transport of integrin β1 within the cell[49]. PKC also modulates integrin-ECM interactions; integrin ligation to fibronectin recruits PKC to surface [50,47,46], and PKC is required for vitronectin-integrin αvβ5 focal adhesion formation and subsequent migration[51].

In addition to modulating migration, integrin signaling also plays a significant role in invasion and metastasis because of its overlap with cell death pathways. In normal tissue control, when there is no suitable ECM ligand present, integrin β1 or β3 recruit cytoplasmic caspase-8 and initiate apoptosis, or programmed cell death[52,53]. If a suitable ligand is present though, indicating appropriate cell-matrix interactions, pro-survival or anti-apoptotic signaling initiates[52]. This may explain why certain cancer types metastasize to certain organs, due to distinct ECM interactions that trigger survival in that location rather than death[52]. Blocking certain integrins, specifically

αvβ3, sufficiently inhibits invasion and induces apoptosis[54]. Successful invasion and migration, therefore, requires anti-apoptotic signaling[52].

Many anti-apoptotic pathways are the same as migration pathways. Anchorage-dependent cells that cannot bind immobilized ECM undergo apoptosis[55], but activated FAK alone is sufficient for anchorage-independent survival[56], indicating its role as a survival-inducing factor. In fact, disturbed FAK signaling in otherwise anchorage-independent breast carcinoma cells induces apoptosis through caspase-8[57]. Downstream targets of FAK, including RAS [58], RAC[59], PI3K[58], ERK[59], are also involved in survival signaling.

Ionizing Radiation

Radiation therapy utilizes high energy radiation from x-rays, gamma rays, or charged particles to shrink a tumor before surgery or, more commonly, to kill any cancer cells remaining after surgery[3]. Radiation therapy can be localized from an external beam or an internally planted device, or it can be delivered systemically[3]. In the exposed cells, the ionizing radiation used in radiation therapy forms free radicals and reactive oxygen species, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl (OH^-) radical, which then cause random damage within the cell by impacting the first cellular component that makes contact[60]. This random mechanism of destruction makes both normal and cancerous cells vulnerable to reproductive death via apoptosis or senescence, although in the latter case it may require one or more replication cycles for the induced damage to kill the cell[60].

Although incredibly complicated, it is important to have a sense of how signaling pathways act downstream of integrins to promote invasion and metastasis because it is likely that these pathways change in different microenvironments, such as that induced by radiation therapy. For example, reactive oxygen species may affect the stability of a tumor by causing degradation of the extracellular matrix proteins or loss of cellular adhesion proteins[61]. In another study, cell invasion, migration, and adhesion were enhanced by ionizing radiation due to changes in matrix metalloproteinase activity[62]. The cell responds to the radiation induced damage via many signaling pathways, including those listed above, which can result in changes in the cell adhesion profile of the cells on many different levels. These include transcriptional and translational control, spatial distribution along the membrane, endocytosis and degradation, and formation of higher order adhesion complexes[63]. Integrin function and subsequent migratory ability is also mediated by glycosylation[64], which has its own set of signaling pathways that could be affected by radiation-induced cell damage. In addition, mutant p53—found in over half of human cancers—promotes invasion and metastasis by increasing the recycling of integrin $\alpha 5\beta 1$ and epidermal growth factor receptor[65]; radiation could easily damage this pathway since it already lacks appropriate control.

Project Rationale and Hypothesis

The MDA-MB-231 cell line used in this project is an invasive human breast adenocarcinoma with mutant p53, an important tumor suppressor. Breast adenocarcinoma originates from the epithelial cells of the glandular tissue (*i.e.* ducts and lobules) of the breast[1]. The hypothesis of this project is that exposure to ionizing radiation will induce changes in the expression of adhesion proteins on cancer cells, and these changes may affect the metastatic potential of the primary tumor. The specific aim was to identify these changes in the breast adenocarcinoma cell line after exposure to high dose ionizing radiation at different points in time. The relative amount of each integrin subunit was determined using Western blot analysis. Functional changes in adhesion were then determined using an adhesion assay in which certain integrins were blocked to determine their normal function.

Materials and Methods

Cell Culture: Breast cancer cell lines MDA-MB-231, which is an invasive human breast adenocarcinoma, was provided by Dr. Jianjian Li (UC-Davis). Cells were cultured in Eagle's Minimal Essential Medium with 10% Fetal Bovine Serum, sodium pyruvate, non-essential amino acids, and penicillin/streptomycin (100 U/mL) at 37°C with 5% CO_2.

Radiation: Cells were irradiated with γ-rays from a Cesium-137 source at indicated doses from 0 to 20 Gy.

Western Blot Analysis: For dose-dependent experiments, cells were given 24 hours to recover. Cells were harvested with trypsin then lysed in NP-40 lysis buffer (20 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM EDTA, 7 mM β-mercaptoethanol, 0.5% NP-40, 5% glycerol) with protease and phosphatase inhibitors. Total protein concentration was measured using BioRad's DC Protein Assay. Equal amounts of protein were loaded into wells of an 8% polyacrylamide gel and resolved by SDS-PAGE, followed by electroblotting onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk in Tris-Buffered Saline/ Tween 20 (TBS-T), and blotted with anti-integrin αv (Cell Signaling), anti-integrin α3 (Santa Cruz), anti-integrin β1 (Santa Cruz), anti-activated integrin β1 (Millipore), anti-Focal Adhesion Kinase (FAK, Cell Signaling), anti-phospho-FAK (Cell Signaling). The membrane was then blotted with its respective horseradish peroxidase-conjugated secondary antibody (anti-goat IgG and anti-mouse IgG from Santa Cruz, anti-rabbit IgG from BioRad). Proteins were

then detected using enhanced chemiluminescent substrate (Thermo), and the signal was developed on a film.

Fibronectin Plating: Following radiation, cells were given 24 hours to recover. To examine how extracellular matrix protein interactions affect the expression of integrins, some cells were incubated on plates coated with fibronectin (Sigma) for 1, 2, and 4 hours. These cells were harvested with a non-enzymatic cell dissociation buffer (Cellstripper from Mediatech), and then subjected to Western blot analysis as described above.

Adhesion Assay: Following radiation, cells were given 24 hours to recover. The effect of ionizing radiation on the adhesion of cells to different ECM proteins was examined using an ECM screening kit (Millipore). For indicated experiments, cells were incubated in a blocking antibody (integrins αv, α1, α2, α3, α5, β1, α2β1 dimer, α5β1 dimer from Millipore) for 10 minutes prior to seeding. Cells (10^5) were plated onto 8-well strips coated with fibronectin, laminin, vitronectin, collagen I, or collagen IV and incubated at 37°C for 2 hours. Cells were washed with Dulbecco's PBS three times and then fixed with 4% formaldehyde for 15 minutes. After a wash with PBS, cells were stained with crystal violet (5 mg/mL) in 2% ethanol for 10 minutes. Cells were then washed three times with PBS and air dried for about 30 minutes. The stain was then solubilized with 2% SDS for 30 minutes, and the absorbance was read at a wavelength of 550 nm.

Results and Discussion

Western blot analysis and adhesion assays were done simultaneously, with the results of any given experiment dictating the variable tested in the next experiment. Therefore, results from each type of experiment may be discussed in either section if deemed relevant.

Determination of the effect of IR on integrin expression

Western blot analysis was used to qualitatively study the integrin expression changes induced by radiation and interactions with the ECM protein fibronectin. Fibronectin was chosen because of its significant adhesive and anti-adhesive interactions with multiple integrins, as determined from the adhesion assays. Antibodies for integrins α5, β3, and β5 could not be detected, and thus results are not shown.

Focal adhesion kinase (FAK) expression is independent of cell density, radiation exposure in the absence of ECM interactions, and radiation exposure in the presence of fibronectin interactions (Fig. 1). Phosphorylated FAK expression increases with increasing cell density and decreases with increasing radiation exposure. Therefore, increasing cell density activates the phosphorylation cascade, while increasing radiation exposure deactivates the phosphorylation cascade.

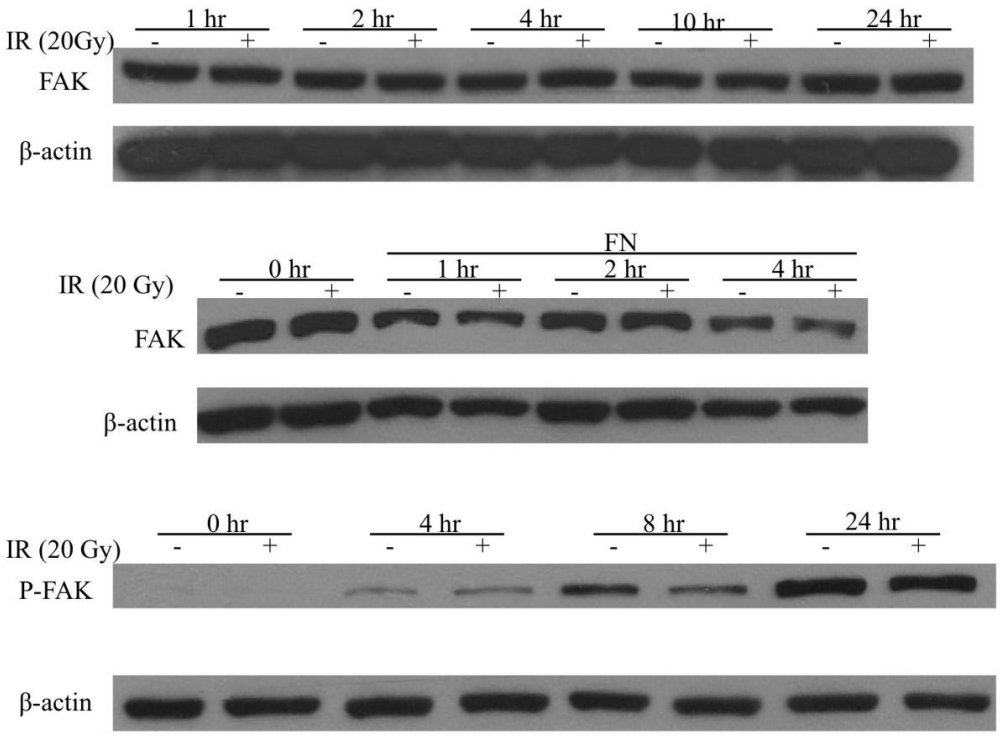

Figure 1. FAK and P-FAK expression. Cells were irradiated (+) with high dose (20 Gy) ionizing radiation and immediately harvested or incubated for 1, 2, 4, 10, or 24 hours on uncoated or fibronectin-coated (FN) plates. FAK expression does not change with time, IR alone, or IR+FN; P-FAK increases with time, decreases with IR alone.

Integrin β1 alone has no largely significant adhesive interaction with any of the ECM proteins, but its expression and activation shows distinct trends. Integrin β1 expression increases with increasing cell density, is independent of radiation exposure in the absence of ECM interactions, decreases with increasing radiation exposure when interacting with fibronectin, and increases with increasing radiation exposure when interacting with bovine serum albumin (BSA) (Fig. 2). Therefore, integrin β1 expression is dependent on density, and its dependence on radiation exposure requires

specific ECM interactions. Activated integrin β1 expression shows the same trends as its inactivated form for cell density and radiation with fibronectin interactions, but expression of the activated form increases with radiation in the absence of ECM interactions. Therefore, radiation exposure in the absence of ECM interactions causes activation of integrin β1 rather than changes in expression levels.

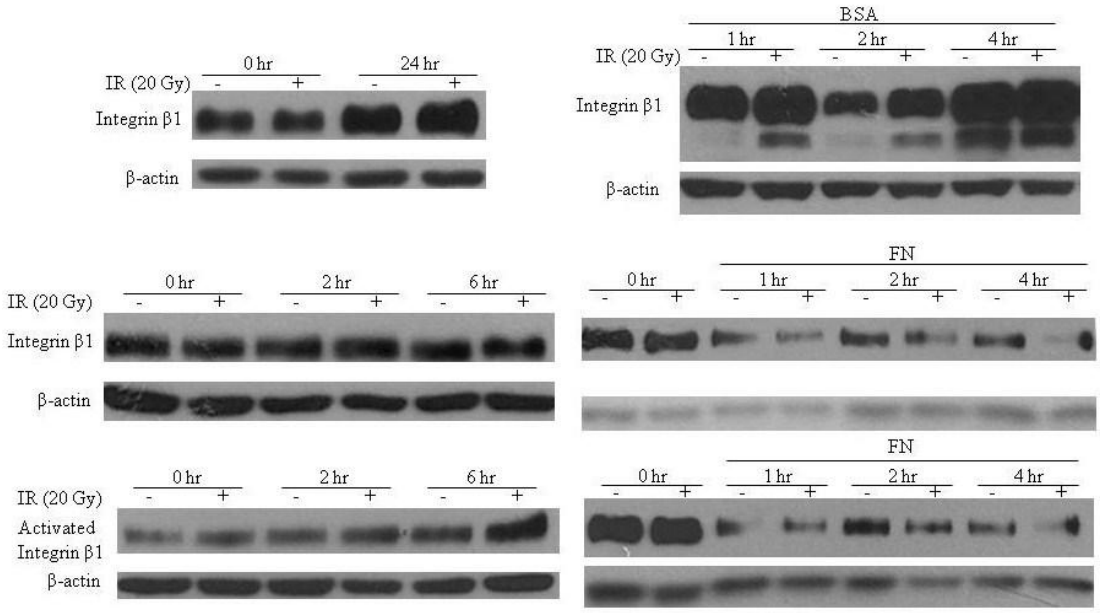

Figure 2. Integrin β1 and activated integrin β1 expression. Cells were irradiated (+) with high dose (20 Gy) ionizing radiation and immediately harvested or incubated for 1, 2, 4, 6, or 24 hours on uncoated, fibronectin-coated (FN), or bovine serum albumen-coated (BSA) plates. Integrin β1 expression increases with time, does not change with IR alone, increases with IR+BSA, and decreases with IR+FN; activated integrin β1 increases with time, increases with IR alone, and decreases with IR+FN.

Integrin α3 displays an anti-adhesive interaction with fibronectin, laminin, collagen I, and collagen IV with an average relative change of 0.12 ± 0.05 for the

control cells and 0.16 ± 0.03 for the IR cells. Western blot analysis shows integrin α3 expression is independent of cell density and is independent of radiation exposure (Fig. 3). Therefore, integrin α3 seems to have stable expression and function across all variables tested, which may explain the relatively similar adhesion across the different ECM proteins.

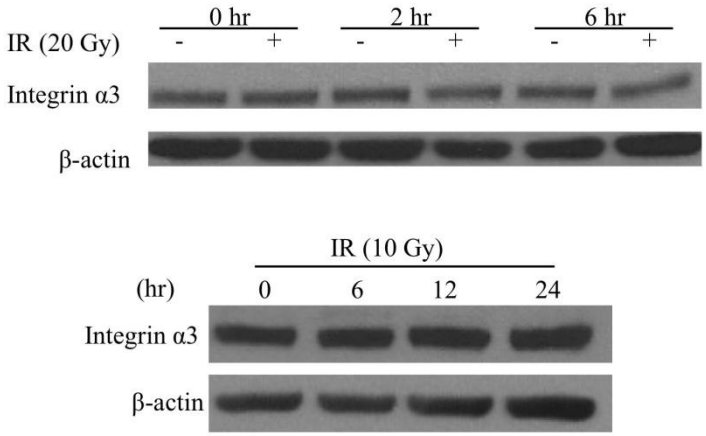

Figure 3. Integrin α3 expression. Cells were irradiated (+) with high dose (10 or 20 Gy) ionizing radiation and immediately harvested or incubated for 2, 6, 12, or 24 hours on uncoated plates. Integrin α3 expression does not change with time or IR alone.

Integrin αv has a significantly adhesive interaction with fibronectin (relative change of -0.13) only in non-irradiated cells, though this result is from a single experiment and only this ECM protein was examined. Integrin αv expression increases with increasing cell density and increases with increasing radiation exposure (Fig. 4). More functional studies should be done to better understand the relationship between integrin αv expression and adhesion interactions.

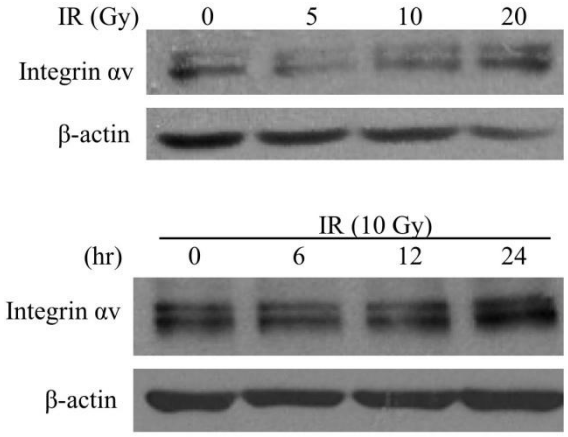

Figure 4. Integrin αv expression. Cells were irradiated with increasing dose (0, 5, 10, or 20 Gy) or high dose (10 Gy) ionizing radiation and immediately harvested or incubated for 6, 12, or 24 hours on uncoated plates. Integrin αv expression increases with time, increases with IR alone.

Integrin β1 (both activated and inactivated forms), integrin αv, integrin α3, and phosphorylated focal adhesion kinase (FAK) increase expression with time in both control and irradiated samples (Table 1). These results suggest their expression is inherently density dependent.

Radiation exposure alone decreases phosphorylation of FAK, increases activation of integrin β1, and increases expression of integrin αv (Table 1). Therefore, ionizing radiation has an effect on various signaling pathways, though the exact mechanism is still unknown.

Integrin β1 (both activated and inactivated forms) shows a change in expression when comparing experiments from radiation exposure alone and radiation exposure combined with fibronectin interactions (Table 1). Therefore, interactions with ECM proteins have a significant effect on the function of integrin β1.

Table 1. Protein Expression Summary.

	Time	IR alone	IR + FN
FAK	no change	no change	no change
P-FAK	increase	decrease	---
Integrin β1	increase	no change	decrease
Activated Integrin β1	increase	increase	decrease
Integrin αv	increase	increase	---
Integrin α3	no change	no change	---

Determination of the effect of IR on integrin-mediated adhesion to ECM proteins

The adhesion assay was used to quantitatively study the functional adhesion changes induced by radiation and ECM protein interactions. Equal numbers of cells are seeded into wells coated in different ECM proteins. After incubation, the wells are washed, removing any non-adhered cells. Adhered cells were then fixed before staining to ensure they were not removed in subsequent washing steps. The amount of crystal violet in the solubilized solution, and therefore the absorbance, is proportional to the number of adhered cells.

Experiments with no error bars were only done once, while those with error bars represent at least two independent experiments. Outliers were omitted from graphic representation using the Grubb's test (alpha = 0.05). Relative change, or relative number of cells, is equal to the difference in absorbance between the IR and control samples divided by the absorbance of the control sample. For the adhesion blocking assay, relative change is equal to the difference in absorbance between the

blocked and un-blocked cells divided by the absorbance of the un-blocked cells, for both IR and control samples. To be considered significant, the relative change must be greater than 0.05 in either the positive or negative direction, and the standard deviation must be entirely positive or negative, respectively. A significant decrease in adhesion following blocking indicates that the blocked integrin has an adhesive interaction with the ECM protein. Conversely, a significant increase in adhesion following blocking indicates that the blocked integrin has an anti-adhesive interaction with the ECM protein.

High dose radiation exposure overall causes increased adhesion of MDA-MB-231 cells to fibronectin, laminin, collagen I, and collagen IV, but no significant change in vitronectin adhesion (Fig. 5). This indicates that radiation-induced damage is stimulating pathways involved in invasion and metastasis.

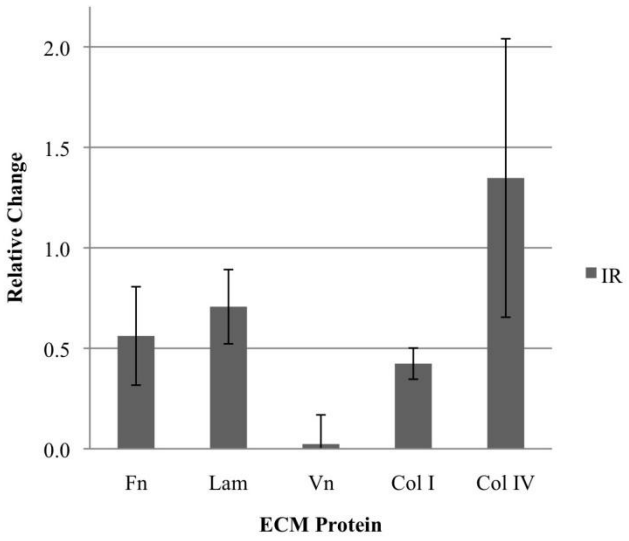

Figure 5. Adhesion to different ECM proteins. Fibronectin (FN), Laminin (Lam), Vitronectin (Vn), Collagen I (Col I), Collagen IV (Col IV)

The most significant adhesive interactions with fibronectin involve integrin α5 (Fig. 6). Blocking α5 alone results in decreased fibronectin adhesion, as does blocking α5 and β1 and—though to a lesser extent—blocking the heterodimer α5β1. Blocking integrin β1 alone results in no change in fibronectin adhesion. It is likely, therefore, that α5 dimerizes with a different β integrin to have an adhesive interaction with fibronectin. Conversely, integrin α3 has a significant anti-adhesive interaction with fibronectin. The adhesive changes seen for the α5β1+α3 experiment appear to be simply additive effects of the α3 alone and α5 alone experiments. Integrins αv, α1, β1, and α2β1 have no largely significant interaction with fibronectin.

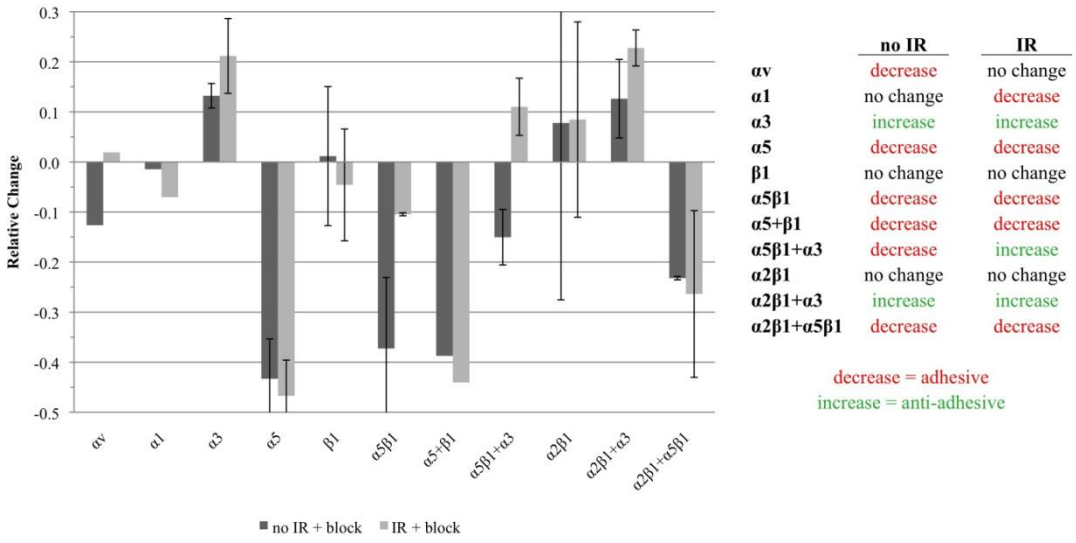

Figure 6. Fibronectin adhesion after integrin blocking. A significant decrease in adhesion following blocking indicates that the blocked integrin has an adhesive interaction with the ECM protein. Conversely, a significant increase in adhesion following blocking indicates that the blocked integrin has an anti-adhesive interaction with the ECM protein.

The most significant adhesive interactions with laminin also involve integrin α5, as well as integrin α1 and dimer α2β1 following radiation exposure (Fig. 7). As seen with fibronectin, blocking integrin β1 alone also results in no change in laminin adhesion, and integrin α3 has an anti-adhesive interaction with laminin. These results are based on a limited number of experiments, however, and more should be done before any conclusions can be drawn.

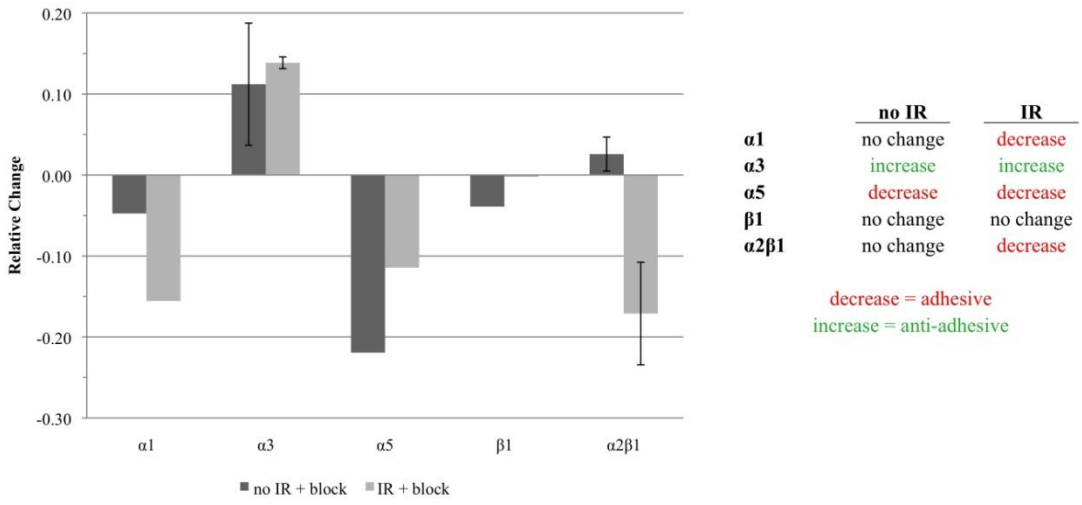

Figure 7. Laminin adhesion after integrin blocking. A significant decrease in adhesion following blocking indicates that the blocked integrin has an adhesive interaction with the ECM protein. Conversely, a significant increase in adhesion following blocking indicates that the blocked integrin has an anti-adhesive interaction with the ECM protein.

The integrin α2β1 heterodimer has no significant interaction with vitronectin (data not shown), but it has a significant adhesive interaction with collagen I (Fig. 8). However, the dimer is required for this interaction, as blocking integrin α2 alone

causes no significant change. Again, integrin α3 appears to have an anti-adhesive interaction with collagen I, but only for irradiated cells. Integrin α5 has no significant interaction with collagen I, and integrin β1 has no largely significant interaction based on a single experiment.

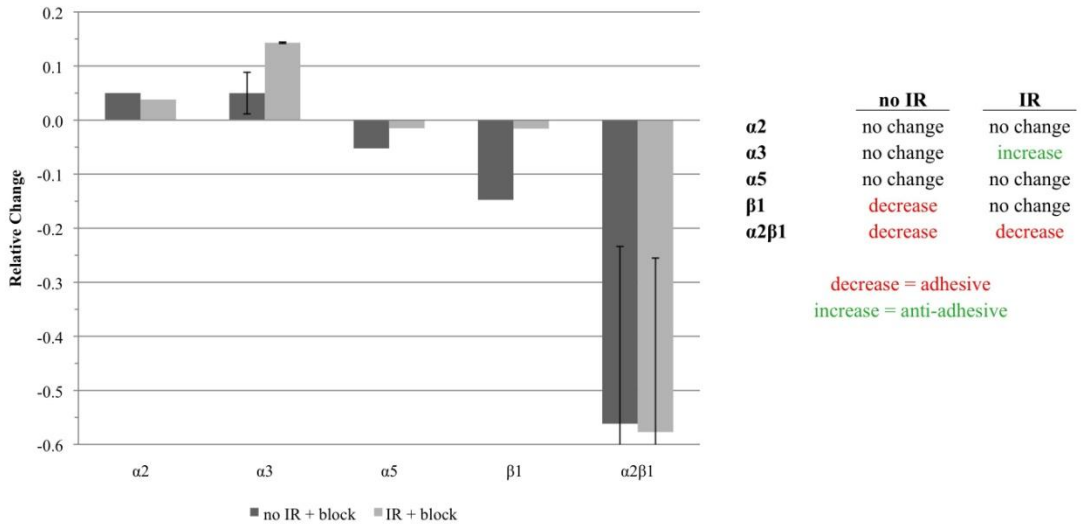

Figure 8. Collagen I adhesion after integrin blocking. A significant decrease in adhesion following blocking indicates that the blocked integrin has an adhesive interaction with the ECM protein. Conversely, a significant increase in adhesion following blocking indicates that the blocked integrin has an anti-adhesive interaction with the ECM protein.

While blocking integrin α1 or integrin α2 alone show no significant change in adhesion on collagen IV, blocking both α1 and α2 results in decreased adhesion (Fig. 9). Therefore, these two integrins may interact in a focal adhesion site. As seen before, blocking integrin β1 alone has no largely significant effect based on a single experiment, and blocking α2β1 results in decreased adhesion. Therefore, the integrin

α2β1 dimer has adhesive interaction and integrin α3 has anti-adhesive interaction on collagen IV, as on collagen I.

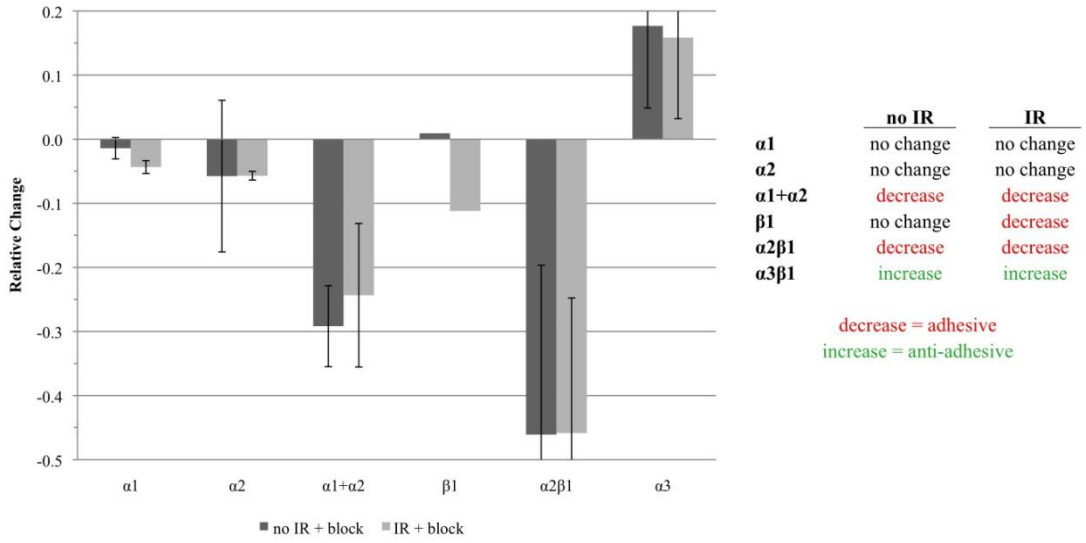

Figure 9. Collagen IV adhesion after integrin blocking. A significant decrease in adhesion following blocking indicates that the blocked integrin has an adhesive interaction with the ECM protein. Conversely, a significant increase in adhesion following blocking indicates that the blocked integrin has an anti-adhesive interaction with the ECM protein.

Collectively, integrin α3 displays an anti-adhesive interaction with fibronectin, laminin, collagen I, and collagen IV. Integrin α5 displays adhesive interaction with fibronectin and laminin. Integrin α2β1 displays adhesive interaction with laminin (only irradiated cells), collagen I, and collagen IV. Finally, integrin β1 alone has no largely significant adhesive interaction with any of the ECM proteins.

Conclusions

The purpose of this project was to obtain preliminary results to determine if ionizing radiation (IR) induces changes in the expression and function of integrins. The results suggest that there is, in fact, significant change occurring following IR exposure in both integrin expression and adhesion, and a profile of these changes has been started for the MDA-MB-231 human breast cancer adenocarcinoma cell line.

Ionizing radiation clearly induces changes in expression of certain integrins, and expression of certain integrins is correlated with initiation of the invasive phenotype. In fact, the functional studies showed that, in this cell line, IR increases adhesion—an initiating step of invasion. Therefore, this study could have serious clinical implications. These experiments likely represent the interactions of the outer cancer cells in a heterogeneous tumor with the ECM, but IR could be inducing the EMT— a more stem-cell like state, which is typically more resistant to chemotherapy and radiation therapy. This, in turn, could be related to the overlapping pathways of integrins and cell survival. Certain integrins induce apoptosis, and loss of these integrins could promote invasion. These conclusions further support the cyclical regulation between the tumor microenvironment and integrins; the former changes the latter, which then changes the former, and so on.

It is important to note that metastasis occurs over time, not in distinct stages. There is also a dynamic interaction of reciprocal regulation between ECM and integrins expressed on cancer cells at each point in time. Once a better understanding of the molecular mechanisms of radiation-induced, integrin-mediated adhesion and

invasion is obtained, it may be possible to inhibit the invasive process prior to radiation therapy, thus minimizing the risk of metastasis. It is highly unlikely, however, that results from this project will apply broadly across all cancers. Because of the tightly integrated interactions between the extracellular matrix, which is tissue specific, and the integrin proteins, different signaling pathways may be active to differing degrees. Therefore, the components affected by ionizing radiation will vary based on the random nature of damage and probability alone; profiles of each tumor subtype will have to be completed before any therapeutic intervention will be possible.

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