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Breast Cancer Cells in Three-dimensional Culture Display an Enhanced Radioresponse after Coordinate Targeting of Integrin α5β1 and Fibronectin

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Abstract

Tactics to selectively enhance cancer radioresponse are of great interest. Cancer cells actively elaborate and remodel their extracellular matrix (ECM) to aid in survival and progression. Previous work has shown that β1-integrin inhibitory antibodies can enhance the growth-inhibitory and apoptotic responses of human breast cancer cell lines to ionizing radiation, either when cells are cultured in three-dimensional laminin-rich ECM (3D lrECM) or grown as xenografts in mice. Here, we show that a specific α heterodimer of β1-integrin preferentially mediates a prosurvival signal in human breast cancer cells that can be specifically targeted for therapy. 3D lrECM culture conditions were used to compare α-integrin heterodimer expression in malignant and nonmalignant cell lines. Under these conditions, we found that expression of α5β1-integrin was upregulated in malignant cells compared with nonmalignant breast cells. Similarly, we found that normal and oncofetal splice variants of fibronectin, the primary ECM ligand of α5β1-integrin, were also strikingly upregulated in malignant cell lines compared with nonmalignant acini. Cell treatment with a peptide that disrupts the interactions of α5β1-integrin with fibronectin promoted apoptosis in malignant cells and further heightened the apoptotic effects of radiation. In support of these results, an analysis of gene expression array data from breast cancer patients revealed an association of high levels of α5-integrin expression with decreased survival. Our findings offer preclinical validation of fibronectin and α5β1-integrin as targets for breast cancer therapy.

Introduction

Cancer cells have the ability to co-opt their microenvironment to create the necessary conditions for growth and survival by eliciting processes such as neoangiogenesis, and actively remodeling the extracellular matrix (ECM). ECM has profound effects on cellular behavior and can facilitate cancer progression (1). In addition, specific ECM components such as fibronectin have been associated with poor prognosis in patients with breast cancer (2).

The primary receptors for ECM ligands are the integrins. Integrins are a large family of heterodimeric ECM receptors that consists of 18α and 8β subunits (3). Each individual member of the integrin family of receptors binds multiple ECM ligands, such as fibronectin, laminin, and collagen, which then activate intracellular signaling pathways (3, 4). The α subunit typically confers specificity for the ligand, whereas the β subunit couples to the downstream signaling pathways (3). In our previous studies, we have shown that the three-dimensional laminin-rich ECM (3D lrECM) culture model allows rapid discrimination between breast cancer cells and nonmalignant epithelial cells, which undergo acinar development in 3D lrECM, but not two-dimensional cultures (5). We applied this model to show that β1-integrin inhibitory antibody, AIIB2, leads to selective apoptosis and decreases proliferation in human breast cancer cells in 3D lrECM and in vivo (6) without toxicity to normal tissues. In addition, we found that combining β1-integrin inhibition with ionizing radiation (IR) allowed for the reduction of IR dose necessary to achieve growth inhibition in vivo.

The present study addresses whether a specific β1-integrin heterodimer is more potent in prosurvival signaling in cancer compared with normal cells and whether this could be more

specifically targeted. Although β1-integrin and its downstream signaling have been implicated in resistance to IR (7–9), specific α subunit partners of β1-integrin and the molecular mechanisms involved in survival signaling after IR have not been fully investigated.

In this study, we show that α5β1-integrin is a major β1 heterodimer that is upregulated in 3D lrECM and after IR exposure in malignant T4-2 and other breast cancer cells. We found that its preferred ECM ligand, fibronectin, is also dramatically upregulated, coincident with the increase in α5β1-integrin. In addition, several malignant cell lines in 3D lrECM preferentially upregulate protein of the fibronectin splice variant, EDA+, compared with nonmalignant cells. Demonstrative of its therapeutic potential, inhibition of α5-integrin and fibronectin interaction by ATN-161 (Ac-PHSCN-NH2) induces apoptosis and enhances IR efficacy in T4-2 and MDA-MB-231 malignant breast cancer cell lines. Finally, we found that high α5-integrin gene expression is associated with decreased survival, providing a potential clinical context for α5β1-integrin inhibition for therapy.

Materials and Methods

Cell culture

HMT-3522 mammary epithelial cells were originally derived from a woman with fibrocystic breast disease and were propagated as described in the Supplementary Data. HMT-3522-T4-2 wild-type cells that were either stably transfected with a constitutively active myristoylated Akt (T4-2 myr-Akt) or empty vector control (T4-2 vc) were previously described (10). 3D lrECM cultures consisted of cells trypsinized from monolayer cultures and plated on top of commercially available growth factor–reduced Basement Membrane Extract (Trevigen) as previously described (6, 11). Cultures were treated with ATN-161 or ATN-163 (kind gifts from Dr. Andrew Mazar, Attenuon, San Diego, CA) or anti–α5-integrin antibodies on day 4 of culture for malignant cell lines. All cultures were analyzed 72 hours after the first treatment.

Real-time PCR analysis

Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel). cDNA was synthesized with the SuperScript first-strand synthesis kit (Invitrogen) from 0.5 to 1.0 μg RNA. Quantitative real-time PCR analysis was performed with the LightCycler System using LightCycler FastStart DNA Master SYBR GreenI (Roche). The primers, the protocol used to amplify total fibronectin and EDA+ fibronectin, and 18S rRNA are described in the Supplementary Data.

Cell surface labeling

After releasing cells from 3D lrECM, cell colonies were incubated with 0.2 mg/mL sulfo-NHS-SS-biotin (ThermoScientific) in PBS for 30 minutes at 4°C, and then the supernatant was replaced with 50 mmol/L glycine/PBS for 15 minutes at 4°C. After washing, cell pellets were lysed in 1% radioimmunoprecipitation assay buffer. Cell lysates were subjected to immunoprecipitation and SDS-PAGE using nonreducing conditions and then transferred onto nitrocellulose membranes. Proteins labeled on the cell surface were detected with streptavidin-horseradish peroxidase (HRP) conjugate (GE Healthcare) at a 1:500 dilution.

Survival analysis

A gene expression data set including the microarray profiles of 295 human breast cancers and the associated clinical data (12) was obtained from Rosetta Inpharmatics. All papatients had stage I or II breast cancer and were younger than 53 years (12). Among the 295 patients, 151 patients had lymph node–negative disease and 144 had lymph node–positive disease (12). For survival analysis, patients were stratified into upper, lower, and interquartiles for the expression of ITGA5. Kaplan-Meier survival curves were calculated and shown using SigmaPlot (version 11.0). Statistical significance was determined by the log-rank test; a P value of <0.05 was considered statistically significant.

Statistical analysis

Results referred to in the remaining text, in Figs. 1 through 6, and in the Supplementary Data are expressed as mean ± SEM. Data were analyzed by Student's t test. P values of <0.05 were considered significant. Significant differences are indicated by * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, and n.s. for not significant.

Other materials and methods

Lysis from 3D lrECM, immunoprecipitation, immunoblotting, immunostaining, apoptosis assay, Akt kinase assay, and antibodies are described in the Supplementary Data.

Results

α5β1-Integrin heterodimers are strikingly upregulated in malignant T4-2 cells compared with nonmalignant S1 cells in 3D lrECM

We have shown previously that β1-integrins are important potential targets for cancer therapy alone and in combination with IR (6, 13). However, whether a specific β1-integrin heterodimer preferentially mediates tumor survival and/or resistance to radiation is not known. To investigate which β1-integrin heterodimer plays a dominant role in malignant breast cells, we compared the expression level of several α-integrin subunits between malignant T4-2 cells and their nonmalignant counterpart, S1 cells. We found that the α5-integrin subunit was dramatically upregulated in T4-2 compared with S1 cells in 3D lrECM (Fig. 1A). We also confirmed that the α5β1-integrin complex is upregulated by the immunoprecipitation of β1-integrins (Fig. 1B) Interestingly, α5-integrin expression was significantly higher in T4-2 cells compared with S1 cells only in 3D lrECM cultures, but not on two-dimensional tissue culture plastic (two-dimensional data not shown).

Elevated α5-integrin gene expression is associated with significantly decreased long-term survival in patients with breast cancer

We previously showed that high β1-integrin and fibronectin expression detected by immunohistochemistry was associated with significantly decreased survival in patients with early-stage invasive breast cancer (2). To our knowledge, no reported studies have found

significant correlations between α5β1-integrin and clinical outcome in breast cancer. To investigate whether α5-integrin expression is associated with survival in breast cancer, we queried a gene expression data set that included the microarray profiles of 295 patients with clinical follow-up (12). We found that high α5-integrin gene expression is significantly associated with decreased survival (Fig. 1C), and of this group, 61.6% were estrogen receptor–positive. These data indicate that α5-integrin is a potentially important target for breast cancer therapy.

Upregulation of fibronectin and its splice variant EDA+ are associated with coordinate upregulation of α5β1-integrin in malignant breast cancer cells in 3D lrECM

Fibronectin is the main ECM ligand of α5β1-integrin, which interacts with α5β1-integrin through its Arg-Gly-Asp and Pro-His-Ser-Arg-Asn synergy sequences (14). α5β1-Integrin and its interaction with fibronectin have been shown to mediate diverse roles in cancer cell survival, proliferation, and invasion (15–18). We previously found that fibronectin-inhibitory antibodies induced the phenotypic reversion of tumorigenic T4-2 cells, leading to the formation of normal acinar structures with correct basal polarity in 3D lrECM (19). Here, we further investigated the relationship between fibronectin and α5β1-integrin expression in T4-2 malignant cells. We found that like α5β1-integrins, total fibronectin was significantly upregulated in T4-2 cells in 3D lrECM. In addition, we did not observe enhanced α5-integrin expression in two-dimensional cultures despite the addition of exogenous fibronectin (Fig. 2A).

We reasoned that malignant T4-2 cells, but not nonmalignant S1 cells, may be producing endogenous fibronectin in 3D lrECM cultures, which could effectively interact with the upregulated α5β1-integrin. When we measured total fibronectin protein levels in the conditioned medium of T4-2 cells in 3D lrECM compared with S1 cells, we found that fibronectin was secreted by T4-2 cells at a 9.4-fold higher level than S1 cells (Fig. 2B). In addition, fibronectin is known to contain several alternatively spliced domains. One species, EDA+ fibronectin, in particular, is expressed during embryogenesis and wound healing (20, 21). Remarkably, we found that this EDA+ segment is upregulated 22-fold in the conditioned medium of T4-2 cells in 3D lrECM, compared with S1 cells (Fig. 2B). We also confirmed that mRNA levels of total fibronectin and EDA+ segment are upregulated in T4-2 cells compared with S1 cells (Fig. 2C).

To investigate whether the upregulation of α5-integrin, β1-integrin, total fibronectin, and EDA+ fibronectin in malignant breast cancer cells is a general phenomenon, we measured their expression levels in several additional breast cancer cell lines. We found that highly aggressive metastatic breast cancer cells (MDA-MB-436, MDA-MB-231, and Hs578T), which are of the basal molecular phenotype (22), showed a robust coordinate upregulation of α5-integrin and β-integrin, total fibronectin, and EDA+ fibronectin (Fig. 2D). Interestingly, by contrast, MCF-7, SKBR-3, MDA-MB-361 cell lines, all of luminal phenotype, did not display a similar robust upregulation of α5β1-integrins or fibronectin in 3D lrECM. These results indicate that malignant breast cells of the basal phenotype preferentially upregulate the fibronectin-α5β1-integrin ligand-receptor pair in 3D lrECM, and synthesize and secrete predominantly the fibronectin EDA+ variant, which is known to play a role in tumorigenesis (23).

Specific inhibition of the α5β1-integrin-fibronectin interaction by ATN-161 induces apoptosis in malignant breast cancer cells in 3D lrECM

α5β1-Integrin interacts with the Arg-Gly-Asp and Pro-His-Ser-Arg-Asn sequences of fibronectin (14). ATN-161 is a small peptide that was previously shown to decrease angiogenesis by disrupting the α5β1-integrin or αvβ3-integrin interaction with the Pro-His-Ser-Arg-Asn synergy region of fibronectin (24–26). We asked whether α5β1-integrin interaction with fibronectin is essential for survival and could be targeted to enhance therapeutic effect in T4-2 cells. We previously found that inhibition of β1-integrin by AIIB2 for 72 hours was effective in killing cancer cells, with little or no effect in nonmalignant S1 colonies (6). Examination of the consequences of specifically inhibiting α5β1-integrin-fibronectin interactions using ATN-161 in the 3D lrECM assay (Fig. 3A) revealed that the growth of T4-2 colonies—but not S1 colonies—was significantly reduced (Fig. 3B). ATN-161–induced apoptosis of T4-2 cells was measured by counting terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive nuclei at concentrations of 0.5 mg/mL (mean = 8.3%, $P < 0.001$) or 2.0 mg/mL (mean = 23.4%, $P < 0.001$) ATN-161 (Fig. 3C). In keeping with its effect on inhibiting α5β1-integrin signaling, ATN-161 treatment also induced the downregulation of α5β1-integrin protein expression in T4-2 cells cultured in 3D lrECM (Fig. 4B). Consistent with previous results using β1-integrin inhibitory antibodies, ATN-161 did not significantly affect apoptosis in nonmalignant S1 cells in 3D lrECM (Fig. 3B and C). Of note, the scrambled peptide, ATN-163, did not affect apoptosis in T4-2 cells (Supplementary Fig. S1).

It has been reported that ATN-161 can also target other integrin heterodimers, specifically αvβ3-integrin (24). We also detected a modestly elevated level of αv-integrins in T4-2 cells compared with S1 cells (Supplementary Fig. S2). To validate α5β1-integrin as a specific target, we tested two separate clones of α5- integrin inhibitory antibodies, IIA1 and P1D6. Consistent with ATN-161 results, inhibition of α5β1-integrin by these inhibitory antibodies effectively induced apoptosis of T4-2 cells, but not S1 cells, in 3D lrECM (Fig. 3D).

Akt activity mediates survival downstream of α5β1-integrin pathway in malignant T4-2 cells in 3D lrECM

We previously showed that Akt played a role in conferring resistance to apoptosis post-IR and in response to β1-integrin inhibitory antibodies, which could be overcome by administering increasing concentrations of inhibitory antibodies (13). Akt serine/threonine protein kinase activity is mediated through the phosphorylation of serine 473 and has been implicated in cell survival by inhibiting apoptosis (27). Here, we verified that Akt kinase activity and Akt phosphorylation were downregulated by α5β1-integrin inhibition (Fig. 4A and B). Akt phosphorylation was not affected by ATN-161 on S1 cells (Supplementary Fig. S3). Furthermore, we tested α5β1-integrin and fibronectin inhibition on T4-2 myr-Akt cells, which express constitutively active Akt (13), and confirmed that T4-2 myr-Akt cells were resistant to ATN-161–induced apoptosis (Fig. 4C). These data indicate that sustained malignant growth and survival in the context of 3D lrECM is mediated at least in part by Akt downstream of α5β1-integrin, and cellular death may be targeted directly through ECM signaling pathways.

α5β1-Integrin heterodimer is upregulated after IR exposure in malignant T4-2 cells in 3D lrECM

β1-Integrin expression has previously been shown to be upregulated by IR in lung cancer cell lines and breast epithelial cells (28, 29), and to mediate cellular resistance to apoptosis after IR (7). In addition, α5β1-integrin interaction with fibronectin has been shown to play an important role in cell survival, including that of breast cancer cells (30–32). Thus, we hypothesized that α5β1-integrins at the cell surface after IR exposure could potentially increase survival signaling by enhanced interaction with fibronectin and thus could be targeted to enhance therapy. The surface expression of α5-integrins was measured by biotin labeling of cell surface proteins on T4-2 cells cultured in 3D lrECM. Total protein lysates were subjected to immunoprecipitation by anti–α5-integrin antibody, followed by immunoblotting using HRPconjugated streptavidin. Surface expression of α5-integrins was most prominent after 2 Gy, and upregulation also occurred after 4 Gy IR on T4-2 cells cultured in 3D lrECM (Fig. 5A). Consistent with this result, localization of α5-integrins was observed also at the plasma membrane after IR by confocal immunofluorescence (Fig. 5B). These data indicate that α5β1-integrin is upregulated significantly by IR exposure, and that enhanced expression was reflected in the level of surface expression, which could potentially be targeted to enhance IR efficacy.

IR exposure in combination with α5β1-integrin inhibition enhances apoptosis in T4-2 and MDA-MB-231 breast cancer cell lines in 3D lrECM

We previously reported that β1 integrin inhibition enhances IR efficacy in several breast cancer cell lines cultured in 3D lrECM (13). Following previous experimental procedures, we treated T4-2 cells cultured in 3D lrECM with 0.5 mg/mL ATN-161 before or after exposure to 2 Gy IR (Fig. 6A). The disorganized colonies formed by T4-2 cells showed a significant increase in apoptosis after IR exposure given either before or after ATN-161 compared with IR alone (Fig. 6B). Importantly the combination of ATN-161 and IR was also effective in MDA-MB-231, a highly aggressive, metastatic breast cancer cell line that was previously reported to respond to ATN-161 treatment with suppression in tumor growth and metastasis in vivo (24). Compared with colonies treated with IR alone, those treated with ATN-161 post-IR showed a significant increase in apoptosis (Fig. 6C). Interestingly, treatment with ATN-161 pre-IR in this case was not effective (Fig. 6C). These data indicate that inhibition of α5β1-integrin enhances the IR effect on the apoptosis of malignant breast cancer cells, and that sequencing may be an important factor in determining the optimal treatment.

Discussion

We previously reported on the therapeutic potential of targeting β1-integrins showing that β1-integrin inhibitory antibodies could selectively enhance apoptosis and cytostasis in malignant breast cancer cells in 3D lrECM and in vivo, and increase the efficacy of IR without toxicity to normal tissues (6, 13). In the present work, we provide a more in-depth investigation of the α5β1-integrin heterodimer and reveal a novel relationship between expression of the α5β1-integrin receptor and its ligand, fibronectin. We show that the α5β1-integrin heterodimer is specifically and strikingly upregulated in malignant T4-2 breast cancer cells in 3D lrECM and after IR, and that total fibronectin as well as the oncofetal EDA+ variant of fibronectin are coordinately upregulated in aggressive breast cancer cell lines of the basal molecular phenotype.

We further show that the α5β1-integrin-fibronectin interaction can be specifically targeted using a specific small peptide inhibitor, ATN-161, and α5-integrin inhibitory antibodies to induce apoptosis through an Akt-mediated mechanism. Importantly, we also find that high α5-integrin gene expression is associated with significantly decreased survival in patients with invasive breast cancer, providing a potential clinical context for α5β1-integrin–targeted therapy.

α5β1-Integrin is associated with more aggressive disease phenotypes in a wide variety of human carcinomas as well as breast cancer (33–35) and, therefore, seems to have a universal function in progression in several cancer types. We found that α5β1-integrin and fibronectin, its primary ligand, are coordinately upregulated in several aggressive breast cell lines of the basal phenotype. The addition of exogenous fibronectin has been previously shown to upregulate α5-integrin expression in Caco2-BBE human intestinal cells in twodimensional culture (36). In the present study, we found that upregulation of α5β1-integrin in T4-2 cells is observed only in the 3D lrECM context and that coating flat surfaces with ECM components was not sufficient to elicit the increased response. Others have shown that a three-dimensional environment is important for α5β1-integrin localization and α5β1-integrin–mediated matrix assembly in fibroblasts (37, 38). Our results highlight the importance of α5β1-integrin–mediated ECM remodeling in tumor progression and the dependence of three-dimensional architecture on this process.

We also found that the EDA+ variant of fibronectin was specifically overexpressed in these cells. Others have shown that EDA+ fibronectin is overexpressed in wound healing and some tumors, although it is poorly represented in the ECM of adult normal tissues (21, 39). In breast cancer, EDA+ fibronectin is expressed in the normal-appearing stroma of ductal carcinoma in situ lesions and strongly elaborated in invasive breast cancer (40). It has been proposed that the EDA domain inclusion may mediate a conformational change in the whole molecule, hence increasing cell-ECM interaction through the binding to α5β1-integrin (41). Thus, we hypothesize that malignant cells increase EDA+ fibronectin to enhance survival signaling through α5β1-integrin. However, the detailed molecular mechanisms of the upregulation of α5β1-integrins and its relation to fibronectin in malignant breast cancer cells are still unknown.

Molecular phenotyping has led to the increased understanding of the developmental origins of some breast cancers and has provided tools to more accurately assess individual risk for metastasis (42). The basal phenotype is more aggressive in patients and is associated with significantly decreased survival (43). We found that the cell lines that showed significant coordinate upregulation of α5β1-integrin and EDA+ fibronectin were of the basal phenotype (22). Interestingly, none of the luminal cell types tested showed the same upregulation. We confirmed that 61.6% of ITGA5-high population in the survival analysis is estrogen receptor positive, which corresponds to the luminal subtype. These results indicate that the association between ITGA5 expression and outcome in breast cancer is independent of molecular subtype. Further investigations to validate these results and investigate the potential underpinnings of these correlations are ongoing.

Previous studies with ATN-161 had focused on its ability to suppress tumor growth by inhibiting angiogenesis in vivo (25, 44) primarily by targeting α5β1 and αvβ3 integrins. Others have previously found that inhibition of αv-integrins may affect primary tumor growth and increase

sensitivity to IR (45). Our study indicates that the primary tumor cell survival signaling may also be targeted using ATN-161, resulting in downregulation of α5β1-integrin through the suppression of Akt activity, which has been implicated in cell survival by inhibiting apoptosis (46). In addition, we and others have shown previously that Akt activity is a promising target in cancer cells downstream of β1-integrin signaling post-IR (13, 47). In the present study, our extended findings support the role of Akt in mediating survival through α5β1-integrin signaling specifically.

β1-Integrin expression is aberrantly upregulated post-IR and has been implicated in survival and resistance to IR in several cancer cell types (7, 48). However, the nature of the specific β1-integrin heterodimers that are upregulated post-IR or their relationship with IR dose has not been well studied. Here, we found that both total and cell surface expression of α5β1-integrin were increased after IR in T4-2 cells, suggesting that the increase in α5β1-integrin signaling contributes to survival signaling post-IR in 3D lrECM. The peak upregulation of α5β1-integrin occurred after 2-Gy exposure, and the degree of upregulation decreased with increasing radiation dose. Others have shown that upregulation of β1-integrins occurs after a single dose of 6 Gy in lung cancer cell lines (29). Upregulation of endothelial αvβ3-integrin was observed after 2 Gy, reaching a plateau at 15 Gy (49), and αIIbβ3-integrin with doses ranging between 0.5 to 2.5 Gy in melanoma cells (50). Onoda and colleagues (50) showed a dose-dependent increase in B16 melanoma cells' adhesion to fibronectin post-IR; they also showed that maximum increase in adhesion occurs after exposure to 0.5 Gy. Thus, our current findings are within the range of dose-response reported by others. However, the nature of response of integrin levels to dose of radiation and the relationship to tissue specific integrin heterodimers need further investigation.

In our study, we found that the addition of ATN-161 treatment following 2 Gy IR resulted in a significant increase in apoptosis of both T4-2 cells and MDA-MB-231 cells compared with single treatments. The sequencing of biological agents with IR can be an important factor in optimizing treatment. To test the effect of sequencing, we treated cultures with ATN-161 before IR exposure. We found that in T4-2 cells, reverse sequencing was not distinguishable from treatment with ATN-161 post-IR. However, when MDA-MB-231 cells were treated with IR following ATN-161, an enhance apoptotic effect was not observed. We hypothesize that the effect of ATN-161 or IR may be influenced by several factors, such as expression level of α5β1-integrin in tumor cells, or the availability of fibronectin in tumor microenvironment, which will be important areas of future investigation.

In summary, in the context of 3D lrECM, malignant breast cancer cells of the basal phenotype strikingly upregulate α5β1-integrin coordinately with its primary ligand, fibronectin in its oncofetal form. Inhibition of α5β1-integrin and fibronectin interaction using ATN-161 and anti–α5-integrin inhibitory antibodies results in apoptosis in malignant T4-2 cells in 3D lrECM. In addition, α5β1-integrins are upregulated in malignant T4-2 cells after IR, and the combination of ATN-161 and 2 Gy IR enhanced apoptosis compared with single treatments in breast cancer cells. Finally, we found that α5-integrin gene overexpression is associated with decreased survival in breast cancer patients. Together, our findings indicate that α5β1-integrin and its ligand fibronectin are important for survival signaling in breast cancer and are important targets for therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. Science 2006;314:298–300.
2. Yao ES, Zhang H, Chen YY, et al. Increased β1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res 2007; 67:659–64.
3. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
4. Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science 1987;238:491–7.
5. Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A 1992;89:9064–8.
6. Park CC, Zhang H, Pallavicini M, et al. β1 Integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res 2006;66:1526–35.
7. Cordes N, Seidler J, Durzok R, Geinitz H, Brakebusch C. β1-Integrinmediated signaling essentially contributes to cell survival after radiation-induced genotoxic injury. Oncogene 2006;25:1378–90.
8. Hodkinson PS, Elliott T, Wong WS, et al. ECM overrides DNA damage-induced cell cycle arrest and apoptosis in small-cell lung cancer cells through β1 integrin-dependent activation of PI3-kinase. Cell Death Differ 2006;13:1776–88.
9. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 1999;93: 1658–67.

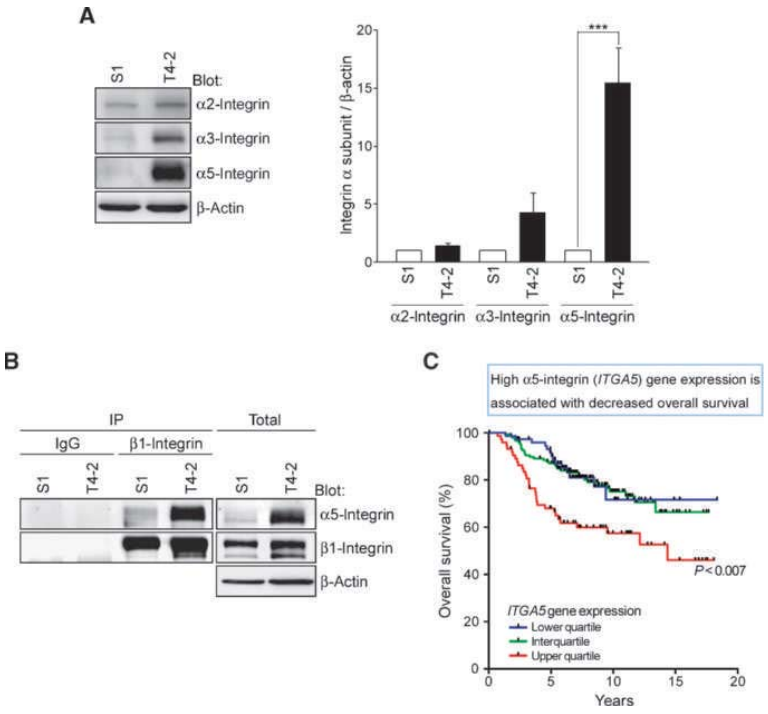
10. Liu H, Radisky DC, Nelson CM, et al. Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. Proc Natl Acad Sci U S A 2006;103:4134–9.
11. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 2007;4:359–65.
12. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347:1999–2009.
13. Park CC, Zhang HJ, Yao ES, Park CJ, Bissell MJ. β1 Integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. Cancer Res 2008;68:4398–405.
14. Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J Biol Chem 1994;269:24756–61.
15. Caswell PT, Spence HJ, Parsons M, et al. Rab25 associates with α5β1 integrin to promote invasive migration in 3D microenvironments. Dev Cell 2007;13:496–510.
16. Maschler S, Wirl G, Spring H, et al. Tumor cell invasiveness correlates with changes in integrin expression and localization. Oncogene 2005;24:2032–41.
17. Nista A, Leonetti C, Bernardini G, Mattioni M, Santoni A. Functional role of α4β1 and α5β1 integrin fibronectin receptors expressed on adriamycin-resistant MCF-7 human mammary carcinoma cells. Int J Cancer 1997;72:133–41.
18. Zeng BX, Fujiwara H, Sato Y, et al. Integrin α5 is involved in fibronectininduced human extravillous trophoblast invasion. J Reprod Immunol 2007;73:1–10.
19. Sandal T, Valyi-Nagy K, Spencer VA, Folberg R, Bissell MJ, Maniotis AJ. Epigenetic reversion of breast carcinoma phenotype is accompanied by changes in DNA sequestration as measured by AluI restriction enzyme. Am J Pathol 2007;170:1739–49.
20. Vartio T, Laitinen L, Narvanen O, et al. Differential expression of the ED sequence-containing form of cellular fibronectin in embryonic and adult human tissues. J Cell Sci 1987;88:419–30.
21. Jarnagin WR, Rockey DC, Koteliansky VE, Wang SS, Bissell DM. Expression of variant fibronectins in wound healing: cellular source and biological activity of the EIIIA segment in rat hepatic fibrogenesis. J Cell Biol 1994;127:2037–48.
22. Neve RM, Chin K, Fridlyand J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 2006;10:515–27.
23. Oyama F, Hirohashi S, Shimosato Y, Titani K, Sekiguchi K. Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver tumors. J Biol Chem 1989; 264:10331–4.
24. Khalili P, Arakelian A, Chen G, et al. A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo. Mol Cancer Ther 2006;5:2271–80.
25. Livant DL, Brabec RK, Pienta KJ, et al. Anti-invasive, antitumorigenic, and antimetastatic activities of the PHSCN sequence in prostate carcinoma. Cancer Res 2000;60:309–20.
26. Stoeltzing O, Liu W, Reinmuth N, et al. Inhibition of integrin α5β1 function with a small peptide (ATN-161) plus continuous 5-FU infusion reduces colorectal liver metastases and improves survival in mice. Int J Cancer 2003;104:496–503.
27. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007;129:1261–74.
28. Park CC, Henshall-Powell RL, Erickson AC, et al. Ionizing radiation induces heritable disruption of epithelial cell interactions. Proc Natl Acad Sci U S A 2003;100:10728–33.

29. Cordes N, Blaese MA, Meineke V, Van Beuningen D. Ionizing radiation induces up-regulation of functional β1-integrin in human lung tumour cell lines in vitro. Int J Radiat Biol 2002;78:347–57.
30. Zhang Z, Vuori K, Reed JC, Ruoslahti E. The α 5 β 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc Natl Acad Sci U S A 1995;92:6161–5.
31. Korah R, Boots M, Wieder R. Integrin α5β1 promotes survival of growth-arrested breast cancer cells: an in vitro paradigm for breast cancer dormancy in bone marrow. Cancer Res 2004;64:4514–22.
32. Maglott A, Bartik P, Cosgun S, et al. The small α5β1 integrin antagonist, SJ749, reduces proliferation and clonogenicity of human astrocytoma cells. Cancer Res 2006;66:6002–7.
33. Sawada K, Mitra AK, Radjabi AR, et al. Loss of E-cadherin promotes ovarian cancer metastasis via α 5-integrin, which is a therapeutic target. Cancer Res 2008;68:2329–39.
34. Saito T, Kimura M, Kawasaki T, Sato S, Tomita Y. Correlation between integrin α 5 expression and the malignant phenotype of transitional cell carcinoma. Br J Cancer 1996;73:327–31.
35. Gong J, Wang D, Sun L, Zborowska E, Willson JK, Brattain MG. Role of α 5 β 1 integrin in determining malignant properties of colon carcinoma cells. Cell Growth Differ 1997;8:83–90.
36. Kolachala VL, Bajaj R, Wang L, et al. Epithelial-derived fibronectin expression, signaling, and function in intestinal inflammation. J Biol Chem 2007;282:32965–73.
37. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cellmatrix adhesions to the third dimension. Science 2001;294:1708–12.
38. Mao Y, Schwarzbauer JE. Stimulatory effects of a three-dimensional microenvironment on cell-mediated fibronectin fibrillogenesis. J Cell Sci 2005;118:4427–36.
39. Kornblihtt AR, Pesce CG, Alonso CR, et al. The fibronectin gene as a model for splicing and transcription studies.FASEBJ 1996;10:248–57.
40. Brown LF, Guidi AJ, Schnitt SJ, et al. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. Clin Cancer Res 1999;5:1041–56.
41. Manabe R, Oh-e N, Sekiguchi K. Alternatively spliced EDA segment regulates fibronectin-dependent cell cycle progression and mitogenic signal transduction. J Biol Chem 1999; 274:5919–24.
42. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
43. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869–74.
44. Danese S, SansM, SpencerDM,et al. Angiogenesis blockade as a new therapeutic approach to experimental colitis. Gut 2007;56:855–62.
45. Cao Q, Cai W, Li T, et al. Combination of integrin siRNA and irradiation for breast cancer therapy. Biochem Biophys Res Commun 2006;351:726–32.
46. Johnson GE, Ivanov VN, Hei TK. Radiosensitization of melanoma cells through combined inhibition of protein regulators of cell survival. Apoptosis 2008;13:790–802.
47. Ning S, Chen Z, Dirks A, et al. Targeting integrins and PI3K/Aktmediated signal transduction pathways enhances radiation-induced anti-angiogenesis. Radiat Res 2007; 168:125–33.

48. Cordes N, Meineke V. Cell adhesion-mediated radioresistance (CAM-RR). Extracellular matrix-dependent improvement of cell survival in human tumor and normal cells in vitro. Strahlenther Onkol 2003;179:337–44.
49. Abdollahi A, Griggs DW, Zieher H, et al. Inhibition of α(v)β3 integrin survival signaling enhances antiangiogenic and antitumor effects of radiotherapy. Clin Cancer Res 2005;11: 6270–9.
50. Onoda JM, Piechocki MP, Honn KV. Radiation-induced increase in expression of the α IIb β 3 integrin in melanoma cells: effects on metastatic potential. Radiat Res 1992;130:281–8.

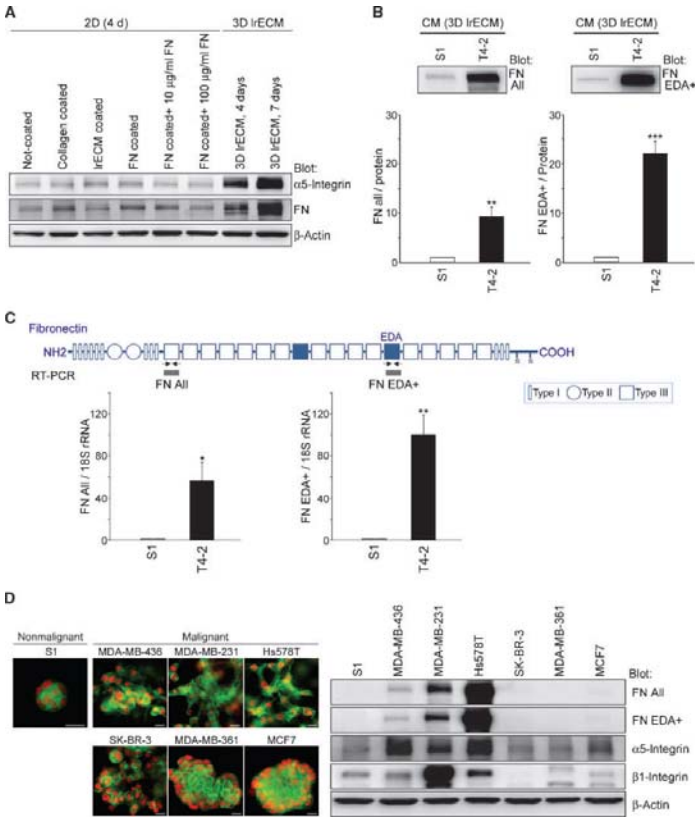
Figures

FIGURE 1



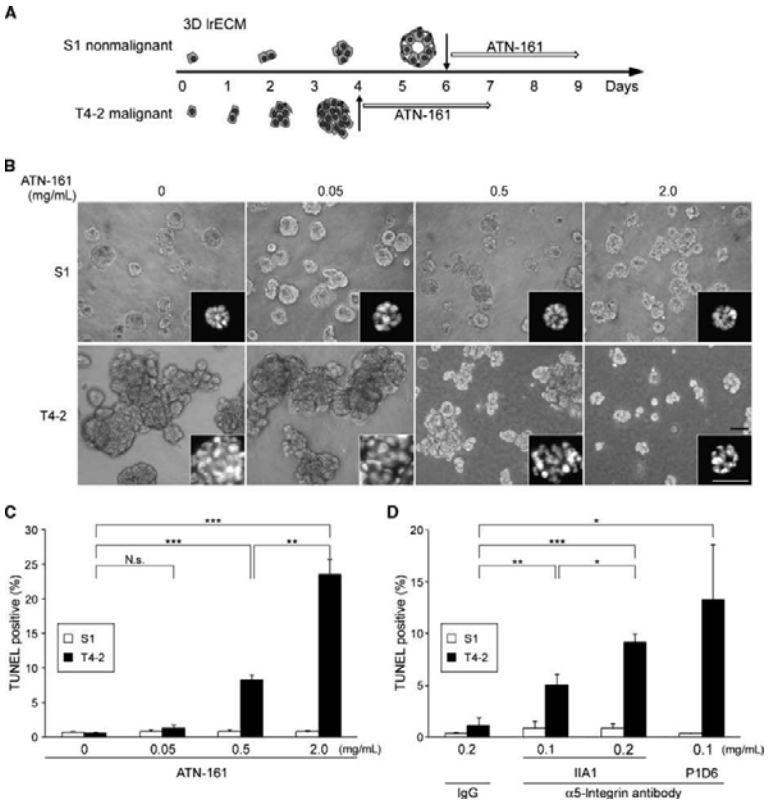
α5β1-Integrin heterodimers are strikingly upregulated in malignant T4-2 cells compared with nonmalignant S1 cells in 3D lrECM. A, Western blot for α2-, α3-, α5-integrin subunits from total cell lysates shows upregulation of α5-integrin in malignant T4-2 cells compared with nonmalignant S1 cells cultured in 3D lrECM. Equal amounts of protein were loaded and subjected to immunoblotting. Columns, mean intensity of Western blot analysis (n = 4); bars, SEM. ***, P < 0.001. B, α5β1-integrin complex are upregulated in T4-2 cells compared with S1 cells cultured in 3D lrECM. Lysates were subjected to immunoprecipitation with anti–β1-integrin and subsequent immunoblotting using antibodies, as indicated. IP, immunoprecipitation. C, elevated α5-integrin gene expression is associated with significantly decreased long-term survival in patients with breast cancer. A Kaplan-Meier survival analysis of 295 human breast cancers stratified by ITGA5 expression is shown. The highest quartile of ITGA5 expression is significantly associated with decreased survival. P values (log-rank) between upper and lower or interquartile are <0.007.

FIGURE 2



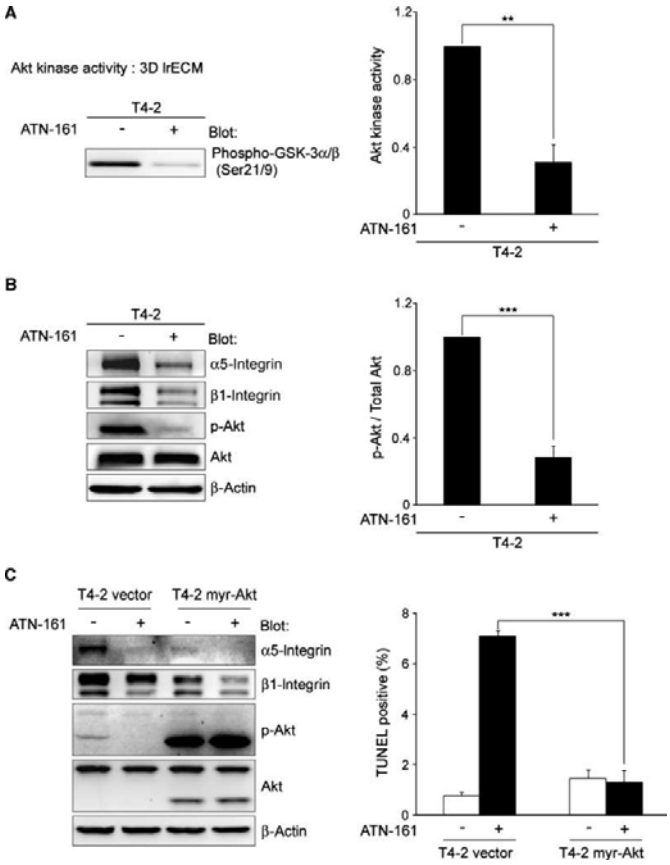
Upregulation of fibronectin and its splice variant EDA+ are associated with coordinate upregulation of α5β1-integrin in malignant breast cancer cells in 3D lrECM. A, upregulation of α5-integrin is not dependent on activation by ECM ligands on two-dimensional culture but required three-dimensional culture conditions. B, fibronectin (FN) secretion is upregulated in T4-2 cells compared with S1 cells in 3D lrECM. Twenty microliters of conditioned-medium (CM) from S1 or T4-2 cells were subjected to immunoblotting using fibronectin antibodies. The signals of immunoblotting were normalized with total protein of S1 or T4-2 cell lysate. Columns, mean (n = 3); bars, SEM. **, $P < 0.01$; ***, $P < 0.001$. C, quantification of mRNA levels of total and EDA+ fibronectin in S1 and T4-2 cells cultured in 3D lrECM assessed by real-time PCR. The levels of total fibronectin are relative to 18S rRNA. Columns, mean (n = 3); bars, SEM. *, $P < 0.05$; **, $P < 0.01$. D, coordinate upregulation of α5β1-integrin and total and EDA+ fibronectin occurs in several highly aggressive metastatic breast cell lines, MDA-MB-436, MDA-MB-231, and Hs578T. Lower levels were observed in less aggressive cell lines, SK-BR-3, MDA-MB-361, and MCF7. Immunofluorescence microscopy shows morphologies of breast cell lines cultured in 3D lrECM. Green, filamentous actin; red, nuclei. Scale bar, 50 μm.

FIGURE 3



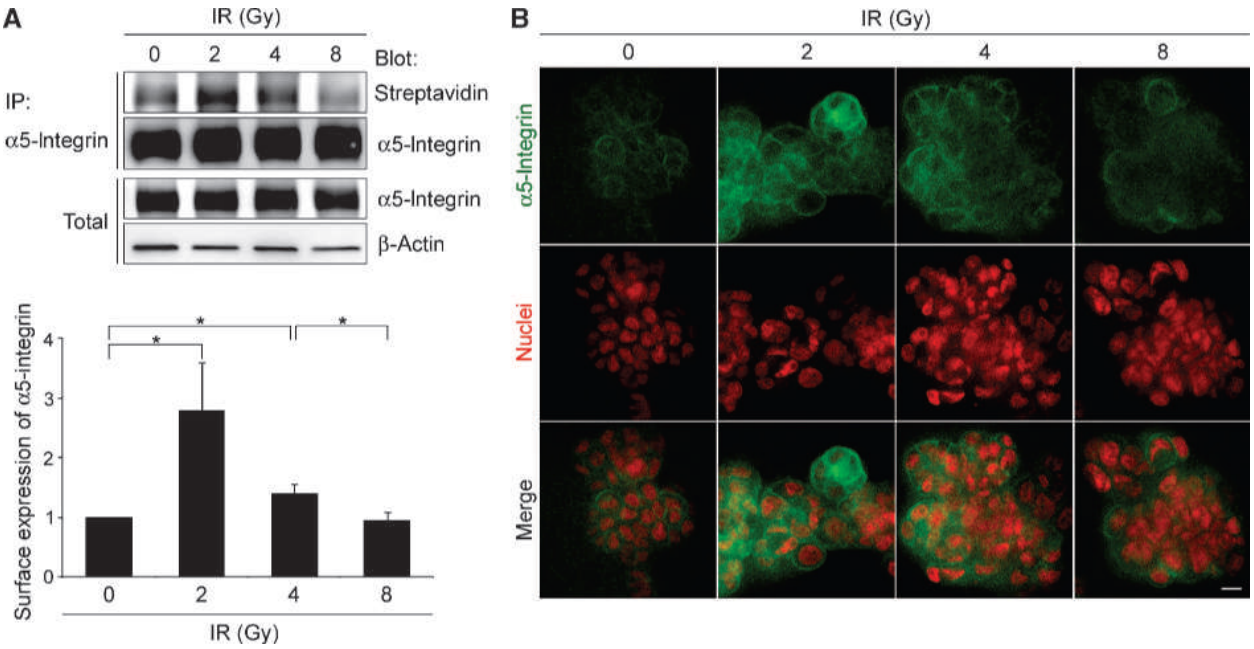
Specific inhibition of the α5β1-integrin-fibronectin interaction induces apoptosis in malignant breast cancer cells in 3D lrECM. A, experimental schema. B, phase-contrast micrographs of S1 and T4-2 cell colonies cultured in 3D lrECM with ATN-161 are shown. Nuclei were stained with 4′,6-diamidino-2-phenylindole (insets). Scale bar, 50 μm. C, T4-2 colonies, but not S1 structures, showed a significant increase in apoptosis, measured by TUNEL assay. Columns, mean (n = 3); bars, SEM. **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant. D, specific inhibition of α5β1-integrin-fibronectin interaction using anti–α5-integrin inhibitory antibodies, IIA1 and P1D6, induces apoptosis in T4-2 cells, but not S1 cells, cultured in 3D lrECM. Apoptosis was measured by TUNEL assay. Columns, mean (n = 3); bars, SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIGURE 4



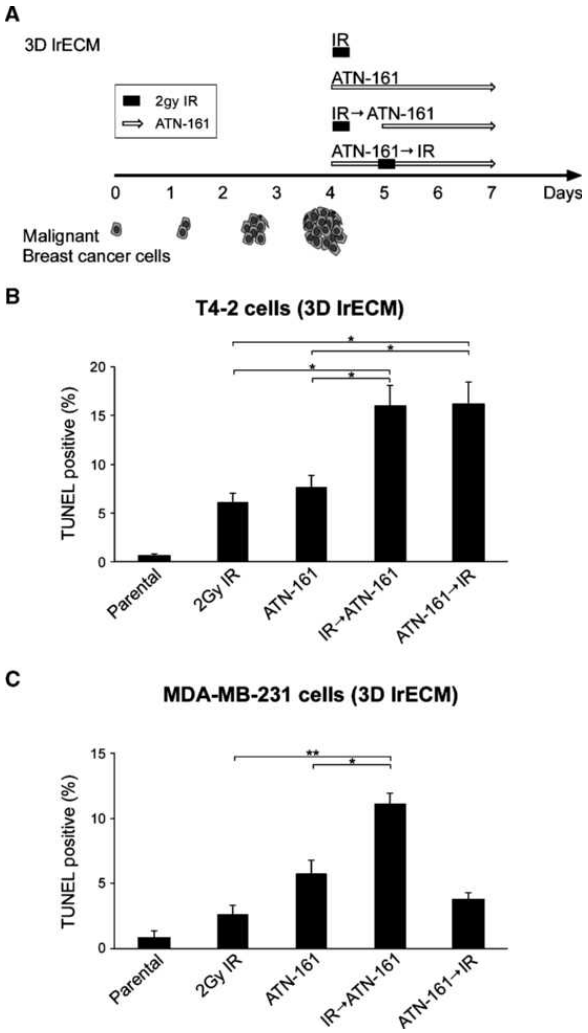
Akt activity mediates survival downstream of α5β1-integrin pathway in malignant T4-2 cells in 3D lrECM. A, Akt kinase activity was downregulated by inhibition of α5β1-integrin and fibronectin interactions. Cells were treated with or without 0.5 mg/mL ATN-161. Akt kinase activity was measured by phosphorylation of the GSK-3α/β fusion protein. Columns, mean (n = 3); bars, SEM. **, $P < 0.01$. B, ATN-161 treatment (0.5 mg/mL) of T4-2 cells in three-dimensional culture resulted in decreased expression of α5- and β1-integrins and pAkt. The level of phosphorylated Akt relative to total Akt is shown. Columns, mean (n = 3); bars, SEM. ***, $P < 0.001$. C, ATN-161–induced apoptosis is totally suppressed in T4-2 cells stably transfected with a constitutively active form of Akt (T4-2 myr-Akt). Columns, mean (n = 3); bars, SEM. ***, $P < 0.001$.

FIGURE 5



α5β1-Integrin heterodimer is upregulated after IR exposure in malignant T4-2 cells in 3D lrECM. A, cell surface expression of α5-integrins is upregulated after 2-4 Gy IR exposures in T4-2 cells cultured in 3D lrECM. Surface proteins of T4-2 cells were labeled with biotin and cell lysates were subjected to immunoprecipitation of α5-integrin and subsequent immunoblotting using HRP-conjugated streptavidin. Cell surface expression of α5-integrins was measured using streptavidin blots. Columns, mean (n = 3); bars, SEM. *, $P < 0.05$. B, immunofluorescence confocal localization of α5-integrins after IR exposure in T4-2 cells in 3D lrECM is shown. Increased cell surface α5-integrin expression is detected after 2 to 4 Gy IR. Green, α5-integrin; red, nuclei. Scale bar, 20 μm.

FIGURE 6



IR exposure in combination with α5β1-integrin inhibition enhances apoptosis in T4-2 and MDA-MB-231 breast cancer cell lines in 3D lrECM. A, experimental schema. Apoptosis of breast cancer cells was measured by TUNEL assay. B, IR exposure in combination with α5β1-integrin inhibition enhances apoptosis in T4-2 cells in 3D lrECM compared with either ATN-161 or IR alone. Columns, mean (n = 3); bars, SEM. *, $P < 0.05$. C, IR exposure followed by α5β1-integrin inhibition enhances apoptosis in MDA-MB-231 cells in 3D lrECM. Columns, mean (n = 3); bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

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