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Regulation of Ionizing Radiation-Induced Adhesion of Breast Cancer Cells to Fibronectin by Alpha5beta1 Integrin

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Abstract

Ionizing radiation (IR) is commonly used for cancer therapy, however, its potential influence on cancer metastatic potential remains controversial. In this study, we elucidated the role of integrins in regulation of IR-altered adhesion between breast cancer cells and extracellular matrix (ECM) proteins, which is a key step in the initial phase of metastasis. Our data suggest that the extent of effect that ionizing radiation had on cell adhesion depended on the genetic background of the breast cancer cells. Ionizing radiation was a better adhesion inducer for p53-mutated cells, such as MDA-MB-231 cells, than for p53 wild-type cells, such as MCF-7 cells. While IR-induced adhesions between MDA-MB-231 cells to fibronectin, laminin, collagen I and collagen IV, only blocking of the adhesion between $\alpha 5\beta 1$ integrin and fibronectin using anti- $\alpha 5\beta 1$ integrin antibody could completely inhibit the radiation-induced adhesion of the cells. A soluble Arg-Gly-Asp peptide, the binding motif for fibronectin binding integrins, could also reduce the adhesion of the cells to fibronectin with or without ionizing radiation exposure. The inhibition of the cell-fibronectin interaction also affected, but did not always correlate with, transwell migration of the cancer cells. In addition, our data showed that the total expression of $\alpha 5$ integrin and surface expression of $\alpha 5\beta 1$ integrin were increased in the cells treated with ionizing radiation. The increased surface expression of $\alpha 5\beta 1$ integrin, along with the adhesion between the cells and fibronectin, could be inhibited by both ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) kinase inhibitors. These results suggested that ATM/ATR-mediated surface expression of $\alpha 5\beta 1$ integrin might play a central role in regulation of ionizing radiation-altered adhesion.

INTRODUCTION

Extracellular matrix (ECM) proteins such as fibronectin (FN), laminin (LN) and collagen (COL) are essential for connecting cells together in tissues, but also for guiding cell movement during wound healing and in the initial step of the metastatic process. These processes are initiated by the binding of adhesion molecules such as integrins to ECM and involve a number of intracellular signaling pathways (1, 2). One essential component of the ECM that controls cancer cell adhesion and migration is FN. Through its tripeptide motif Arg-Gly-Asp, FN interacts with FN-binding receptors, such as $\alpha 5\beta 1$ integrin on the cell surface. Upon engagement of FN, $\alpha 5\beta 1$ activates an associated focal adhesion kinase-

dependent intracellular signaling pathway (2) and thereby regulates cancer cell invasion (3, 4).

Radiotherapy is widely used in the treatment of various cancers, however, the effects of ionizing radiation (IR) on cancer metastatic potential remain unclear. Integrins have emerged as important players in cancer metastatic behavior (5). Moreover, ionizing radiation has been shown to upregulate the expression of $\alpha v\beta 3$ or $\alpha 5\beta 1$ on glioma cells and colorectal cancer cells, respectively (6, 7), as well as the expression and sialylation of $\beta 1$ integrin (8, 9). In addition, it was also reported that $\alpha 5\beta 1$ is involved in radiation-induced invasion of pancreatic cancer cells (10). However, the extent to which IR could alter adhesive strength of cancer cells to ECM and the role of integrin-ECM protein interaction in regulation of cancer migration is not well known. In this study, we investigated the correlation of expression and functional activation of integrins, adhesion between cancer cells and each individual ECM protein and the invasiveness of cancer cells after irradiation. Our data suggest a novel mechanism of ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) mediated $\alpha 5\beta 1$ integrin expression in the regulation of metastatic potential of breast cancer cells in response to ionizing radiation.

MATERIALS AND METHODS

Cell Culture

Human breast cancer cell lines MDA-MB-231 were kindly provided by Dr. J. J. Li (UC Davis, Davis, CA), and MDA-MB-468, MCF-7, ZR-75-1, T47D, Hs578t, BT-20 were kindly provided by Dr. M. M. Burdick (Ohio University, Athens, OH). MDA-MB-231 cells were cultured in Eagle's minimum essential medium [(MEME) Corning, Manassas, VA] containing 10% fetal bovine serum [(FBS) Denville, Metuchen, NJ], 2 mM glutamine, 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), 1 mM sodium pyruvate and 1× nonessential amino acids (Corning); MCF-7, ZR-75-1, T47D and Hs578t cells were cultured in Dulbecco's modified Eagle's medium [(DMEM) Corning] with 10% FBS; BT-20 were cultured in Eagle's MEM with Earle's balanced salts solution [(MEM/EBSS) Corning] with 10% FBS. All of the cells were incubated at 37°C in 5% CO₂ and 95% humidified air.

Drug Treatments and Gamma Irradiation

To induce functional blocks with either RGD peptide or antiintegrin antibodies, the cells were detached from the plate, suspended into 5×10^5 /mL in serum-free MEME supplied with or without a corresponding blocking antibody (10 µg/mL) or RGD peptide (cat. no. sc-201176, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. For treatment of ATM or ATR kinase inhibitor, the cells were pretreated with DMSO, 10 µM of CGK-733 (Sigma-Aldrich, St. Louis, MO), VE-821 (Selleckchem, Houston, TX) or KU-55933 (Selleckchem) for 2 h before exposure to ionizing radiation. The irradiated cells were incubated for 24 h in the presence of inhibitors. The cells were irradiated with single fraction of 10 Gy using a ¹³⁷Cs irradiator (J. L. Shepherd Associates, San Fernando, CA).

Cell Adhesion Assay

The cells were detached and resuspended with serum-free MEME. The cells suspensions (5×10^4 cells in 100 μ l) were immediately transferred to ECM protein-coated wells (Millipore, Darmstadt, Germany). After incubation at 37°C for 2 h, the cells were washed three times, then fixed in 4% formaldehyde and stained with 0.2% crystal violet in 10% ethanol. The stained cells were then resuspended by 2% SDS and the amount of solubilized stain was determined by absorbance at 570 nm using a Spectra-max plate reader (Molecular Devices Corp., Sunnyvale, CA).

Migration Assay

Cell migration assays were performed according to the manufacturer's instructions. The transwell filters [Polycarbonate Membrane Transwell® Inserts (8 μ m pore), Corning] were coated with 20 μ g/mL fibronectin overnight and stored at 4°C. The filters were then blocked with 0.5% bovine serum albumin (BSA) for 1 h before use. Each lower chamber was filled with 0.6 mL DMEM containing 10% FBS. The cells (10^5) were seeded onto each filter, incubated for 2 h at 37°C, then irradiated with 10 Gy of ionizing radiation. After 24 h incubation, cells that migrated into the lower side of the membrane were fixed and stained with crystal violet for 10 min. The cells were counted under a microscope at 5 different fields.

Flow Cytometry

Surface expression of integrins on MDA-MB-231 cells was evaluated by flow cytometry. The cells were pelleted by centrifugation, resuspended in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 10 min. Cells (1×10^6) from each sample were rinsed, blocked with 5% BSA and incubated with anti- β 1 integrin (CD29, cat. no. sc-9970, Santa Cruz Biotechnology), anti- α 5 integrin (JBS5, cat. no. sc-59762, Santa Cruz Biotechnology), anti- α 5 β 1 integrin (HA5, MAB1999, Millipore), anti- α 2 β 1 integrin (BHA2.1, MAB1999, Millipore) or anti-activated β 1 integrin (HUTS-4, MAB2079Z, Millipore) for 1 h in the dark, followed by a FITC-conjugated secondary antibody. After washing with 5% BSA twice, cells were suspended in 0.5 mL PBS and were analyzed using a FACSsort flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Western Blotting

MDA-MB-231 cells were lysed in 2% NP-40 buffer (2% NP-40, 80 mM NaCl, 0.1% SDS) containing protease inhibitor cocktail set III (Millipore). After 15 min incubation on ice the lysates were centrifuged and the supernatants were collected. The proteins (20 μ g) were electrophoresed on an 8% SDS-PAGE, transferred to a nitrocellulose membrane and blocked with 5% nonfat milk in trisbuffered saline with Tween (TBS-T) (USB, Cleveland, OH) for 1 h. Blots were incubated with anti-human α 5 integrin (sc-10729), antihuman α 2 integrin (sc-9089), anti-human β 1 integrin (sc-6622) (all three with Santa Cruz Biotechnology), or anti-beta actin antibody (A5316, Sigma-Aldrich, St. Louis, MO) and incubated overnight at 4°C. Membranes were washed then with TBS-T three times and probed with corresponding secondary antibodies for 0.5 h at room temperature. Blots were then washed with TBS-T three times and with tris-buffered saline (TBS) twice. The signals

were detected using West Pico Supersignal chemiluminescent substrate (Pierce, Rockford, IL).

Statistical Analysis

Student's *t* test was performed to compare data in two different groups and considered statistically significant at $P < 0.05$.

RESULTS

The Differential Effect of Ionizing Radiation on Breast Carcinoma Cell Lines with Various Genetic Backgrounds

We first determined the effect of IR (10 Gy) on the avidity between breast cancer cells and each of the ECM proteins, including fibronectin (FN), laminin (LN), vitronectin (VN), collagen I (Col I) and collagen IV (Col IV). Seven breast carcinoma cell lines with various expression status of p53, ER, PR and HER2 (Table 1) were used in the study. Our data show that IR can positively or negatively impact avidity between breast cancer cells and ECM proteins (Table 1). Almost all of the five p53 negative cell lines (MDA-MB-231, MDA-MB-468, T47D, Hs578t and BT-20) showed increased avidity to ECM proteins after irradiation (Table 1). The two p53 positive cell lines (MCF-7 and ZR-75-1) behaved differently. While ionizing radiation exposure reduced the avidity of ZR-75-1 to all tested ECM proteins, irradiation of MCF-7 decreased avidity to VT and Col I, but increased avidity to FN and Col IV (Table 1). Our results suggest that ionizing radiation-altered avidity of breast carcinoma to ECM proteins is dependent on protein expression, such as p53 status, of the cancer cells.

$\alpha 5\beta 1$ Mediates Ionizing Radiation-Induced Adhesion between MDA-MB-231 Cells and ECM Proteins

To further assess the mechanism of ionizing radiation-induced adhesion of breast cancer cells and ECM, we determined the contributions of integrins, which have been shown to mediate cell-ECM interaction (11), in ionizing radiation-induced (10 Gy) adhesion between breast carcinoma and ECM proteins. MDA-MB-231 cells, which our studies indicate are highly inducible by radiation, but not the most inducible (Table 1), were used in the experiment. Our data show that ionizing radiation exposure increases the avidity of the cancer cells to FN, LN, Col I and Col IV (Fig. 1A–D). Function-blocking studies indicate that the antibody against $\alpha 5\beta 1$ integrin, which is a known receptor of FN, can efficiently block the ionizing radiation-induced adhesion between the cancer cells and FN (Fig. 1A). Antibodies against $\alpha 5$ or $\beta 1$ integrin also reduced the adhesion induced by irradiation (Fig. 1A). However, interestingly the antibody against $\alpha 2\beta 1$ integrin, which is a known receptor of LN, Col I and Col IV, reduced the adhesion between the cancer cells and the ECM proteins with or without ionizing radiation exposure (10 Gy) (Fig. 1B–D). Antibodies against $\alpha 2$ or $\beta 1$ integrin also reduced the adhesion between the cells and ECM at various degrees (Fig. 1B–D). In contrast, the antibody against $\alpha 3$ integrin, which is a polyspecific FN, LN, Col receptor (12), showed no statistically significance difference in reduce adhesion between the cancer cells and any of the ECM proteins after irradiation (Fig. 1A–D). These results suggest that while $\alpha 5\beta 1$ -mediated cell-FN interaction is responsible for the

increased avidity of MDA-MB-231 cells to the ECM after irradiation, $\alpha 2\beta 1$ -mediated cell-ECM interaction contributes to adhesion between cells and ECM with or without ionizing radiation exposure.

$\alpha 5\beta 1$ Integrin-Fibronectin Interaction Regulates the Invasiveness of MDA-MB-231 Cell

To confirm the role of FN in ionizing radiation-induced adhesion between MDA-MB-231 and FN, we investigated the effect of RGD-containing peptide (GRGDNP), which is critical for fibronectin recognition of integrins, on the avidity of MDA-MB-231 cells to FN (11). Our data show that GRGDNP reduced not only ionizing radiation-induced avidity, but also the background avidity of the cells to FN in a dose-dependent manner (Fig. 2A). These results suggest that the interaction between integrin and FN plays a critical role in controlling adhesion of MDA-MB-231 cells with or without ionizing radiation exposure.

To correlate the avidity to invasiveness of the cancer cells, we determined the extent of effect of GRGDNP on FN-coated transwell migration of MDA-MB-231 cells. Our data show that the cell migration was decreased by 41% after irradiation (10 Gy) even though its avidity for FN was increased by 76.7% (Fig. 2, panel B vs. panel A). Treating the cells with GRGDNP reduced the migration with or without ionizing radiation exposure in a RGD dose-dependent manner (Fig. 2B). Our results suggest that while the RGD-mediated radiation-induced adhesion of the cells to FN reduces the invasiveness, the background adhesion of the cells to FN may promote migration of the cancer cells. To further examine the correlation between cell-FN adhesion and cell migration, we determined the effect of anti- $\alpha 5\beta 1$ antibody on the migration of MDA-MB-231 cells. Our data show that while the antibody has no statistically significant effect on the background cell adhesion (Fig. 1A, control, NT vs. $\alpha 5\beta 1$), the anti- $\alpha 5\beta 1$ antibody promotes migration of the cancer cells without ionizing radiation treatment (Fig. 1C, control, NT vs. 20 $\mu\text{g/mL}$). Interestingly, while the antibody almost completely inhibited the ionizing radiation-induced adhesion between the cells and FN (Fig. 1A, IR, NT vs. $\alpha 5\beta 1$), it may slightly enhanced (not statistically significant) the migration of the cells on FN-coated transwell (Fig. 1C, IR, NT vs. 20 $\mu\text{g/mL}$). These results indicate that while both RGD-containing peptide and anti- $\alpha 5\beta 1$ antibody can reduce the adhesion between the cells and FN, their effects on the migration of the cancer cells are variable.

Ionizing Radiation-Induced Surface Expression of Integrins on MDA-MB-231 but not on MCF-7 Cells

To determine whether the increased adhesive strength of the cells is correlated to an increased surface expression of adhesion molecules, we determined the surface expression levels of $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins in MDA-MB-231 as well as MCF-7 cells at 24 h postirradiation (10 Gy) using flow cytometry. Our data indicate that both $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins were increased approximately 30% on the surface of MDA-MB-231 (Fig. 3A, top panels) (Fig. 3B) but did not changed on the surface of MCF-7 cells (Fig. 3A, bottom panels). Our data further shows that the increased levels of $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins were correlated with the increased surface level of $\beta 1$ integrin and activated $\beta 1$ integrin [Fig. 3A (middle panels) and B]. Noticeably, the increased level of $\beta 1$ integrin was not statistically

significantly different from the increased level of activated $\beta 1$ integrin (Fig. 3B), indicating that almost all ionizing radiation-induced $\beta 1$ on the cell surface was activated.

To determine whether the increase of surface levels of integrins is correlated with an upregulation of expression of the integrins, we analyzed the total levels of $\alpha 2$, $\alpha 5$ and $\beta 1$ integrins in MDA-MB-231 cells using Western blot analysis. Our data show that while the expression of $\beta 1$ integrin was not significantly changed, the expression of $\alpha 5$ integrin was increased in a time-dependent manner (Fig. 3C) after IR exposure.

Both ATM and ATR Kinases Mediate Ionizing Radiation-Induced Expression of $\alpha 5\beta 1$ Integrin and Adhesion of MDA-MB-231 Cells

ATM and ATR protein kinases are a critical intermediate in a number of cellular responses to ionizing radiation, including phosphorylation of p53 and gene expressions (13, 14). To investigate whether ionizing radiation-induced expression of $\alpha 5\beta 1$ integrin and thus cancer cell adhesion relies on activation of the ATM and/or ATR kinase, we examined the effect of inhibitors of ATM (KU-55933), ATR (VE-821) and ATM/ATR (CGK-733) (15) on cell surface expression of $\alpha 5\beta 1$ integrin on MDA-MB-231 cells after IR exposure. Our data show that all three ATM and/or ATR inhibitors reduced (KU-55933) or eliminated (VE-821 and CGK-733) the ionizing radiation-induced cell surface expression of $\alpha 5\beta 1$ integrin (Fig. 4A), which also reflected in the inhibition of the adhesion between the cells and FN with or without ionizing radiation exposure (Fig. 4B). These data demonstrate that both ATM and ATR kinases are involved in regulation of $\alpha 5\beta 1$ -mediated adhesion between breast cancer cells and FN with or without ionizing radiation exposure.

DISCUSSION

Cancer metastasis is a complex process and consists of a series of distinct steps that result in the transfer tumor cells from a primary site to a distant locations (16). The process of metastasis is highly dependent on the interaction of various cell adhesion molecules between tumor cells and endothelial cells or ECM. Ionizing radiation has been shown to alter the expression and function of certain cell adhesion molecules, including integrins (7, 9). While some clinical studies showed that radiation could induced cancer metastatic potential under certain circumstances (17), the role of ionizing radiation exposure on cancer metastasis remains controversial (18). In this study, we used breast cancer cell lines with different genetic backgrounds to study the extent of the effect of ionizing radiation on expression and functional activation of integrins, adhesion between cancer cells and each individual ECM protein, as well as the invasiveness of cancer cells. Our data indicate that the adhesive strengths between breast cancer cells and each individual ECM protein could be positively or negatively altered by IR exposure (Table 1). One interesting observation from our studies was that the adhesion between the breast cancer cells and ECM proteins was less affected in cells with wild-type p53, while it increased in cells with mutated p53 (Table 1). These results suggest that the metastatic potential of breast cancer cells after radiotherapy could be dependent on their genetic background and could perhaps help explain the controversial conclusions regarding the effect of IR on cancer metastasis that were observed in clinical studies (18).

To further determine the molecular mechanism of ionizing radiation-induced ECM interaction of breast cancer cells, we determined the role of integrins, known as ECM receptors, in mediating ionizing radiation-induced ECM protein binding of MDA-MB-231 cells which we clearly showed the adhesion of which was upregulated by IR (Table 1). Our data indicated that functional blocking of either $\alpha 2\beta 1$ or $\alpha 5\beta 1$ by corresponding antibodies could reduce adhesive strength between MDA-MB-231 cells and the individual ECM proteins (Fig. 1). However, while $\alpha 2\beta 1$ antibody reduced the adhesion with and without IR (Fig. 1B, C and D), $\alpha 5\beta 1$ antibody only reduced the adhesion induced by IR (Fig. 1A). Furthermore, incubating MDA-MB-231 cells with RGD motif-containing peptide reduced the cell adhesion with and without IR treatment (Fig. 2). These results suggest that $\alpha 5\beta 1$ integrin plays a critical role in regulation of MDA-MB-231 cell adhesion.

Since ionizing radiation-induced expression of $\alpha 5\beta 1$ integrin has been suggested as having an important role in the regulation of cancer cell invasiveness (10, 19), we determined whether inhibition of the interaction between $\alpha 5\beta 1$ integrin and FN could also change the level of invasiveness of MDA-MB-231 cells. Our data show that ionizing radiation-induced cell adhesion is correlated with a reduced MDA-MB-231 cells transwell migration, suggesting a decrease in metastatic potential and also that the regulation of cell motility maybe mediated by $\alpha 5\beta 1$ (Fig. 1, panels: A vs. C). Surprisingly, inhibition of ionizing radiation-induced adhesion, which was carried out by treating the cells with RGD peptide, did not restore the cell migration reduced by irradiation; instead, the RGD treatment further inhibited the migration of the cells with or without ionizing radiation exposure (Fig. 2, panels: A vs. B) and suggests that the interplay between adhesion and migration is regulated by adhesiveness, which is modulated by the affinity and avidity of integrins for their ECM ligands (20). It has been reported that the speed of cell migration is governed by cell-substratum adhesiveness (21), and that migration is allowed to reach a maximum rate at the optimal levels of adhesiveness. At the low and high level of adhesiveness, integrin activation causes a decrease in migration because weakly attached cells cannot generate sufficient traction to move, while cells with high levels of adhesiveness cannot sufficiently detach to move (20, 22). In contrast to the cells treated with RGD peptide, the cells functionally blocked with $\alpha 5\beta 1$ integrin antibody, which can completely inhibited ionizing radiation-induced adhesion between the cells and FN (Fig. 1A), showed a slight increase in migration (Fig. 2C, NT vs. 20 $\mu\text{g/mL}$). The conflicting result may be due to other RGD binding proteins, such as $\alpha v\beta 3$ integrin, or other involve mediating the invasiveness of cancer cells.

To elucidate the potential molecular mechanism for cell adhesion in response to IR, we determined the effect of IR on the surface and total expressions of $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins since functional blocking of these integrins reduced the cell adhesion (Fig. 1). Our data indicate that an increased surface expression and activation of $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins after IR (Fig. 3A and B) was correlated to the increased adhesion of the cancer cells (Table 1, MDA-MB-231 and MCF-7 cells), even though total integrin expression did not necessarily change (Fig. 3C). The data in our study suggest a mechanism involving ATM/ATR kinase-mediated DNA damage signaling pathway in adhesion of MDA-MB-231 cells to FN (Fig. 4). We show that inhibitors of ATM and/or ATR blocked the ionizing radiation-induced cell surface expression of $\alpha 5\beta 1$ integrin and reduced the adhesion between breast cancer cells and FN (Fig. 4A and B). However, because the adhesive behaviors of the ATM and/or ATR

inhibitor-treated cells with or without ionizing radiation exposure were similar to the RGD treated cells, our results suggest that ATM/ATR kinase mediates the cell adhesion at least partially through regulation of cell surface expression of integrins, including $\alpha 5\beta 1$ integrin. Interestingly, the extent of the effect of the inhibitors on $\alpha 5\beta 1$ expression does not completely correlate with the cell adhesion with or without ionizing radiation exposure. Interestingly, inhibition of ATR (VE-821) or ATM/ATR (CGK-733) eliminated ionizing radiation-induced expression, but not background expression of $\alpha 5\beta 1$ integrin, however, inhibition of ATM (KU-55933) reduced both ionizing radiation-induced expression and background expression of $\alpha 5\beta 1$ integrin (Fig. 4A). Therefore, while inhibition of ATM (KU-55933) or ATR (VE-821) only reduced ionizing radiation-induced cell adhesion, inhibition of both ATM and ATR (CGK-733) reduced cell adhesion with or without ionizing radiation exposure (Fig. 4B). These results indicate that even though both the ATM and ATR regulated surface expression of $\alpha 5\beta 1$ integrin and cell adhesion after IR treatment, the signaling pathways that were involved in the regulations mediated by ATM or ATR do not completely overlap with each other. In addition, ATM appeared to only mediate cell adhesion of irradiated cells but not control cells through expression of $\alpha 5\beta 1$ integrin. The precise mechanisms underlying ATM/ATR-mediated cell adhesion and expression of integrin are under further investigation. Taken together, our results suggest that ATM/ATR-mediated activation of $\alpha 5\beta 1$ integrin plays a critical role in regulation of certain breast cancer cell adhesions to FN after IR exposure. These findings could potentially lead to the identification of targets for preventing metastasis of breast cancers with certain genetic backgrounds.

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A. Cells bound to FN **B. Cells bound to LN** **C. Cells bound to Col I** **D. Cells bound to Col IV** **FIG. 1.**

The effect of IR on adhesion of MDA-MB-231 cells to ECM proteins. The cells were irradiated (10 Gy) or sham irradiated. At 24 h postirradiation, the cells were incubated with each of the function blocking antibodies as indicated and placed on an ECM coated strip. After removing the unattached cells, the cells were stained with crystal violet, resuspended with 2% SDS, and cell adhesion was determined by absorbance at 570 nm. The wells on the ECM strip were coated with FN (panel A), LN (panel B), Col I (panel C) and Col V (panel D). Relative cell adhesion was determined by the absorbance of the treated cells normalized to the absorbance of the control cells adhering to ECM-coated plate, and the value from control cells was arbitrarily set at 1. Data represent the mean $\pm$ SEM of three independent experiments, each done in triplicate. * $P < 0.05$ IR vs. control, ** $P < 0.05$ IR with function blocking vs. IR without function blocking.

FIG. 2.

The effect of RGD peptide on ionizing radiation-altered adhesion and migration of MDA-MB-231 cells. Panel A: The cells were irradiated (10 Gy) or sham irradiated. At 24 h postirradiation, the cells were incubated with RGD peptide as indicated. The cells were placed on an FN-coated strip and the attached cells were stained with crystal violet. The cell adhesion was determined by absorbance at 570 nm. Relative cell adhesion was determined by the absorbance of the treated cells normalized to the absorbance of the control cells adhering to ECM-coated plate, and the value from control cells was arbitrarily set at 1. Panel

B: The cells were incubated with RGD peptide and then placed on an FN-coated filters. After incubating for 2 h, the cells were irradiated with 10 Gy of ionizing radiation or sham irradiated and incubated for 24 h. Cells that migrated to the lower side of the membrane were stained with crystal violet and counted under a microscope. Data represent the mean $\pm$ SEM of three independent experiments, each done in triplicate. $*P < 0.05$ IR vs. control, $**P < 0.05$ control vs. RGD, $***P < 0.05$ IR vs. IR with RGD. Panel C: The cells were preincubated with isotype antibody or antibody against $\alpha 5\beta 1$ integrin for 30 min, and placed on an FN-coated filters. After 2 h, the treated cells were irradiated with 10 Gy of IR or sham irradiated. At 24 h post incubation, cells that migrated to the lower side of the membrane were stained with crystal violet and counted under microscope. Relative cell migration was determined by the number of the treated cells normalized to the number of the control cells adhering to the lower side of the membrane, and the value from control cells was arbitrarily set at 1. Data represent the mean $\pm$ SEM of three independent experiments, each done in triplicate. $*P < 0.05$ IR vs. control, $**P < 0.05$ IR vs. $\alpha 5\beta 1$, $***P < 0.05$ $\alpha 5\beta 1$ vs. IR with $\alpha 5\beta 1$.

A. Surface Expression of Integrins

B. Percent Increase of Integrin after IR

C. Integrin Expression after IR

FIG. 3.

The effect of ionizing radiation on MDA-MB-231 cell surface expression of integrins. The cells were irradiated (10 Gy) or sham irradiated and then incubated for 24 h. Panel A: The cells were suspended and incubated with anti-α5, β1, activated β1 (HUT-4), α2β1 or α5β1. Surface expression of each integrin was determined by flow cytometry. Isotype control antibody was used as a negative control antibody. The histograms represent a typical appearance of three independent experiments. Panel B: The average percentage change of cell surface level of integrin after irradiation. Panel C: The irradiated cells were lysed. The expression level of integrin in total lysate was detected by Western blotting analysis.

A. Surface Expression of $\alpha 5\beta 1$ Integrins

B. Cell Adhesion to FN

FIG. 4.

The role of ATM kinase on MDA-MB-231 cell adhesion. The cells were pretreated with 10 μ M of CGK-733, KU-55933, VE-821 or DMSO for 2 h. The treated cells were exposed to 10 Gy irradiation and incubated for 24 h. Panel A: The cells were suspended and incubated with anti- $\alpha 5\beta 1$ antibody. Surface expression of $\alpha 5\beta 1$ integrin was determined by flow cytometry. Isotype control antibody was used as a negative control antibody. The histograms represent a typical appearance of three independent experiments. Panel B: The cells were placed on an FN-coated strips. The attached cells were stained with crystal violet and cell adhesion was determined by absorbance at 570 nm. Relative cell adhesion was determined by the absorbance of the treated cells normalized to the absorbance of the control cells adhering to FN-coated plate and the value from control cells was arbitrarily set at 1. Data represent the mean $\pm$ SEM of three independent experiments, each done in triplicate. * P < 0.001 control vs. IR, ** P < 0.001 control vs. CGK-733, *** P < 0.001 IR vs. IR with CGK-733, KU-55933 or VE-821.

TABLE 1

Adhesion of Breast Carcinoma Cells to ECM Proteins

MCF-7			ZR-75-1			MDA-MB-231					
p53	Wild-type			Wild-type			Mutated				
	ER	PR	HER2	ER	PR	HER2	ER	PR	HER2		
Mark ^a	(6)	(6)	(0-1)	(3)	(4)	(2)	(0)	(0)	(0-1)		
(level)											
IR	-	+	CHG% ^b	-	+	CHG%	-	+	CHG%		
FN	1.000	1.213	21.30	1.000	0.805	-19.50	1.000	1.767	76.70		
LM	1.108	1.108	0.00	1.118	0.882	-21.11	0.870	1.689	94.14		
VN	0.358	0.305	-14.80	0.068	0.056	-17.65	0.097	0.108	11.34		
Col I	1.288	1.271	-1.32	0.966	0.650	-32.71	1.264	1.812	43.35		
Col IV	0.365	0.538	47.40	0.817	0.650	-20.44	0.808	1.325	63.99		
MDA-MB-468			T47D			Hs578t			Hs578t		
Mutated			Mutated			Mutated			Mutated		
ER	PR	HER2	ER	PR	HER2	ER	PR	HER2	ER	PR	HER2
(0)	(0)	(3)	(0)	(0)	(0-1)	(0)	(0)	(0-1)	(0)	(0)	(0-1)
-	+	CHG%	-	+	CHG%	-	+	CHG%	-	+	CHG%
1.000	2.372	137.18	1.000	1.081	8.10	1.000	1.640	64.00	1.000	1.252	25.20
0.872	2.154	147.01	1.733	1.954	12.75	1.024	1.588	55.08	0.849	0.983	15.78
0.285	0.417	46.32	0.249	0.271	8.84	0.131	0.175	33.59	0.140	0.128	-8.57
0.822	1.564	90.22	2.534	2.645	4.38	1.029	1.550	50.63	1.123	1.381	22.97
0.768	1.636	112.99	1.920	2.051	6.82	1.304	1.559	19.56	0.798	0.898	12.53

Notes. The cells were irradiated with ionizing radiation (10 Gy). After 24 h incubation, 5 × 10⁵ cells were placed on an ECM protein-coated plate and incubated for 2 h. Representative data from at least three independent experiments are shown.

^a Subik K. *et al.* Breast Cancer (Auckl) 2010;4:35-41.

^b CHG% = Percentage change of the numbers of adherent cells with or without ionizing radiation exposure.