Identification of the Anti-angiogenic Site within Vascular Basement Membrane-derived Tumstatin*

Yohei Maeshima‡‡‡, Mark Manfredi¶¶, Corinne Reimer¶¶, Kathryn A. Holthaus‡, Helmut Hopfer†*, Babi R. Chandamuri‡, Surender Kharbanda¶¶, and Raghu Kalluri‡‡‡‡

From the Department of Medicine and the Cancer Center, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215 and ‡‡‡‡ILEX Oncology, Inc., Products, Research and Development Division, Boston, Massachusetts 02215

Components of vascular basement membrane are involved in regulating angiogenesis. Recently, tumstatin (the NC1 domain of α3 chain of type IV collagen) was identified as possessing anti-angiogenic activity. In the present study, the anti-angiogenic activity of tumstatin was localized to the putative 54–132-amino acid Tum-5 domain, and the activity mediated by αvβ3 integrin interaction in an RGD-independent manner. The recombinant Tum-5 produced in *Escherichia coli* and *Pichia Pastoris* specifically inhibited proliferation and caused apoptosis of endothelial cells with no significant effect on nonendothelial cells. Tum-5 also inhibited tube formation of endothelial cells on Matrigel and induced G1 endothelial cell cycle arrest. Moreover, anti-angiogenic effect of Tum-5 was also examined in vivo using both a Matrigel plug assay in C57BL/6 mice and human prostate cancer (PC-3) xenografts in nude mice. The in vivo results demonstrate that Tum-5 at 1 mg/kg significantly inhibited growth of PC-3 tumors in association with a decrease in CD31 positive vasculature. These in vivo studies also show that, at molar equivalents, human Tum-5 is at least 10-fold more active than human endostatin. In addition, these studies for the first time suggest that through the action of endogenous inhibitors, αvβ3 integrin may also function as a negative regulator of angiogenesis. Taken together, these findings demonstrate that Tum-5, a domain derived from tumstatin, is an effective inhibitor of tumor-associated angiogenesis and a promising candidate for the treatment of cancer.

Angiogenesis, the development of new blood vessels from pre-existing ones, is required for tumor growth and metastasis (1, 2). The tumor cell component and the endothelium of blood vessels function in concert to coordinate the expansion of tumor tissue (1). These events are mediated via an angiogenic switch generating an overwhelming pro-angiogenic stimulus. Recent studies from several different laboratories have shown that endogenous inhibitors of angiogenesis are produced de novo to orchestrate a systematic regulation of tumor uptake and growth (3–7).

Basement membranes are organized as thin layers of specialized extracellular matrix that provide the supporting scaffold for epithelial and endothelial cells (8). Basement membranes not only provide a mechanical support but also influence cellular behavior such as differentiation, proliferation, and migration of various cells including endothelial cells. Vascular basement membrane constitutes an insoluble structural wall of newly formed capillaries and is speculated to play an important role in regulating pro- and anti-angiogenic events (6, 9, 10). Type IV collagen is one of the major macromolecular constituents of basement membranes (11) and is expressed as six distinct α-chains, namely, α1–α6 (12). These α-chains are assembled into triple helices that further form a network to provide a scaffold for other macromolecules to interact with the basement membrane. These α-chains are composed of three domains, the N-terminal 7 S domain, the middle triple helical domain, and the C-terminal globular noncollagenous domain (NC1) (13). Type IV collagen is thought to be important in endothelial cell proliferation and behavior during the angiogenic process (5, 6, 9). The NC1 domain of type IV collagen plays a crucial role in the assembly of type IV collagen to form trimers and thus influences basement membrane organization, which is important for new blood vessel formation (9, 11, 14). Synthetic peptides (amino acids 185–203) derived from NC1 domain of α3 chain of type IV collagen (α3IV/NC1) have been shown to inhibit the proliferation of melanoma *in vitro* (15) and have been found to bind to αvβ3 integrin and CD47/IAP (16).

Recently, we identified that α3/IV/NC1, termed “tumstatin,” possessed a novel anti-angiogenic activity (7, 17). Integrin αvβ3 is potentially associated with angiogenic vascular cells and plays a critical role in angiogenesis and in promotion of endothelial cell survival (18, 19). In this regard, we recently identified that tumstatin binds to αvβ3 integrin in a RGD-independent manner, and this binding is essential for its anti-angiogenic activity (20). In the present study, the putative 54–132-amino

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‡‡‡ Recipient of the 1999 Research Award for Young Scientists from the Inoue Foundation for Science of Japan.

§§§ Consultant for Ilex Oncology, Inc. To whom correspondence should be addressed: Nephrology Div., Dept. of Medicine, RW 563a, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-0445; Fax: 617-975-5663; E-mail: rkalluri@caregroup.harvard.edu.

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1 The abbreviations used are: NC1, noncollagenous 1; C-PAE, bovine pulmonary arterial endothelial; HUVEC, human umbilical vein endothelial cells; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; BrdUrd, bromodeoxyuridine; MTThe 2,5-dihydropyrazole-4-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; MVD, microvessel density; GP, Goodpasture.

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The BrdUrd incorporation assay was conducted using the BrdUrd proliferation assay kit according to manufacturer's instructions (Calbiochem) with some modifications. Briefly, C-PAE cells were seeded onto 96-well plates in DMEM containing 10% FCS. The next day the medium was replaced with DMEM containing 2% FCS with or without full-length tumstatin (293 cell expressed). Plates were then incubated for 46 h at which time cells were pulsed for 2 h with BrdUrd (10 nm). The cells/DNA were then fixed to the wells, reacted with anti-BrdUrd primary and secondary antibodies and then developed using a colorimetric reaction. The plates were read at A 450 nm on a Molecular Devices plate reader. All groups represent triplicate samples.

**MTT Assay**—Cell viability was assessed by MTT (Chemicon) assay according to the manufacturer's instructions. C-PAE cells were plated and preincubated with the antibody for 1 h before Tum-5 (final concentration, 20 µg/ml) was added.

**Competition Proliferation Assay**—C-PAE cells were plated onto 96-well plates and serum-depleted as described above. Tum-5 (final concentration, 20 µg/ml) was added to tubes containing 2% FCS and recombinant Tum-5 or Tum-5/126C-A. After 21 h, the cells were harvested and fixed for 48 h. Proliferation assays were performed using the methylene blue staining method as previously described (5). In experiments with anti-α,β3 integrin antibody, cells were plated and preincubated with the antibody for 1 h before Tum-5 (final concentration, 20 µg/ml) was added.

**Annexin V-FITC Assay**—Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine, was used to detect apoptosis (29). Briefly, cells (0.5 × 10⁶/well) were seeded onto a 6-well plate coated with fibronectin in 5% FCS and recombinant Tum-5 or Tum-5/126C-A. After 21 h, the cells were harvested and then fixed in 70% ice-cold ethanol. The fixed cells were rehydrated at room temperature for 30 min in PBS containing 2% FCS and 0.1% Tween-20 and then centrifuged and resuspended in 1 ml of the same buffer. RNase digestion (5 µg/ml) was carried out at 37 °C for 1 h, followed by staining with propidium iodide (5 µg/ml). The cells were analyzed using a Coulter EPICS XL-MCL flow cytometer.

**Endothelial Tube Formation Assay**—The endothelial tube formation assay was performed as previously described (5). After 18 h of treatment, floating and attached cells were harvested and processed as described elsewhere (5). The supernatant and attached cells were combined, and an equal number of cells (4 × 10⁵ cells/ml) was processed following the manufacturer’s instruction (CLONTECH). A specific inhibitor of caspase-3, DEVD-fmk, was used for specificity. The absorbance was measured in a microplate reader (Bio-Rad) at 450 nm. Similarly nonendothelial cells (PC-3) were used and analyzed. This assay was repeated three times.

**Cell Attachment Assay**—This assay was performed as previously described (5). Matrigel-precoated 96-well plates were used, and HUVECs (5,000 cells/well) were seeded in the presence of Tum-5 or human endostatin. Control cells were incubated with PBS. Cells were incubated for 24 h at 37 °C, and the number of cells attached was counted using a microscope (magnification of 3.3× objective). Three wells were viewed, and the number of tubes/well was counted and averaged by two investigators blinded for the experimental protocols.

**Cell Attachment Assay**—This assay was performed as previously described (20, 30). 96-well plates were coated with 10 µg/ml of Tum-5 or vitronectin (Collaborative Biomedical Products) at a concentration of 20 µg/ml overnight. Plates were blocked with 10% FCS and rediluted with 2% FCS and recombinant Tum-5. After 21 h, the cells were harvested and maintained as previously described (5). Polyclonix B (Sigma, 5 µg/ml) was used to inactivate any endothelin that was still present (28), although all samples tested were less than 50 enzyme units/ng in endothelin, a concentration that is not enough to affect endothelial cell growth (data not shown). WM-164 cells were analyzed using a similar protocol.

**Materials and Methods**

**Production of Recombinant Deletion Mutants of Human Tumstatin and Tum-5**—The sequence encoding deletion mutants (Tum-1–5) of tumstatin was amplified using polymerase chain reaction from the α2β3INKI/pIDS vector as previously described (7, 21). The resulting cDNA fragment was ligated into pET28a+ (Novagen, Madison, WI). Expression of recombinant protein in E. coli and purification using nickel-nitrilotriacetic acid-agarose column (Qiagen) was performed as previously described (7). Recombinant Tum-5 was expressed in yeast using P. pastoris with a method previously described (22). pPICZαA was used for the subcloning of Tum-5 so that Tum-5 would be fused to C-terminal 6-histidine tag. Site-directed mutagenesis (C126 to A126) was induced to facilitate enhanced secretion of Tum-5 (5/126C-A). Human endostatin was expressed in yeast using P. pastoris with a previously described method (22). In some experiments, Tum-5 was processed for reduction and alkylated as described elsewhere (23, 24).

**Synthetic Peptides**—Synthetic peptides CDRGDCFVF (RDG-4C) and control peptides CNGRC were kindly provided by ILEX Oncology, Inc (San Antonio, TX). These peptides were synthesized and characterized as previously described (25).

**Immunoblotting**—Recombinant Tum-5 was analyzed by SDS-PAGE and immunoblotting as previously described (26). Goat anti-rabbit IgG and anti-human IgG antibody conjugated with horseradish peroxidase were purchased from Sigma. Monoclonal anti-polystyhdine tag antibody was purchased from Invitrogen, and monoclonal anti-polystyphhistidine tag antibody conjugated with peroxidase was purchased from Sigma.

**Cell Lines and Culture**—Bovine pulmonary arterial endothelial cells (C-PAE), human umbilical vein endothelial cells (HUVEC), human prostate adenocarcinoma cell line (PC-3), and NIH3T3 fibroblasts were all obtained from American Type Culture Collection. These cell lines were maintained in DMEM (C-PAE, Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin; in EGM-2 (HUVEC; Clonetics, San Diego, CA); or in F12K (PC-3; Cellgro). The melanoma cell line WM-164 was obtained from Dr. Meenhard Herlyn at the Wistar Institute (Philadelphia, PA) and maintained as previously described (20, 27). DU-145 was purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS.

**Proiferation Assay**—A suspension of C-PAE cells (7,000 cells/well, passage 2–6) in DMEM containing 0.5% FCS was added onto 96-well plates precoated with fibronectin (Sigma, 10 µg/ml). After 24 h, medium was changed to DMEM containing 20% FCS and recombinant Tum-5. Then, after 36–48 h, methylene blue staining was performed as previously described (5). Polyclonix B (Sigma, 5 µg/ml) was used to inactivate any endothelin that was still present (28), although all samples tested were less than 50 enzyme units/ng in endothelin, a concentration that is not enough to affect endothelial cell growth (data not shown). WM-164 cells were analyzed using a similar protocol.
Monoclonal antibody to human α3β1 integrin (clone LM609) was purchased from Chemicon.

**Direct Binding Assay for α3β1 Integrin**—Direct ELISA was performed as previously described (31). Tumstatin (293 cell expressed) or Tum-5 was coated onto a 96-well plate in triplicate (100 ng/well), and an equal molar amount of binding protein α3β1 integrin (Chemicon) was added. Binding was established with monoclonal antibodies to α3β1 integrin (clone LM609, Chemicon). The ELISA was developed with an alkaline phosphatase secondary antibody and read in a plate reader at absorbance of 405 nm.

**Matrigel Plug Assay**—In vivo Matrigel plug assay was performed as previously described (7). 5–6-week-old male C57BL6 mice (Jackson Laboratories, Bar Harbor, ME) were obtained. All animal studies were reviewed and approved by the animal care and use committee of Beth Israel Deaconess Medical Center and are in accordance with the guidelines of the Department of Health and Human Services. Matrigel (Collaborative Biomolecules) was mixed with 20 units/ml of heparin (Pierce), 50 ng/ml of vascular endothelial growth factor (R&D), and 5 μg/ml of Tum-5 or 10 μg/ml of Tum-1. The control group did not receive recombinant proteins. The Matrigel mixture was injected subcutaneously, and after 6 days mice were sacrificed, and the Matrigel plugs were removed and fixed in 4% paraformaldehyde. The plugs were embedded in paraffin, sectioned, and hematoxylin and eosin stained. Sections were examined by light microscopy, and the number of blood vessels from 4–7 high power fields (∼400) were counted and averaged. All sections were coded and observed by an investigator who was blinded for study protocols. Each group consists of four Matrigel plugs.

**In Vivo Tumor Studies**—Male athymic nude NCRNU mice, 5–6 weeks old, weighing ~25 g were implanted with 2 × 10⁶ PC-3 cells into the dorsal subcutis. The tumors were measured using Vernier calipers, and the volume was calculated using the standard formula (width² × length × 0.52) (3, 4). The tumors were allowed to grow to ~50 mm³, and animals were then pair-matched into groups of 6 mice. Initial doses were given on the day of pair-matching (Day 1). Tum-5, Tum-5/126C-A, or human endostatin was intraperitoneally injected twice daily at doses ranging from 1–20 mg/kg for 20 days in sterile PBS. Only soluble proteins were used. In addition, continuous delivery using Alzet minipumps surgically implanted subcutaneously were used in one treatment (Tum-5) group. Mice were weighed twice weekly, and tumor measurements were taken twice weekly, starting on Day 1. Estimated mean tumor volumes were plotted as a ratio (V/V₀), where V = tumor volume on day of measurement and V₀ = initial tumor volume. Upon termination (Day 21), the mice were weighed and sacrificed, and their tumors were excised and examined by light microscopy and CD31 immunostaining. In this model, (the mean treated tumor weight)/(mean control tumor weight) × 100% was subtracted from 100% to give the tumor growth inhibition for each group. In all experiments, the control group received vehicle injection.

**CD31 Immunostaining**—Intratumoral microvessel density (MVD) was analyzed on frozen sections of PC-3 tumor xenografts using a rat anti-mouse CD-31 monoclonal antibody (Pharmingen, San Diego, CA) with a standard streptavidin-biotin-peroxidase detection system ( Vectastain ABC Elite kit). Endogenous peroxidase activity was blocked using 1% H₂O₂/methanol for 30 min, and slides were then subjected to antigen retrieval by incubating with proteinase K for 30 min at room temperature. Anti-mouse CD31 antibody was diluted 1:20 in PBS-T (PBS containing 0.1% Tween-20) and incubated for 2 h after sections were blocked with 5% normal goat serum/PBS-T. Normal rat IgG was used as a negative control. Immunoperoxidase staining was carried out utilizing the Vectastain ABC Elite reagent kit (Vector Labs, Burlingame, CA). Sections were counterstained with Methyl green. MVD was assessed at first by scanning the tumor at low power, then identifying three areas at the tumor periphery that contain the maximum number of discrete microvessels, and then counting individual microvessels on a lower magnification (40×) field. The mean microvessel density was compared among treatment groups and analyzed using Student’s t-test.

**Statistical Analysis**—All values are expressed as means ± S.E. Analysis of variance with a one-tailed Student’s t-test was used to identify significant differences in multiple comparisons. A level of p < 0.05 was considered statistically significant.

**RESULTS**

**Expression and Purification of Tum-5**—Tum-5 was produced as a fusion protein with a C-terminal 6-histidine tag in E. coli using the expression plasmid pET 28a and in yeast using plasmid pPICZaA. Tum-5 was expressed to encompass amino acids 45–132 to include the putative amino acids 54–132 antiangiogenic domain of tumstatin. The E. coli expressed protein was isolated predominantly as soluble protein after a refolding process, and SDS-PAGE analysis revealed a monomeric band at 12 kDa (Fig. 1A). The eluted fraction represented by lane 8 was used in the experiments described in the present study.

Tum-5 protein contains 5 cysteines, and to decrease aggregation caused by the presence of 5th pair cysteine residue, we generated Tum-5/126C-A, in which the cysteine residue at position 126 of tumstatin was mutated to alanine. Tum-5/126C-A expressed in E. coli was detected at the same molecular weight size as Tum-5 (Coomassie Blue staining; Fig. 1B). E. coli expressed Tum-5, and E. coli and yeast (P. pastoris) expressed Tum-5/126C-A were immunodetectable by anti-histidine tag antibody (Fig. 1, C and D). Tumstatin consists of 244 amino acids including 12 amino acids from the triple helical portion located in the N-terminal portion, and 232 amino acids derived from the NC1 domain. Goodpasture (GP) syndrome is an autoimmune disease characterized by pulmonary hemorrhage and/or rapidly progressing glomerulonephritis (32–34). These symptoms are caused by the disruption of glomerular and alveolar basement membrane through immune injury associated with autoantibodies targeted against epitopes on α5(IV) NC1 (32, 33). Recently, the epitopes were identified in the N-terminal portion of the NC1 domain (26, 35) and was further confined to be within the N-terminal 40 amino acids (36, 37). Tum-5 consists of 45–132 amino acids of tumstatin outside of the GP autoepitope. To further confirm that Tum-5 is not potentially detectable by GP autoantibody, antisera from pa-
Patients with GP syndrome were used in Western blotting experiments. GP antisera detected 293 cell expressed full-length tumstatin with high sensitivity, although they failed to detect both E. coli expressed Tum-5 and yeast expressed Tum-5/126C-A, as shown for representative GP serum (Fig. 1E). These data suggest that Tum-5 and Tum-5/126C-A produced in the present study do not contain GP autoepitope, excluding the possibility that these recombinant proteins might induce an autoimmune disorder.

Anti-proliferative Effect of Tum-5—The anti-proliferative effect of Tum-5 and Tum-5/126C-A on C-PAE cells was examined in the present study. Using both BrdUrd incorporation assay (Fig. 2A) and methylene blue proliferation assay (Fig. 2B), Tum-5 (Fig. 2, A and B) and Tum-5/126C-A (data not shown) revealed a comparable dose-dependent inhibition. This inhibition is comparable with full-length tumstatin (Fig. 2A). Tum-5 (Fig. 2B) and Tum-5/126C-A produced both in E. coli and yeast (data not shown) significantly inhibited 20% FCS-stimulated cell proliferation in a dose-dependent manner with an ED50 10–12 μg/ml. This anti-proliferative activity was not observed on several nonendothelial cell lines including WM-164 cells (Fig. 2C).

Cell Cycle Analysis—To further evaluate the inhibition of endothelial cell proliferation by Tum-5, its effect on cell cycle progression was analyzed. In control cells (0 h), 5.8% of cells were in S phase (Fig. 2D). When the cells were stimulated with 5% FCS for 21 h, there was a 3.7-fold increase in the percentage of cells in S phase (21.5%). Treatment with Tum-5 decreased the percentage of cells in S phase to 6.0% (basal level). This effect of Tum-5 was dose-dependent (1 μg/ml,
less potent in inhibiting tube formation when compared with man Tum-5, 2.1 protein was: bovine serum albumin control, 22.7 6

caspase-3 activity, comparable with maximal effect of TNF-

Effect of Tum-5 on Cell Viability—Tum-5 and Tum-5/126C-A significantly decreased cell viability in a dose-dependent manner with an ED50 at 12 µg/ml, as assessed by the MTT assay (Fig. 2, E and H). Tum-5/126C-A decreased cell viability as potent as Tum-5, once again strongly suggesting that the terminal cysteine of Tum-5 is not essential for the anti-angiogenic activity (Fig. 2E). Both of these recombinant proteins did not exhibit any effect on nonendothelial cells (PC-3, DU-145) (Fig. 2, F and G). These results further suggest that Tum-5 is an endothelial cell-specific angiogenesis inhibitor.

Role of Secondary Structure (Disulfide Bonds) on the Activity of Tum-5—To establish the contribution of disulfide bonds and the associated secondary structure on the anti-angiogenic activity of Tum-5, we reduced and alkylated Tum-5. Reduction of Tum-5 led to a retarded migration of the protein band on SDS-PAGE gel, suggesting the loss of disulfide bonds (data not shown). Cell viability assays with reduced and nonreduced Tum-5 suggest that reduction of disulfide bonds does not have any significant effect on the activity of Tum-5, implicating that this activity is potentially contained within the primary sequence of Tum-5 (Fig. 2H).

Endothelial Cell Apoptosis—The induction of apoptosis in endothelial cells by Tum-5 was examined using annexin V-FITC as previously described (7). This assay detects the externalization of membrane phosphatidyl serine, which occurs early during apoptosis, because a FITC conjugate of annexin V binds naturally to phosphatidylserine (29). Tum-5 at 5 µg/ml revealed a distinct shift of annexin fluorescence peak after 18 h (Fig. 3A). The shift in fluorescence intensity was similar for Tum-5, Tum-5/126C-A (data not shown), and the positive control TNF-α (data not shown).

Tum-5 Increases the Activity of Pro-apoptotic Enzyme Caspase-3—Caspase-3 (CPP32), an intracellular protease activated at an early stage of apoptosis, initiates cellular breakdown by degrading structural and DNA repair proteins (38, 39). The protease activity of caspase-3 was measured spectrophotometrically by detection of the chromophore (p-nitroanilide) cleaved from the labeled substrate (DEVD-pNA). Tum-5 (10 µg/ml)-treated cells exhibited a 4.5-fold increase in caspase-3 activity, comparable with maximal effect of TNF-α (Fig. 3B). A specific inhibitor of caspase-3, DEVD-fmk, decreased the protease activity to base line, indicating that the increase in the measured activity was specific for caspase-3 activity. In nonendothelial cells (PC-3), there was no difference in caspase-3 activity between control and Tum-5-treated cells (Fig. 3C).

Effect of Tum-5 on Endothelial Cell Tube Formation—When HUVECs are cultured on Matrigel matrix, they rapidly align and form hollow tube-like structures (40). Human Tum-5 significantly inhibited endothelial tube formation in a dose-dependent manner as compared with control (Fig. 4, A–C). The percentage of tube formation after treatment with 5 µg/ml of protein was: bovine serum albumin control, 22.7 ± 3.1%; human Tum-5, 2.1 ± 2.0%. Yeast produced human endostatin was less potent in inhibiting tube formation when compared with yeast produced human Tum-5/126C-A at molar equivalents (Fig. 4C).

Cell Attachment to Tum-5 Is Not Inhibited by RGD Sequence—Synthetic peptides RGD-4C and control peptides CNGRC were previously reported to bind to vascular endothelial cells (25). RGD peptide at 5 µg/ml inhibits attachment of endothelial cells onto vitronectin-coated plates, whereas CNGRC peptides do not exhibit this property (Fig. 5A). Incubation of endothelial cells with Tum-5 did not inhibit cell attachment onto vitronectin-coated plates (Fig. 5A). When endothelial cells were incubated with RGD peptide (10 µg/ml) or
CNGRC peptide, attachment of C-PAEs to Tum-5-coated plates was not decreased (Fig. 5B). This further suggests the notion that Tum-5 binds to αvβ3 integrin in a RGD-independent manner and thus independent of vitronectin binding. As expected, incubation of cells with soluble Tum-5 decreased cell attachment onto Tum-5-coated plates (Fig. 5B).

Tum-5 Binds to Endothelial Cells via αvβ3 and β1 Integrin—We examined the attachment of HUVECs and C-PAEs to Tum-5-coated plates in the presence of integrin blocking antibodies. As shown in Fig. 5C, αvβ3 antibody inhibited the attachment of HUVECs by 51.0% and β1 antibody blocked by 48.4%, as compared with control IgG treatment. Cell attachment was also decreased by β3 integrin antibody, and interestingly, αv integrin antibody had no effect on cell attachment. Incubating cells with both αvβ3 and β1 integrin antibodies further decreased cell attachment (but not complete inhibition) as compared with the inhibitory effect by individual antibodies. This suggests that additional binding sites for endothelial cells may still be present on Tum-5. Comparable inhibition was also observed using C-PAE cells instead of HUVECs (data not shown). Collectively, these results suggest that Tum-5 binds to αvβ3 and β1-containing integrin on endothelial cells. Interaction of Tum-5 with αvβ3 integrin is mediated predominantly by β3 integrin subunit. In the previous study, we showed that the β3-containing integrin binding to tumstatin is αvβ3 (20).

Direct Binding Assay for Tum-5 Binding to αvβ3 Integrin—Binding of Tum-5 or tumstatin to αvβ3 integrin was assessed by direct ELISA. ELISA plates were coated with Tum-5 or tumstatin and incubated with equal molar concentration of αvβ3 integrin. As shown in Fig. 5D, our results show that Tum-5 and tumstatin significantly bind to αvβ3 integrin, whereas control mouse IgG showed no inhibition. Direct binding assay for Tum-5 or tumstatin was assessed as described under “Materials and Methods.”

Reversal of Anti-proliferative Effect of Tum-5 by Soluble αvβ3 Integrin Protein and Anti-αvβ3 Integrin Antibody—To further establish the role of αvβ3 integrin for Tum-5 activity, competi-
coated plates were incubated with by 65.9%. Treatment with significantly reversed Tum-5-induced anti-proliferative effect by methylene blue staining. Anti-proliferative effect of Tum-5 and growth factors. After 48 h, cell proliferation was examined 30 min and then added to C-PAEs stimulated with 20% FCS averaged. Tum-5 (5 dosage of Tum-5 was incubated with C-PAEs on vitronectin-coated plates (Fig. 5C). In previous studies, we have shown that control (Fig. 6C). The number of vessels per high power field was: Tum-1, 0.47 ± 0.16; Tum-5, 0.80 ± 0.16; and control, 8.81 ± 0.35 (Fig. 6D).

Effect of Human Tum-5 on Angiogenesis in Matrigel Plugs in C57BL/6 Mice—To evaluate the in vivo effect of human Tum-5 on the formation of new capillaries, we performed a Matrigel plug assay in mice as previously described (7). A 91% reduction in the number of blood vessels was observed with 5 μg/ml of human Tum-5 (Fig. 6D) on day 6 as compared with the PBS control (Fig. 6B). Human Tum-1, lacking the N-terminal 53 amino acids of tumstatin (7), inhibits neovascularization by

Tum-5 suppresses tumor growth in PC-3 xenograft mice model. Twice daily intraperitoneal injections of human Tum-5 (1 mg/kg) or human Tum-5/126C-A (1 mg/kg) inhibited the growth of PC-3 xenografts as compared with the PBS control. Treatment with human endostatin (20 mg/kg) did not show significant decrease of tumor growth. Continuous administration of Tum-5 (1 mg/kg/day) by using a subcutaneous osmotic pump was also effective in inhibiting tumor growth. This experiment was started when the tumor volumes were around 50 mm³. Each point represents the mean ± S.E. of six mice. Frozen tumor sections were stained by anti-CD31 antibody, and the number of vessels were counted and averaged. A, control. B, Tum-5 (E. coli expressed) intraperitoneal injection (200× magnification). Arrows indicate the CD31 positive blood vessels. D, there were significantly less blood vessels observed in the tumor section of Tum-5-treated group as compared with control (p = 0.046).

Effect of Human Tum-5 on the Growth of Human Xenograft Tumors in Nude Mice—We examined the effect of soluble human Tum-5 and Tum-5/126C-A on established primary human tumor models in nude mouse. No evidence of toxicity was observed in any groups, as judged by weight change. Both human Tum-5 and human Tum-5/126C-A significantly inhibited the growth of PC-3 human prostate carcinoma xenografts (Fig. 7A). Human Tum-5 at 1 mg/kg had a tumor growth inhibition of 74.1% (p = 0.02), and human Tum-5/126C-A had a tumor growth inhibition of 92.0% (p = 0.001) as compared with the vehicle-injected control group. Continuous delivery of human Tum-5 (1 mg/kg, over 24 h) using Alzet mini-pumps implanted in the dorsal subcutis also showed significant tumor growth inhibition (70.1%, p = 0.03). Human endostatin delivered at a dose of 20 mg/kg (twice daily, bolus intraperitoneal) revealed insignificant tumor growth inhibition as compared with the vehicle alone treated or Tum-5 group. These experiments suggest that at molar equivalents, human Tum-5 is at least 10-fold more potent than human endostatin in controlling the growth of human prostate xenograft tumors in mice. Further studies are needed to firmly establish the head to head potency of human endostatin in comparison with human Tum-5.

Tum-5 Decreased Neovascularization in Human PC-3 Xenograft Tumor in Nude Mice—We examined the effect of soluble human Tum-5 on intratumoral MVD in PC-3 xenograft tumors by CD31 immunostaining. Tum-5 intraperitoneal injection (Fig. 7C) significantly decreased MVD as compared with
vehicle injected group (Fig. 7B). The number of CD31-positive blood vessels per low power field \((40\times)\) was, Tum-5, 6.33 \(\pm\) 0.54, and control, 9.44 \(\pm\) 1.05, \(p = 0.047\) (Fig. 7D). These studies, of course, only include tumors that were still present upon the full course treatment of Tum-5. Groups treated with Tum-5/126C-A and the Tum-5-pump group showed a similar decrease of MVD (data not shown).

**DISCUSSION**

Angiogenesis is essential for the progression of various pathological disorders including diabetic retinopathy and rheumatoid arthritis, as well as tumor growth and metastasis (1). The switch to an angiogenic phenotype requires both up-regulation of angiogenic stimulators and down-regulation of angiogenesis inhibitors (1). Vascular endothelial growth factor and basic fibroblast growth factor are among the major inducers of angiogenesis. To date, a number of angiogenesis inhibitors have been identified, and certain factors such as angiostatin (3), endostatin (4), canstatin (5), arresten (6), and tumstatin (7) are tumor-associated angiogenesis inhibitors that are generated in vivo.

The predominant interest in our laboratory is focused on understanding the anti-angiogenic cues originating from vascular basement membrane. Our prevailing hypothesis centers on the notion that changes in the extracellular matrix and vascular basement membrane during the inductive and resolution phase of angiogenesis play an important role in regulating the formation/creation of new blood vessels. These changes are speculated to be modulated by growth factors produced by proliferating tumor cells (10). In pursuit of understanding these dynamic changes associated with angiogenesis, our laboratory recently identified a novel anti-angiogenic protein domain derived from the \(\alpha_3\) chain of type IV collagen, associated with vascular basement membrane (7). This protein domain, named tumstatin for its ability to cause tumor “stasis,” is an inhibitor of endothelial cell proliferation and causes endothelial cell specific apoptosis (7). Subsequently, our laboratory (20) and studies by Petitclerc et al. (17) show that tumstatin (\(\alpha_3\)IV/NC1) binds to endothelial cells via \(\alpha_3\beta_3\) integrin. We further show that the binding to \(\alpha_3\beta_3\) is pivotal for the anti-angiogenic activity associated with tumstatin and that the activity is restricted to amino acids 54–132 within the 244-amino acid tumstatin using deletion mutagenesis (20). Additionally, we show that the \(\alpha_3\beta_3\) binding to tumstatin is mediated via a mechanism independent of the RGD-containing amino acid sequence (20). Petitclerc et al. (17) also show that tumstatin (\(\alpha_3\)IV/NC1) binds to endothelial cells via \(\alpha_3\beta_3\) integrin but speculate that it is possibly via the RGD sequence present in the N terminus of the \(\alpha_3\)IV/NC1 domain. This RGD sequence does not constitute the NC1 domain sequence but is derived from the triple helical region and included in an original clone described by Neilson et al. (21). Petitclerc et al. (17) used this clone to recombinantly produce tumstatin (\(\alpha_3\)IV/NC1) in 293 embryonic kidney cells. When this sequence is removed using site-directed mutagenesis, the \(\alpha_3\beta_3\) binding is preserved, strongly suggesting a RGD-independent binding (20).

Deletion mutagenesis identified a 54–132-amino acid region as pivotal for the anti-angiogenesis activity of tumstatin. In the present study, we recombinantly produced this molecule (Tum-5) in pET28a bacterial expression system and in Pichia A yeast (\(P.\) pastoris) system and evaluated for potential anti-angiogenic activity. Our experiments suggest that the anti-angiogenic activity of tumstatin is localized to this region using both in vitro and in vivo assays. This activity is dependent on \(\alpha_3\beta_3\) integrin on endothelial cells, as is the activity of the parent tumstatin molecule.

In this study, the anti-angiogenic activity of Tum-5 was compared with recombinant full-length tumstatin expressed in 293-HEK cells in BrdUrd incorporation assay. The activity of Tum-5 was almost equivalent to full-length tumstatin. These results suggest that Tum-5 maintains equal anti-angiogenic activity even upon 64% truncation. When Tum-5 was produced in \(E.\) coli or yeast, it resulted in generation of Tum-5 predominantly in aggregate form. Thus, to improve our production yield, we replaced cysteine 126 with alanine to enable better expression and solubility of Tum-5 domain and produce more soluble protein for preclinical studies. This mutation resulted in enhanced protein expression, but Tum-5/126C-A still possessed comparable anti-angiogenic activity in vitro and in vivo, as compared with Tum-5 and parent tumstatin. Collectively, these data suggest that terminal cysteine of Tum-5 is not required in exerting its anti-angiogenic activity. Our experiments to understand the mechanism of action for Tum-5 are consistent with previous studies with tumstatin, which document endothelial cell-specific apoptosis associated with \(G_1\) arrest of endothelial cell cycle. Whether cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, and transcription factors such as E2F are involved (41) needs further investigation.

Integrin binding assays using Tum-5 show that previously reported \(\alpha_3\beta_3\) binding property of tumstatin, as well as \(\beta_1\) binding, is still preserved. The result of competition proliferation assay using soluble \(\alpha_3\beta_3\) integrin protein and anti-\(\alpha_3\beta_3\) integrin antibody (LM609) with Tum-5 suggest the involvement of \(\alpha_3\beta_3\) integrin binding in the anti-angiogenic activity of Tum-5. In this regard, LM609 has been shown to inhibit angiogenesis (18). Cell binding and proliferation assays in the present study suggest that Tum-5 binds to endothelial cells independent of an RGD sequence and vitronectin binding. Conceivably, tumstatin and Tum-5 can bind to \(\alpha_3\beta_3\) integrin on endothelial cells, whereas the cells are still attached to vitronectin and fibronectin. Such binding induces anti-proliferative and pro-apoptotic effect by endogenous angiogenesis inhibitors such as tumstatin. This may potentially explain the reason for the potent effect of tumstatin on proliferating endothelial cells.

In vivo experiments in C57BL/6 mice using Matrigel plugs show that Tum-5 is effective in inhibiting serum-, vascular endothelial growth factor-, and basic fibroblast growth factor-induced neovascularization. Studies using PC-3 xenograft tumors demonstrate in vivo efficacy of Tum-5 in inhibiting, and in a few instances, regressing pre-established tumors. When Tum-5 was administered through a subcutaneous osmotic pump, tumor growth was inhibited to a similar extent as two intraperitoneal injections every day. These experiments suggest that human Tum-5 may have a favorable half-life, potentially making continuous infusion not necessary for maximal anti-tumor effect. Human endostatin was not effective in inhibiting tumor growth at 20 mg/kg, in comparison with human Tum-5, which inhibited tumor growth even at 1 mg/kg. This is consistent with the result of the tube formation assay, which showed less effect with human endostatin as compared with human Tum-5. These results strongly suggest that at molar equivalent concentrations, human endostatin is much less effective in inhibiting tumor growth when compared with Tum-5.

Our studies indicate that tumstatin binds to \(\alpha_3\beta_3\) integrin, independent of vitronectin binding, to \(\alpha_3\beta_3\) integrin. It is speculated that vitronectin binding to \(\alpha_3\beta_3\) integrin may be responsible for the cell survival/proliferative signal to endothelial cells (42). It is hence conceivable that tumstatin binding to \(\alpha_3\beta_3\) integrin, in this context, may counteract such survival/proliferative signals and drive the endothelial cell toward apoptosis.
Our studies constitute the first report of a potential novel role for \( \alpha_5 \beta_1 \) integrin in negative regulation of angiogenesis. Such a novel function for \( \alpha_5 \beta_1 \) integrin may implicate its potential role in controlling and inhibiting tumor growth.

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Identification of the Anti-angiogenic Site within Vascular Basement Membrane-derived Tumstatin
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