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Research Article

β_1 Integrin Cytoplasmic Variants Differentially Regulate Expression of the Antiangiogenic Extracellular Matrix Protein Thrombospondin 1

Hira Lal Goel,[1] Loredana Moro,[1] Joanne E. Murphy-Ullrich,[3] Chung-Cheng Hsieh,[1] Chin-Lee Wu,[4] Zhong Jiang,[2] and Lucia R. Languino[1]

[1]Department of Cancer Biology, Prostate Cancer Discovery and Development Program, and [2]Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts; [3]Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama; and [4]Departments of Pathology and Urology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Abstract

β_1 integrins play an important role in regulating cell proliferation and survival. Using small interfering RNA or an inhibitory antibody to β_1, we show here that, *in vivo*, β_1 integrins are essential for prostate cancer growth. Among the five known β_1 integrin cytoplasmic variants, two have been shown to differentially affect prostate cell functions. The β_{1A} variant promotes normal and cancer cell proliferation, whereas the β_{1C} variant, which is down-regulated in prostate cancer, inhibits tumor growth and appears to have a dominant effect on β_{1A}. To investigate the mechanism by which β_{1C} inhibits the tumorigenic potential of β_{1A}, we analyzed changes in gene expression in cells transfected with either β_{1C} or β_{1A}. The results show that β_{1C} expression increases the levels of an extracellular matrix protein, thrombospondin 1 (TSP1), an angiogenesis inhibitor. TSP1 protein levels are increased upon β_{1C} expression in prostate cancer cells as well as in β_1-null GD25 cells. We show that TSP1 does not affect proliferation, apoptosis, or anchorage-independent growth of prostate cancer cells. In contrast, the newly synthesized TSP1, secreted by prostate cancer cells expressing β_{1C}, prevents proliferation of endothelial cells. In conclusion, our novel findings indicate that expression of the β_{1C} integrin variant in prostate glands prevents cancer progression by up-regulation of TSP1 levels and inhibition of angiogenesis. [Cancer Res 2009;69(13):5374–82]

Introduction

Prostate cancer develops through a series of defined states: prostatic intraepithelial neoplasia, high-grade prostatic intraepithelial neoplastic lesions, invasive cancer, and an androgen-independent state (1, 2). These defined states arise through multiple alterations in normal cell functions, including transcription, translation, and post-translational processes (3).

Among the alterations described in prostate cancer, aberrant expression and abnormal functions of integrins and of their extracellular matrix (ECM) ligands have been suggested to play a pivotal role in prostate cancer progression (4, 5). In prostate diseases, cells express an abnormal integrin repertoire and are surrounded by a markedly different ECM compared with normal prostate cells (6, 7). These changes have profound consequences given the ability of each integrin to regulate specific cell functions. At this time, 24 integrin heterodimers, 18 α and 8 β subunits, have been described (8). Five β_1 variant subunits, β_{1A}, β_{1B}, β_{1C}, $\beta_{1C\text{-}2}$, and β_{1D}, generated by alternative splicing, have been described. Among these, two variants, β_{1C} and β_{1A}, have been shown to be expressed in prostatic epithelium. β_{1C} is expressed in normal prostatic epithelial cells but is markedly down-regulated in adenocarcinoma (9, 10); it inhibits cell proliferation and tumor growth, whereas the β_{1A} variant is up-regulated and promotes normal and cancer cell proliferation (6, 11).

Another crucial factor promoting tumor growth is its abnormal angiogenic response (12). Tumor angiogenesis plays a critical role in the progression and growth of cancer cells by providing nutrients and other growth factors; thus, blocking angiogenesis in prostate as well as in other tissues is a therapeutic strategy (13–15). Angiogenesis is inhibited by ECM proteins such as thrombospondin 1 (TSP1; refs. 16, 17). TSP1 is secreted by a wide variety of epithelial and mesenchymal cells and has been shown to bind $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, and $\alpha_V\beta_3$ integrins (16, 17). The antiangiogenic effect of TSP1 is partially mediated by its inhibitory effects on endothelial cell proliferation and also neovascularization *in vivo* (16). Besides inhibiting proliferation, TSP1 has also been shown to induce apoptosis of primary human brain microvascular endothelial cells (18). A recent study defines a novel role of TSP1 in preventing anoikis via activation of Akt (19).

In human prostate cancer, TSP1 expression is progressively decreased when normal epithelium is compared with benign prostate hyperplasia and cancer specimens and when low-grade cancer is compared with high-grade cancer (20). In addition, prostate cancer specimens show an inverse relationship between TSP1 immunoreactivity and Gleason score (20). Decreased expression of TSP1 is especially evident in the stroma surrounding cancer areas, where decreased expression is correlated with progressive disease (20). Using 73 prostate tissue specimens (32 patients with benign prostate hyperplasia, 7 with prostatic intraepithelial neoplasia, and 34 with cancer), Vallbo and colleagues show that TSP1 is expressed in benign prostate hyperplasia, down-regulated in prostatic intraepithelial neoplasia, and absent in prostate cancer tissue (21). In human prostate cancer, the tumor growth-inhibitory role of TSP1 begins to be investigated. TSP1 inhibits growth of LNCaP xenograft and microvessel density (22). Although *in vitro* TSP1 does not exert

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for L. Moro: Institute of Biomembranes and Bioenergetics, National Research Council, Via Amendola 165/A, 70126 Bari, Italy.

Requests for reprints: Lucia R. Languino, Department of Cancer Biology, Prostate Cancer Discovery and Development Program, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605. Phone: 508-856-1606; Fax: 508-856-1310; E-mail: lucia.languino@umassmed.edu.

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any significant growth-inhibitory activity on DU145 cells, *in vivo* DU145 xenografts injected with TSP1-expressing plasmid show an extensive area of necrosis compared with control tumors (23). Despite compelling evidence showing widespread down-regulation of TSP1 during prostate malignant progression, the factors that regulate TSP1 levels are not established nor a role for integrins in TSP1 down-regulation has ever been reported.

In the present study, we describe a novel mechanism of prostate cancer progression mediated by integrins and TSP1.

Materials and Methods

Reagents and antibodies. Reagents used for this study include Lipofectamine 2000 and Oligofectamine (Invitrogen), cycloheximide (Sigma), Matrigel (BD Biosciences), and tumor necrosis factor-α (R&D Systems). TSP1 was purified from human platelets as described before (24). Fibronectin was purified from human plasma. The following mouse monoclonal antibodies were used: human β_1 integrin TS2/16 (American Type Culture Collection) used for fluorescence-activated cell sorting (FACS); P4C10 (Chemicon) and AIIB2 (Aragen Bioscience) used for inhibition assays; clone 18 (BD Bioscience) used for immunoblotting; chicken β_1, W1B10 (Sigma), used for FACS; hemagglutinin 12CA5 (American Type Culture Collection); TSP1:133 previously described (25) and clone A4.1 (Thermo Scientific; ref. 25); and laminin α5 chain, 4C7, kindly provided by Dr. Eva Engvall (26). The following rabbit polyclonal antibodies were used: c-Jun, H-79; and ERK1, C-16 (Santa Cruz Biotechnology). Normal purified rabbit IgG, mouse IgG, mouse IgM (Sigma), or rat IgG (Pierce) were used as controls.

Cell lines and transfectants. Mouse cell line GD25, which lacks expression of the β_1 family of integrin heterodimers due to disruption of the β_1 integrin subunit gene, was transfected with either human β_{1A} or β_{1C} under the control of a doxycycline-regulated promoter and described previously (27). These transfectants were cultured as described (27).

PC3 parental or PC3 transfectants expressing chimeric β_{1A} (clones A1 and A2), β_{1C} (clones C1 and C2) integrin (chicken extracellular and human intracellular), or pTet (clone 6 and pool) were generated using the tetracycline (tet)-regulated expression system and cultured as described before (28).

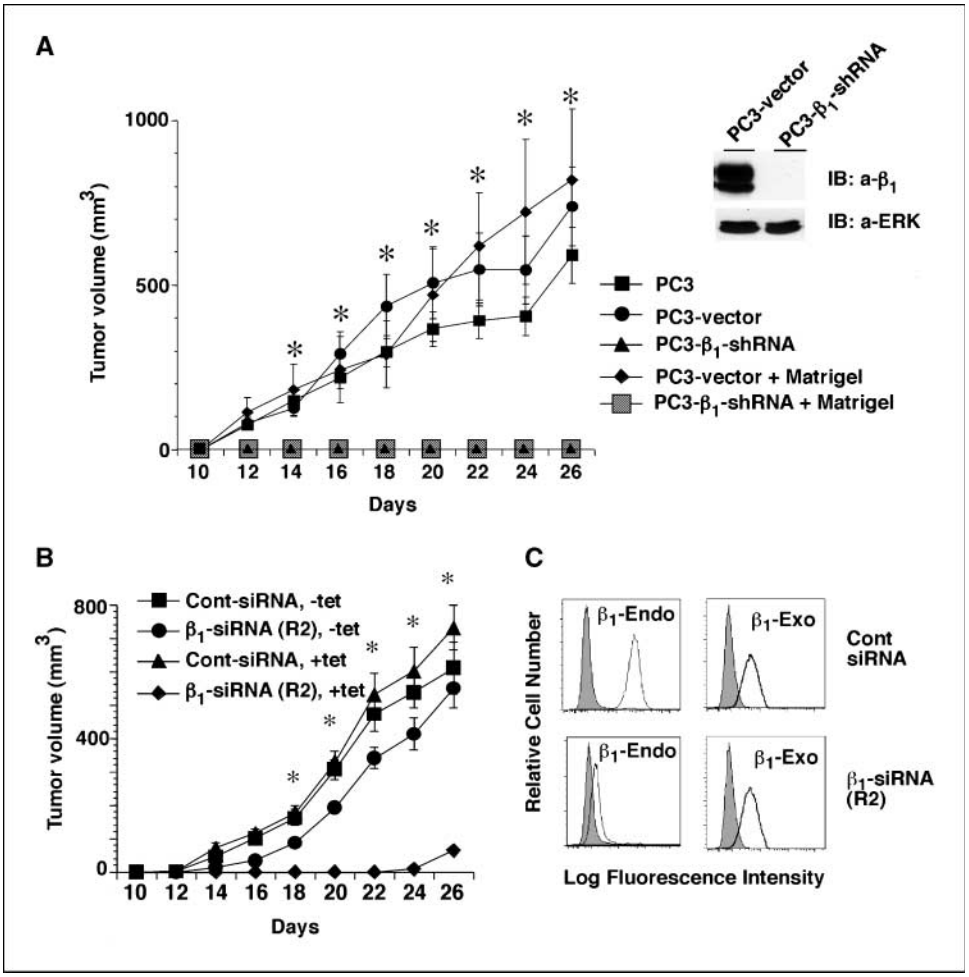

Figure 1. β_1 integrin expression is essential for prostate tumor growth. *A, left,* PC3, PC3-β_1-shRNA, or PC3-vector cells were injected subcutaneously in nude mice. Two groups of mice were also injected with PC3-β_1-shRNA or PC3-vector cells in the presence of Matrigel. The graph shows kinetics of tumor growth. Tumor growth was measured up to 26 d and is expressed as tumor volume in mm^3. The difference in tumor volume between cells transfected with vector or β_1 shRNA from 14 to 26 d after injection are statistically significant (*, $P \leq 0.0001$). *Right,* PC3-β_1-shRNA or PC3-vector cells were lysed and immunoblotted using antibody to β_1 or ERK1. These experiments were repeated twice with similar results. *B* and *C,* PC3 transfectants expressing chimeric exogenous (*exo*) β_{1A} (chicken extracellular and human intracellular) were transfected with β_1 siRNA (siRNA sequence R2), to down-regulate endogenous (*endo*) β_1, or with control siRNA (100 nmol/L). Cells were cultured in the presence or absence of tetracycline and injected subcutaneously in nude mice. Cells express one of the following four combinations of β_1: both endogenous and exogenous (control-siRNA; *−tet*), endogenous only (control-siRNA; *+tet*), exogenous only (β_1-siRNA; *−tet*), and none of the two (β_1-siRNA; *+tet*). The graphs show kinetics of tumor growth measured as described above. *, $P \leq 0.0001$ from 14 to 26 d after injection (*B*). Mean ± SE obtained using 11 animals (*A*) or 5 animals (*B*). Cells transfected with β_{1A} siRNA (siRNA sequence R2) or control siRNA were cultured in the absence of tetracycline. Cells were processed for FACS analysis to determine the expression of endogenous (human) and exogenous (chimera) β_1 using antibody to human β_1 (TS2/16; *thin black line*), chicken β_1 (W1B10; *thick black line*), or, as a negative control, 12CA5 (*filled gray*; *C*).

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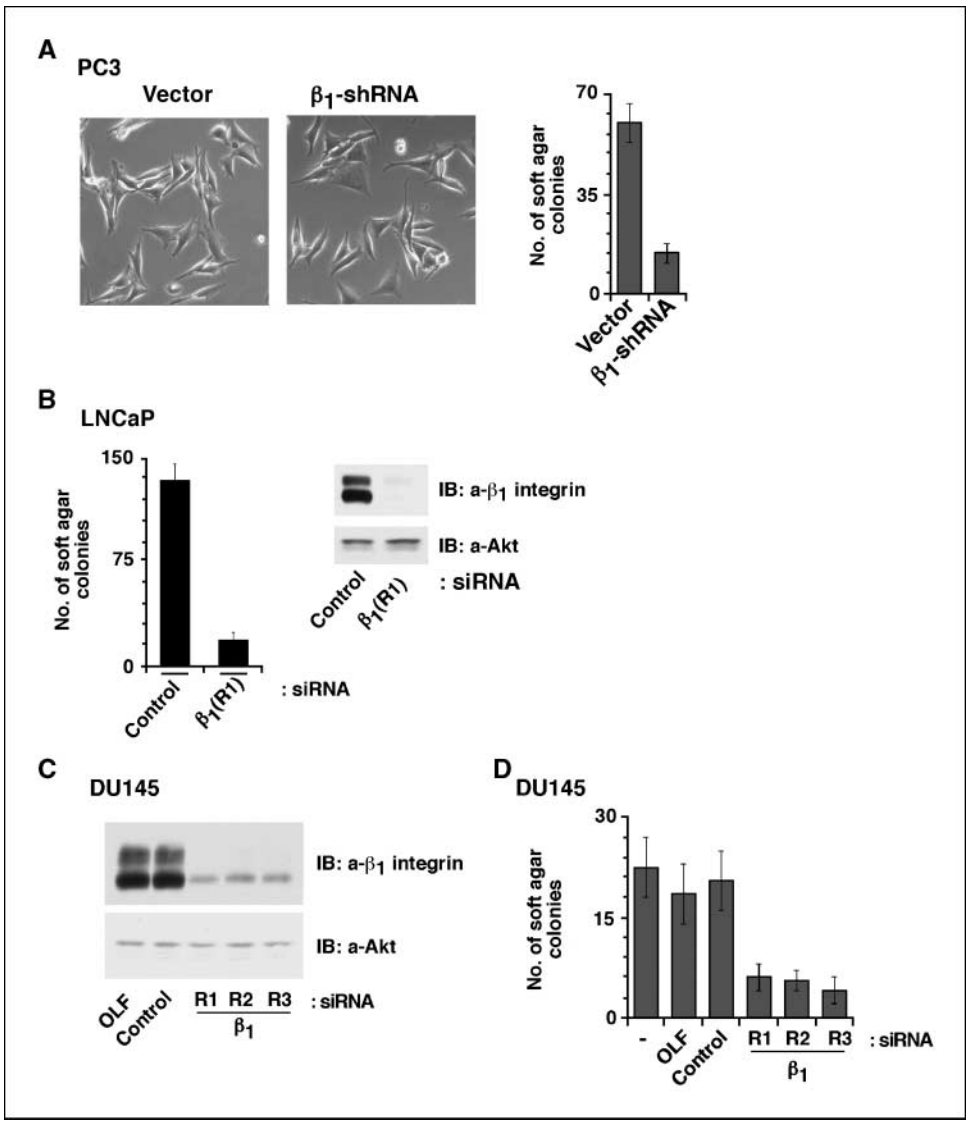

Figure 2. Down-regulation of β_1 integrins inhibits growth of androgen-dependent and androgen-independent cells. *A,* PC3-β_1-shRNA or PC3-vector cells were plated on tissue culture plates. Cells on tissue culture plates were observed under phase-contrast microscope (*left*); alternatively, cells were processed to measure anchorage-independent growth (*right*). *B,* LNCaP cells were transiently transfected with either β_{1A} siRNA (siRNA sequence R1) or control siRNA. Cells were processed to measure anchorage-independent growth (*left*); alternatively, the cells were lysed and immunoblotted with an antibody to β_1 or as a loading control, Akt (*right*). *C* and *D,* DU145 cells were transiently transfected with three β_1 siRNAs (R1, R2, and R3) or control siRNA or Oligofectamine (*OLF*) alone. Cells were lysed and immunoblotted with an antibody to β_1 integrins or as a loading control, Akt (*C*); alternatively, the cells were processed to measure anchorage-independent growth (*D*). Triplicate observations were done. All the results were reproduced in three separate experiments.

PC3 and DU145 cells were stably transfected with plasmids containing either pEGFP or pEGFP-β_1-short hairpin RNA (shRNA; ref. 29) using Lipofectamine 2000 (30). G418-resistant clones were pooled to generate a population.

Human umbilical vein endothelial cells (HUVEC; Clonetics) and LNCaP cells were cultured as described (31, 32).

FACS analysis. Cells were detached and FACS analysis was used to detect surface expression of exogenous (chimera) or endogenous (human) β_1 in PC3 transfectants using W1B10, TS2/16, 12CA5, or mouse IgG (28). Surface expression of β_{1A} or β_{1C} in GD25 transfectants was analyzed by FACS using TS2/16 or, as negative control, 12CA5 (30).

Prostate xenografts. PC3 transfectants (PC3-β_1-shRNA or PC3-vector) were detached, washed, and resuspended in RPMI or RPMI containing Matrigel (50%). Cells (1×10^6) were inoculated subcutaneously into the right flank of 6- to 8-week-old male athymic BALB/c mice (National Cancer Institute-Frederick).

PC3 stable cell transfectants expressing chimeric β_{1A} integrin (chicken extracellular and human intracellular) were transiently transfected with either human β_1 small interfering RNA (siRNA; R2; ref. 29) or control siRNA and cultured in the presence or absence of tetracycline for 48 h. Cells were detached and inoculated subcutaneously into the right flank of athymic BALB/c mice as described above. Mice were given water supplemented with either 5% sucrose to induce β_{1A} expression or 5% sucrose plus 100 μg/mL tetracycline.

PC3 cells were detached, washed, and resuspended in RPMI. Cells (1×10^6) were inoculated subcutaneously into the right flank of 7-week-old male athymic *nu/nu* mice (Charles River). AIIB2 or nonspecific rat IgG was injected intraperitoneally (5 mg/kg) biweekly once the tumor reached 100 mm^3 (33). Subcutaneous tumors from mice described above were isolated, fixed, and embedded in paraffin. Apoptosis was measured using Apoptag cell death detection kit (Chemicon).

In all cases, tumor size was determined using a caliper every other day and tumor volume was calculated as described (11).

RNA isolation and analysis. Gene expression profiles of β_{1A}- or β_{1C}-GD25 stable cell transfectants were generated using 1.2 Atlas Mouse cDNA Expression Arrays (Clontech) according to the manufacturer's instructions (27, 30). Briefly, GD25 stable cell lines were starved for 48 h. During the last 24 h, cells were kept in the presence of 2 μg/mL doxycycline and then detached, washed, and plated for 5 h on fibronectin (5 μg/mL) in serum-free medium. The above conditions used for studying gene expression profiles of β_{1A}- or β_{1C}-GD25 stable cell transfectants were selected to allow comparable cell adhesion, as shown previously (27).

Immunoblotting. Cell lysates as well as concentrated conditioned medium were immunoblotted as described before (34).

Anchorage-independent growth assay. This study used β_1 siRNA (R1, R2, and R3) or nonspecific control siRNA (Dharmacon). The siRNA specific for β_{1A} [R1 (35)] or siRNA for all β_1 integrin subunits [R2 (29) and R3 (36)]

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were described before. DU145 or LNCaP cells were transfected with siRNA and processed to analyze anchorage-independent growth as described (35). PC3-β_1-shRNA or PC3-vector cells were plated on soft agar as described before (35).

β_{1C}-PC3 transfectants were cultured as described (28). Cells were plated on soft agar in the presence of either mouse IgM or a-TSP (TSP blocking antibody; clone A4.1). The antibodies were added twice a week. The numbers of colonies larger than 100 μm were counted.

Cell proliferation assay. β_{1C}-PC3 transfectants were detached, plated on fibronectin, and cultured in the presence or absence of tetracycline and TSP1. After 24 h incubation, cells were processed to measure cell proliferation using sulforhodamine B (Sigma) assay as described (11).

HUVECs (5,000 per well) were plated on a 96-well plate and incubated overnight at 37°C for attachment in complete medium. For each treatment, cells were plated in triplicate. Cells were treated with conditioned medium from β_{1C}-PC3 or β_{1A}-PC3 or TSP1 (10 μg/mL). Cell proliferation was measured using sulforhodamine B as described above. In some experiments, TSP blocking antibody, clone A4.1 was added at a concentration of 20 μg/mL (25, 37).

Apoptosis assay. β_{1C}-PC3 cells were induced as described above. Cells were incubated for 42 h in the presence of cycloheximide (3 μg/mL), tumor necrosis factor-α (150 ng/mL), and TSP1 (2 μg/mL; ref. 28). Cells were processed for DNA fragmentation assay using Cell Death Detection ELISA kit (Roche). DU145 cells transfected with three β_1 siRNAs or control siRNA or DU145 stable transfectants expressing β_1-shRNA or vector alone were detached and processed for apoptosis detection as described above.

Statistical analysis. Data on cell proliferation, apoptosis, and anchorage-independent growth and for *in vivo* tumor growth were expressed as

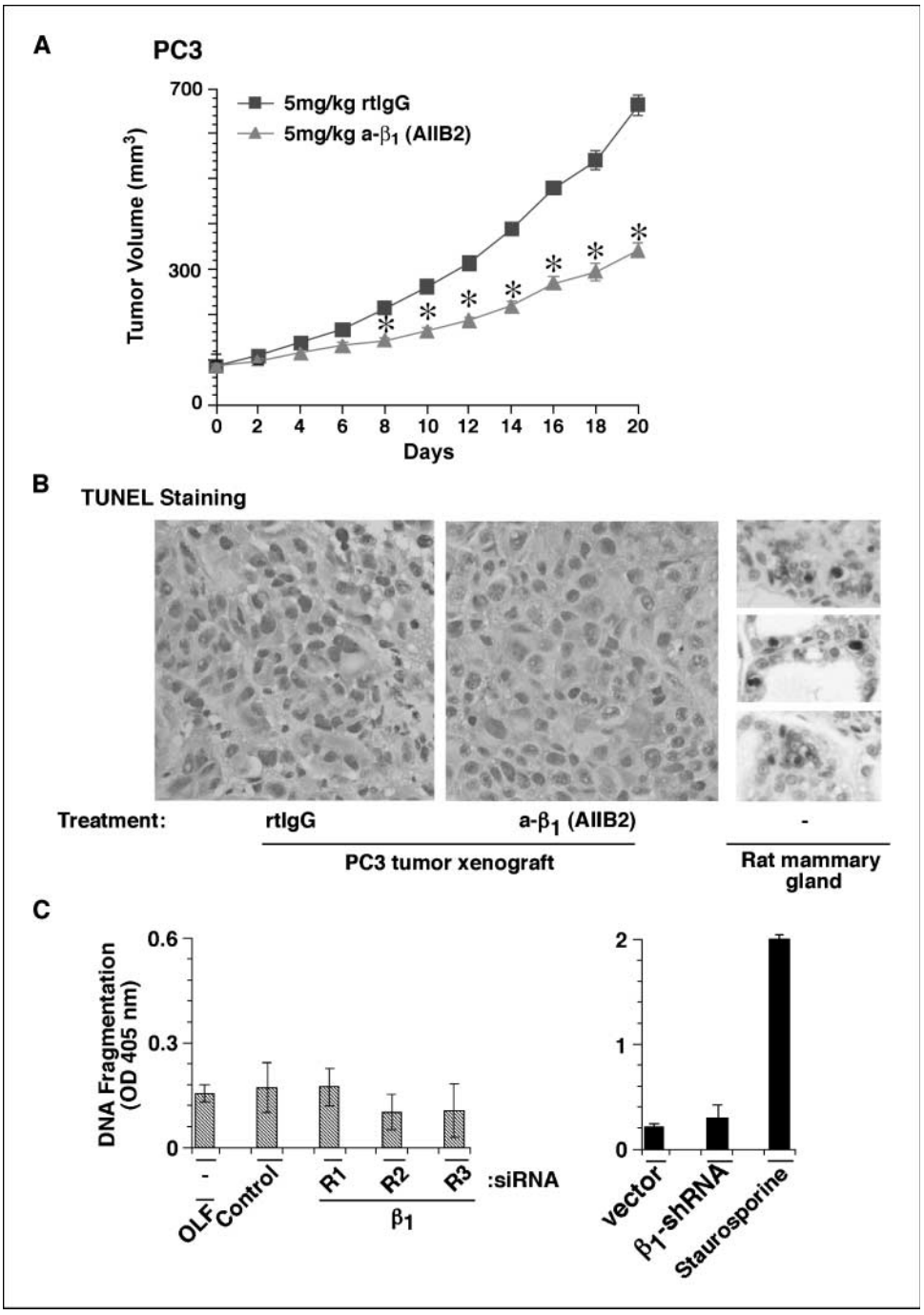

Figure 3. β_1 integrin inhibition does not induce apoptosis *in vivo*. *A,* PC3 xenograft (100 mm^3)-bearing nude mice were injected with either AIIB2 or rat IgG (*rtIgG*) biweekly. Tumor growth was measured up to 20 d and is expressed as tumor volume in mm^3. Mean ± SE of 6 animals per group. The differences in tumor volume in the range of 10 to 20 d after injection are statistically significant between mice injected with AIIB2 and mice injected with rat IgG (*, $P \leq 0.001$). The graph shows kinetics of tumor growth. *B,* subcutaneous tumors from mice described in *A* were dissected and fixed in 10% neutral buffered formalin. The fixed tissues were then embedded in paraffin. A section from female rat mammary gland, 3 to 5 d after weaning of rat pups, was used as a positive control. Apoptosis was measured using Apoptag cell death detection kit from Chemicon. *C,* DU145 cells transiently transfected with three β_1 siRNAs (R1, R2, and R3) or control siRNA or Oligofectamine alone (*left*) or DU145 stable transfectants expressing β_1-shRNA or vector alone (*right*) were detached and processed for DNA fragmentation assay. DU145 cells treated with staurosporine (1 μmol/L) were used as a positive control for apoptosis.

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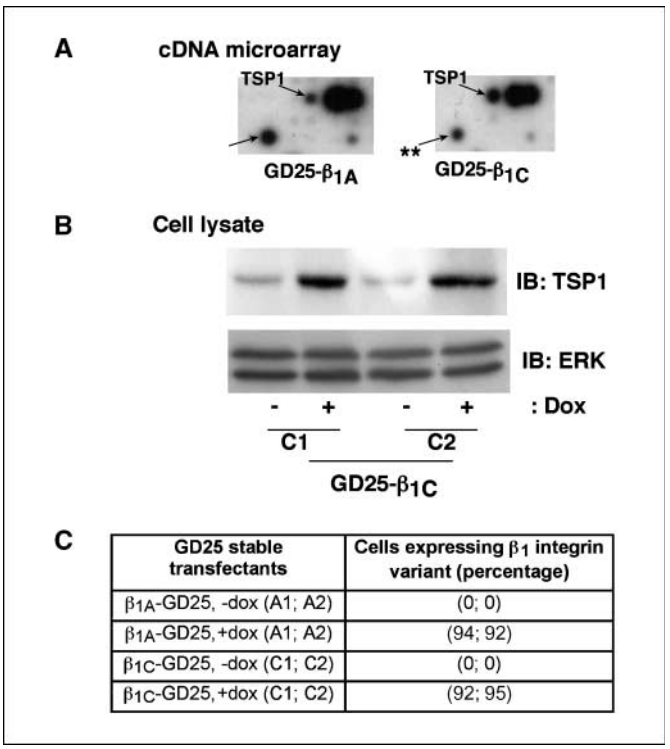
mean ± SE. Statistical analysis between different groups was conducted using the Student's *t* test. All *P* values were based on two-tailed tests.

Results

β_1 integrins regulate tumor growth. β_{1A} and β_{1C} integrins are differentially expressed and redistributed in prostate cancer progression in human and transgenic adenocarcinoma of mouse prostate mice (35, 38, 39). To study the role of β_1 integrins during prostate cancer growth *in vivo*, we generated PC3 stable cell transfectants expressing either a β_1 shRNA or vector alone. We injected these PC3 transfectants (PC3-β_1-shRNA or PC3-vector) in nude mice and find that down-regulation of β_1 integrins completely inhibits the ability of these cells to form tumors (Fig. 1*A, left*). The results show a dramatic inhibition of tumor growth in the absence of β_1 integrins (11 of 11 mice failed to develop tumors), whereas PC3-vector transfectants form large tumors (Fig. 1*A*). Mice injected with PC3-β_1-shRNA transfectants were followed for 60 days, and they never develop tumors (data not shown). Because β_1 plays a crucial role in basement membrane organization, it was investigated whether this effect on tumor growth due to β_1 inhibition was a consequence of an incomplete basement membrane (40, 41). Subcutaneous injection of PC3-β_1-shRNA transfectants resuspended in Matrigel does not rescue the ability of these cells to grow in nude mice, suggesting that the failure to form tumors does not result from lack of supporting basement membrane components. We confirmed down-regulation of β_1 in β_1-shRNA-expressing cells by immunoblotting (Fig. 1*A, right*).

We further investigated whether expression of β_1 integrins in PC3-β_1-shRNA cells could rescue tumor growth. For these experiments, we transfected siRNA targeting human β_1 in cells expressing the chicken/human chimera β_{1A} integrin. We find that expression of β_1 rescues the effect observed in PC3-β_1-shRNA cells on tumor growth (Fig. 1*B*). As shown in Fig. 1*C*, β_1 siRNA specifically down-regulates the endogenous human β_1 without affecting the exogenous chimeric β_{1A} integrin. These results suggest that the β_{1A} integrin plays a crucial role in prostate cancer tumorigenesis. β_1 down-regulation does not affect proliferation, adhesion (data not shown), or morphology of PC3 cells in tissue culture plates but significantly inhibits anchorage-independent growth of these cells (Fig. 2*A*). We also analyzed the effect of β_1 down-regulation on anchorage-independent growth in androgen-dependent LNCaP cells. Using siRNA to β_1, we achieved complete down-regulation of β_1, which causes a significant inhibition of anchorage-independent growth of LNCaP cells (Fig. 2*B*). A similar effect was observed in another androgen-independent cell line, DU145. We further confirmed the effect of β_1 down-regulation by using three different siRNAs to β_1 in DU145 cells (Fig. 2*C* and *D*). These results show that β_1 down-regulation impairs the ability of androgen-dependent and androgen-independent prostate cancer cells to grow in an anchorage-independent manner.

To study the effect of inhibiting β_1 on growth of preformed prostate tumors, we implanted PC3 cells in nude mice. An inhibitory antibody to β_1 (AIIB2) or an irrelevant control antibody (rat IgG) was injected intraperitoneally when the tumor volume reached 100 mm^3. Compared with tumors propagated in animals that received rat IgG, there is a significant decrease in the volume of AIIB2-treated tumors (Fig. 3*A*). Furthermore, we analyzed whether inhibition or down-regulation of β_1 integrins induces apoptosis, but we do not detect any apoptotic cells in prostate tumors from mice treated with AIIB2 or rat IgG (Fig. 3*B*). A section from rat mammary gland, after weaning, shows apoptotic nuclei and was used as a positive control (Fig. 3*B*). Similarly, no apoptosis is detected in DU145 (Fig. 3*C*) and PC3 (data not shown) cells on β_1 down-regulation. These results show that β_1 integrin inhibition prevents prostate tumor growth without inducing apoptosis.



C

GD25 stable transfectants	Cells expressing β_1 integrin variant (percentage)
β_{1A}-GD25, -dox (A1; A2)	(0; 0)
β_{1A}-GD25, +dox (A1; A2)	(94; 92)
β_{1C}-GD25, -dox (C1; C2)	(0; 0)
β_{1C}-GD25, +dox (C1; C2)	(92; 95)

Figure 4. β_{1C} integrin expression increases TSP1 mRNA and protein levels in β_1-null GD25 cells. *A,* β_{1C}- and β_{1A}-GD25 transfectants were induced to express β_{1C} or β_{1A} and processed for cDNA array expression analysis. *Arrows,* spots corresponding to TSP1 cDNA or another gene, which is down-regulated (**) in β_{1C}-expressing cells. Similar results were obtained in two separate experiments. *B,* β_{1C}-GD25 transfectants (clones C1 and C2) were cultured as described above. Cells were lysed and immunoblotted using antibody to TSP1 or ERK1. *C,* β_{1C}-GD25 (clones C1 and C2) or β_{1A}-GD25 (clones A1 and A2) transfectants induced to express β_{1C} or β_{1A} were detached and analyzed by FACS using either TS2/16 or 12CA5, as in Fig.1. The table summarizes the results of the FACS analysis.

β_{1C} integrin regulates expression of TSP1. To study the mechanism by which β_{1C} blocks prostate tumor growth, we analyzed the gene expression profile of GD25 cells expressing either the β_{1C} or β_{1A} integrin variants. These stable transfectants express β_{1C} or β_{1A} integrin variants under the control of doxycycline (30). Among the genes, which are differentially regulated, TSP1 levels are significantly increased on β_{1C} expression compared with β_{1A} (Fig. 4*A*). In contrast, other genes are down-regulated (one representative spot is shown as **). To confirm these results, we performed immunoblotting using an antibody to TSP1. β_{1C} causes a significant increase in TSP1 protein expression (Fig. 4*B*), whereas β_{1A} integrin expression does not change TSP1 levels (data not shown). The GD25 stable transfectants used for the above-described experiments show comparable surface expression of β_{1A} or β_{1C} variants (Fig. 4*C*). These results show that TSP1 is specifically up-regulated in cells expressing the β_{1C} variant.

The β_{1C} integrin variant has been shown previously to inhibit prostate tumor growth (11). We investigated the effect of the β_{1C} integrin variant on TSP1 expression in prostate cancer cells. We used PC3 stable transfectants expressing either chimeric β_{1C} or β_{1A}

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integrin (chicken extracellular and human intracellular; ref. 28). These cells were induced to express β_1 variants by removing tetracycline, and expression of TSP1 in cell lysates and in conditioned medium was analyzed. We find increased TSP1 expression upon the attachment of β_{1C} transfectants to fibronectin (Fig. 5*A, left*; data not shown). Conditioned medium from cells expressing β_{1C} also shows up-regulation of TSP1 (Fig. 5*A, right*). However, TSP1 expression in cell lysates as well as in conditioned medium is not affected upon the attachment of β_{1A} transfectants to fibronectin (Fig. 5*A*). In contrast, the levels of laminin α5, another ECM protein shown to inhibit migration, invasion, and angiogenesis (42), are not affected by β_{1C} expression (Fig. 5*B*). We also analyzed the expression of c-Jun, which has been shown to inhibit TSP1 levels (43, 44). The results show that β_{1C} expression does not affect c-Jun protein levels (Fig. 5*B*). Moreover, tetracycline removal does not affect TSP1 levels in cells transfected with vector alone (pTet, pool, and clone 6) or in parental PC3 cells (Fig. 5*C*). We confirmed surface expression of the transfected chimeric β_{1C} (clones C1 and C2) after tetracycline withdrawal using FACS analysis (Fig. 5*D*). These results indicate that β_{1C} induces expression and secretion of TSP1 in prostate cancer cells.

TSP1 does not affect proliferation or apoptosis of β_{1C}-expressing PC3 cells. To investigate whether secreted TSP1 blocks PC3 cell growth in an autocrine manner, β_{1C}-PC3 transfectants were cultured in the presence or absence of tetracycline and cell proliferation was studied at different doses of TSP1. As shown in Fig. 6*A*, TSP1 does not affect proliferation of PC3 cells in the presence or absence of β_{1C} integrin. In addition, TSP1 does not affect apoptosis of β_{1C}-PC3 cells induced by cycloheximide and tumor necrosis factor-α treatment (Fig. 6*B*). We confirmed the homogeneity of two different preparations of TSP1, used in this study, by SDS-PAGE (Supplementary Fig. S1A). In addition, we confirm that PC3 cells adhere to TSP1 and this attachment is inhibited by pretreatment with an inhibitory antibody to β_1 integrins (Supplementary Fig. S1B and C). Overall these results suggest that increased levels of TSP1 in response to β_{1C} expression do not affect prostate cancer cell proliferation or apoptosis.

TSP1 secreted by β_{1C}-expressing PC3 cells inhibits proliferation of HUVECs. Because TSP1 is known to inhibit angiogenesis and proliferation of endothelial cells (37), we investigated the effect of conditioned medium from PC3 cells expressing β_{1C} or β_{1A} integrin on the proliferation of HUVECs. As shown in Fig. 6*C*, we find significant inhibition (range, 36-45%) of HUVEC proliferation by conditioned medium of β_{1C}-PC3 transfectants compared with β_{1A}-PC3 transfectants. An antibody specific for TSP1, clone A4.1, has been shown to inhibit the antiangiogenic activity of TSP1 (25, 37); we observe that TSP1 blocks HUVECs proliferation and this inhibitory effect is rescued by clone A4.1 (data not shown;

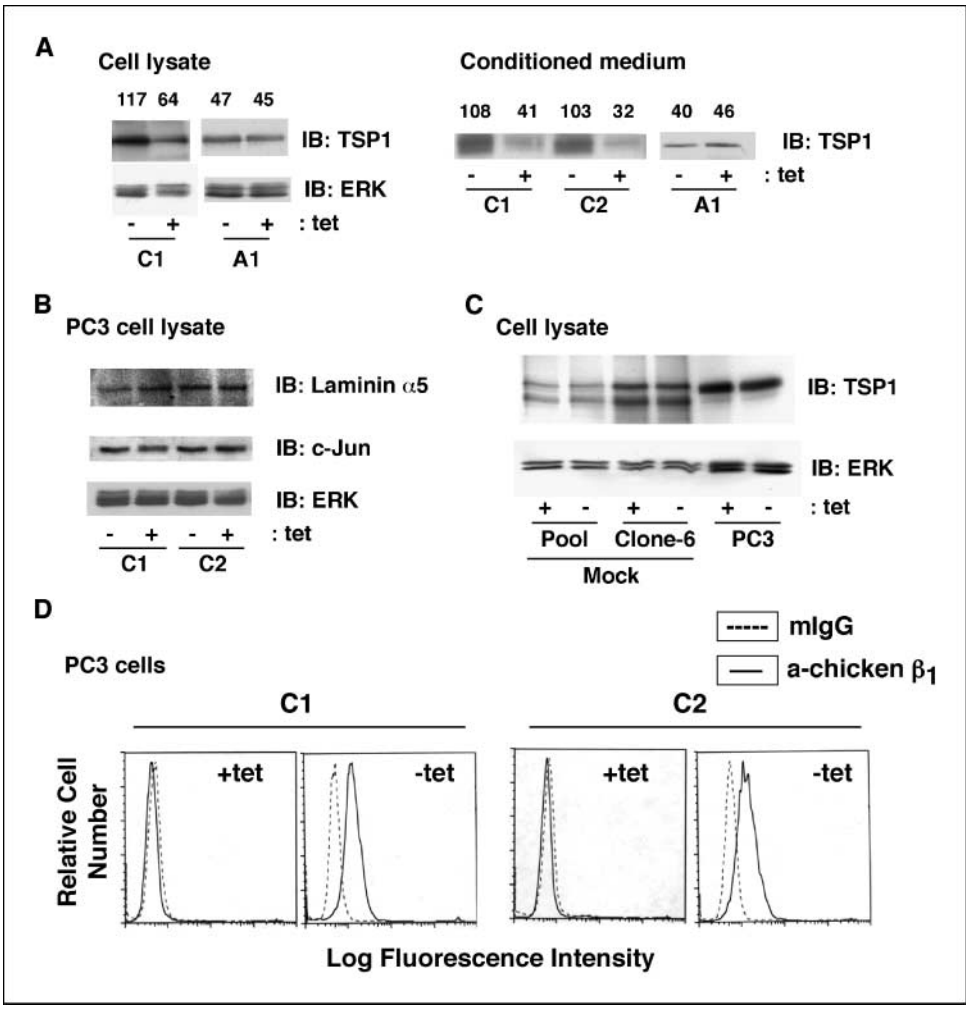

Figure 5. β_{1C} integrin expression increases TSP1 protein levels in PC3 cells. *A* and *B*, β_{1C}-PC3 (clones C1 and C2) or β_{1A}-PC3 (clone A1) cells cultured in presence or absence of tetracycline were detached, plated on fibronectin, and attached cells were cultured in growth medium in the presence or absence of tetracycline. Cells were lysed or conditioned medium was collected and concentrated. Cell lysates as well as conditioned medium was immunoblotted using antibody to TSP1, ERK1 (*A*), laminin α5, c-Jun, or ERK1 (*B*). Densitometric values are shown for each lane. *C*, PC3 cells stably transfected with pTet either pooled (pool) or used as a separate clone (clone 6) were cultured in the presence or absence of tetracycline, lysed, and immunoblotted using antibody to TSP1 or ERK1. *D*, β_{1C}-PC3 (clones C1 and C2) cells were cultured in the presence or absence of tetracycline. Cells were detached and analyzed by FACS using either W1B10 or mouse IgG.

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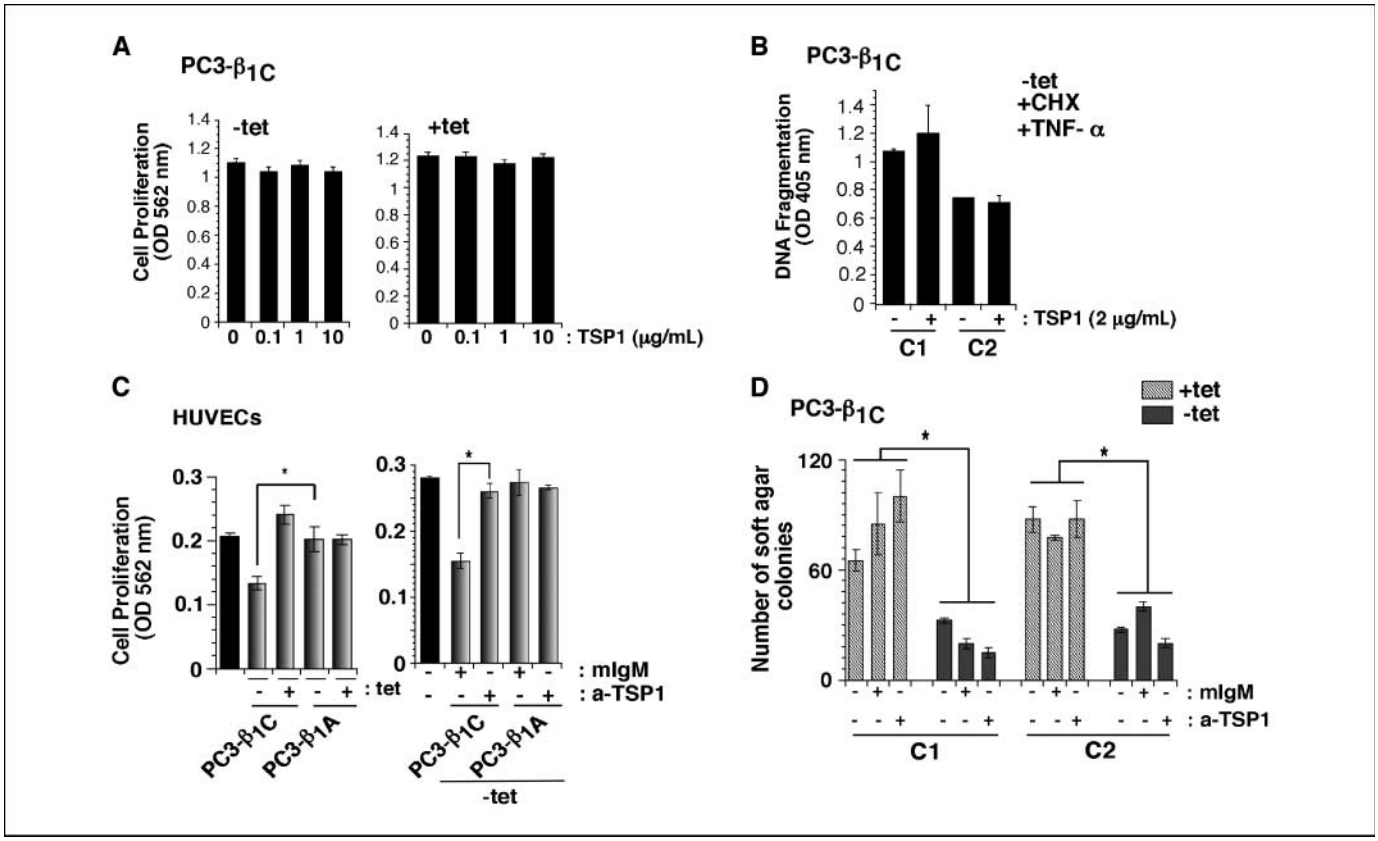

Figure 6. TSP1 inhibits proliferation of HUVECs. *A* and *B,* β_{1C}-PC3 cells were cultured in either the absence (*-tet*; *left*) or the presence (*+tet*; *right*) of tetracycline. Cells were incubated in the presence or absence of TSP1 (0, 0.1, 1, or 10 μg/mL). After 24 h of incubation, cells were processed to measure cell proliferation using sulforhodamine B assay (*A*). Cells were detached and plated on fibronectin and attached cells were incubated in the presence of cycloheximide, tumor necrosis factor-α, or TSP1 (2 μg/mL). Cells were detached and processed for DNA fragmentation assay (*B*). *C,* HUVECs were plated on 96-well plates and incubated overnight for attachment. Cells were washed with PBS and incubated in serum-free medium for 6 h. HUVECs were treated with conditioned medium from β_{1C}-PC3 (clone C1; *-tet* or *+tet*) or β_{1A}-PC3 (clone A2; *-tet* or *+tet*) transfectants for 24 h. Cell proliferation was measured using sulforhodamine B. TSP1 secreted by β_{1C}-PC3 cells inhibits proliferation of HUVECs (*, $P < 0.05$; *left*). *C, right,* cells were plated and treated with conditioned medium from β_{1C}-PC3 (*-tet*) or β_{1A}-PC3 (*-tet*) as described above in the presence of either mouse IgM or TSP antibody (A4.1; 20 μg/mL) and proliferation was measured using sulforhodamine B after 48 h. The TSP1 inhibitory antibody prevents the effect of β_{1C}-PC3 conditioned medium on proliferation of HUVECs (*, $P < 0.05$). *Gray columns,* conditioned medium; *black columns,* HUVEC culture medium. *D,* β_{1C}-PC3 transfectants (clones C1 and C2) were cultured in the presence or absence of tetracycline. Cells were processed to measure anchorage-independent growth in the presence of either mouse IgM or a-TSP (TSP blocking antibody; clone A4.1). The expression of β_{1C} suppresses anchorage-independent growth of PC3 cells (*, $P < 0.05$).

refs. 25, 37). Our data show that addition of A4.1 antibody prevents the effect of β_{1C}-PC3 conditioned medium on proliferation of HUVECs (Fig. 6*C*). To investigate the effect of TSP1 inhibition on the ability of β_{1C} to suppress tumor growth, we performed an anchorage-independent growth assay using PC3 cells expressing β_{1C}. Our results show that expression of β_{1C} suppresses anchorage-independent growth of PC3 cells. However, inhibition of secreted TSP1 could not rescue PC3 cells from the blocking effect of β_{1C} (Fig. 6*D*). We find similar results when anchorage-independent growth of β_{1C}-PC3 was analyzed in the presence of β_{1C}-PC3 conditioned medium (data not shown) previously tested for its ability to prevent endothelial cell proliferation via TSP1 (Fig. 6*C*). Our data show that TSP1 inhibition does not rescue anchorage-independent growth of β_{1C}-expressing PC3 cells, thus supporting our hypothesis that TSP1 secreted by PC3-β_{1C} cells acts by inhibiting proliferation of endothelial cells rather than affecting prostate cancer cells.

Discussion

The present study describes a novel mechanism mediated by the β_1 integrins that may contribute to prostate cancer progression. The results describe for the first time the ability of integrins to regulate the levels of TSP1, an angiogenesis inhibitor. Specifically these findings characterize the β_{1C} integrin variant, a tumor growth inhibitor, as a positive modulator of TSP1 expression.

In the first part of the study, we show that β_1 down-regulation inhibits prostate cancer growth *in vivo*. The finding that β_1 down-regulation inhibits prostate cancer growth *in vivo* had never been reported. This needed to be investigated since *in vitro* β_1 down-regulation in aggressive PC3 prostate cancer cells does not prevent cell proliferation in response to serum (35) and β_1 expression may either prevent (45) or promote (46) tumor growth. We then extended our study to several prostate cancer cell lines and confirm that β_1 down-regulation impairs the ability of androgen-dependent and androgen-independent prostate cancer cells to grow in an anchorage-independent manner. Although we cannot exclude that these β_1 receptors become inactive in poorly differentiated tumors (35), these findings highlight a crucial role for β_1 in androgen-dependent and androgen-independent prostate cancer growth.

In the second part of the study, we focused our attention on two of the five known β_1 integrin cytoplasmic variants, β_{1C} and β_{1A}. These variants are known to differentially affect prostate cell

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functions. The β_{1C} variant, which is down-regulated in prostate cancer and is coexpressed with the β_{1A} integrin variant (9), inhibits cell proliferation and tumor growth and appears to have a dominant effect on the β_{1A} variant, which instead promotes normal and cancer cell proliferation. To investigate the mechanism by which β_{1C} inhibits the tumorigenic potential of β_{1A}, we analyzed changes in gene expression in cells transfected with either β_{1C} or β_{1A}. We show here that TSP1 is a downstream effector of β_{1C} and acts in a paracrine manner by inhibiting endothelial cell proliferation rather than affecting cancer cell proliferation or apoptosis. The differential response of prostate cancer and endothelial cells may be ascribed to the different repertoire of integrins in the two different cell types. Among the receptors for TSP1, both PC3 and HUVECs express predominantly β_1 and β_3 integrins (16, 17); however, these integrins may heterodimerize with different α-subunits in these cell types and thus provide different ligand specificity.

Our observation that changes in RNA and protein levels of TSP1 in prostate cancer cells occur in response to β_{1C} expression is novel and is also highly specific. We show that the levels of laminin α5, another ECM protein, known to inhibit migration, invasion, and angiogenesis (42) are not affected. The described gene regulation of an ECM protein in response to β_{1C} expression seems, therefore, to be specific for TSP1. Other integrins share this ability to regulate gene expression; among others, $\alpha_V\beta_3$ expression has been documented to increase cdc2 levels (32). Similarly, several other molecules (cyclin A, cyclin D1, cyclin E-cdk2 kinase activity, gelatinase, metalloproteinases, and immediate-early response genes) have been shown to be up-regulated in response to integrin expression, although the engagement of these receptors is also required to generate a significant response (32, 47).

Among the possible TSP1 regulators activated by β_{1C}, the following potential downstream effectors of integrins are likely to contribute to this pathway. We have shown earlier that β_{1C} inhibits activation of ERK (35); thus, ERK inhibition may be responsible for TSP1 regulation, although a role for ERK in this pathway remains to be investigated. Additional pathways may be involved. First, β_{1C} may affect TSP1 expression via down-regulation of vascular endothelial growth factor, a factor known to stimulate angiogenesis but also to mediate apoptosis of endothelial cells in the presence of transforming growth factor-β1 (48). This is conceivable because an inverse correlation between expression of TSP1 and vascular endothelial growth factor in prostate cancer specimens has been shown (49) and remains to be investigated. Second, the tumor suppressor protein p53 is known to be regulated by integrins (50). In cultured fibroblasts, loss of the wild-type allele of p53 results in reduced expression of TSP1, whereas reexpression of p53 in these cells restores TSP1 mRNA levels (51). Correlation between reduced TSP1 expression with p53 alterations was also observed in patients with invasive transition carcinoma of the bladder (52). Because PC3 cells used in this study are p53-null, we can rule out the possible involvement of p53 in the regulation of TSP1 levels. Third, several oncogenes such as ras and src, known to be activated by integrins, cause reduction in TSP1 mRNA levels and may act as downstream effectors of integrins (16, 53). Finally, c-jun inhibits TSP1 mRNA levels but is unlikely to be responsible for TSP1 down-regulation (43, 44), because our data show that c-Jun expression levels remain unchanged in the presence of β_{1C} integrin.

Taken together, our results suggest that selective loss of specific integrin variants, particularly of the β_{1C} integrin, may facilitate neoplastic transformation not only by inducing a burst in cell proliferation (54) but also by promoting changes in the microenvironment that would favor angiogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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β_1 Integrin Cytoplasmic Variants Differentially Regulate Expression of the Antiangiogenic Extracellular Matrix Protein Thrombospondin 1

Hira Lal Goel, Loredana Moro, Joanne E. Murphy-Ullrich, et al.

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