Type IIB Procollagen \( \text{NH}_2 \)-propeptide Induces Death of Tumor Cells via Interaction with Integrins \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \)

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Cartilage is resistant to tumor invasion. In the present study, we found that the \( \text{NH}_2 \)-propeptide of the cartilage-characteristic collagen, type IIB, PIIBNP, is capable of killing tumor cells. The \( \text{NH}_2 \)-propeptide is liberated into the extracellular matrix prior to deposition of the collagen fibrils. This peptide adheres to and kills cells from chondrosarcoma and cervical and breast cancer cell lines via the integrins \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \). Adhesion is abrogated by blocking with anti \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) antibodies. When \( \alpha_\nu \) is suppressed by small interfering RNA, adhesion and cell killing are blocked. Normal chondrocytes from developing cartilage do not express \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins and are thus protected from cell death. Morphological, DNA, and biochemical evidence indicates that the cell death is not by apoptosis but probably by necrosis. In an assay for invasion, PIIBNP reduced the number of cells crossing the membrane. In vivo, in a tumor model for breast cancer, PIIBNP was consistently able to reduce the size of the tumor.

Cartilage is a unique tissue in being composed of only one cell type, chondrocytes. In addition, cartilage is avascular and resistant to tumor invasion, although the mechanism by which this occurs is unknown. Cartilage ECM is predominantly made up of fibrillar type IIB collagen and the large proteoglycan aggrecan. The fibrillar collagens, types I, II, III, V, and XI, are synthesized as procollagens containing \( \text{NH}_2 \)- and COOH-terminal extension peptides that are removed prior to deposition of the collagen monomers into fibrils in the extracellular matrix. During the chondrogenesis phase of endochondral bone development, large amounts of type IIB collagen are synthesized as the tissue is established. Thereafter, in the cartilaginous growth plate, the cells become hypertrophic, change their predominant collagen synthesis to type X collagen, and eventually die by apoptosis. The hypertrophic cartilage is vascularized and subsequently removed by specialized osteoclasts, and the tissue is replaced by bone synthesized by osteoblasts. By this process, the cartilage provides an anlagen for bone formation.

Type I procollagen is unique among the fibrillar collagens in containing vicinal RGD motifs in the \( \text{NH}_2 \)-terminal propeptide domain encoded by exon 6 of the \( \text{COL2A1} \) gene. RGD peptides serve as the primary integrin recognition sites in extracellular matrix proteins and, as such, play an important role in regulating cell/matrix interactions required for proper cell function. Integrins are cell adhesion molecules that consist of two non-covalently associated subunits \( \alpha \) and \( \beta \). Integrins are receptors for many ECM matrix proteins, such as for collagens (\( \alpha_\beta \beta \), \( \alpha_\beta \beta \), \( \alpha_\beta \alpha \), \( \alpha_\beta \alpha \), \( \alpha_\beta \gamma \), \( \alpha_\beta \gamma \)), laminin (\( \alpha_\beta \beta \), \( \alpha_\beta \beta \), \( \alpha_\beta \gamma \)), osteopontin (\( \alpha_\beta \beta \), \( \alpha_\beta \gamma \), \( \alpha_\beta \gamma \)), and vitronectin (\( \alpha_\beta \beta \), \( \alpha_\beta \gamma \), \( \alpha_\beta \gamma \), \( \alpha_\beta \gamma \)). The binding of substrate to integrins on the cell surface stimulates intracellular signaling to affect gene expression (outside-in signaling), and the cell can alter the expression and affinity of integrins (inside-out signaling). This bidirectional signaling controls cellular activity, such as cell-cell and cell-matrix adhesion, internalization, and degradation of matrix molecules, as well as cell migration, proliferation, and apoptosis.

Type II procollagen can be synthesized in two splice forms, type IIA and type IIB. Type IIA contains an additional exon (exon 2) in the \( \text{NH}_2 \)-propeptide that encodes a von Willebrand factor C domain and is synthesized by many embryonic tissues, such as basement membrane and prechondrogenic mesenchyme. Unlike most fibrillar collagens, type IIA procollagen is not processed to remove the \( \text{NH}_2 \)-propeptide, and the entire pN-procollagen is deposited into the ECM. It functions to bind growth factors like BMP-2 and transforming growth factor-\( \beta \) via the von Willebrand factor C domain encoded by exon 2. For the type IIB \( \text{NH}_2 \)-propeptide made in cartilage, there is no known or even predicted function. Because the RGDRGKD sequence, among fibrillar collagens, is unique to type II collagen, conserved throughout mammalian species, and liberated from the procollagen molecule in vivo, we sought to determine the function of this RGD domain of the type IIB procollagen \( \text{NH}_2 \)-propeptide. We hypothesized that the function of RGD-encoded regions in type II procollagen \( \text{NH}_2 \)-propeptide may be unique to cartilage and required to induce and maintain important biological properties of the tissue. Here we report that the \( \text{NH}_2 \)-propeptide of type IIB procollagen (PIIBNP) is capable of inducing death of a chondrosarcoma cell line and other tumor cells, and the interaction with cells is dependent on integrins \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \). Because normal chondrocytes do not express these integrin receptors or express

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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2 The abbreviations used are: ECM, extracellular matrix; GST, glutathione S-transferase; PIIBNP, \( \text{NH}_2 \)-propeptide of type IIB procollagen; mPIIBNP, PIIBNP with two RGD motifs mutated to RAD; siRNA, small inhibitory RNA; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; En, embryonic day n.

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them at low levels, we propose that during development, chondrocytes are resistant to the influence of the type IIB collagen NH$_2$-propeptide that they synthesize.

Because these data suggest that PIIBNP might be a useful therapeutic agent for cancer, we tested the anti-invasive property of PIIBNP and established a breast cancer model in mice to demonstrate that PIIBNP is able to reduce tumor size in vivo.

**EXPERIMENTAL PROCEDURES**

**Histological Analysis**—Detection of fibrillar type II collagen, integrin α$_v$, and PIIBNP proteins by immunohistochemistry was performed on 5-μm-thick paraffin-embedded sections of E15.5 mouse embryos using a procedure described previously (15).

**Protein Expression, Purification, and Limulus Amebocyte Lysate Test**—Total RNA was isolated from 54-day human fetal embryonic tissue obtained from the Central Laboratory for Human Embryology, University of Washington. Reverse transcription-PCR was carried out to obtain a 207-bp cDNA and a 315-bp cDNA encoding type II collagen NH$_2$-propeptide protein exon 2 (16) and exons 3–8 (14), respectively. The cDNAs were cloned into pGEX-4T-2 vector (Clontech), and GST fusion proteins were expressed using BL21 (DE3) host strains. The recombinant proteins were purified by affinity chromatography and filtered with a 0.1-μm low binding filter (Millipore). The purity of proteins was confirmed by SDS-PAGE. The proteins were tested for lipopolysaccharide using a limulus amebocyte lysate kit (Associates of Cape Cod).

**Identification of PIIBNP from Developing Human and Mouse Limbs**—Human embryonic (days 56 and 57) and mouse E14.5 limbs were used. Samples were frozen, pulverized, mixed with SDS plus dithiothreitol loading buffer, boiled, and then loaded on a 4–20% SDS gel (Pierce). The samples were then blotted to Immobilon FL (Millipore) at 90 V for 1 h at room temperature. The blot was then washed with PBS and blocked with 5% milk (Bio-Rad) in PBS for 1 h at room temperature with shaking. Primary antibody was then added in PBS with 0.1% Tween 20 (Sigma) (PBS-T) for 1 h at room temperature with shaking. The blot was washed three times for 10 min each at room temperature with PBS-T. Infrared secondary antibody (LICOR) was then added and the blot was washed three times for 10 min each at room temperature. Protein A-agarose (Sigma) previously bound with 0.1% SDS. Absorbance at 595 nm was recorded on a Max plate reader. Synthetic blocking peptides GRGDNP and RADNP (Biomol International) and antibodies to α$_v$β$_3$ and α$_v$β$_5$ integrin were preincubated with cells for 30 min before they were added to the wells.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using a QuickChange kit (Stratagene) to mutate the RGDRGD sequence in GST-PIIBNP. The mutated plasmid was confirmed by DNA sequencing and transformed into BL21 (DE3) host strain to express mutated PIIBNP (mPIIBNP; RADRAD).

**siRNA Interference**—hCh-1 cells were grown to 60–80% confluence in 6-well plates in Opti-MEM medium (Invitrogen) without antibiotics and serum. The sequence of siRNAs, sense and antisense oligonucleotides, and the method were from Graef et al. (20). The final concentration of oligonucleotides was 0.05 μM. Transfection was performed using a Lipofectamine kit, and cells were stimulated with 200 ng/ml phorbol 12-myristate 13-acetate after a 4-h incubation with oligonucleotides, as described (20). Total RNA was collected and reverse transcribed with Superscript RT II transcriptase. 5 μl of the cDNA were used for semiquantitative (α-$^{32}$P)dCTP PCR for α$_v$ integrin and glyceraldehyde-3-phosphate dehydrogenase; the former were amplified for 26 cycles and the latter for 20 cycles. Samples were run on 6% SDS-PAGE, dried, and exposed to a PhosphoImager screen (Storm, ABI). Bands were quantified with ImageQuant software.

**Integrin α$_v$ Immunoprecipitation**—hCh-1 cells were lysed in buffer containing 1% Triton X-100 and a proteinase inhibitor mixture (Sigma). Cell homogenates were mixed with GST, PIIBNP, or mutant PIIBNP and incubated for 1 h at room temperature. Protein A-agarose (Sigma) previously bound with integrin α$_v$ antibody was incubated with cell homogenate mixture for 4 h at 4 °C with gentle shaking. After washing (three times for 5 min each), the precipitated material was analyzed by SDS-PAGE and detected with chicken anti-PIIBNP antibody.

**Immunoblotting**—Western blotting was performed as described (21). Blots were probed with the following primary antibodies: rabbit anti-α$_v$, α$_v$-β$_3$, α$_v$-β$_5$, β$_1$, β$_3$, and β$_5$ integrin and goat anti-actin (Santa Cruz Biotechnology, Inc.) and chicken anti-PIIBNP antiserum (17).
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Cell Surface Labeling and Pull-down Assay—hCh-1 cells were incubated with EZ-link Sulfo-NHS-Biotin reagent for 30 min according to the product instructions (Sigma). After three washes with 0.1 M glycine in PBS, the cells were lysed with buffer containing Triton X-100. The lysate was incubated with glutathione-derivatized agarose beads previously bound with PIIBNP or mutant PIIBNP for 1 h at room temperature. After washing, the bound proteins were separated by SDS-PAGE and transferred to a nylon membrane that was subsequently blocked with 5% nonfat dry milk for 1 h. The membrane was washed and incubated with antibodies and then incubated with a secondary antibody coupled to horseradish peroxidase. The antibody complex was visualized by enhanced chemiluminescence.

Cell Viability Assay—hCh-1, MDA-MB231, or Hela cells were plated, treated with fusion protein, and allowed to incubate for the desired amount of time. When the assay was complete, the medium was removed. The cells were trypsinized, combined with the medium, and centrifuged at 4 °C for 5 min at 1500 rpm. The cell pellet was resuspended in 200 μl of new medium, mixed with an equal volume of trypan blue (Sigma). Both live and dead cells were counted using a hemocytometer.

DNA Laddering—Tissue culture plates were coated with type I collagen. hCh-1 cells were plated at a density of 1 × 10$^6$ cells/ml in RPMI (Cellgrow) containing 10% fetal bovine serum (Invitrogen). The cells were treated with staurosporine (Sigma), PIIBNP, or mutant PIIBNP in serum-free medium. The cells were washed with PBS, harvested by trypsinization, centrifuged to resuspend in Hanks’ balanced salt solution, and fixed in 70% ethanol for 24 h at −20 °C. The cell pellets were resuspended in phosphate citrate buffer. The supernatant was transferred to a new tube and incubated with Nonidet P-40 and RNase for 30 min at 37 °C. Proteinase K was added for an additional 30 min at 37 °C. Samples were resolved by 1.5% agarose gel electrophoresis.

Cell Morphology Analysis—Cell morphology was visualized under a light microscope (NIKON ECLIPSE E800) with a differential interference contrast objective (×40). Images were digitally photographed using a Q capture Retiga 2000R camera (×400).

Lactate Dehydrogenase (LDH) Release Assay—Lactose dehydrogenase release assays were performed using a CytoTox 96 non-radioactive assay kit (Promega). Briefly, The hCh-1 cells were cultured in a 96-well plate overnight in a complete RPMI 1640 medium. The cells were then treated with 10 μM GST, PIIBNP, mPIIBNP, RGD, or RAD peptide in serum-free medium for 90 h. The plate was centrifuged for 4 min, and 50 μl of the supernatant from each well was transferred to a new flat bottom 96-well plate. 50 μl of reconstituted substrate mix was added to each well. The plate was covered and placed at room temperature for 30 min. After adding 50 μl of stopping solution to each well, the absorbance at 490 nm was recorded in a plate reader.

Transwell Invasion Assay—Transwell invasion assays were adapted from previously described work (22) (MB231 or hCh-1). Briefly, the inserts were washed twice with serum-free medium. Matrigel (20 μl/insert; 1:6 dilution with medium) was polymerized and equilibrated at 37 °C and 5% CO$_2$ for 30 min. After equilibration, inserts were put into a 24-well plate containing 300 μl of serum-free medium in each well. 200 μl of media containing 10$^5$ cells/with or without 5 μM GST, PIIBNP, or mPIIBNP were added to each insert. The invasion assay was carried out in cell culture incubator for 30 h for MBN231 and 48 h for hCh-1. The cells were fixed with 4% formaldehyde in PBS and stained with 0.2% toluidine blue for 10 min. After washing five times by dipping the chambers in water, the cells at the top of the Matrigel membrane were removed with several Q-tips. The invading cells were visualized (×20 magnification), photographed (at least 12 fields/condition), and counted using Image J software.

Neutral Red Retention Assay—The neutral red retention assay was adapted from previous studies (23). Briefly, hCh-1 cells were seeded in a clear 96-well plate at 1 × 10$^4$ cells/well overnight. The medium was removed and replaced with serum-free medium containing 5 μM GST, PIIBNP, or mPIIBNP for 72 h. The medium was removed and replaced with new medium containing 40 μg/ml neutral red. After a 1-h incubation at room temperature, the neutral red taken up by the cells was extracted in 100 μl of 1% acetic acid, 50% ethanol for 15 min with shaking at room temperature, and the absorbance was read at 540 nm. Lysosomal integrity was calculated as a percentage of the absorbance of the untreated control.

Tumor Xenograft Growth in Vivo—All of the experimental animal procedures on nude mice were approved by the University Laboratory Animal Care Committee of Washington University and were performed according to federal regulations for animal research. NOD/SCID mice (a kind gift of Dr. Matthew Ellis, Washington University), at 6–9 weeks of age were injected subcutaneously on the dorsal surface with 10 million MDA-MB231 breast cancer cells. Tumor growth was monitored by palpation, and the onset of when tumors were detectable was noted. Tumor size was measured with calipers, and tumor volume was calculated using a reported method (24). When tumors grew to 0.5 cm in diameter, 6 nmol of PIIBNP or GST were introduced into mice by daily subcutaneous injection for 2 weeks. When tumors grew to sacrifice size (tumor diameter of >2 cm), the tumors were removed and weighed. Representative data were obtained from three mice per experimental group, and the entire experiment was repeated in two independent trials (n = 6 in each experimental group).

RESULTS

PIIBNP Adheres to Cells via Integrins—Type II procollagen is unique among the fibrillar collagens in containing vicinal RGD peptides in the NH$_2$-propeptide domain (Fig. 1A). In order to investigate conservation across species, DNA sequence data of the type II NH$_2$-propeptide for human, dog, chicken, horse, rat, Xenopus, and zebrafish were collected from GenBank$^\text{TM}$ and analyzed using the gene analysis tool DNAsar/MagAlign (supplemental Fig. 1). The RGDGRGD sequence in type II NH$_2$-propeptide was conserved across mammalian species; however, only the first RGD was conserved in chicken (RGRDGE), and the second RGD was conserved in Xenopus (RGERGD). In zebrafish, neither RGD is conserved (RGERGA). These differences may reflect the role of cartilage in evolution of endochondral bone formation from zebrafish to xenopus to mammals,
where there are significant differences in growth plate formation and bone development.

To undertake these studies, we selected a cell line that was of human origin in order to provide a homogeneous system for testing the function of the human PIIBNP. We chose to use a human cell line developed in our laboratory from the cartilaginous portion of a high grade chondrosarcoma, called hCh-1 (18). This tumor cell line has many properties of chondrocytes in that the morphology of the cells is polygonal, and they express cartilage-characteristic minor collagens and high levels of aggrecan; however, like most chondrosarcoma cell lines, the hCh-1 does not synthesize type II collagen (18). These characteristics are appropriate for our study of the function of type II collagen NH2-propeptide, because there is no endogenous type II collagen to confound the interpretation of the experiments.

To investigate the protein-cell interaction, hCh-1 cell adhesion assays were performed as described under “Experimental Procedures.” GST fusion proteins containing PIIBNP, a region of the COL2A1 gene that does not contain RGD, exon 2, and GST alone were tested in the cell adhesion assay. The recombinant fusion proteins used in this study are diagramed in Fig. 1B in trimeric form (although monomers are produced in the GST fusion protein system), and an example of the purified recombinant protein expression is shown in Fig. 1C. No contaminating proteins were seen on SDS-PAGE, and there was no contamination with lipopolysaccharide, as determined by a limulus amebocyte lysate test.

**FIGURE 1.** PIIBNP binds to cell surface in an RGD-dependent manner. A, sequence for type IIB NH2-propeptide. ↓, N-proteinase cleavage site; *, cross-link formation site in N-telopeptide. The RGDRGD motif is shown in boldface type, and the GKXY sequence is underlined. Carets delineate exons 3–8. B, the type IIA NH2-propeptide (PIIANP) and protein structure contain the eight exons that are represented by numbers, and type IIB NH2-propeptide (PIIBNP) contains seven exons. C, GST fusion proteins were expressed in bacteria and purified as described under “Experimental Procedures.” Protein purity was assayed using a 4–20% SDS-polyacrylamide gel stained with Coomassie Blue. D, 96-well plates were coated with increasing concentrations of GST ( ), recombinant exon 2 ( ), or GST-PIIBNP ( ) at 4 °C overnight and blocked with 0.5% bovine serum albumin, and hCh-1 cells were cultured in the plate for the adhesion assay as described under “Experimental Procedures.” The value of absorbance at 595 nm represents the number of cells bound to the proteins. Absorbance values are represented as mean ± S.D. (n = 8). The smooth curves are fitted linear regression or moving average curves. E, RGDRGD motif in PIIBNP was mutated to RADRAD (mPIIBNP). hCh-1 cell adhesion to GST, PIIBNP, and mutant PIIBNP was analyzed as described above. Mutant PIIBNP significantly reduced cell adhesion ( , p < 0.001 compared with PIIBNP, n = 18). F, hCh-1 cells were incubated with either synthetic RGD or RAD peptide at a concentration of 0–100 μM for 15 min at room temperature and then added into the wells for the adhesion assay. Absorbance values are represented as mean ± S.D. (n = 8). The smooth curves are fitted moving average curves.

In order to determine whether the cell attachment function was mediated by the RGD sequence, the two RGD triplets were mutated to two RAD triplets by point mutations in the PIIBNP recombinant protein. With the RGDRGD sequence mutated to RADRAD, cell attachment was reduced by 90% (Fig. 1E). As additional evidence for specificity of cell adhesion, competition assays were performed. The synthetic RGD peptide (GRGDNP) inhibited the attachment by 50% at a concentration of ~1.5 μM for PIIBNP.
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concentration of 10 μM (Fig. 1F). In contrast, the non-RGD peptide showed no inhibition at a peptide concentration up to 40 μM. At a concentration of more than 40 μM, the non-RGD peptide also inhibited cell adhesion somewhat, but the inhibition was insignificant compared with that by RGD peptide. These data strongly suggest that the RGD motif in PIIBNP mediates the cell attachment.

PIIBNP Adheres to Integrins α$_v$β$_3$ and α$_v$β$_5$—Reverse transcription-PCR analysis was used to determine which integrins were synthesized by the hCh-1 cells. Positive signal was observed for α$_v$, α$_3$, α$_5$, α$_6$, β$_1$, β$_2$, and β$_3$, with no detectable signal for α$_4$ and α$_9$ (data not shown). A weak signal for α$_10$ was detected. To identify the integrins that adhere to the PIIBNP, hCh-1 cell surface proteins were labeled with biotin, and cell membrane proteins were solubilized with lysis buffer containing Triton X-100. The cell membrane proteins were mixed with glutathione-agarose beads that had previously been loaded with recombinant PIIBNP, mutant PIIBNP, or GST. The beads were washed, and proteins associated with the GST fusion proteins were eluted with SDS loading buffer, analyzed by SDS-PAGE, and blotted with horseradish peroxidase-conjugated streptavidin (Fig. 2A). Proteins were identified by Western blotting against various integrin antibodies. As indicated in Fig. 2A, PIIBNP pulled down integrins α$_v$, β$_3$, and β$_5$ but not β$_1$, α$_3$, α$_4$, or α$_9$ (data not shown). No integrins were found in the protein associated with the mutant PIIBNP or GST.

To further confirm the binding specificity of PIIBNP to α$_v$ integrin, immunoprecipitation of integrin α$_v$-binding proteins was performed using a polyclonal integrin α$_v$ antibody. Triton X-100-solubilized cell membrane proteins were incubated with PIIBNP, GST, or mutant PIIBNP for 1 h at room temperature. Proteins associated with integrin α$_v$ were immunoprecipitated using a polyclonal α$_v$ antibody bound to protein A-agarose beads. The proteins bound to antibody were analyzed by SDS-PAGE and Western blotting with PIIBNP antibody. The Western blot results in Fig. 2B (top) show that α$_v$ integrin antibody immunoprecipitates PIIBNP, but not mutant PIIBNP or GST, confirming that there is an interaction between PIIBNP and integrin α$_v$. The results from Coomassie Blue staining of SDS-PAGE further show this interaction by demonstrating that only PIIBNP is co-immunoprecipitated with α$_v$ (bottom).

To confirm the roles of α$_v$β$_3$ and α$_v$β$_5$ as the receptors for PIIBNP, antibody inhibition of cell adhesion was performed. Antibodies to integrins β$_1$, α$_3$, α$_5$, β$_2$, and β$_3$, were incubated with cells prior to cell adhesion assays. As shown in Fig. 2C, cell adhesion to PIIBNP was reduced by 65% (α$_v$β$_3$), 50% (α$_v$β$_5$), 90% (α$_v$β$_3$ plus α$_v$β$_5$), and 50% (α$_v$) in the presence of equivalent concentrations of integrin α$_v$-containing antibodies. In contrast, antibody to integrin β$_1$ did not inhibit cell adhesion to PIIBNP, confirming that β$_1$ integrin is not involved in the cell adhesion to PIIBNP.

Cell Adhesion to PIIBNP Requires Integrin α$_v$—If integrins α$_v$β$_3$ and α$_v$β$_5$ are the receptors for PIIBNP, suppression of the common integrin subunit, integrin α$_v$, should reduce cell attachment to PIIBNP. To test this hypothesis, siRNA specific to integrin α$_v$ was synthesized, and hCh-1 cells were treated with siRNA prior to cell adhesion assay by the method of Graef et al. (20). As shown in Fig. 3, siRNA suppressed integrin α$_v$ expression both at the mRNA (Fig. 3A) and protein (Fig. 3B) levels. Treatment with α$_v$ integrin siRNA led to more than 70% reduction of cell adhesion to PIIBNP as compared with unrelated siRNA-treated control (Fig. 3C).

PIIBNP Induces Cell Death in Tumor Cells—In order to begin to define functions for the integrin binding of PIIBNP, recombinant proteins were added to hCh-1 cells. We found that 4 μM GST-PIIBNP, but not mutant PIIBNP or GST, induced 37% cell death after 90 h of incubation (Fig. 4A). In the presence of PIIBNP, some cells were partially detached from the matrix and many had a granular appearance. Cell death was determined by permeability to trypan blue dye. GST and GST-mutant PIIBNP did not cause cell death and the cells remained flattened on the culture dish. PIIBNP-induced hCh-1 cell death was dose-dependent (Fig. 4B). To determine whether PIIBNP can reduce survival of cancer cells other than the chondrosarcoma line, two additional cancer cell lines were used: HeLa cells derived from a cervical carcinoma and MDA-MB231 cells, a breast cancer cell line. PIIBNP induced death of both HeLa and MB231 cells. When PIIBNP was incubated with MDA-MB231 cells for
40 h, the cell numbers were reduced by more than 50% compared with GST control (data not shown); the increase in percentage of cell death was dose-dependent (Fig. 4C) and time-dependent (beginning at 12 h at 3 μM concentration; data not shown). Cell death occurred on a variety of substrate matrices over time, indicating that detachment-mediated cell death (anokis) did not occur. None of these matrices predominantly uses the αvβ3 and αvβ5 integrins for cell attachment. In other words, whether cultured on fibronectin (which adheres to α5β1, α4β1, and αvβ3 integrins), Matrigel ECM (primarily laminin and collagen IV, which adheres to α6β1 and α2β1 integrins), or type I collagen (which adheres to α1β1, α2β1, and α5β1 integrins), PIIBNP was able to induce cell death in the same time period (Fig. 4D).

Cell Death Requires Integrin αv—Based on these studies, we hypothesized that PIIBNP adhesion to the cell surface integrins αvβ3 and αvβ5 was a prerequisite for induction of cell death. To test this hypothesis, we treated hCh-1 cells with inhibitor siRNA specific to integrin αv prior to cell viability assay. Cell viability assays indicated that PIIBNP induced death of control siRNA hCh-1 cells but not in anti-αv siRNA-treated hCh-1 cells that no longer expressed integrin αv (Fig. 4E). These results strongly suggested that the PIIBNP-induced cell death requires the RGD sequence and the cellular integrin αvβ3 or αvβ5. To substantiate the correlation of PIIBNP-induced cell death with integrin expression, integrin αv expression in different cell lines was probed by Western blotting (Fig. 4F). Cell viability assays showed that PIIBNP induced cell death of HeLa cells that express primarily αvβ3 integrin (25) and MDA-MB231 breast cancer cells that express both αvβ3 and αvβ5 integrin (25) (Fig. 4D). The normal human chondrocytes did not express αv (Fig. 4F).

Normal Chondrocytes Have Reduced or Undetectable Receptor Integrins for PIIBNP and Thus May Be Protected—If PIIBNP is able to kill cells, how are chondrocytes protected when they synthesize large amounts of type IIB procollagen? We first demonstrated that normal chondrocytes were not killed by PIIBNP (Fig. 5A). Chondrocytes are shown from a 6-year-old normal human donor (obtained from Dr. Davis Atkisson, ISTO Technologies, St. Louis, MO). Cell viability assays indicated...
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A 6-year-old chondrocytes (90 hours)

Fat stem cells (76 hours)

B

FIGURE 5. PIIBNP does not induce death of normal cells with reduced expression of integrin α₅β₁ and α₅β₅. A, 6-year-old chondrocytes and human fat stem cells were cultured in a 24-well plate and incubated with 4 µM PIIBNP in serum-free medium for 90 h (chondrocytes) and 76 h (fat stem cells). Cells were imaged by light microscopy using a Q Capture Retiga 2000R camera (×20 magnification), and cell viability was confirmed by a trypan blue exclusion assay. B, total RNA was isolated from hCh-1 cells, MDA-MB231 cells, and 6-year-old chondrocytes. Reverse transcription-PCR was performed as described elsewhere (53). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

expression of type II collagen (Fig. 6A). The α₅ expression is confined to the synovial cells lining the developing synovial joint cavity and expressed in the hypertrophic cartilage immediately upon the increase in cell volume (Fig. 6, B and D). Consequently, as cartilage is established, the integrin receptors for PIIBNP, α₅β₁ and α₅β₅ are not available on chondrocytes for binding. The expression of α₅ in the hypertrophic cartilage may be related to events involving bone formation, the change in collagen expression from type II to type X, calcification, and invasion by osteoclasts and endothelial cells, and it may be related to the eventual death of the cells. However, this hypothesis is speculative at this time.

In order to reasonably hypothesize a function for the PIIBNP in cartilage, we sought to determine whether the NH₂-propeptide, PIIBNP, could be detected in cartilage. Experiments on the biosynthesis of type IIB procollagen strongly suggest that the NH₂-propeptide is removed prior to deposition of the fibril in the ECM (1). In extracts from developing cartilage, a protein that no trypan blue staining cells were detected in most of the wells when treated with PIIBNP, mPIIBNP, or GST for 90 h. We have also tested chondrocytes from a 12-year-old donor and articular chondrocytes from two young horses (provided by Dr. Lisa Fortier (Cornell University Veterinary School)). Other cell types tested and not killed were human adipose stem cells (Fig. 5A), the murine chondroprogenitor cell line ATDC5, mouse osteoblasts, bone marrow macrophages, the RAW264.7 cell line, and the MC3T3 osteoblast cell line (data not shown). Susceptibility to cell death by PIIBNP correlates with the expression of the integrin receptors, as shown in reverse transcription-PCR analysis of integrin gene expression by some of the cells used in this study (Fig. 5B). In the sample of normal young chondrocytes shown here, there is expression of α₅, but much less expression of β₃ and β₅ than in cancer cells. In the young human chondrocytes shown in Fig. 4F, there was no detection of integrin α₅.

PIIBNP, but Not Integrin α₅, Is Expressed in Developing Cartilage—As mentioned previously and shown in Fig. 5A, normal young human chondrocytes are not killed by PIIBNP. In order to further explore the reason why normal chondrocytes are protected from PIIBNP, we investigated expression of α₅ in developing cartilage by immunohistochemistry. α₅ is present in very few tissues in the mouse embryo and is not present in developing cartilage at E14.5 (Fig. 6, A and B) and E17.5 (Fig. 6, C and D). The developing cartilage is easily detected by the high concentration of type IIB procollagen NH₂-propeptide. The antitumor agent staurosporine was used to induce apoptosis for comparison. After 12 h of treatment, microscopic examination revealed that GST- and mutant PIIBNP-treated hCh-1 cells
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61 µm$^2$ (Fig. 7, E–H). DNA laddering assays showed that DNA degradation occurred in staurosporine-treated cells, but not in PIIBNP-treated cells (Fig. 7J). These experiments indicate that apoptosis is not the mechanism of cell death in hCh-1 cells.

As positive tests for necrosis, we assayed LDH release from the cells and the concentration of lysosomes. Only in the PIIBNP-treated cells was the LDH increased (Fig. 7J). LDH was not released when the apoptosis was induced by treatment with RGD peptides, shown by us and others to induce cell death, probably by a cell detachment-mediated mechanism (26, 27). Programmed necrosis is characterized by a number of cell events caused by calcium permeability, calpain activation leading to lysosomal disruption among other events. We assayed for the number of lysosomes in both hCh-1 cells and MDA-MB231 cells by the method of Kennedy et al. (23). In the presence of 5 µM PIIBNP, lysosomes in MDA-MB231 were reduced by ~25% (Fig. 7K), and the lysosomes in hCh-1 were reduced by 20% (data not shown). These results suggest that PIIBNP induces cell death via a programmed necrosis pathway rather than an apoptosis pathway.

**PIIBNP is Anti-invasive**—Finally, in order to determine whether there is potential for a therapeutic application of PIIBNP, we tested the effect of PIIBNP on tumor cell invasion using a modified Boyden chamber technique with Matrigel-coated membranes (22). In this assay, tumor cells must overcome a reconstructed basement membrane by a sequential process of proteolytic digestion of the substrate and active migration. The results are shown in Fig. 8A; PIIBNP reduced the number of MDA-MB231 breast cancer cells crossing the membrane by 60%, whereas the mutant PIIBNP was equivalent to GST alone.

**PIIBNP Suppresses Tumor Growth in Mice**—To test the effect of PIIBNP in vivo, a well accepted tumor model was established in nude mice. In this model, MDA-MB231 breast cancer cells are injected into the dorsal surface of NOD/SCID mice, where they form solid tumors (28). In toxicology studies prior to the tumor experiment, we found that within 2 months, 6 nmol of recombinant proteins did not have any toxic effect on NOD/SCID animals (data not shown). Two sets of experiments have been performed with PIIBNP, both with three animals in each of two groups for a total of 12 animals: six treated with GST-PIIBNP and six treated with GST alone. When MDA-MB231 cells were injected into the dorsal surface of the mice, tumors measuring 0.5 cm in diameter were formed within 3 days. Mice were divided into two groups and injected in the same site daily with 6 nmol of GST-PIIBNP or GST for 12 consecutive days as per the established model. As indicated in Fig. 8B, PIIBNP effectively suppressed tumor growth. The mice were sacrificed when tumors on mice in the control group grew to 2 cm in diameter. All tumors were retrieved and tumor weights are presented in Fig. 8C. The tumor size was reduced by greater than 75% in all animals treated with PIIBNP compared with GST control.

**DISCUSSION**

Type IIB collagen is the predominant collagen in hyaline cartilage and the endochondral bone growth plate, comprising 50% of the protein within the ECM. Type IIB collagen is syn-
predicted and secreted via the endoplasmic reticulum, Golgi, and secretory granules (14). The COOH- and NH2-propeptides are removed prior to deposition of the fibrillar domain in the ECM, but the exact location of their removal is not known. The COOH-propeptide is thought to be removed by the astacin proteinase, BMP-1 (29), whereas the NH2-propeptide is removed by an NH2-proteinase now known as ADAMTS-3 (30). The function of the fibrillar portion of the collagen in the ECM is to provide structural integrity to the tissue; however, there are no known functions for the NH2- or COOH-propeptides. The COOH-propeptide, also named chondrocalcin, has been isolated from cartilaginous growth plates, and it has been suggested that it is involved in mineralization of the tissue (31). Here we demonstrate that type II NH2-propeptide is unique among the fibrillar collagen NH2-propeptides in containing vicinal RGD motifs encoded by exon 6. We show that type IIB procollagen NH2-propeptide, PIIBNP, mediates cell attachment via integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) via RGD, reduces invasion, and induces cell death in tumor cell lines that express these integrins, human chondrosarcoma (hCh-1), breast cancer (MDA-MB2310), and cervical cancer (HeLa). In vivo, PIIBNP inhibits breast cancer tumor growth in a model of breast cancer. These results strongly suggest that the integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) are receptors for PIIBNP and that ligation of PIIBNP conducts a death signal.

Prechondrogenic mesenchyme and many other embryonic tissues synthesize the other splice form of type II collagen, IIA. The NH2-propeptide of IIA propeptide is not removed during matrix assembly, being deposited as the pN-procollagen (14). When the recombinant type IIA NH2-propeptide was tested for the integrin binding and cell killing, it was able to adhere to cell surface but did not kill cells. Integrins play important physiological roles in endochondral bone development. For example, integrin \( \alpha_v \beta_1 \) influences proliferation, differentiation, and apoptosis of hypertrophic chondrocytes (32). The natural presence of PIIBNP in developing cartilage and specific binding of the PIIBNP to \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins sug-

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FIGURE 8. PIIBNP inhibits MDA-MB231 breast cancer tumor invasion in vitro and growth in NOD/SCID mice. A, invasion assay. MB231 breast cancer cells were allowed to invade three-dimensional matrigel-coated membranes in the presence of 5 μM GST, PIIBNP, or mPIIBNP for 30 h prior to fixation and quantification of invasion. Toluidine blue-stained cells are shown in the top panel. In the bottom panel, data are expressed as mean numbers of invading cells per high power field (20×) ± S.D. from a minimum of 12 fields/condition. Statistical significance of means was determined using Student’s t-test (*, p < 0.005 relative to mPIIBNP or GST control). B, in vivo tumor assay. When the tumors grew to 0.5 cm in diameter, the mice were divided into two groups with three mice in each. Mice received 6 nmol of PIIBNP or GST/mouse/day by subcutaneous injection into the tumor for 2 weeks. This experiment has been repeated with identical results. Dashed lines, circumference of the tumor. C, when the largest tumor size in the control group reached to 2 cm in diameter, the mice were sacrificed, and tumors were removed. The tumor weights were represented as mean ± S.D. (*, p < 0.01 as compared with GST control, n = 6).

gests specific functions for this integrin signaling. These integrins are characteristic of endothelial cells (33), osteoclasts (34), synovial cells (35), and many tumor cells (36) and have been used to target anti-angiogenic and anti-tumor agents (37, 38). In this light, our initial choice of the human chondrosarcoma cell line, hCh-1, for these investigations proved to be critical; these cells have high levels of expression of αvβ3 and αvβ5 integrins, thus allowing for the identification of the integrins that bind to PIIBNP. Although adult articular chondrocytes have been reported to express αvβ3 and αvβ5 (39), the expression level is very low. In young human growth plate (7–15 years), integrins αvβ3 and αvβ5 are not expressed in developing cartilage. Rather, it has been reported that integrin αvβ3 was found only in osteoclasts and integrin αvβ5 only in the hypertrophic zone (40). These results agree well with our finding that αvβ3 and αvβ5 integrins were not detected in normal young primary chondrocytes used here (Fig. 4F), and integrin αv is undetectable in developing mouse cartilage of long bones (Fig. 3B).

The ability of PIIBNP to induce death of cells with high expression of αvβ3 and αvβ5 integrins may be crucial to protect normal articular chondrocytes and prevent vascular and osteoclast invasion in developing cartilage. Normal young chondrocytes from humans and horses were not killed. Using αv antibody, we show that embryonic chondrocytes do not express αv during chondrogenesis or as mature chondrocytes but do express αv upon hypertrophy at the time of the switch in collagen synthesis from type II to type X collagen. We have made a preliminary report that PIIBNP can inhibit angiogenesis in vivo and endothelial tube formation in vitro (41) and have shown that PIIBNP can also kill osteoclasts via the αvβ3 integrin but not osteoblasts. Consequently, the data strongly suggest that the normal function of the PIIBNP is targeted to cells such as endothelial, osteoclast, and synovial cells rather than the chondrocytes. By virtue of the pattern of type II collagen synthesis (highest during cartilage formation) and the ability of PIIBNP to eliminate endothelial cells and osteoclasts from the developing cartilage, we speculate that PIIBNP is an excellent candidate for contributing to the molecular mechanism by which growing cartilage remains avascular and intact.

Other fragments of extracellular matrix proteins have been shown to induce cell death, particularly when released from a larger protein moiety. For example, canstatin, the noncollagenous domain of collagen type IV α-chains, functions as an angiogenic inhibitor. Canstatin triggers tumor cell death through a cross-talk with integrin αvβ3 and αvβ5; cell death is through an apoptotic mechanism (42).

PIIBNP binds to cell surface integrins αvβ3 and αvβ5 but apparently, in hCh-1 cells, does not induce apoptosis. For hCh-1 cells, we did not see positive evidence of apoptosis but did see positive morphological and biochemical evidence of necrosis. In other words, the cells exhibited membrane disruption, the medium was positive for cytosolic enzyme activity as

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measured by LDH (43), and there was a reduction in lysosomes. Classic necrosis is characterized as a rapid loss of plasmid membrane integrity, organelle swelling, and mitochondrial dysfunctions and the lack of typical apoptotic features such as DNA cleavage and nuclear condensation. The cell death falls into the category of necrosis as judged by LDH leakage, lysosomal integrity loss, and the lack of DNA cleavage and nuclear condensation, but it takes more than 40 h to induce the cell death, indicating that there is a regulated mechanism behind the PIIBNP-induced cell death.

The other fibrillar collagens, types I, III, and V, are also synthesized with NH$_2$- and COOH-propeptides that are removed prior to deposition of fibrils in the ECM. Function has been attributed to the liberated type I NH$_2$-propeptide, which has been suggested to be involved in feedback inhibition of collagen synthesis (44) and, based on the similarity to type IIA procollagen NH$_2$-propeptide, has recently been shown to bind to BMP2 and transforming growth factor-$eta$1 and to act intracellularly (45). This mechanism has nothing to do with integrin binding but functions via the von Willebrand factor C domain of type I and type IIA collagens.

In our study, we have used recombinant GST-PIIBNP to study its function in detail, although a histidine-tagged PIIBNP also acts similarly (data not shown). It is likely that this fusion protein is similar to that found in the tissue in terms of the availability of RGD sequences to bind to the cell surface because we detected monomeric PIIBNP in both human and mouse developing cartilage (Fig. 5E). The NH$_2$-propeptide does not have any glycosylated residues but potentially has four hydroxyproline residues near the COOH terminus. The lysine cross-link is located COOH-terminal to the ADAMTS-3 cleavage site. Our results indicate that monomeric NH$_2$-propeptide can be identified in cartilage. Because there are two RGD sequences next to each other, it is likely that they would be available for binding, particularly because they are near an interruption in the Gly-X-Y sequence and embedded in sequence that would not be tightly wound in a collagen helix (see Fig. 1). It is likely that the PIIBNP would be free in the ECM and available for signaling for a number of reasons. First, there is one NH$_2$-propeptide removed from every procollagen molecule synthesized; consequently, during periods of high collagen synthesis, particularly during development of cartilage and the cartilaginous growth plate, large amounts of NH$_2$-propeptide will be produced. Second, for the type I NH$_2$-propeptide, there is evidence that it is present in the ECM because it has been isolated from bone, where it is synthesized in abundance (46). Last, we show evidence for the presence of PIIBNP in the tissue by biochemical analysis. It appears that the predominant form of PIIBNP in the tissue is the monomeric form. This was true for developing cartilage from both human and mouse embryonic limbs, where a high level of type IIB procollagen synthesis occurs. The presence of monomers in the matrix indicates that our recombinant protein reflects the structure of the NH$_2$-propeptide after cleavage from the type IIB procollagen molecule. There are no disulfide bridges in the type IIB procollagen NH$_2$-propeptide, and the lysine cross-link located in the telopeptide remains on the mature collagen fibril (17). Consequently, this result makes it highly likely that the RGDs of the NH$_2$-propeptide, in vivo, will be available for function in the developing endochondral bone. In support of the function in vivo, new preliminary data from co-culture of hCh-1 cells with embryonic cartilage showed the same tumor cell killing effect as the recombinant PIIBNP (data not shown), further suggesting that recombinant PIIBNP is functionally similar to PIIBNP in vivo.

Certain implications arise from this work. First, cells that are not welcome in cartilage (i.e. tumor cells, endothelial cells, and osteoclasts) express $\alpha_\beta_3$ and $\alpha_\beta_5$ integrins. Consequently, the type II collagen NH$_2$-propeptide, when liberated from the procollagen, may be one of the protein fragments that establish cartilage anlagen and cartilage growth plates as avascular. Second, because many tumor cells express $\alpha_\beta_3$ and $\alpha_\beta_5$ integrins and our experiments show that PIIBNP induces chondrosarcoma, HeLa cervical carcinoma, and MDA-MB231 breast cancer cell death, PIIBNP could be a useful therapeutic agent in the treatment of tumors, especially for those that lack effective adjuvant chemotherapy, such as chondrosarcoma and triple negative breast cancers. Chondrosarcoma is the third most common primary malignancy of bone after myeloma and osteosarcoma. The tumors are resistant to commonly used radiation and chemotherapy; therefore, wide surgical excision is currently the best available treatment for intermediate to high grade tumors. Therefore, PIIBNP may be a potential anti-tumor reagent for chondrosarcoma.

Since the 1970s, investigators have sought the mechanism by which cartilage remains free of tumors and blood vessels (47, 48). Candidates have been identified that have some anti-tumor or anti-angiogenic properties, such as the 25-kDa NC1 domain of chondromodulin-1 (49), TIMP-2 (50), troponin-1 (51), and perlcan (52). Many of these anti-tumor agents are also anti-angiogenic and thus inhibit tumor growth. We propose that PIIBNP would be a good candidate for one of the anti-tumor agents in cartilage for the following reasons: 1) it is naturally produced at high levels during organogenesis of cartilage, and 2) it kills specific cell types that would invade and degrade cartilage. Last, PIIBNP is active in vivo in a breast cancer model system, demonstrating potential for harnessing its activity as a therapeutic agent.

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