Abstract

The αvβ3 integrin is involved in various physiologic and pathologic processes such as wound healing, angiogenesis, tumor growth, and metastasis. The impact of αvβ3 integrin on the radiosensitivity of prostate cancer cells and the molecular mechanism controlling cell survival in response to ionizing radiation (IR) was investigated. Both LNCaP cells stably transfected with αvβ3 integrin and PC-3 cells that contain endogenous β3 integrin were used. This study demonstrated that αvβ3 integrin increases survival of αvβ3-LNCaP cells upon IR while small hairpin RNA (shRNA)-mediated knockdown of αvβ3 integrin in PC-3 cells sensitizes to radiation. Expression of αvβ3 integrin in LNCaP cells also enhances anchorage-independent cell growth while knockdown of αvβ3 integrin in PC-3 cells inhibits anchorage-independent cell growth.

Introduction

Prostate cancer is the most common noncutaneous malignant disease and the second expected cause of cancer-related death among men in the United States in 2018 (1). Radiotherapy is an important primary treatment modality for localized prostate cancer, and recent advances in radiosurgery and intensity-modulated radiotherapy have allowed dose escalation (i.e., 76–80 Gy) to improve biochemical failure rate and decrease metastasis (2). Despite these advances, intermediate- and high-risk populations of patients with prostate cancer continue to relapse after definitive radiotherapy (3). One possible reason for failure after radiotherapy may be due to intrinsic radioresistance of a small subpopulation of prostate tumor clonogen within the primary tumor. Therefore, the research on the influence of specific tumor signal response to radiation and cell survival is important for advancing the care of patients with prostate cancer (4, 5).

Integrin belongs to a family of at least 24 heterodimeric cell surface receptors that consist of noncovalently associated α and β subunits (6). These receptors influence cell functions, including adhesion, differentiation, proliferation, migration, and cell survival. Alteration of integrin expression in cancer cells correlates with tumor growth, progression, invasiveness, and metastatic potential. In particular, αvβ3 integrin remains one of the most actively investigated members of the integrin family because it has been shown to promote angiogenesis, tumor growth, and metastasis (7, 8). Its expression correlates strongly with malignancy in many tumor types including prostate cancer. Expression of αvβ3 integrin has been shown in prostate adenocarcinoma, as well as in the invasive prostate cancer PC-3 cell line, whereas it is absent in normal prostate epithelial cells and the less aggressive LNCaP cell line (9). Overexpression of αvβ3 integrin in LNCaP prostate cancer cells upregulatescdc2 level and increases cell migration (10).

In the past decade, there has been emerging evidence to suggest that αvβ3 integrin may promote radioresistance of a tumor. In 2005, Gnerber and colleagues reported that patients with cervical cancer with αvβ3 expression had significantly worse local control, metastasis, and survival after curative radiotherapy (11). Also in 2005, Abdollahi and colleagues demonstrated that S247 (an αvβ3 petidomimetic antagonist) potentiates antiangiogenic effect of ionizing radiation (IR) on endothelial cells and xenograft tumors (12). In 2006, Albert and colleagues demonstrated that cilengitide (αvβ3 cyclic peptideantagonist) increased sensitivity of human endothelial cells and non–small cell lung cancer (NSCLC) cells in vitro (13). In an orthotopic rat glioma xenograft model, application of a single dose of cilengitide (4 mg/kg), 4–12 hours prior...
to radiation potentiates radiation efficacy (14). Although phase II clinical trial of cilengitide in patients with nonmetastatic castration-resistant prostate cancer shows no detectable clinical activity (15), application of cyclic RGD peptide with liposomal drug delivery system enhances therapeutic efficacy in treating prostate cancer bone metastasis, implying a complex prostate cancer response to the integrin antagonist (16).

Survivin belongs to a family of inhibitors of apoptosis (IAP; ref. 17). It plays an important role in mitosis, inhibition of apoptosis and autophagy, repair of DNA breaks, and resistance to chemother/peptide (18) or radiotherapy (19, 20). Notably, survivin is overexpressed in many types of cancer cells including prostate cancer while absent in normal differentiated tissues (21). Thus, survivin expression level is found to be positively correlated with tumor progression and inversely correlated with the overall survival in patients after treatment (22, 23). The purpose of this study is thus to investigate whether αvβ3 integrin can promote intrinsic radioresistance of prostate cancer cells and to determine whether the survivin is involved in the regulation of cell survival controlled by αvβ3 integrin.

Materials and Methods

Antibodies and reagents

The following antibodies were used for immunoblotting analysis: anti-ERK1 (Santa Cruz Biotechnology, Inc.), anti-AKT (Cell Signaling Technology, Inc.), anti-Bcl-xL and anti-XIAP (BD Biosciences), anti-survivin (Novus Biologicals Inc.), anti-αvβ3 integrin AP-3 (ATCC), anti-αv integrin (NKI-M9) and anti-β3 integrin (TS2/16; Thermo Fisher Scientific). Cyclo (-Arg-Gly-Asp-D-Phe-Val; cRGD), and the control Cyclo (-Arg-Ala-Asp-D-Phe-Val; cRAD) peptides were from Bachem. Survivin-derived (S4) double-stranded RNA oligonucleotide and control siRNA (VIII) were from GE Healthcare Dharmacon Inc.. Power-Prep HP plasmid purification system was from OriGene. BSA, Lipofectamine 2000, and Opti-MEM were purchased from Invitrogen.

Cell line and transfectants

Human prostate cancer cells PC-3, LNCaP, and LNCaP stable cell lines expressing pRc/CMV vector alone (mock-LNCaP) or vector containing full-length β3 integrin (αvβ3-LNCaP) were described previously (9). Cells were cultured in RPMI1640 medium supplemented with 5% heat-inactivated FBS (from Gemini Bio-Products, Inc.), 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Invitrogen), PC-3- or LNCaP-stable cell lines infected with lentivirus carrying shRNA were maintained in the presence of 0.5 or 1 µg/mL of puromycin, respectively. LNCaP-β3 integrin transfectants were maintained in the presence of 100 µg/mL of geneticin. Integrin surface expression was analyzed by FACS analysis as described previously (9). Cells were maintained in the culture medium at 37°C with 5% CO2.

FACS analysis

One-color FACS analysis was performed using nonpermeabilized cell suspensions with one of the following mAbs to human integrins: TS2/16 to β3, NKI-M9 to αv, and AP-3 to β3 while 12CA5 (ATCC) to hemagglutinin (HA) or mouse IgG as negative controls. The cells were incubated with goat anti-mouse FITC-conjugated secondary antibody (40 µg/mL; Cappel) or goat anti-mouse PE-conjugated secondary antibody (The Jackson Laboratory) at 4°C for 30 minutes for FACS staining. FACS analysis and sorting were performed using FACSort (Becton Dickinson).

Immunoblot analysis

Cells were collected using trypsin-EDTA and lysed with the lysis buffer containing: 50 mM/L Tris (pH 7.5; American Bioanalytical), 1% NP40 (Calbiochem), 150 mM/L NaCl, 1 mM/L sodium pyrophosphate, 50 µm/L NaF, and 2 µm/L EDTA (pH 8), 1 µm/L PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, and 1 mM/L sodium orthovanadate (all from Sigma Chemical Co.). Protein concentrations were determined using the BCA protein assay reagent (Pierce Chemical Co.), and proteins in lysates were resolved by 10% or 15% SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes (Schleicher and Schuell). Quantitative analysis was conducted using a computing densitometer (Molecular Dynamics) or ImageLab (Bio-Rad).

Radiation exposure

Cells were irradiated at room temperature using a 6 MeV Varian 2300CD linear accelerator (Varian Medical Systems). Single doses of 2–10 Gy were delivered at 3 Gy/minute with a 1-cm surface bolus application to ensure dose uniformity at a depth of 100-cm source to tray distance. Nonirradiated cells were given a sham irradiation as controls.

Cell survival assay

We used clonogenic assay and colorimetric sulforhodamine B (SRB) assay to evaluate survival of cells in response to radiation. For SRB assay, cells were harvested, resuspended, counted, and irradiated at room temperature over the dose range of 2–10 Gy. Appropriate cells with optimal seeding density were then immediately plated into a 96-well plate and incubated for 6–7 days before determination of the survival by SRB assay. SRB assay was performed as described previously (24, 25). Optical density was measured at 562 nm (Bio-Rad 550 microplate reader). For survival curve, surviving fractions were calculated as: OD of irradiated cells/OD of nonirradiated cells. For inhibition assays, cRGD or cRAD (control) were added into cell culture 1 hour before irradiation, and fresh peptides with media were exchanged every 3 days. To determine synergistic effect of peptide in combination with IR, survival curves were corrected for the drug effect of peptide itself: surviving fraction = OD of irradiated cells with the peptide/OD of nonirradiated cells with the peptide. For clonogenic assay, cells were exposed to IR at doses from 2 to 10 Gy and seeded in either 6-cm dish or 6-well plates. Cells were fixed with formalin and stained with crystal violet 10–12 days post IR. The surviving fraction was scored with the colony containing more than 50 cells.

Anchorage-independent growth assay

Cell growth in soft agar was evaluated as described previously (26). Cells were harvested with trypsin/EDTA. Cells were exposed to IR with dose ranges of 2–10 Gy at room temperature. After radiation, cells were seeded onto soft agar. A total of 5 × 104 cells were suspended in the complete medium containing 0.3% low-melt agarose (Invitrogen). The mixture was then plated into 60-mm tissue culture plates containing 0.5% agar in complete medium. Cells were allowed to grow for 11–14 days, and colonies with the size equal to or greater than 100 µm were...
counted. Duplicate plates were prepared, and 20 fields per plate were counted. For inhibition assays, the cells were incubated in the presence of cRGD or control cRAD 1 hour prior to irradiation, and fresh peptides were added every 3 days.

**Cell adhesion assay**

Adhesion assays were performed as described previously (9). Vitronectin (VN) and fibronectin (FN) were purified as described previously (27, 28). Cell adhesion was scored after purifying the absorbance at 630 nm. Inhibition assay was performed with incubating cells in the presence of either cRGD or cRAD (as control).

**Transfection, gene silencing, and plasmid construction**

Silencing survivin using siRNA transfection was performed as described previously (29). Briefly, mock- and α3β1-LNCaP cells were seeded in 6-well plates at a density of 2 × 10^5 cells per well. Cells were transfected with either control siRNA (VIII) or survivin siRNA (S4) at a concentration of 10 nmol/L using Lipofectamine 2000. Survivin expression was analyzed using immunoblot analysis.

Meanwhile, we also silenced 1) survivin expression in αβ3-LNCaP cells using pLKO.1 lentivirus carrying shRNA; 2) β3-integrin expression in PC-3 cells using pGIPZ lentivirus carrying shRNA (UMASS Medical School RNAi Core, Worcester, MA). The mature antisense sequences of survivin shRNA were 5'-TTCITGAAATGAGAATCCGG-3' (SV-1) or 5'-AACITGCTTCTTGACAGAAGC-3' (SV-2). The mature antisense sequences of β3 integrin shRNA were 5'-ACAGTTCAGTATGACCCGG-3' (β3-Sh1) or 5'-TCACITCTCITATGAAAGGC-3' (β3-Sh2). The lentivirus containing vectors without the target shRNA were used as a control. The cells were infected with lentivirus in the presence of 10 μg/mL polybrene, selected with the puromycin, and maintained in the puromycin-containing culture medium.

The wild-type full-length survivin cDNA was excised in the pcdAN3.1 carrying a HA tag as described previously (30). Plasmid DNA was prepared using PowerPrep HP plasmid purification system (Origene) for cell transfection. For transient transfection experiments, cells were mixed with plasmids and Lipofectamine (Invitrogen) using the manufacturer’s protocol.

**Statistical analysis**

For the SRB assay, the survival curves were calculated as: mean OD of treated cells/mean OD of untreated control cells. The results are expressed as mean ± SD. All experiments in this study were repeated at least two times, confirmed with at least two clones of each transfectant cell line. Significance of differences was analyzed using the Student’s t test. Statistical significance is considered to be P < 0.01.

For anchorage-independent cell growth, significance of differences was analyzed using the Student’s t test. P value smaller than 0.01 or 0.05 are considered statistically significant.

**Results**

**α3β1 expression in LNCaP transfecants remains unchanged after IR**

To determine whether α3β1 can promote intrinsic radiosistance of prostate cancer cells, we used LNCaP transfecants stably transfected with β3 integrin (Fig. 1A) as described previously (9). To exclude confounding factors of alteration of other integrin subtypes in cells, we also surveyed αv and β3 integrin and determined whether there is a difference between mock- and α3β1-LNCaP cells. Our data showed that no difference of β3 integrin between mock- and α3β1-LNCaP cells. However, there is a significant increase of α3 integrin level in α3β1-LNCaP than in mock-LNCaP (Supplementary Fig. S1). Because radiation has been shown to upregulate α3β1 in endothelial cells, NSCLC, and glioblastoma cells (31, 32), we examined this integrin expression in the transfecants after radiation. No change of α3β1 expression level was observed in cells upon IR as shown by immunoblotting (Fig. 1B).

**α3β1 increases survival of prostate cancer cells upon IR**

The standard for radiosensitivity testing in vitro is the clonogenic assay by scoring colonies upon IR. However, LNCaP cells cannot be assessed with this traditional clonogenic assay because of cell density and poor plating efficiency (33). We performed clonogenic soft-agar assay and the colorimetric assay to analyze the radiosensitivity of cells transfected with α3β1-LNCaP as compared with their mock counterparts. The colorimetric assay has been adopted in situations where colony counting cannot be performed, and Pauwels and colleagues have previously demonstrated that the colorimetric SRB assay generates similar radiation–dose response curve and radiosensitivity parameters as the clonogenic assay (25). Significantly improved survival of α3β1-LNCaP was observed with radiation dose from 2 Gy to 10 Gy (P < 0.01; Fig. 1C left). To eliminate the possibility of transfecnt artifact, two different clones of either mock-LNCaP or α3β1-LNCaP were evaluated and show similar results.

PC-3, another prostate cancer cell line, contains endogenous β3 integrin. To see whether enhanced survival in α3β1-LNCaP cells was due to the expression of β3 integrin, we downregulated β3 integrin in PC-3 cells using lentivirus-containing β3 integrin shRNA (Sh1 and Sh2 cells). Cells with β3 integrin downregulated up to 70% (Sh2; Fig. 1E) showed a significantly increased survival of cells in response to IR (P < 0.01). However, radiation significantly decreased colony formation in both mock-LNCaP and α3β1-LNCaP cells (P < 0.01). However, mock-LNCaP irradiated with 5 Gy completely failed to generate colonies in the soft agar, whereas irradiated α3β1-LNCaP cells were still able to form colonies in the agar (Fig. 2A). The difference of colony formation between irradiated mock- and α3β1-LNCaP is statistically significant (P < 0.01).

Meanwhile, we also performed on clonogenic soft-agar assay using PC-3 cells infected with lentivirus carrying β3 integrin shRNA. Downregulation of β3 integrin in PC-3 cells leads to significantly enhanced radiosensitivity in PC-3 cells at high dose (Fig. 2B), which is again replicated in two different clones of PC-3 cells.
cRGD increases radiosensitivity of prostate cancer cells

cRGD is the first-generation $\alpha_\text{v}\beta_3$-selective cyclic peptide antagonist. Cilengitide was derived with similar purpose (with N-methylation modification) for increased antitumor activity (34). We used cRGD peptide for our in vitro testing and to illustrate $\alpha_\text{v}\beta_3$-specific relationship for radioresistance. We confirmed the specificity of the peptide to $\alpha_\text{v}\beta_3$ by blocking adhesion of $\alpha_\text{v}\beta_3$-LNCaP cells to VN but not to FN, which is generally $\alpha_\text{v}\beta_1$ mediated. On the other hand, control peptide, cRAD, does not affect $\alpha_\text{v}\beta_3$-mediated adhesion to VN (Fig. 3A). When the cells were treated with the peptides before irradiation and incubated afterward, the SRB assay revealed that cRGD has a synergistic effect with IR independent of the drug effect, with statistical significance at 2 and 4 Gy ($P < 0.01$; Fig. 3C). However, the peptide alone also had profound effect on survival, which might be at least partially due to its effect on adhesion (Fig. 3B)

Figure 1.

$\alpha_\text{v}\beta_3$ integrin expression increases survival of LNCaP cells in response to IR. A, Surface expression of ectopic $\alpha_\text{v}\beta_3$ integrin in $\alpha_\text{v}\beta_3$-LNCaP, mock-LNCaP, and parental LNCaP cells were analyzed using FACS. Cells were incubated with mAb specific to $\alpha_\text{v}\beta_3$ integrin (AP3) and mAb specific to HA epitope (12CA5) as a negative control. B, Cells were treated with 0 or 5 Gy and were lysed 48 hours after radiation. Proteins in cell lysates were separated in 10% polyacrylamide gels, immunoblotted, and probed with Ab to carboxyl end of $\beta_3$ integrin (41). ERK1/2 is used as a loading control. C, Survival curves of mock-LNCaP and $\alpha_\text{v}\beta_3$-LNCaP were determined using colorimetric SRB assay. Cells (1,000/well) were plated in 96-well plates at each radiation dose. Cells were incubated for 6–7 days before the SRB assay. Surviving fractions were calculated as: OD of irradiated cells/OD of nonirradiated cells. Each dose was carried out with 6 replicates, and the results are expressed as mean ± SEM. Two different clones for either mock-LNCaP or $\alpha_\text{v}\beta_3$-LNCaP were also tested to yield similar results. Each experiment was repeated at least two times. D, Survival curves of PC-3-NS, PC-3-$\beta_3$-sh1, and PC-3-$\beta_3$-sh2 were determined using clonogenic assay. Cells were plated in 6-cm plates at each radiation dose. Cells were incubated for 10–12 days in the culture. Survival of cells was scored with colonies with more than 50 cells. Each experiment was repeated three times. E, Downregulation of $\beta_3$ integrin using shRNA containing lentivirus was confirmed by using immunoblot analysis with antibody specific to $\beta_3$ integrin. $\beta$-Actin was used as a control.
manner (Fig. 3F and G). Unlike αvβ3-LNCaP cells, 2 μmol/L cRGD has little effect on PC-3 cell survival at the lower doses of radiation (2–6 Gy) and the increased dose of cRGD (3 μmol/L) can sensitize PC-3 cells to 6 Gy (Supplementary Fig. S4) suggesting the heterogeneous response of cRGD treatment in different cell types in response to IR.

αvβ3 prevents radiation-induced downregulation of survivin

Apoptosis is an active process characterized by programmed cell death in which a cascade of events is triggered in response to IR. In head and neck squamous cancer cells, cilengitide has been found to enhance radiation sensitivity through downregulation of Bcl-2 expression (35). Survivin is a member of the IAP gene family that plays an important role in antiapoptosis and cell-cycle division (17). Thus, we further investigated whether survivin is involved in the αvβ3-mediated survival of cells upon IR by looking at the expression levels of IAP members including survivin and XIAP as well as Bcl-XL. When mock-LNCaP cells were exposed to 5 Gy of IR, survivin expression was significantly downregulated (~4-fold) 48 hours postirradiation while maintained in irradiated αvβ3-LNCaP cells (Fig. 4A). In contrast, the expression of XIAP (another important member of IAP) and Bcl-XL (4 member of Bcl-2 family) were either stable or increased after radiation in both mock- and αvβ3-LNCaP cells (Fig. 4B and C). Taken together, expression of αvβ3 integrin in LNCaP cells prevents downregulation of survivin of cells in response to IR.

We further investigated the effect of IR on survivin level using PC-3 cells with downregulated β3 integrin (Sh1 and Sh2). Unlike αvβ3-LNCaP cells, PC-3 cells did not show a change of survivin level when cells were exposed to 5 Gy of radiation (Fig. 4D). However, when the IR dose was increased to 10 Gy, PC-3 cells showed a significantly reduced level (30%–80%) of survivin (Fig. 4E).

RNA interference of survivin enhances the radiation-induced inhibition of anchorage-independent growth of αvβ3-LNCaP Cells

To investigate the role of survivin in αvβ3-mediated survival of LNCaP cells in response to IR, we disrupted survivin expression using siRNA or shRNA. For survivin siRNA, a titration experiment was performed to determine the appropriate siRNA dosages (ranging from 5 to 20 nmol/L) for radiation experiment. Survivin siRNA inhibited survivin levels in αvβ3-LNCaP at a dose-dependent manner (Supplementary Fig. S5A). We chose 10 nmol/L siRNA as the optimal concentration for further experiment because 10 nmol/L siRNA significantly reduced survivin level with minimal toxicity and partially inhibited cell growth (Supplementary Fig. S5B). The cells stably transfected with survivin shRNA also showed a significant downregulation of survivin level (Fig. 5B, right).

We further studied whether downregulation of survivin can enhance sensitivity of cells upon IR. Soft-agar assay was used to determine anchorage-independent cell growth. After 10–12 days of incubation of cells treated by either survivin siRNA or shRNA, colonies larger than 100 μm were scored under a microscope. Although IR had a minor effect on the survivin level in either control (NS) or siRNA-infected cells (Fig. 5A, right), IR or survivin siRNA/shRNA alone partially reduced colony formation in αvβ3-LNCaP cells. The combination of IR and survivin siRNA/shRNA completely inhibits anchorage-independent growth in αvβ3-LNCaP cells (Fig. 5A and B, left).
Overexpression of survivin rescues cell survival in response to IR

Downregulation of β3 integrin enhanced loss of survivin in PC-3 cells (Fig. 4E) and subsequently survival of cells (Fig. 1D) in response to IR. We further asked whether overexpression of survivin in PC-3-αβ3-shRNA cells could rescue the survival of cells upon radiation. Cells were transiently transfected with either the plasmid containing wild-type survivin (SV-WT) or

Figure 3.
cRGD increases radiosensitivity of αβ3-LNCaP and PC-3 cells. A, cRGD peptide blocks αβ3 integrin–mediated adhesion to VN, but not to FN (P < 0.01). Adhesion of αβ3-LNCaP cells to FN or VN was performed in the presence or absence of cRGD peptide (1.74 μmol/L) or control peptide cRAD (1.74 μmol/L). Triplicate experiments were performed. B, SRB assay also showed that cRGD alone caused reduction of survival of αβ3-LNCaP cells. Surviving fraction because of different treatments was calculated as OD of treated cells/OD of cells treated with control cRAD. cRGD (2 μmol/L) alone caused significant reduction of cell survival (P < 0.01). For comparison, the absolute surviving fraction of cells treated with IR (4 Gy) or combination of cRGD and IR (4 Gy) were shown. C, cRGD produced synergistic inhibition of αβ3-LNCaP cell survival in combination with IR at dose-dependent manner. αβ3-LNCaP cells were collected, counted, and incubated with either cRGD or control cRAD for 1 hour prior to IR. Immediately after IR, cells were plated in 96-well plates and incubated at 37°C in 5% CO2 in the presence of the peptide. A fresh peptide-containing medium was exchanged every 3 days during the incubation. To determine the synergistic effect of peptide in combination with IR, survival curves were corrected for the drug effect of the peptide itself: surviving fraction = OD of irradiated cells with the peptide/OD of nonirradiated cells with the peptide. Results were achieved in two experiments with two different clones of αβ3-LNCaP. D, cRGD in combination with IR completely inhibits anchorage-independent growth of αβ3-LNCaP cells. αβ3-LNCaP or mock-LNCaP cells were incubated in the presence or absence of 2 μmol/L cRGD 1 hour prior to irradiation. After either 0 or 5 Gy, 5 × 104 cells were seeded in 6-cm soft-agar dishes with duplicates. The experiment was repeated twice with two different clones of αβ3-LNCaP with similar results. E, Representative images of anchorage-independent growth of LNCaP transfectants (0 Gy vs. 5 Gy) are presented. The scale bar (equivalent to 400 μm) was added using a software Freehand. F, Soft-agar assay was used to determine anchorage-independent cell growth. PC-3 cells were incubated in the presence or absence of 2 μmol/L cRGD 1 hour prior to irradiation up to 8 Gy. The experiment was repeated twice. The differences between cRGD and cRAD (control) treatments are significant at high dose of IR (P < 0.01). G, Representative images of anchorage-independent growth of PC-3 cells (0 Gy vs. 8 Gy) are presented. The scale bar equals to 400 μm as measured using a microscope.
empty plasmid vector as a control. As shown in Fig. 5C, cells transfected with SV-WT showed a significantly higher survival at the doses higher than 6 Gy. The survivin levels in cells postradiation were significantly lower (70% lower) in control cells compared with cells that overexpressed SV-WT (30% lower; Fig. 5D). This suggests that the expression of survivin in αvβ3 knockdown cells promotes cell survival in response to IR.

**Discussion**

The αvβ3 integrin is attracting increased attention as a target for cancer therapy; however, this is often related to its role in angiogenesis (12, 13). The effect of integrins on tumor response with conventional treatment modalities including radiotherapy is not understood, and the molecular signaling mechanism affecting cellular response to IR has not been defined. Our study demonstrates a novel finding that introduction of αvβ3 integrin significantly increases the radioresistance of prostate cancer cells. Inhibition of αvβ3 integrin by its antagonist cRGD reduces the radio-protective effect of the integrin. Furthermore, we showed that αvβ3 integrin regulates survivin level upon IR. Finally, disruption of survivin expression using siRNA significantly abolishes αvβ3 integrin-mediated radioresistance of prostate cancer cells. Thus, our study proposes a novel approach to target αvβ3 integrin prior to and/or during radiotherapy for patients with prostate cancer with integrin expression. Cilengitide, the cyclic peptide antagonist of αvβ3 integrin, has demonstrated a very tolerable toxicity profile in phase I trial, although the drug produced minimal tumor response in the limited number of patients tested (36). In our previous study, our laboratory has demonstrated that IR exhibits profound inhibitory effect on β3 integrin expression of prostate cancer cells and has impact on the adhesion of androgen-dependent prostate cancer cells treated with bicalutamide (37, 38). Inhibition of β3 integrin enhances the cytotoxicity in both prostate cancer and breast cancer cells exposed to IR (39, 40). Therefore, combining integrin-targeted drug with radiotherapy can potentially produce a synergistic effect. However, simply inhibiting integrin with antagonist may not be sufficient. Selectively targeting molecules of downstream signaling pathways may
further enhance radiation kill of tumors and prevent cancer cell repopulation.

The relationship between αvβ3 integrin and survivin is novel from a mechanistic perspective. Survivin is a member of the IAP gene family. It plays an important role in the regulation of cellular apoptosis and cell-cycle division (17). It is highly expressed in most malignant tissues, and its expression has been linked to resistance to radiotherapy and chemotherapy. Chakravarti and colleagues have previously shown that radioresistant glioblastoma cell lines have higher level of survivin with additional upregulation after irradiation. The inhibition of survivin function with dominant-negative survivin increases the radiation response of the more radioresistant cell lines. They reported that the mechanism of survivin-mediated radioresistance is caspase
independent and related to enhanced double-strand DNA repair (20). In our data, αβ3 integrin maintained survivin expression after IR, which would be otherwise downregulated in the absence of the αβ3 integrin. In contrast, αβ3 integrin itself does not increase survivin levels in the absence of radiation (41). Radiation-induced increase of survivin levels is found in several tumor cells which also endogenously express αβ3 integrin. By maintaining survivin expression after irradiation (as shown in Fig. 4), αβ3 integrin increases the likelihood of cells to survive posttreatment and leads to a more radioreistant phenotype (as shown in Figs. 1 and 2). This is further supported by our rescue experiment whereby reintroduction of survivin into PC-3 cells that were treated with αβ3 shRNA enhances cell survival in response to radiation. To our knowledge, such observation linking αβ3 integrin with survivin has not been reported, and the pronounced effect seen only after irradiation may open a new area of translational research. For example, upregulation of survivin may work in synchrony with G2-checkpoint regulation to enhance cellular survival because (i) αβ3-LNCaP cells show significant increase in G2–M population after IR; (ii) G2 delay of a variety of cell lines is associated with increasing radioreistance (42); and (iii) αβ3 integrin upregulates CDC2, which is the key regulator for maintaining G2 arrest (10).

The link between integrin-mediated signaling pathway and survivin expression has been found in a variety of cell types. In keratinocyte stem cells, inhibition of β1 integrin signaling using anti-β1 antibody downregulates SV40 WT expression and induces anoikis (43). Treatment of Keratin 8(-/-) mice with the anti-β1 integrin antibody upregulates survivin expression with decreased activation of caspases (44). In pancreatic cancer cells chemoresentant to gemcitabine, the constitutive FAK activation induced by laminin (a β1 integrin ligand) has been shown to be required for AKT activation that mediates an increased expression of survivin (45). In osteosarcoma MG64 cells, knockdown of survivin expression inhibits the invasion and migration of osteosarcoma in vitro and the expression of α5 integrin on cell surface (46). In melanoma, knockdown of α5 integrin with siRNA abrogates the enhanced tumor growth of melanoma cells overexpressed with survivin. Xenograft of survivin-overexpressing cells in mice demonstrated increased α5 integrin level in both the primary tumor and metastatic colonies in the pulmonary parenchyma (47). However, how integrin regulates survivin expression is still not understood. In prostate cancer, adhesion of PC-3 cells to fibronectin via β1 integrin upregulates survivin expression. This prevents cells from apoptosis induced by TNFα in which the regulation of survivin level is mediated by protein kinase B/AKT mechanism (48). Moreover, overexpression of β3 integrin leads to enhancement of survivin level through INK pathway while inhibition of INK activity with JBD significantly abolished the expression of survivin (41). In glioma cells, αβ3 integrin cross-talks with p53 pathway in regulation of survivin and PEA-15 (49). Although similar mechanisms may be predicted for this pathway, the regulation of β3 integrin on survivin level in prostate cancer cells in response to IR remains to be explored.

In conclusion, the data observed in androgen-dependent prostate cancer cell lines with endogenous β3 integrin expression provide evidence for αβ3 integrin as a promoter of radioreistance through regulation of survivin expression that subsequently may contribute to the radioreistance of prostate cancer. Our results and other articles suggest the complexity of prostate cancers in which the differential expression of molecular profile, that is, integrins and antiapoptotic molecules, plays an important role in regulation of various survival pathways in the heterogeneous subpopulations of prostate cancer cells. Future investigation on the signaling pathways downstream of integrins and regulation of antiapoptotic molecules should be continued to guide the design of novel therapeutic treatments with different combination of radiotherapy, including αβ3 peptide antagonist and survivin antagonist.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Wang, J. Huang, M. Vue, H.L. Goel, L.R. Languino, T.J. FitzGerald
Development of methodology: T. Wang, J. Huang, M. Vue, H.L. Goel, L.R. Languino, T.J. FitzGerald
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Wang, J. Huang, M.R. Alavian, L.R. Languino
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Wang, J. Huang, M. Vue, M.R. Alavian, L.R. Languino, T.J. FitzGerald
Writing, review, and/or revision of the manuscript: T. Wang, J. Huang, M. Vue, M.R. Alavian, H.L. Goel, D.C. Altieri, L.R. Languino, T.J. FitzGerald
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Wang, L.R. Languino, T.J. FitzGerald
Study supervision: T. Wang, L.R. Languino

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