\(\alpha_\beta_3\) INTEGRIN MEDIATES RADIORESISTANCE OF PROSTATE CANCER CELLS THROUGH REGULATION OF SURVIVIN

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Abstract

The αvβ3 integrin is involved in various physiological and pathological processes such as wound healing, angiogenesis, tumor growth and metastasis. The impact of αvβ3 integrin on the radiosensitivity of prostate cancer (PrCa) 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 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. The αβ3 antagonist, cRGD, significantly increases radiosensitivity in both αvβ3-LNCaP and PC-3 cells. Moreover, αvβ3 integrin prevents radiation-induced down-regulation of survivin. Inhibition of survivin expression by siRNA or shRNA enhances IR-induced inhibition of anchorage-independent cell growth. Overexpression of wild-type survivin in PC-3 cells treated with αvβ3 integrin shRNA increases survival of cells upon IR. These findings reveal that αvβ3 integrin promotes radioresistance and regulates survivin levels in response to IR.

Implications: Future translational research on targeting αvβ3 integrin and survivin may reveal novel approaches as an adjunct to radiation therapy for PrCa patients.
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 prostate cancer patients 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 prostate cancer patients (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 since 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 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 up-regulates cdc2 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, Gruber et al. reported that cervical cancer patients with αvβ3 expression had significantly worse local control, metastasis and survival after curative radiotherapy (11). Also in 2005, Abdollahi et al. demonstrated that S247 (an αvβ3 petidomimetic antagonist) potentiates anti-angiogenic effect of ionizing radiation (IR) on endothelial cells and xenograft tumors (12). In 2006, Albert et al. demonstrated that cilengitide (αvβ3 cyclic peptide antagonist) increased sensitivity of human endothelial cells and non-small cell lung cancer cells in vitro (13). In an orthotopic rat glioma xenograft model, application of a single dose of
cilengitide (4 mg/kg) 4-12 hrs prior to radiation potentiates radiation efficacy (14). Although phase II clinical trial of cilengitide in patients with non-metastatic castration resistant PrCa shows no detectable clinical activity (15), application of cyclic RGD peptide with liposomal drug delivery system enhances therapeutic efficacy in treating PrCa bone metastasis, implying a complex PrCa response to the integrin antagonist (16).

Survivin belongs to a family of inhibitors of apoptosis (17). It plays an important role in mitosis, inhibition of apoptosis and autophagy, repair of DNA breaks, and resistance to chemo- (18) or radio-therapy (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 the current 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 (Abs) were used for immunoblotting analysis (IB): 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-β3 integrin AP-3 (ATCC). anti-αv integrin (NKI-M9) and anti-β1 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 (Beidendorf, Switzerland). Survivin-derived (S4) double-stranded RNA oligonucleotide and control siRNA (VIII) were from GE Healthcare Dharmacon Inc. (Lafayette, CO). PowerPrep HP plasmid purification system was from Origene (Rockville, MD). BSA, Lipofectamine 2000 and Opti-MEM medium were purchased from Invitrogen (Carlsbad, California).

**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
previously described (9). Cells were cultured in RPMI 1640 medium supplemented with 5% heat inactivated fetal bovine serum (FBS, from Gemini Bio-Products, Inc., Calabasas, CA), 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, California). PC-3 or LNCaP stable cell lines infected with Lentivirus carrying sh-RNA 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 Fluorescence activated cell sorter (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 monoclonal antibodies to human integrins: TS2/16 to β1, NKI-M9 to αv AP-3 to β3 while 12CA5 (ATCC) to hemagglutinin or mouse IgG as negative controls. The cells were incubated with goat anti-mouse FITC-conjugated secondary antibody (40 μg/ml; Cappel, Durham, NC) or goat anti-mouse PE conjugated secondary antibody (Jackson Laboratory) at 4°C for 30 minutes for FACS staining. FACS analysis and sorting were performed using FACSort (Becton Dickinson).

**Immunoblot (IB)**

Cells were collected using trypsin-EDTA and lysed with the lysis buffer containing: 50 mM Tris (pH 7.5; American Bioanalytical, Natick, MA), 1% NP40 (Calbiochem, La Jolla, CA), 150 mM NaCl, 1 mM sodium pyrophosphate, 50 mM NaF, and 2 mM EDTA (pH 8), 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM sodium orthovanadate (all from Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined using the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL), and proteins in lysates were resolved by 10% or 15% SDS-PAGE under reducing conditions. Proteins were transferred to PVDF membranes (Schleicher and Schuell, Keene, NH). Quantitative analysis was conducted using a computing densitometer (Molecular Dynamics, Sunnyvale, CA) or Imagelab (Biorad Co., Hercules, CA).
Radiation exposure

Cells were irradiated at room temperature using a 6 MeV Varian 2300CD linear accelerator (Varian Medical Systems, Palo Alto, CA). Single doses of 2 to 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. Non-irradiated cells were given a sham irradiation as controls.

Cell survival assay

We used clonogenic assay and colorimetric sulforhomaine 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 to 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 (24, 25). Optical density was measured at 562 nm (Biorad 550 microplate reader). For survival curve, surviving fractions were calculated as: OD of irradiated cells / OD of non-irradiated 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 non-irradiated 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 (26). Cells were harvested with Trypsin/EDTA. Cells were exposed to ionizing radiation with dose ranges of 2 to 10 Gy at room temperature. After radiation, cells were seeded onto soft agar as shown in anchorage-independent growth assay. 50 x 10^3 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 (27, 28). Cell adhesion was scored after measuring 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 plasmids construction**

Silencing survivin using siRNA transfection was performed as described previously (29). Briefly, mock- and αvβ3-LNCaP cells were seeded in 6-well plates at a density of 2 x 10^5 cells per well. Cells were transfected with either control siRNA (VIII) or survivin siRNA (S4) at a concentration of 10 nM using Lipofectamine 2000. Survivin expression was analyzed using IB.

Meanwhile, we also silenced survivin expression in αvβ3-LNCaP cells using pLKO.1 lentivirus carrying shRNA or 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’-TTCTTGAATGTAGAGATGCGG-3’ (SV-1) or 5’-AACTGCTTCTTGACAGAAAGG-3’ (SV-2). The mature antisense sequences of β3-integrin shRNA were 5’-ACAGTTGCAGTAGCGCCG-3’ (β3-Sh1) or 5’-TCACTTCTCATTGAAGC-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 inserted in the pcDAN3.1 carrying a HA tag as described before (30). Plasmid DNA was prepared using PowerPrep HP plasmid purification system (Origene) for cell transfection. For transient transfection experiments, cells were mixed with plasmids with Lipofectamine (Invitrogen) using the protocol provided by the manufacturer.

**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 ± standard error of mean (SEM). All experiments in this study were repeated at least 2 times, confirmed with at least 2 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. Statistical significance is considered for P<0.01.

**Results**

**α₃β₃ expression in LNCaP transfectants remains unchanged after IR**

To determine whether α₃β₃ can promote intrinsic radioresistance of prostate cancer cells, we used LNCaP transfectants stably transfected with β₃ integrin (Fig. 1A) as previously described (9). To exclude confounding factors of alteration of other integrin subtypes in cells, we also surveyed αᵥ and β₁ integrin and determined if there is a difference between mock- and α₃β₃-LNCaP cells. Our data showed that no difference of β₁ integrin between mock- and α₃β₃-LNCaP cells. However, there is a significant increase of αᵥ integrin level in α₃β₃-LNCaP than in mock-LNCaP (Supplemental Fig. 1). Since radiation has been shown to up-regulate α₃β₃ in endothelial cells, non-small cell lung cancer and glioblastoma cells (31, 32), we examined this integrin expression in the transfectants after radiation. No change of αᵥβ₃ expression level was observed in cells upon IR as shown IB (Fig. 1B).

**α₃β₃ increases survival of PrCa 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 due to 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 α₃β₃-LNCaP as compared to their mock counterparts. The colorimetric assay has been adopted in situations where colony counting cannot be performed, and Pauwels et al. have previously demonstrated that the colorimetric sulphorhodamine B (SRB) assay generates similar radiation-dose response curve and radiosensitivity parameters as the clonogenic assay (25). Significantly improved survival of α₃β₃-LNCaP was observed with radiation dose from 2 Gy to 10 Gy (P<0.01) (Fig. 1C.
left panel). To eliminate the possibility of transfectant artifact, two different clones of either mock-LNCaP or α₁β₃-LNCaP were evaluated and show similar results.

PC-3, another prostate cancer cell line, contains endogenous β₃ integrin. To see if enhanced survival in α₁β₃-LNCaP cells was due to the overexpression of β₃ integrin, we down-regulated β₃ integrin in PC-3 cells using lentivirus containing β₃ integrin shRNA (Sh1 and Sh2 cells). Cells with β₃ integrin down-regulated up to 70% (Sh2, Fig. 1E) showed a significant sensitivity to IR starting at a dose as low as 4 Gy (Fig. 1D). Cells with β₃ integrin down-regulated for -52% showed a significant sensitivity to IR at higher doses (Sh1, Fig. 1D). The result suggested the role of β₃ integrin in prevention of cell death from IR damage. There is no significant difference of α₁- or β₁-integrin level among PC-3 β₃ integrin shRNA treated and the control cells (Supplemental Fig. 2)

To further validate the impact of α₁β₃ on radioresistance and to eliminate the effect of adhesion, clonogenic soft agar assay was analyzed anchorage-independent cell growth upon IR. Overexpression of α₁β₃ integrin in LNCaP cells significantly increased the survival of cells in response to IR when cells were exposed to IR up to 10 Gy (P<0.01)(Fig. 2A). Radiation significantly decreased colony formation in both transfected cell lines (P<0.01). However, mock-LNCaP irradiated with 5 Gy completely failed to generate colonies in the soft agar, whereas irradiated α₁β₃-LNCaP cells were still able to form colonies in the agar (Fig. 2A). The difference of colony formation between irradiated mock- and α₁β₃-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. Down-regulation 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 PrCA cells
cRGD is the first generation α₁β₃ selective cyclic peptide antagonist. Cilengitide was derived with similar purpose (with N-methylation modification) for increased anti-tumor activity (34). We used cRGD peptide for our in vitro testing and to illustrate α₁β₃ specific relationship for radioresistance. We confirmed the specificity of the peptide to α₁β₃ by blocking adhesion of α₁β₃-LNCaP cells to VN but not to FN, which is generally α₁β₁ mediated. On the other hand,
control peptide, cRAD, does not affect αvβ3-mediate 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). To exclude the confounding effect of adhesion, we performed soft agar assay using cRGD to assess for its effect on anchorage-independent growth of αvβ3-LNCaP upon 5 Gy of IR. cRGD alone or IR with control cRAD did not completely inhibit colony formation, but combination of cRGD and IR produced dramatic inhibition of colony formation, which is statistically significant (P<0.01) as compared to the control cRAD combined with IR (Fig. 3D and 3E). Similarly, we also performed the soft agar assay on β3-integrin expressing PC-3 cells treated with 2 μM cRGD or cRAD in combination with IR. cRGD significantly enhanced sensitivity of cells upon IR at a high dose of 8 Gy as a dose-dependent manner (Fig. 3F and 3G). Unlike αvβ3-LNCaP cells, 2 μM 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 μM) can sensitize PC-3 cells to 6 Gy (Supplemental Fig. 4) suggesting the heterogeneous response of cRGD treatment in different cell types in response to ionizing radiation.

αvβ3 prevents radiation-induced down-regulation 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 down-regulation of Bcl-2 expression (35). Survivin is a member of the inhibitory of apoptosis (IAP) gene family which plays an important role in anti-apoptosis and cell cycle division (17). Thus, we further investigated if 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 down-regulated (approximately 4 fold) 48 hours post irradiation while maintained in irradiated αvβ3-LNCaP cells (Fig. 4A). In contrast, the expression of XIAP (another important member of IAP) and Bcl-XL (a member of Bcl-2 family) were either stable or increased after radiation in both mock- and αvβ3-LNCAP cells.
(Fig. 4B and 4C). Taken together, expression of αv3 integrin in LNCaP cells prevents down-regulation of survivin of cells in response to IR.

We further investigated the effect of IR on survivin level using PC-3 cells with down-regulated 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

In order to investigate the role of survivin in αvβ3-mediated survival of LNCaP cells in response to IR, we disrupted survivin expression using small interference RNA (siRNA) or small hairpin RNA (shRNA). For survivin siRNA, a titration experiment was performed to determine the appropriate siRNA dosages (ranging from 5 to 20 nM) for radiation experiment. Survivin siRNA inhibited survivin levels in αvβ3-LNCaP at a dose-dependent manner (Supplemental Fig. 5A). We chose 10 nM siRNA as the optimal concentration for further experiment because 10 nM siRNA significantly reduce survivin level with minimal toxicity and partially inhibit cell growth (Supplemental Fig. 5B). The cells stably transfected with survivin shRNA also showed a significant down-regulation of survivin level (Fig. 5B right panel).

We further studied if down-regulation 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 +/- IR, 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 panel), IR or survivin siRNA/shRNA alone partially reduce 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 left panel and 5B left panel).

Overexpression of survivin rescues cell survival in response to IR

Down-regulation 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 empty plasmid vector as a control. As shown in Fig. 5C, cells transfected with wild type survivin showed a significantly higher survival at the doses higher than 6 Gy. The survivin levels in cells post radiation were significantly lower (70% lower) in control cells in comparison to the cells that overexpressed wild type survivin (30% lower) (Fig. 5D). This suggests that the expression of survivin in αβ3 knockdown cells promotes cell survival in response to IR.

Discussion
The αβ3 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 αβ3 integrin significantly increases the radioresistance of prostate cancer cells. Inhibition of αβ3 integrin by its antagonist cRGD reduces the radioprotective effect of the integrin. Furthermore, we showed that αβ3 integrin regulates survivin level upon IR. Finally, disruption of survivin expression using siRNA significantly abolishes αβ3 integrin mediated radioresistance of prostate cancer cells. Thus, our study proposes a novel approach to target αβ3 integrin prior to and/or during radiotherapy for prostate cancer patients with integrin expression. Cilengitide, the cyclic peptide antagonist of αβ3 integrin, has demonstrated a very tolerable toxicity profile in phase I trial, though 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 β1 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 β1 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 $\alpha_v\beta_3$ integrin and survivin is novel from a mechanistic perspective. Survivin is a member of the inhibitory of apoptosis (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 et al. have previously shown that radioresistant glioblastoma cell lines have higher level of survivin with additional up-regulation 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, $\alpha_v\beta_3$ integrin maintained survivin expression after IR, which would be otherwise down-regulated in the absence of the $\alpha_v\beta_3$ integrin. In contrast, $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ integrin. By maintaining survivin expression after irradiation (as shown in Fig. 4), $\alpha_v\beta_3$ integrin increases the likelihood of cells to survive post treatment and leads to a more radioresistant phenotype (As shown in Fig. 1 and 2). This is further supported by our rescue experiment whereby reintroduction of survivin into PC-3 cells that were treated with $\alpha_v\beta_3$ shRNA enhances cell survival in response to radiation. To our knowledge, such observation linking $\alpha_v\beta_3$ 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, up-regulation of survivin may work in synchrony with $G_2$ checkpoint regulation to enhance cellular survival because (1) $\alpha_v\beta_3$-LNCaP cells show significant increase in $G_2/M$ population after IR; (2) $G_2$ delay of a variety of cell lines is associated with increasing radioresistance (42) and; (3) $\alpha_v\beta_3$ integrin up-regulates CDC2 which is the key regulator for maintaining $G_2$ 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 $\beta_1$ integrin signaling using anti-$\beta_1$ antibody down-regulates wild type survivin expression and induces anoikis (43). Treatment of Keratin 8(-/-) mice with the anti-$\beta_1$-integrin antibody up-regulates survivin expression with decreased activation of caspases (44). In pancreatic cancer cells chemoresistant to gemcitabine, the constitutive FAK activation induced by laminin (a $\beta_1$ integrin ligand) has been shown to be required for AKT activation that mediates an increased expression of survivin.
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 up-regulates survivin expression. This prevents cells from apoptosis induced by TNF-α in which the regulation of survivin level is mediated by protein kinase B/AKT mechanism (48). Moreover, overexpression of β6 integrin leads to enhancement of survivin level through JNK pathway while inhibition of JNK activity with JBD significantly abolished the expression of survivin (41). In glioma cells, α5β1 integrin crosstalks 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 β1 integrin expression provide evidence for αvβ3 integrin as a promoter of radioresistance through regulation of survivin expression that subsequently may contribute to the radioresistance of prostate cancer. Our results and other publications suggest the complexity of prostate cancers in which the differential expression of molecular profile, i.e. integrins and anti-apoptotic 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 anti-apoptotic molecules should be continued to guide the design of novel therapeutic treatments with different combination of radiotherapy, including αvβ3 peptide antagonist and survivin antagonist.

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References

Figure legends

Figure 1. $\alpha_\beta_3$ integrin expression increases survival of LNCaP cells to in response to IR.
(A) Surface expression of ectopic $\alpha_\beta_3$ integrin in $\alpha_\beta_3$-LNCaP, mock-LNCaP and parental LNCaP cells were analyzed using FACS. Cells were incubated with mAb specific to $\alpha_\beta_3$ 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 hrs 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 $\alpha_\beta_3$-LNCaP were determined using colorimetric SRB assay. Cells (1000/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 non-irradiated 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 $\alpha_\beta_3$-LNCaP were also tested to yield similar results. Each experiment was repeated at least 2 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 3 times. (E) Down-regulation of $\beta_3$-integrin using shRNA containing lentivirus was confirmed by using Immunoblot with antibody specific to $\beta_3$-integrin. $\beta$-actin was used as a control.

Figure 2. $\alpha_\beta_3$ integrin expression increases anchorage-independent growth of LNCaP or PC-3 cells and radioresistance to IR. (A) Soft-agar assay was used to determine anchorage-independent cell growth (left panel). Both mock- and $\alpha_\beta_3$-LNCaP cells were irradiated up to 10 Gy. The experiment was repeated 2 times with two clones of $\alpha_\beta_3$-LNCaP with similar results. The differences between irradiated and non-irradiated cells for both mock and $\alpha_\beta_3$-LNCaP are significant (P<0.01). Representatives of anchorage-independent growth of LNCaP transfectants were presented (Right panel). (B) Soft-agar assay was used to determine anchorage-independent cell growth. PC-3-NS, PC-3-$\beta_3$-sh1 and PC-3-$\beta_3$-sh2 were irradiated up to 8 Gy. The experiment was repeated 2 times. The differences between irradiated and non-irradiated cells for PC-3-NS and PC-3-$\beta_3$-shRNA cells and PC-3-$\beta_3$-sh2 are significant (P<0.01) at high dose of IR (Left panel). Representative images of anchorage-independent growth of LNCaP transfectants are
Figure 3. cRGD increases radiosensitivity of α₃β₃-LNCaP and PC-3 cells. (A) cRGD peptide blocks α₃β₃ integrin mediated adhesion to VN, but not to FN (P<0.01). Adhesion of α₃β₃-LNCaP cells to FN or VN was performed in the presence or absence of cRGD peptide (1.74 μM) or control peptide cRAD (1.74 μM). Triplicate experiments were performed. (B) SRB assay also showed that cRGD alone caused reduction of survival of α₃β₃-LNCaP cells. Surviving fraction due to different treatments was calculated as OD of treated cells / OD of cells treated with control cRAD. cRGD (2 μM) 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 α₃β₃-LNCaP cell survival in combination with IR at dose-dependent manner. α₃β₃-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 contained 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 non-irradiated cells with the peptide. Results were achieved in two experiments using two clones of α₃β₃-LNCaP. (D) cRGD in combination with IR completely inhibits anchorage-independent growth of α₃β₃-LNCaP cells. α₃β₃-LNCaP or mock-LNCaP cells were incubated in the presence or absence of 2 μM cRGD 1 hour prior to irradiation. After either 0 or 5 Gy, 50,000 cells were seeded in a 6-cm soft agar dishes with duplicates. The experiment was repeated twice with 2 different clones of α₃β₃-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 μ) 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 μM 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 μ as measured using a microscope.

**Figure 4. αvβ3 integrin prevents down-regulation of survivin in αvβ3-LNCaP cells upon IR.** Overexpression of αvβ3 integrin prevents radiation-induced down-regulation of survivin. Cells were exposed to 0 Gy or 5 Gy and harvested 48 hrs post-radiation. Proteins in cell lysates were separated in 15% polyacrylamide gels, immunoblotted and probed with pAb specific to survivin (A), XIAP (B) or Bcl-XL (C). Experiment was confirmed at least two times with two different clones of the transfectants. ERK 1/2 was used as a loading control. (D) Down-regulation of β3-integrin does not change the survivin level at 5 Gy hrs post-radiation as shown in IB. α-tub was used as a loading control. (E) Down-regulation of β3-integrin in PC-3 cells significantly reduces the survivin level and XIAP levels at 10 Gy 24-48 hrs post radiation as shown in IB. α-tubulin was used as a loading control. Bcl-XL does not show changes.

**Figure 5. Survivin siRNA or shRNA significantly enhances radiosensitivity of cells in anchorage-independent growth.** (A) Left panel: IR or survivin siRNA alone partially decreases colony formation in αvβ3-LNCaP cells. The combination of IR and survivin siRNA inhibits anchorage-independent growth (colony formation) of αvβ3-LNCaP cells. Results are displayed as mean±SEM. Two independent experiments were performed, and similar results were achieved. The differences of colony size and number between irradiated and non-irradiated survivin siRNA transfected αvβ3-LNCaP cells are statistically significant (*P<0.01). Right panel: Survivin siRNA (10 nM) down-regulates survivin expression in αvβ3-LNCaP cells upon IR. The control siRNA (NS) was used to compare the effect of survivin siRNA. All survivin siRNA transfected cells were exposed to 0 or 5 Gy 48 hours after transfection. Akt was used as a loading control. (B) left panel: IR or survivin shRNA alone partially decreases colony formation in αvβ3-LNCaP cells. The combination of IR and survivin shRNA inhibits anchorage-independent growth (colony formation) of αvβ3-LNCaP cells. Results are displayed as mean±SEM. Two independent experiments were performed, and similar results were achieved. The differences of colony number between irradiated and non-irradiated survivin shRNA transfected αvβ3-LNCaP cells are statistically significant (*P<0.01). Right panel: Survivin shRNA down-regulates survivin expression in αvβ3-LNCaP cells upon IR. The control shRNA (NS) was used to compare the effect of survivin shRNA. (C) Survival curves of PC-3-β3-sh cells overexpressed with the
plasmids containing either wild-typed survivin (SV-WT) or empty vector PCDNA3-HA (Control) 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 post-radiation. Survival of cells was scored with the colonies containing more than 50 cells. (D) Overexpression of wild-typed survivin in PC-3-β3-sh cells significantly rescues the survivin level 10 Gy 24-48 hrs post-radiation as shown in IB. α-tubulin is used as a loading control.
Figure 2

(A) LNCaP

Surviving Fraction

Connection between Surviving Fraction and Dose (Gy) for Mock and αvβ3 conditions. Dose groups: 0 Gy, 5 Gy, and 10 Gy (Mock), and 0 Gy, 5 Gy (αvβ3).

(B) PC-3

Surviving Fraction

Surviving Fraction in response to dose (Gy) for NS, β3-Sh1, and β3-Sh2 conditions. Dose groups: 0 Gy, 8 Gy, and 0 Gy.
Figure 4: Western blot analysis of LNCaP and PC-3 cell lines treated with mock, αvβ3, IR(Gy), Bcl-XL, Survivin, XIAP, and ERK1/2. 

A. LNCaP cell line after treatment with mock and αvβ3 followed by IR(Gy) for 0 and 5 Gy. 

B. LNCaP cell line after treatment with mock and αvβ3 followed by IR(Gy) for 0 and 5 Gy. 

C. LNCaP cell line after treatment with mock and αvβ3 followed by IR(Gy) for 0 and 5 Gy. 

D. PC-3 cell line after treatment with mock, β3-Sh1, and β3-Sh2 followed by IR(Gy) for 0, 5, and 0 Gy. 

E. PC-3 cell line after treatment with mock, β3-Sh1, and β3-Sh2 followed by IR(Gy) for 0, 24, and 48 hours. 

Note: Relative intensity values are indicated for each protein band.
**Figure 5**

**A**

![Graph showing colony number with survivin siRNA and IR(Gy) impact](image)

- *P < 0.01
- Colony Number
- siRNA: NS, Survivin
- IR(Gy): 0, 5

**B**

![Graph showing colony number with sh-RNA and IR(Gy) impact](image)

- *P < 0.01
- Colony Number
- sh-RNA: NS, SV-1, SV-2
- IR(Gy): 0, 5

**C**

![Graph showing surviving fraction with dose and survivin](image)

- *P < 0.05
- Surviving Fraction
- Dose (Gy)

**D**

![Graph showing relative intensity with time post IR](image)

- Relative intensity
- Survivin
- α-Tubulin
- Time post IR (hr)

*Images and data from Wang & Huang et al.*
αvβ3 INTEGRIN MEDIATES RADIORESISTANCE OF PROSTATE CANCER CELLS THROUGH REGULATION OF SURVIVIN

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