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Tumorigenesis and Neoplastic Progression

Plasmin-Cleaved β-2-Glycoprotein 1 Is an Inhibitor of Angiogenesis

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β-2-Glycoprotein 1, an abundant plasma glycoprotein, binds anionic cell surfaces and functions as a regulator of thrombosis. Here, we show that cleavage of the kringle domain at Lys317/Thr318 switches its function to a regulator of angiogenesis. *In vitro*, the cleaved protein specifically inhibited the proliferation and migration of endothelial cells. The protein was without effect on preformed endothelial cell tubes. *In vivo*, the cleaved protein inhibited neovascularization into subcutaneously implanted Matrigel and Gelfoam sponge implants and the growth of orthotopically injected tumors. Collectively, these data indicate that plasmin-cleaved β-2-glycoprotein 1 is a potent antiangiogenic and antitumor molecule of potential therapeutic significance. ***(Am J Pathol 2007, 171:1659–1669; DOI: 10.2353/ajpath.2007.070146)***

There is increasing evidence of a strong interrelationship between thrombosis, fibrinolysis, and angiogenesis that is controlled by synchronized cross talk between zymogens and their cleavage products.[1] For example, during coagulation, the proteolytic activation of prothrombin produces thrombin and prothrombin fragments I and II, which triggers clotting and regulates endothelial cell (EC) growth,[2] respectively. In fibrinolysis, fibrin-catalyzed cleavage of plasminogen produces clot-digesting plasmin and the antiangiogenic molecule angiostatin.[3,4]

β2-Glycoprotien 1 (β2GP1), also known as apolipoprotein H, is a single-chain plasma glycoprotein composed of 326 amino acid residues that forms four complement control protein modules (domains I through IV) and a distinct C-terminal kringle domain (domain V).[5–8] Kringle domain V carries a lysine-rich sequence motif (C281KNKEKKC288) that binds negatively charged lipids[9,10] and a hydrophobic loop (313LAFW316) that embeds the protein into anionic lipid-containing target membranes.[6,11–13] Because of these properties, the protein inhibits ADP-induced platelet aggregation[14–16] and competes for the assembly of coagulation cascade proteins on procoagulant cell surfaces.[17–23] Other studies have shown that it binds EC[24] and protects cells against nitric oxide-mediated apoptosis[25] and atherosclerosis.[26,27] Interestingly, varying levels of a proteolytically cleaved form (Lys317/Thr318 cleavage site) of the protein (nicked β2GP1) have been found in the plasma of leukemia patients[28] and patients treated with streptokinase.[29] Because cleavage at Lys317/Thr318 abrogates the protein's ability to bind anionic surfaces,[9,10] a decrease in the ratios of intact to nicked forms of β2GP1 might influence the thrombotic events commonly seen in these patients. Many enzymes involved in coagulation and fibrinolysis (factor Xa, factor XI, plasmin, and elastase) cleave β2GP1 at Lys317/Thr318, suggesting that activation of fibrinolysis contributes to an increasingly diminished role of β2GP1 in thrombosis.[29,30] On the other hand, plasmin cleavage of the intact protein (iβ2GP1) to the nicked form (nβ2GP1) results in a gain of function that also regulates thrombus formation by accelerating thrombin-dependent factor XI activation[23,31] and fibrinolysis by inhibiting plasminogen/tissue plasminogen activator (t-PA)-mediated activation of plasminogen.[32] These findings, together with observations on the relationship between kringle structures and antiangiogenic activity,[33] raise the possibility that iβ2GP1-to-nβ2GP1 transitions result in a kringle domain alteration that dramatically switches its function from regulating thrombosis to regulating fibrinolysis and angiogenesis. Indeed, recent studies raised the possibility that nβ2GP1 functions as an antiangiogenic molecule *in vivo*.[34]

It this article, we demonstrate that nβ2GP1 inhibits EC proliferation *in vitro*, inhibits neovascularization into subcutaneously implanted Matrigel and Gelfoam plugs, and blocks tumor growth in a mouse model system. Taken

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together, these data provide evidence in support of the concept that nβ2GP1 plays a regulatory role in EC physiology and angiogenesis.

Materials and Methods

Animals, Cells, and Reagents

Male C57Bl/6 and BALB/c mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Tramp C2RE3 prostate adenocarcinoma cells (TRAMP) were provided by J. Killion, M. D. Anderson Cancer Center. These cells were derived from TRAMP C3 cells[35] by selection for aggressively growing tumors after repeated orthotopic injections. The cells were maintained *in vitro* in minimal essential media containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Bovine aortic endothelial cells (BAECs) were cultured in bovine endothelial growth medium (Cell Applications, Inc., San Diego, CA). Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 200 containing low serum growth supplement (Cascade Biologicals, Portland, OR). Plasmin and its chromogenic substrate, S-2251, were purchased from Chromogenix (Lexington, MA). Human serum albumin (HSA) was from Alpha Therapeutics (Los Angeles, CA), and annexin 2 antibodies (clone 5) and Matrigel were from BD Biosciences (Bedford, MA). Mouse CD31 antibodies (clone CO.3R1D4) were from Serotec (Raleigh, NC). Other chemicals and chromatographic media were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-human angiostatin (Oncogene Research Products, San Diego, CA) exhibited significant cross-reactivity with plasminogen and plasmin. Proteins used in this study were routinely tested to ensure the absence of lipopolysaccharide contamination using the Pyrochrome LAL reagent (Associates of Cape Cod Inc., East Falmouth, MA) assay. Immobilized plasmin was prepared by incubating 1 mg of plasmin in ice-cold PBS with 1 ml of Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA). The coupling was allowed to proceed at 4°C for 2 hours, after which uncoupled reagents were removed by repeated washings with PBS. Polyclonal antibodies to iβ2GP1 were produced in rabbits by multiple intradermal injections of 0.5 mg of β2GP1 in complete Freund's adjuvant in multiple intradermal sites, followed by two boosters (0.25 mg of protein) at 2-week intervals in incomplete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection. IgG was purified from the immune serum by protein G affinity chromatography.

^{51}Cr-Labeled Mouse Red Blood Cells

Syngeneic mouse red blood cells were labeled with ^{51}Cr by incubation at 37°C for 4 hours with 0.25 mCi of Na^{51}chromate (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in Hepes-buffered saline (pH 7.4) containing 30 mmol/L glucose. Unbound ^{51}Cr was removed by repeated washings with the same buffer. The cells were resuspended to a 25% hematocrit in the same buffer before injection.

Purification of iβ2GP1

Intact β2GP1 was purified from pooled human plasma as described previously.[36,37] In brief, whole blood collected from healthy volunteers (Gulf Coast Regional Blood Center, Houston, TX) was centrifuged at 2500 $\times$ *g* for 10 minutes to sediment the blood cells. The supernatant (plasma) was then chilled on ice, and perchloric acid [1.5% (v/v)] was added dropwise with continuous stirring. The plasma was incubated on ice for 15 minutes, followed by centrifugation at 20,000 $\times$ *g* for 15 minutes to sediment the precipitated proteins. The supernatant containing β2GP1 was brought to pH 7.0 with saturated sodium bicarbonate and dialyzed against Tris buffer (50 mmol/L Tris, pH 8.0) containing 20 mmol/L NaCl. The dialysate was passed over a DEAE-Sephacel column equilibrated with the same buffer. The flow-through was collected and passed over a Hi-Trap Heparin-Sepharose affinity column. The column was washed with Tris buffer containing 20 mmol/L NaCl, and the bound β2GP1 was eluted with the same buffer containing 250 mmol/L NaCl. Purity was assessed by gel electrophoresis and Western blotting with rabbit anti-human iβ2GP1. The identity of the protein was confirmed by N-terminal sequencing.

Preparation of nβ2GP1

Intact β2GP1 was incubated with immobilized plasmin at 37°C for 17 hours. The beads were removed by centrifugation and the supernatant recovered. Cleavage was verified by an electrophoretic shift under reducing conditions and by N-terminal sequencing, which revealed a second N terminus corresponding to the Lys317/Thr318 cleavage site. Western blotting of the purified product indicated that the nβ2GP1 preparations were plasmin-free and did not contain autoproteolytic products (no reactivity with plasmin or angiostatin antibodies).

Immunofluorescence Analysis of β2GP1 Binding to EC

BAECs and TRAMP cells were incubated with iβ2GP1 or nβ2GP1 (4 μmol/L) on ice for 30 minutes. The cells were then washed with PBS and incubated for an additional 30 minutes on ice with 2 μg of biotinylated rabbit anti-human β2GP1 IgG, followed by incubation with 50 ng of fluorescein isothiocyanate (FITC)-streptavidin. Binding was determined by flow cytometric analysis using cells incubated only with the primary antibody and FITC-streptavidin as negative controls. For the competition experiments with annexin 2 antibody, BAECs were cultured on glass coverslips for 24 hours and incubated on ice for 1 hour with iβ2GP1 or nβ2GP1 (4 μmol/L) in the absence or presence of annexin II or CD31 (negative control) antibodies (0.33 μmol/L). The cells were then washed, fixed with 2% paraformaldehyde, and stained with biotinylated rabbit anti-human β2GP1 IgG (2 μg), followed by phycoerythrin-conjugated streptavidin (100 ng).

Assay for Plasmin Activity

BAECs or HUVECs were cultured to 80% confluence. One milliliter of conditioned or fresh (negative control) medium was transferred to cuvettes, and the change in absorbance at 405 nm was recorded following the addition of the chromogenic plasmin substrate S-2251 (0.3 mmol/L).

Cell Proliferation Assay

[^{3}H]Thymidine Incorporation

BAECs and TRAMP C2RE3 cells were cultured in complete medium containing 0.5 μCi of [^{3}H]thymidine and 4 μmol/L HSA (control), iβ2GP1, or nβ2GP1. After 72 hours, the cells were washed three times with PBS, twice with 5% trichloroacetic acid, and solubilized in 0.2% SDS. The cell lysate was resuspended in 5 ml of scintillation cocktail for liquid scintillation counting.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay

HUVECs were cultured in 96-well plates in medium containing 4 μmol/L HSA (control), iβ2GP1, or nβ2GP1 (4 μmol/L). After 72 hours, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2.5 mg/ml) was added to each well and incubated for 2 hours at 37°C. The medium was then removed and the formazan crystals solubilized in 50 μl of dimethyl sulfoxide before spectrophotometric quantification (A = 560 nm). Cell proliferation was expressed as the percentage of controls.

Migration Assays

Boyden Chamber Assay

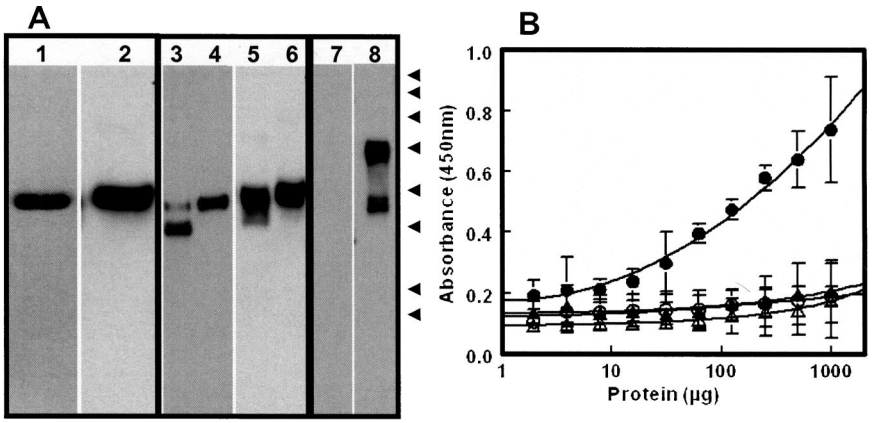
Cells were plated at ~70% confluency on 6.5-mm Transwell polycarbonate membranes (8-μm pore size; Corning, Acton, MA). Vascular endothelial growth factor (VEGF) (25 ng/ml) was added to the lower chamber, and iβ2GP1 or nβ2GP1 (4 μmol/L) was added to the upper chamber. After 5 hours at 37°C, the cells on the upper surface were removed by scraping. The polycarbonate filters were then stained with Hema-Diff reagent (StatLab Medical Products, Inc. Lewisville, TX). Results are expressed as the mean ± SD of 10 individual experiments.

Scratch Assay

Cells were cultured on 24-well tissue culture plates to confluency. Cells in the center of the wells were removed by scratching with a 1-ml pipette tip.[38] The remaining adherent cells were washed twice with PBS, incubated with iβ2GP1 or nβ2GP1 (4 μmol/L) in BAEC medium for 8 hours, and photographed. Motility was measured by counting the number of cells that repopulated the cleared area. Results are expressed as the number of cells/mm^2 ± SD and are the mean of four individual experiments.

Tube Disruption Assay

Forty-eight-well tissue culture plates were coated with 250 μl of Matrigel (8.9 mg/ml) for 2 hours at 37°C. Cells (7.5×10^4) were plated in BAEC medium for 96 hours to allow for tube formation. The preformed tubes were then



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		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Amino acid sequence of secreted iβ2GP1		Gly	Arg	Thr	Cys	Pro	Lys	Pro	Asp	Asp	Leu	Pro	Phe	Ser	Thr	....
(Accession: NP_000033)		313	314	315	316	317	318	319	320	321	322	323	324	325	326	
	....	Leu	Ala	Phe	Trp	**Lys**	**Thr**	Asp	Ala	Ser	Asp	Val	Lys	Pro	Cys	
		1	2	3	4	5	6	7	8	9	10					
N-terminal analysis of purified iβ2GP1	N-terminus # 1	Gly	Arg	Thr	X	Pro	Lys	Pro	Asp	Asp	Leu					
N-terminal analysis of plasmin-treated iβ2GP1	N-terminus # 1	Gly	Arg	Thr	X	Pro	Lys	Pro	Asp	Asp	Leu					
	N-terminus # 2	Thr	Asp	Ala	Ser	Asp	Val	Lys	Pro	X						

Figure 1. Analysis of purified iβ2GP1 and nβ2GP1. **A:** SDS-PAGE analysis of iβ2GP1 and nβ2GP1; **lane 1**, Coomassie Brilliant Blue staining of iβ2GP1; **lane 2**, Western blot analysis of iβ2GP1 with polyclonal anti-β2GP1 IgG; **lanes 3** to **6**, Western blot analysis of nβ2GP1 (**lanes 3** and **5**) and iβ2GP1 (**lanes 4** and **6**) in reducing (**lanes 3** and **4**) and nonreducing (**lanes 5** and **6**) SDS-PAGE; **lane 7**, Western blotting of purified nβ2gp1 with mouse monoclonal plasmin antibodies; and **lane 8**, plasmin positive control. **Arrowheads** represent molecular mass markers (250, 150, 100, 75, 50, 37, 20, and 10 kDa). **B:** Binding of intact and nβ2GP1 to PS. Ninety-six-well enzyme-linked immunosorbent assay plates were coated with phosphatidylcholine or PS in ethanol (20 μg/ml) and incubated with serial dilutions of intact and nβ2GP1. Protein binding was assessed with rabbit anti-β2GP1 and peroxidase-conjugated anti-rabbit IgG. ○ and △ PC; ● and ▲ PS; circles, iβ2GP1; triangles, nβ2GP1. **C:** N-Terminal sequence analysis of iβ2GP1 and nβ2GP1. The amino acids in bold show the plasmin-cut site in iβ2GP1.

incubated with HSA, iβ2GP1, or nβ2GP1 (4 μmol/L) for 24 hours and assessed for tube integrity by microscopy.

Neovascularization Assays

The effect of iβ2GP1 and nβ2GP1 on neovascularization was determined by two independent assays.

Gelfoam Implant

Sterile Gelfoam absorbable sponges (Pharmacia & Upjohn, Peapack, NJ) were cut into 5 × 5 × 7-mm pieces and hydrated overnight with PBS. Agarose (0.4%, 100 μl) containing VEGF (2 pmol/implant) and nβ2GP1 (0.2 μmol) or HSA (0.2 μmol, control) was pipetted onto each sponge. After 1 hour at room temperature, the gel foams were placed into a subcutaneous pocket as described previously.[39] Vascularization into the implants was quantified after 2 weeks by assessing blood volume after i.v. injection of ^{51}Cr-labeled syngeneic red blood cells several minutes before recovery of the implants. Blood volume was calculated from the specific activity of the blood (cpm/μl blood/g implant).

Matrigel Plug

Matrigel (1.5 ml) was mixed on ice with VEGF (0.7 pmol) in the presence or absence of iβ2GP1 or nβ2GP1 (6 nmol). BALB/c mice (three per group) were injected intradermally with 0.5 ml of the Matrigel. Two weeks later, ^{51}Cr-labeled syngeneic red blood cells were injected i.v. several minutes before recovery of the implants. Blood volume was calculated as described for the gel foams.

Murine Prostate Cancer Model

TRAMP C2RE3 cells (2×10^4) were implanted orthotopically into the prostate of 6-week-old, C57Bl/6 mice. The mice were randomly assigned to different groups. nβ2GP1 or iβ2GP1 (1.7 mg/0.2 ml pump) was administered to the mice with Alzet 2002 mini-osmotic pumps (delivery rate of 3.6 mg/kg/day; Durect Corp., Cupertino, CA) that were implanted i.p. and s.c. on days 1 and 14, respectively (spent pumps were not removed). Mice in the chemotherapy and combination therapy groups were also administered docetaxel intraperitoneally at 8 mg/kg once a week for 4 weeks beginning on day 3. Animals were sacrificed on day 28, and the tumors were harvested, weighed, and quick-frozen for immunohistochemistry. Frozen sections were stained for CD31-positive EC with rat anti-CD31 antibody (BD Biosciences) followed by Texas Red-conjugated goat anti-rat IgG (Jackson ImmunoResearch). Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining performed according to manufacturer's (Promega Corporation, Madison, WI) instructions.

Analysis of β2GP1 Degradation Products

BAECs were grown for 72 hours in the absence or presence of intact or nβ2GP1 (4 μmol/L). The supernatants were centrifuged to remove cell debris and incubated with rabbit anti-human β2GP1. The antibody and bound antigens were concentrated by pull-down with protein G-Sepharose beads. The beads were washed and solubilized with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and resolved by gel electrophoresis. For Western blotting, the proteins were transferred to polyvinylidene difluoride membranes and probed with the same antibodies followed by peroxidase-conjugated anti-rabbit IgG.

Immunoprecipitation

One microgram of Glu-plasminogen, plasmin, or angiostatin was incubated with 10 μg of iβ2GP1 or nβ2GP1 for 1 hour at 20°C, followed by incubation for 1 hour with control rabbit IgG or rabbit anti-β2GP1 IgG (20 μg) and protein G-Sepharose (20 μl). Captured IgG (and bound proteins) were centrifuged, and the supernatant (un-

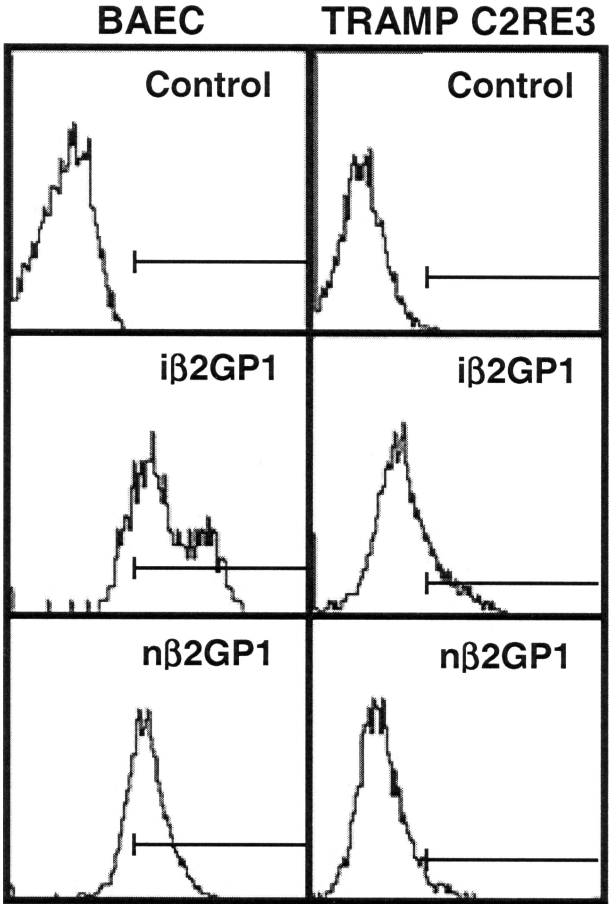

Figure 2. Binding of iβ2GP1 and nβ2GP1 to endothelial cells. BAECs and TRAMP cells were incubated with iβ2GP1 or nβ2GP1 on ice for 30 minutes followed by incubation with biotinylated anti-β2GP1 IgG and FITC-streptavidin as described in Materials and Methods. Control cells were incubated only with primary antibody and FITC-streptavidin. Data are representative of three independent experiments.

bound protein) was mixed with SDS-PAGE sample buffer for Western blot analysis. The protein G beads were washed twice with PBS, centrifuged through 30% sucrose in PBS, and resuspended in SDS-PAGE sample buffer.

Results

Purification of iβ2GP1 and Preparation of nβ2GP1

Intact β2GP1 was purified from pooled human plasma by perchloric acid treatment followed by ion exchange and heparin affinity chromatography as described in Materials and Methods. SDS-PAGE and Western blot analysis showed that the purity of the protein was >98% (Figure 1). Nicked β2GP1 was prepared by incubating iβ2GP1 for 17 hours at 37°C with immobilized plasmin. SDS-PAGE analysis of the product under reducing conditions showed that >98% of the protein was cleaved (Figure 1A). N-Terminal sequencing revealed that the protein was cleaved at amino acids 317 and 318 (Lys-Thr). Western blotting of the final preparation with plasmin antibodies indicated that the nβ2GP1 was plasmin-free. Solid-phase enzyme-linked immunosorbent assay binding assays were performed to determine the binding of the nicked isoform to anionic phosphatidylserine (PS). Figure 1B shows that the ability of iβ2GP1 to bind PS was lost after plasmin treatment. Control experiments showed that neither isoform bound neutral phosphatidylcholine.

β2GP1 Binds EC and Inhibits Proliferation and Migration in Vitro

Previous studies have shown that iβ2GP1 binds to the surface of EC through annexin 2 expressed at the cell surface.[24] To determine the binding of iβ2GP1 and nβ2GP1, BAECs were incubated with the proteins for 30 minutes. Fluorescence-activated cell sorting analysis after incubation of the cells with biotinylated anti-human-β2GP1 followed by FITC-conjugated streptavidin showed that both iβ2GP1 and nβ2GP1 bound to >75% of the EC (Figure 2). Unlike EC, only the intact protein bound to TRAMP C2RE3 cells.

Several studies were performed to determine the influence of iβ2GP1 and nβ2GP1 on EC function. First, the effect of β2GP1 on EC migration was determined by assessing cell mobility using the Boyden chamber transwell migration assay. Figure 3, A and B, shows that nβ2GP1, but not iβ2GP1, significantly inhibited the migration of cells to the lower chamber. Additional evidence supporting this observation was obtained with the *in vitro* wound healing model system.[38] Confluent EC monolayers incubated with iβ2GP1 or nβ2GP1 were scraped, and migration of cells into the denuded sections was monitored at various time intervals. Figure 3, C and D, shows that, whereas control and iβ2GP1-treated ECs repopulated the denuded area within 8 hours, incubation in the presence of nβ2GP1 resulted in ~60% inhibition in cell migration. Control experiments showed that nβ2GP1 did not inhibit migration of TRAMP cells, suggesting that the inhibitory effect of nβ2GP1 was EC-specific (Figure 3C). In contrast to the apparent specific effect of nβ2GP1 on BAEC migration, both iβ2GP1 and nβ2GP1 significantly

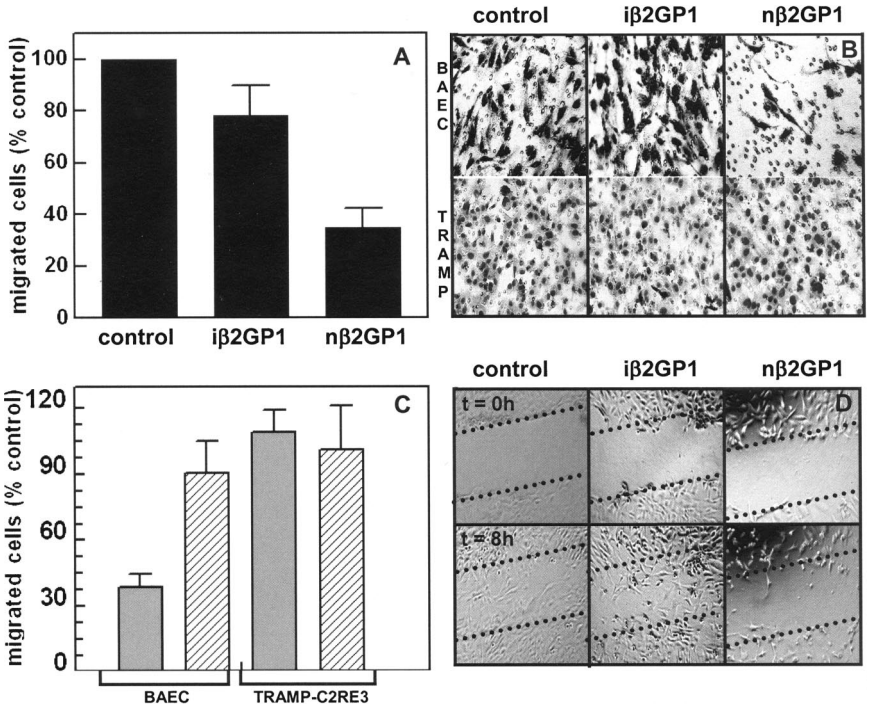

Figure 3. nβ2GP1 inhibits EC migration. **A:** BAECs were incubated with iβ2GP1 or nβ2GP1 (4 μmol/L) for 5 hours, and cells that migrated to the VEGF-containing lower side of the polycarbonate membrane were counted. Results are expressed as the mean ± SD of 10 experiments. **B:** Representative photomicrographs of migrated BAECs (**top panels**) and TRAMP C2RE3 (**bottom panels**) cells. **C:** Confluent BAECs and TRAMP C2RE3 monolayers were denuded by scraping and incubated for 5 hours with 4 μmol/L nβ2GP1 (shaded bars) or iβ2GP1 (hatched bars). Results are expressed as the mean ± SD of four experiments. **D:** Typical photomicrograph of denuded BAEC monolayers at 0 hours (**top panels**) and 8 hours (**bottom panels**).

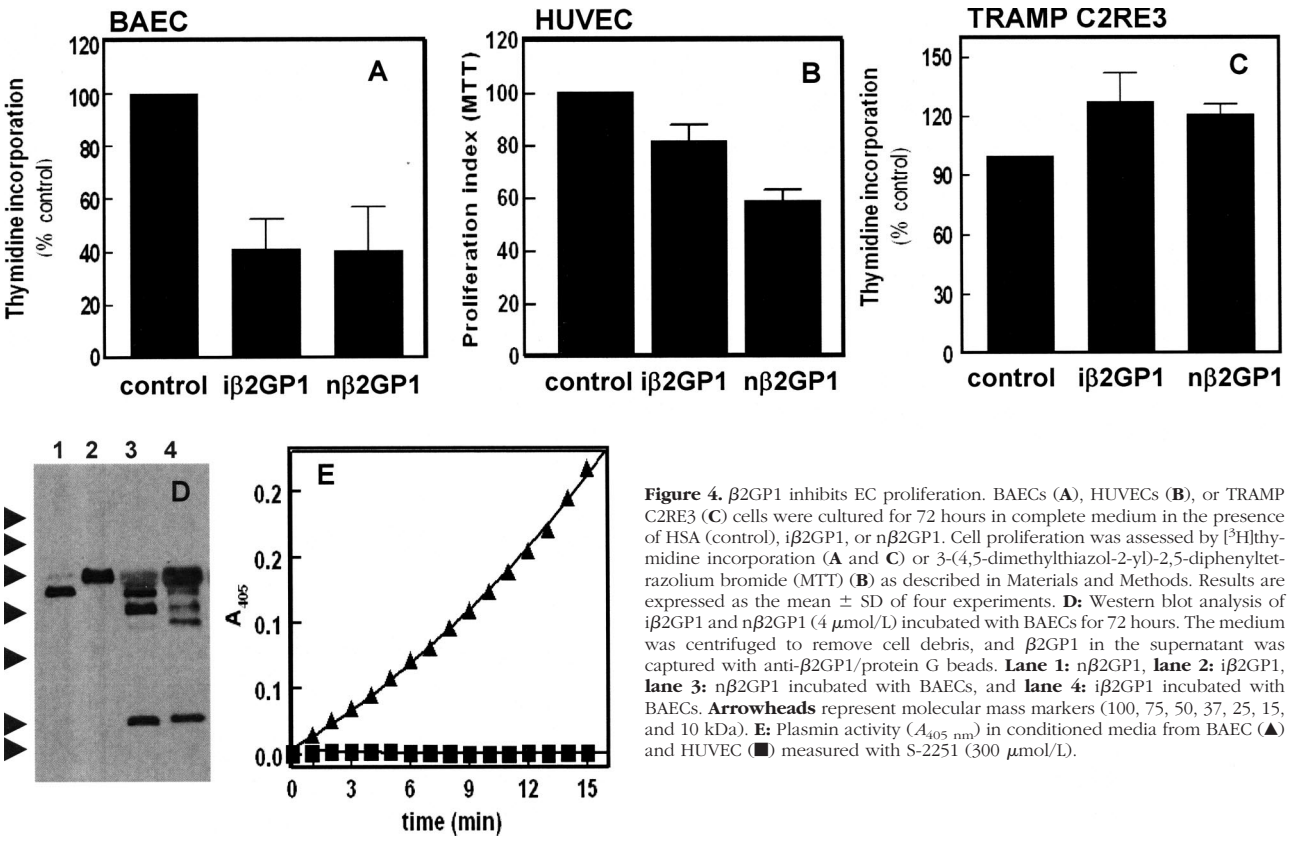

Figure 4. β2GP1 inhibits EC proliferation. BAECs (**A**), HUVECs (**B**), or TRAMP C2RE3 (**C**) cells were cultured for 72 hours in complete medium in the presence of HSA (control), iβ2GP1, or nβ2GP1. Cell proliferation was assessed by [^{3}H]thymidine incorporation (**A** and **C**) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (**B**) as described in Materials and Methods. Results are expressed as the mean ± SD of four experiments. **D:** Western blot analysis of iβ2GP1 and nβ2GP1 (4 μmol/L) incubated with BAECs for 72 hours. The medium was centrifuged to remove cell debris, and β2GP1 in the supernatant was captured with anti-β2GP1/protein G beads. **Lane 1:** nβ2GP1, **lane 2:** iβ2GP1, **lane 3:** nβ2GP1 incubated with BAECs, and **lane 4:** iβ2GP1 incubated with BAECs. **Arrowheads** represent molecular mass markers (100, 75, 50, 37, 25, 15, and 10 kDa). **E:** Plasmin activity ($A_{405\ nm}$) in conditioned media from BAEC (▲) and HUVEC (■) measured with S-2251 (300 μmol/L).

inhibited BAEC proliferation (Figure 4A). Growth of TRAMP C2RE3 cells (and PC3, LnCap, MCF7, MDA-MB-231, and MDA-MB-435 cells, data not shown) was unaffected when incubated with either protein (Figure 4C). In contrast to BAECs, HUVECs were sensitive to the inhibitory effect of the nβ2GP1, but not iβ2GP1 (Figure 4B).

Considering the specific inhibition of nβ2GP1 on EC migration, the inhibitory effect of the intact protein on the proliferation of BAECs (but not HUVECs) was unexpected. Because the proliferation experiments were performed over prolonged incubation times, we tested the BAEC supernatants for potential β2GP1 degradation products by Western blotting using β2GP1 antibodies. Figure 4D shows that both the nicked and intact proteins were degraded into several distinct products. The nicked protein produced two major degradation products at 37 and 15 kDa, with a minor product at ~30 kDa. With the exception of a band consistent with the nicked isoform at ~40 kDa, the intact protein produced degradation products identical to those formed by the nicked protein. It seems likely, therefore, that the ability of iβ2GP1 to inhibit BAEC proliferation was due to its *in situ* conversion to the nicked isoform (together with other degradation products) as a result of BAEC-derived proteolytic activity. Indeed, analysis of culture supernatants revealed significant plasmin activity in BAEC- but not HUVEC-conditioned medium (Figure 4E). Taken together, these data suggest that the inability of iβ2GP1 to inhibit the proliferation of HUVECs was because the protein was not cleaved to the active nicked isoform. Thus, the inability of the intact protein to inhibit BAEC migration (Figure 3A) was probably due to the very limited proteolysis of the protein within the time frame of these experiments (5 hours) as opposed to the extended incubation times in the proliferation assay (72 hours). Taken together, these data suggest that nβ2GP1 specifically functions as an inhibitor of EC growth and migration. It is important to note that neither protein was able to disrupt preformed EC tubes *in vitro* (Figure 5).

Inhibition of Neovascularization by nβ2GP1

Because nβ2GP1 inhibits EC migration and proliferation *in vitro*, we tested its potential to function as an inhibitor of angiogenesis *in vivo*. Intact and nβ2GP1 was incorporated together with VEGF into Matrigel (Figure 6A) or Gelfoam plugs (Figure 6B) and implanted subcutaneously into BALB/c mice. Two weeks later, the mice were injected with ^{51}Cr-labeled syngeneic red blood cells to quantify blood volume within the implants. Figure 6 shows that control implants seemed to be highly vascularized, whereas both the nβ2GP1- and iβ2GP1-containing implants were relatively clear. Indeed, assessment of blood volume within the implants showed that vascularization was reduced ~10-fold and threefold in the Gelfoam and Matrigel implants, respectively. The absence of neovascularization in the iβ2GP1-containing implants suggests that the protein was cleaved to the nicked isoform *in situ*.

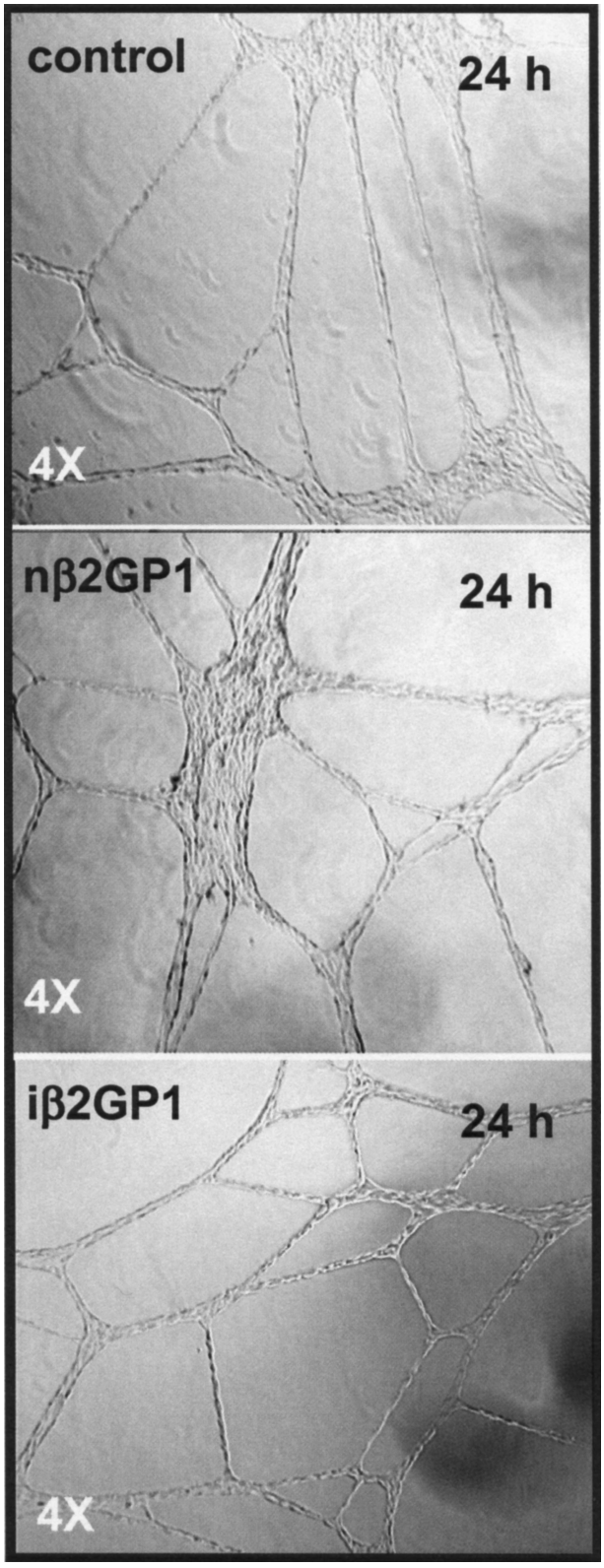

Figure 5. β2GP1 does not disrupt pre-existing EC tubes. EC tubes formed on Matrigel-coated plates were incubated with iβ2GP1 or nβ2GP1 (4 μmol/L) for 24 hours. Note that the tube structure remains largely intact.

Inhibition of Orthotopically Implanted TRAMP Prostate Cancer Tumor Growth by nβ2GP1

To test the ability of β2GP1 to inhibit tumor growth, TRAMP C2RE3 prostate carcinoma cells were injected orthotopically. Groups of animals were treated by i.p. implantation of 14-day Alzet pumps containing HSA (control group), iβ2GP1, or nβ2GP1. To ensure continued release of the test proteins over the time course of the study, an additional pump was implanted s.c. at day 14. The experiment was terminated at day 28, and tumor growth was assessed. The data presented in Figure 7 show a 56% reduction in tumor volumes in mice treated with nβ2GP1. The extent of inhibition was comparable with that obtained with docetaxel alone. CD31 and TUNEL staining of thin sections from tumors recovered from the nβ2GP1-treated animals revealed significant tumor cell apoptosis (Figure 7, photomicrograph). Although treatment with docetaxel resulted in inhibition in tumor growth, multiple thin sections through the residual tumor failed to reveal large numbers of apoptotic cells. The reason for this is unclear but could be related to the route of drug administration (i.p.), which would affect its bioavailability. Similar to recent data obtained with doxorubicin,[40] our results suggest that inhibition of tumor growth was due to cytostasis, not apoptosis. Irrespective of the treatment group, multiple serial sections through the tumors did not reveal apoptotic ECs. Taken together with the inhibition of angiogenesis in the *in vivo* Matrigel and Gelfoam assays, these data raise the possibility that the reduction in tumor growth is a result of inhibition of vascular expansion.

Binding of iβ2GP1 and nβ2GP1 to Annexin 2, Plasminogen, and Its Cleavage Products

It has been previously shown that annexin 2 on EC binds t-PA and plasminogen,[41–43] a process that is critical to the localized production of plasmin for dissolution of fibrin clots and wound healing. Interestingly, plasmin also catalyzes proteolysis of the extracellular matrix, a process that is crucial to EC growth and expansion. Because annexin 2 also binds β2GP1,[24,44] it is possible that iβ2GP1 and nβ2GP1 regulate the binding of plasminogen to annexin 2 and/or the activity of plasmin. To determine whether β2GP1 binds to plasminogen or its cleavage products, iβ2GP1 and nβ2GP1 were incubated with plasminogen, plasmin, and angiostatin. Complexes were captured with anti-β2GP1 and protein G-Sepharose. Western blot analysis of the supernatants and pellets showed that nβ2GP1 bound to plasminogen exclusively, whereas the intact isoform did not bind plasminogen or any its cleavage products (Figure 8A).

Previous studies have shown that iβ2GP1 binds to HUVECs through annexin 2. To determine whether annexin 2 also serves as a "receptor" for nβ2GP1, we tested the ability of annexin 2 antibodies to block the binding of iβ2GP1 and nβ2GP1 to BAECs. Figure 8B shows that annexin 2 antibodies significantly reduced the ability of both proteins to bind BAECs. Control experiments using

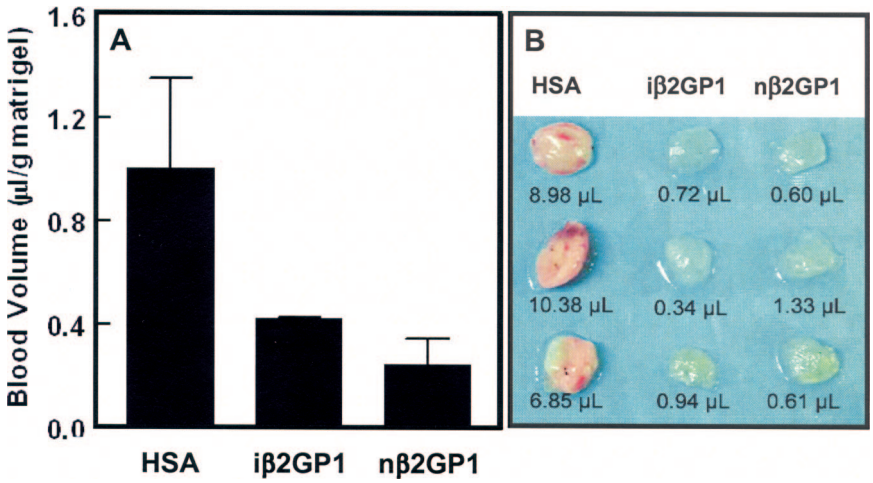

Figure 6. nβ2GP1 inhibits neovascularization into subcutaneous implants. Matrigel (**A**) or Gelfoam sponge (**B**) containing VEGF and HSA or nβ2GP1 were placed s.c. in BALB/c mice (10 mice/group). The implants were recovered 2 weeks later. Blood volume (μl/g implant) was calculated from the specific activity of ^{51}Cr-labeled red blood cells in the implants. The data shown are the mean ± SD of three experiments.

anti-CD31 (Figure 8B) or nonspecific mouse IgG (not shown) did not inhibit binding.

Discussion

There is increasing evidence that proteins that participate in coagulation and fibrinolysis also regulate angiogenesis.[1] Many of these "dual function" proteins are conformationally altered cleavage products of parent molecules that regulate thrombosis. For example, during coagulation, the zymogen prothrombin is cleaved to produce thrombin and prothrombin fragments (I and II), which promote clotting and arrest EC growth, respectively.[2] Likewise, high-molecular weight kininogen plays a role in contact activation of coagulation,[45] whereas its kallikrein cleavage product promotes EC apoptosis.[46] Similar properties have been found for apolipoprotein (a)[38,47,48] and histidine proline-rich glycoprotein.[49–51] During fibrinolysis, the zymogen plasminogen is activated to produce plasmin, which initiates dissolution of fibrin and

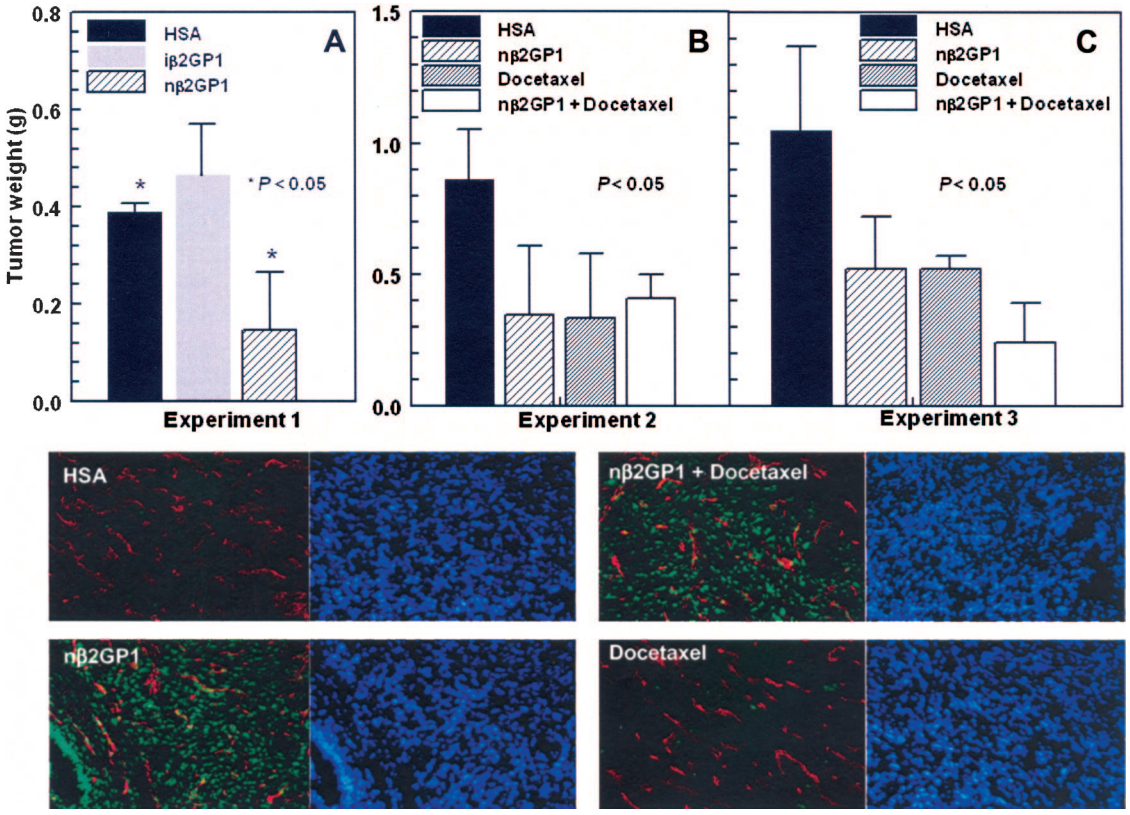

Figure 7. nβ2GP1 reduces tumor burden in an orthotopic murine prostate cancer model. **A**, **B**, **C**: Mice (10 mice/group/experiment) injected orthotopically with TRAMP C2RE3 (2×10^4 cells) were treated as indicated. iβ2GP1 or nβ2GP1 was administered through Alzet mini osmotic pumps implanted on days 1 and 14. Docetaxel was administered once a week i.p. Results are mean ± SD of three experiments. Photomicrographs: Representative thin sections were stained for CD31 (red), TUNEL (green), and total cells with 4,6-diamidino-2-phenylindole (DAPI) (blue).

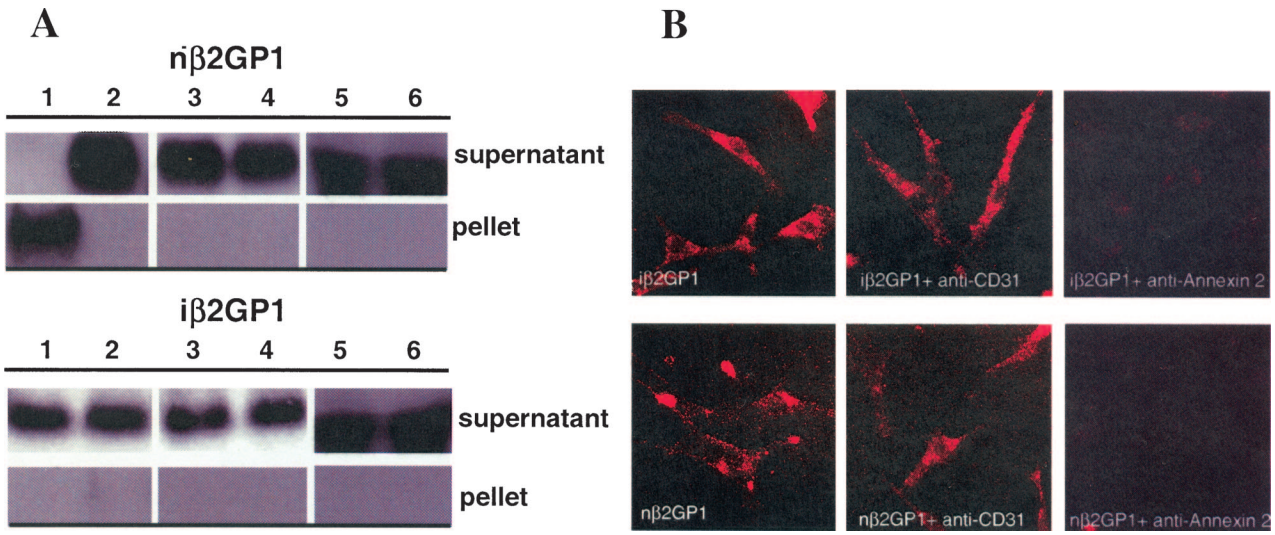

Figure 8. Binding of iβ2GP1 and nβ2GP1 to annexin 2 and plasminogen and its cleavage products. **A:** iβ2GP1 and nβ2GP1 were incubated with the target proteins, plasminogen, plasmin, or angiostatin. The mixture was then incubated with anti-β2GP1 and Protein G-Sepharose to capture β2GP1 and any bound target proteins. Proteins in the supernatant and protein G bead pellets were resolved by SDS-PAGE and analyzed by Western blotting using plasminogen, plasmin, or angiostatin antibodies. Odd-numbered lanes represent incubation with anti-β2GP1, and even-numbered lanes represent incubation with control IgG. **Lanes 1** and **2:** Plasminogen, **lanes 3** and **4:** plasmin, and **lanes 5** and **6:** angiostatin. Data are representative of three independent experiments. **B:** BAECs were plated on glass coverslips overnight and incubated on ice for 1 hour with iβ2GP1 or nβ2GP1 (4 μmol/L) in the absence or presence of anti-annexin II or anti CD31 (0.33 μmol/L). The cells were then washed, fixed with 2% paraformaldehyde, and stained with biotinylated rabbit anti-human β2GP1 IgG/streptavidin-PE.

revascularization. Concurrent with EC proliferation (vascularization), autocatalytic inactivation of plasmin produces angiostatin that inhibits further angiogenesis.[4] Interestingly, all of these antiangiogenic fragments are characterized by one or more disulfide-linked kringle domain structures.[33]

β2GP1 (apolipoprotein H) is a kringle domain-containing plasma glycoprotein that binds to EC through annexin 2[24] and functions as an EC survival factor.[25] Similar to other multifunctional coagulation proteins, cleavage of β2GP1 induces a conformational change[52] that alters the regulatory properties of the protein.[32] Because of the structural similarities in the kringle domain of β2GP1 with other antiangiogenic fragments, we investigated the potential function of this protein as a regulator of angiogenesis. Our results show that nβ2GP1 is a potent inhibitor of EC proliferation and migration *in vitro*. This property was dependent on the nβ2GP1 isoform because prolonged incubation of iβ2GP1 with BAECs gave similar results due to the *in situ* generation of nβ2GP1 by the action of cell-derived proteases secreted into the medium.

These data raised the possibility that nβ2GP1 might function as an endogenous regulator of angiogenesis. To test this, two independent *in vivo* experiments were performed: i) VEGF-dependent neovascularization into subcutaneously implanted Matrigel and Gelfoam plugs, and ii) the growth of orthotopically implanted prostate cancer cells. The data presented in Figure 6 show that the inclusion of both the intact and nicked isoforms of β2GP1 into the Matrigel and Gelfoam plugs significantly inhibited vascularization. In contrast, only the nicked isoform inhibited tumor growth. Based on our *in vitro* data, the ability of the Matrigel/Gelfoam-embedded iβ2GP1 to inhibit neovascularization was probably due to localized production of plasmin at the implant/wound site, which led to the *in situ* generation of the active nicked isoform. For the tumor inhibition studies, iβ2GP1 was implanted into 14-day Alzet osmotic pumps where the diffusion port was placed distal to the incision site. Pump placement, combined with the fact that solutes (including plasmin) cannot diffuse into the pumps, probably precluded plasmin-dependent cleavage of iβ2GP1 to nβ2GP1. Immunohistochemical studies of tumor thin sections revealed numerous TUNEL-positive tumor cells in the nβ2GP1 treatment groups (Figure 7). Interestingly, only a small fraction of the CD31-positive EC appeared also to be TUNEL-positive. This suggests that the observed antitumor effect of nβ2GP1 is because of inhibition of vascular expansion that is critical to tumor survival and not ablation of preexisting vasculature.

Because the antiangiogenic activity of β2GP1 is dependent on site-specific cleavage within domain V, its activity probably requires a conformationally altered kringle domain that binds to an EC-specific cell surface moiety/receptor. The data shown in Figure 8 provide evidence that, similar to angiostatin,[53] both iβ2GP1 and nβ2GP1 bind to EC through annexin 2.[24,34,44] Because only the nicked isoform inhibited angiogenesis (Figure 6), it is possible that nβ2GP1 competes with iβ2GP1 for binding to annexin 2 on the EC surface. We also show that nβ2GP1, but not iβ2GP1, binds plasminogen. Because annexin 2 regulates plasmin production by forming an annexin 2/t-Pa/plasminogen complex,[41–43] the binding of nβ2GP1 to annexin 2 on EC could initiate a negative feedback loop that blocks assembly of the annexin 2/t-Pa/plasminogen complex, thereby precluding plasmin production and concomitant neovascularization.[32] In principle, such a mechanism provides a self-limiting autoregulatory negative feedback loop *in situ*. This model is in agreement with recent data indicating an important role for annexin 2 in neoangiogenesis.[54] In addition to its possible role in inhibiting the generation of plasmin at the EC expansion front, nβ2GP1 might also exert direct inhibitory effects

on EC growth through (unidentified) downstream events that alter cell cycle regulatory pathways.[34]

Collectively, the data reported here support the concept that nβ2GP1 functions as an antiangiogenic molecule that likely suppresses tumor expansion through a specific EC-dependent pathway. The apparent resistance of pre-existing vasculature to the protein makes it a potential candidate for antiangiogenic therapy that warrants further investigation.

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