Distinct Antitumor Properties of a Type IV Collagen Domain Derived from Basement Membrane*

Yohei Maeshima‡, Pablo C. Colorado, Adriana Torre, Kathryn A. Holthaus, James A. Grunkemeyer§, Mark B. Ericksen, Helmut Hopfer¶, Yingwen Xiao, Isaac E. Stillman, and Raghu Kalluri

From the Department of Medicine/Pathology and the Cancer Center, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Vascular basement membrane is an important structural component of blood vessels. During angiogenesis this membrane undergoes many alterations and these changes are speculated to influence the formation of new capillaries. Type IV collagen is a major component of vascular basement membrane, and recently we identified a fragment of type IV collagen α2 chain with specific anti-angiogenic properties (Kamphaus, G. D., Colorado, P. C., Panka, D. J., Hopfer, H., Ramchandran, R., Torre, A., Maeshima, Y., Mier, J. W., Sukhatme, V. P., and Kalluri, R. (2000) J. Biol. Chem. 275, 1209–1215). In the present study we characterize two different antitumor activities associated with the noncollagenous 1 (NC1) domain of the α3 chain of type IV collagen. This domain was previously discovered to possess a C-terminal peptide sequence (amino acids 185–203) that inhibits melanoma cell proliferation (Han, J., Ohno, N., Pasco, S., Monboisse, J. C., Borel, J. P., and Kefalides, N. A. (1997) J. Biol. Chem. 272, 20395–20401). In the present study, we identify the anti-angiogenic capacity of this domain using several in vitro and in vivo assays. The α3(IV)NC1 inhibited in vitro neovascularization in matrigel plug assays and suppressed tumor growth of human renal cell carcinoma (786-O) and prostate carcinoma (PC-3) in mouse xenograft models associated with in vivo endothelial cell-specific apoptosis. The anti-angiogenic activity was localized to amino acids 54–132 using deletion mutagenesis. This anti-angiogenic region is separate from the 185–203 amino acid region responsible for the antitumor cell activity. Additionally, our experiments indicate that the antitumor cell activity is not realized until the peptide region is exposed by truncation of the α3(IV)NC1 domain, a requirement not essential for the anti-angiogenic activity of this domain. Collectively, these results effectively highlight the distinct and unique antitumor properties of the α3(IV)NC1 domain and the potential use of this molecule for inhibition of tumor growth.

The development of new blood vessels from pre-existing ones is generally referred to as angiogenesis (1). In the adult, new blood vessels arise via angiogenesis, a process critical for normal physiological events such as wound repair, the ovarian cycle, and endometrium remodeling (2). However, uncontrolled neovascularization is associated with a number of pathological disorders including diabetic retinopathy, rheumatoid arthritis, as well as tumor growth, and metastasis (3, 4). Tumor growth and metastasis require angiogenesis (1), and this process is pivotal to the survival and subsequent growth of solid tumors beyond a few mm³ in size (3). Expansion of tumor mass occurs not only by perfusion of blood through the tumor but also by paracrine stimulation of tumor cells by several growth factors and matrix proteins produced by the new capillary endothelium (3). Recently, a number of angiogenesis inhibitors have been identified, namely angiotatin, endostatin, restin, and pigment epithelium-derived factor (5–8).

Basement membranes are thin layers of a specialized extracellular matrix that provide the supporting structure on which epithelial and endothelial cells grow and that surround muscle, fat, etc. (9). They are always associated with cells, and it has been well demonstrated that basement membranes do not only provide a mechanical support but also influence cellular behavior such as differentiation and proliferation. The major macromolecular constituents of basement membranes are type IV collagen, laminin, heparan sulfate proteoglycans, fibronectin, and entactin (10). 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 (11). In general, type IV collagen promotes cell adhesion, migration, differentiation, and growth (11). Type IV collagen is expressed as six distinct α-chains, namely, α1-α6 (12), assembles into triple helices, and further forms a network to provide a scaffold for other macromolecules in basement membranes. 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). The α1 and α2 isoforms are ubiquitously present in...

* This work was supported in part by Grants DK-51711 and DK-55001 from the National Institutes of Health (to R. K.), the 1998 Hershey Prostate Cancer Research Award (to R. K.), the 1998 American Society of Nephrology Carl Gottschalk Research Award (to R. K.), the 1998 National Kidney Foundation Murray award (to R. K.), the 1998 Beth Israel Deaconess Medical Center Enterprise Award (to R. K.), and research funds from the Beth Israel Deaconess Medical Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

§ Supported by National Institutes of Health/NIDDK Grant F32 DK09946.

¶ Supported by Deutsche Forschungsgemeinschaft Grant HO 2138/1-1.

‖ Equity holder and consultant for Ilex Oncology, Inc., which is involved in the clinical development of tumstatin. To whom correspondence should be addressed: Nephrology Div., Dept. of Medicine, BWH 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.

The abbreviations used are: NC1, noncollagenous 1; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; PAGE, polyacrylamide...
human basement membranes (9). The other four isoforms exhibit more tissue and organ-specific distributions (14, 15). The distribution of the α3 (IV) chain is limited to certain basement membranes, such as glomerular basement membrane, several basement membranes of the cochlea, ocular basement membrane of the anterior lens capsule, Descemet’s membrane, ovarian and testicular basement membrane (16), and alveolar capillary basement membrane (15, 17, 18). This chain is absent from epidermal basement membranes of the skin and the vascular basement membrane of liver (15).

The α3(IV) NC1 domain has been shown to bind and inhibit the proliferation of melanoma and other epithelial tumor cell lines in vitro (19). Han et al. (19) localized the binding site for melanoma cells to amino acids 185–203 of the α3(IV) NC1 domain. Monoclonal and polyclonal antibodies raised against this site were able to block melanoma cell adhesion and inhibition of proliferation (19). They also found that the specific sequence, -SNS-, located within amino acids 189–191, was required for both the melanoma cell adhesion and inhibition of proliferation (19). Additionally, these investigators did not use the isolated human α3(IV) NC1 domain in these studies (19). In these studies, the 185–203 α3(IV) NC1 synthetic peptide was not tested on other cell types, including endothelial cells.

Recent studies have illustrated the anti-angiogenic properties associated with inhibitors of collagen synthesis, supporting the notion that basement membrane collagen assembly and organization is important for blood vessel formation (20, 21). Furthermore, the NC1 domain of type IV collagen is speculated to play a crucial role in the assembly of type IV collagen to form trimers and thus influence basement membrane organization and modulation of cell behavior (10, 11, 22–24). These prior observations coupled with the identification of endostatin as the type XVIII collagen NC1 domain (6) prompted us to examine the anti-angiogenic property of the NC1 domain of type IV collagen. In this regard, recently our laboratory identified a novel type IV collagen-associated inhibitor of angiogenesis and tumor growth, termed canstatin. Canstatin (NC1 domain of α2 chain) was identified as an endothelial cell-specific apoptotic agent (25).

In the present study, we demonstrate the pivotal role of the NC1 domain of the α3 chain of human type IV collagen (13, 26) produced as a recombinant protein, in inhibiting the proliferation of capillary endothelial cells and blood vessel formation using in vitro and in vivo models of angiogenesis and tumor growth and also in inducing endothelial cell-specific apoptosis. We named this domain “tumstatin” (for its unique property of causing “tumor stasis”) to add another member to the newly discovered family of endogenous inhibitors of angiogenesis derived from larger proteins, such as angiotatin, endostatin, restin, and canstatin (5, 6, 25). Among the six NC1 domains of type IV collagen, three exhibited promising anti-angiogenic activity with distinct mechanisms of action (25, 27). The NC1 domain of the α3 chain (tumstatin) was most potent in inhibiting the proliferation of endothelial cells and causing apoptosis when compared with the other α(IV) chain NC1 domains. This newly discovered anti-angiogenic property of tumstatin, coupled with the previously reported antitumor cell activity, makes tumstatin a potentially useful therapeutic molecule in inhibiting tumor growth.
described previously (25). Polymyxin B (Sigma) at a final concentration of 5 μg/ml was used to inactive endotoxin (32). Briefly, C-PAE cells (passages 2–4) were grown to confluence and kept contact inhibited for 48 h. 786-O, PC-3, HPE, and WM-164 cells were used as nonendothelial controls. Cells were trypsinized and a suspension of 12,500 cells in DMEM was added to each well of a 24-well plate precoated with fibronectin. The cells were incubated for 24 h at 37 °C, and medium was replaced with DMEM containing 20% FCS. Unstimulated control cells were incubated with medium containing 0.1% FCS. Cells were treated with various concentrations of tumstatin or deletion mutants. All wells received 1 μCi of [3H]thymidine 12 h after the beginning of treatment. After 24 h, the thymidine was added using a scintillation counter. The methylene blue staining method was performed as described previously (25). All groups represent three samples.

**Endothelial Tube Assay—**Endothelial tube assay was performed as described previously (25). Matrigel (Collaborative Biомolecules) was added (320 μl) to each well of a 24-well plate and allowed to polymerize. A suspension of 25,000 MAE cells in EGM-2 without antibiotics was seeded into each well. The cells were treated with either tumstatin, BSA, or the 7 S domain of a lipid-binding protein with a high affinity for phosphatidylserine was added (320 μg/ml) to each well of a 24-well plate and allowed to polymerize. The next day a varying concentration of tumstatin or deletion control cells were incubated with medium containing 0.1% FCS. Cells were treated with various concentrations of tumstatin or deletion mutants. All wells received 1 μCi of [3H]thymidine 12 h after the beginning of treatment. After 24 h, the thymidine was added using a scintillation counter. The methylene blue staining method was performed as described previously (25). All groups represent three samples.

**Annexin V-FITC Assay—**Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine was used to detect apoptosis (34). This assay was performed as described previously (25). CPAE cells (0.5 × 10⁶/well) were seeded onto a 6-well plate in 10% FCS-supplemented DMEM. On the next day the fresh medium containing 10% FCS was added together with tumstatin ranging from 0.02 to 20 μg/ml or 80 ng/ml TNF-α. Control cells received an equal volume of PBS. After 18 h of treatment, medium containing floating cells was collected, and attached cells were trypsinized and centrifuged together with floating cells at 2000 × g. The cells were then washed in PBS and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Annexin V-FITC (CLONTECH) was added to a final concentration of 150 μg/ml, and the cells were incubated in the darkness for 10 min. The cells were washed again in PBS and resuspended in binding buffer. Annexin V-FITC-labeled cells were analyzed using a Becton-Dickinson cytometer. For each treatment 15,000 cells were counted and stored in list-mode. These data were then analyzed using Cell Quest software (Becton-Dickinson).

**TUNEL Method to Detect DNA Fragmentation in Tissue Sections—**DNA fragmentation associated with apoptosis was detected in situ by the addition of digoxigenin-labeled nucleotides to free 3'-hydroxyl groups in DNA using the ApopTag In Situ apoptosis detection kit following the manufacturer's instruction (INTERGEN). The number of TUNEL-positive cells/blood vessel was counted, and the mean number of positive nuclei/10 high power fields was calculated. The number of apoptotic cells was counted. An endothelial cell was considered morphologically apoptotic when it displayed loss of cell volume and chromatin condensation along the nuclear membrane with intensely basophilic staining (36).

**RESULTS**

**Expression and Purification of Human Tumstatin—**Human tumstatin was produced in *E. coli* using an expression plasmid, pET22b or pET 28a, as a fusion protein with a C-terminal six histidine tag. The *E. coli*-expressed protein was isolated predominantly as a soluble protein, and SDS-PAGE analysis revealed a monomeric band at 30 kDa (Fig. 1A). The additional 3 kDa arises from polylinker and histidine tag sequences. Therefore, because of the ease in production, we used *E. coli*-expressed protein for all of the studies. In

**Expression and Purification of Human Tumstatin—**Human tumstatin was produced in *E. coli* using an expression plasmid, pET22b or pET 28a, as a fusion protein with a C-terminal six histidine tag. The *E. coli*-expressed protein was isolated predominantly as a soluble protein, and SDS-PAGE analysis revealed a monomeric band at 30 kDa (Fig. 1A). The additional 3 kDa arises from polylinker and histidine tag sequences. Therefore, because of the ease in production, we used *E. coli*-expressed protein for all of the studies. In
some experiments, we also used 293 cell-expressed protein to confirm our results obtained by using E. coli-expressed protein.

**Antiproliferative Effect of Tumstatin on Endothelial Cells—**

The antiproliferative effect of tumstatin on C-PAE cells was examined by $[^3H]$thymidine incorporation assay using E. coli-produced soluble protein. Tumstatin significantly inhibited 20% FCS-stimulated $[^3H]$thymidine incorporation in a dose-dependent manner with an ED$_{50}$ of $\approx 0.01 \mu g/ml$ (Fig. 2A). No significant antiproliferative effect was observed with PC-3, 786-O, or primary human prostate epithelial cells (Fig. 2B). When polymyxin B was used to inactivate endotoxins, the antiproliferative effect of tumstatin on C-PAE cells was not affected (Fig. 2E). Tumstatin had no effect on the proliferation of WM-164 melanoma cells even at a concentration of 20 $\mu g/ml$ (Fig. 2F). In previous studies, the proliferation of WM-164 melanoma cells was inhibited by synthetic peptide 185–203 derived from the tumstatin sequence (19). In these studies, full-length human tumstatin (a3(IV) NC1) was not used (19).

**Effect of Tumstatin on Endothelial Cell Tube Formation—**

When mouse aortic endothelial cells are cultured on matrigel, they rapidly align and form hollow tube-like structures (37). Tumstatin, produced in E. coli, significantly inhibited endothelial tube formation in a dose-dependent manner as compared with BSA controls (Fig. 3, A and C). The percentage of tube formation after treatment with 1 $\mu g/ml$ protein was 98.0 $\pm$ 4.0 for BSA and 14.0 $\pm$ 4.0 for tumstatin. Similar results were also obtained using tumstatin produced in 293 cells (data not shown). The $7\,S$ domain of the $\alpha_3$ chain of type IV collagen had no effect on endothelial tube formation (Fig. 3B) and proliferation of mouse aortic endothelial cells (data not shown). However, we must point out that some recent studies have suggested that planar models of spontaneous angiogenesis (endothelial tube assays) stimulate invasive angiogenesis poorly (38).

**Effect of Tumstatin on Inducing Endothelial Cell Apoptosis—**

In the early stage of apoptosis, translocation of the membrane phospholipid phosphatidylserine from the inner surface of plasma membrane to outside is observed (34, 39, 40). Externalized phosphatidylserine can be detected by staining with a FITC conjugate of annexin V that binds naturally to phosphatidylserine (34). Tumstatin at 20 $\mu g/ml$ showed a distinct shift of annexin fluorescence peak after 18 h (Fig. 3D). The shift in fluorescence intensity was similar for tumstatin at 20 $\mu g/ml$ and the positive control TNF-$\alpha$ (80 ng/ml) (data not shown). Tumstatin at 2 $\mu g/ml$ also showed a mild shift in annexin fluorescence intensity, but concentrations below 0.2 $\mu g/ml$ did not demonstrate any annexin V positivity (data not shown). This shift of peak intensity was not observed when nonendothelial cells (PC-3) were used (data not shown).

**Tumstatin Increases the Activity of Caspase-3—**

Caspase-3 (CPP32) is an intracellular protease activated at the early stage of apoptosis and initiates cellular breakdown by degrading structural and DNA repair proteins (41, 42). The protease activity of caspase-3 was measured spectrophotometrically by detection of the chromophore ($p$-nitroanilide) cleaved from the labeled substrate (DEVD-$p$-nitroanilide). Tumstatin (20 $\mu g/ml$)-treated cells exhibited a 1.6-fold increase in caspase-3 activity, whereas TNF-$\alpha$ gave a comparable (1.7-fold) increase compared with control (Fig. 3E). A specific inhibitor of caspase-3, DEVD-fmk, decreased the protease activity to baseline indicating that the increase in the measured activity was specific for caspase-3. In nonendothelial cells (PC-3), there was no difference in caspase-3 activity between control and tumstatin-treated cells (Fig. 3F).

**Effect of Tumstatin on Angiogenesis in Matrigel Plug Assay—**

To evaluate the in vivo effect of E. coli-produced soluble tumstatin on the formation of new capillaries, we performed a matrigel plug assay in mice (43). A 67% reduction in the number of blood vessels was observed with a dose of 1 $\mu g/ml$ tumstatin (Fig. 4C) on day 14 as compared with PBS control (Fig. 4D).
The number of vessels/high power field was 2.25 ± 6.1.32 for tumstatin and 7.50 ± 6.2.17 for control (Fig. 4A).

**Effect of Tumstatin on the Growth of Tumors in Mouse Xenograft Model**—We examined the effect of tumstatin on established primary human tumor models in nude mice. Human tumstatin, produced in *E. coli*, significantly inhibited the growth of PC-3 human prostate carcinoma xenografts (Fig. 4D). Human tumstatin at 20 mg/kg inhibited tumor growth similar to mouse endostatin (20 mg/kg) (Fig. 4D). Significant inhibitory effect on tumor growth was observed on day 10 (control 202.8 ± 50.0 mm³, tumstatin 82.9 ± 25.2 mm³, endostatin 68.9 ± 16.7 mm³). Additionally, tumstatin at as little as 6 mg/kg inhibited the growth of 786-O human renal cell carcinoma xenografts as compared with the BSA control (Fig. 4E). A significant inhibitory effect on tumor growth was observed on day 10 (control 1096 ± 179.7 mm³, tumstatin 619 ± 120.7 mm³).

**Tumstatin Induces Endothelial Cell Apoptosis in Vivo**—We evaluated the effect of tumstatin in inducing endothelial cell apoptosis in vivo using tumor (PC-3) tissue sections. The number of TUNEL-positive apoptotic cells in vessel walls was significantly increased in the tumstatin-treated group (Fig. 4H) at day 10 as compared with controls (Fig. 4G) (control 2.0 ± 1.0, tumstatin 13.0 ± 1.5; Fig. 4F). Apoptosis in situ was also evaluated by the conventional cell morphology using light microscopy. The number of apoptotic nuclei in the blood vessels of the tumstatin-treated group (Fig. 4J) was also significantly higher than the control group (Fig. 4I).

**Effect of Truncated Tumstatin (tum-1) on Progression of 786-O Tumors in Mouse Xenograft Model**—Goodpasture syndrome is an autoimmune disease characterized by pulmonary
hemorrhage and/or rapidly progressing glomerulonephritis (17, 44). These symptoms are caused by the disruption of glomerular and alveolar basement membrane through immune injury associated with autoantibody against α3 (IV) NC1 (17, 44). Recently, the most probable disease-related pathogenic epitope was identified in the N-terminal portion (30, 45) and was further confined to be within the N-terminal 40 amino acids (46, 47). We produced truncated tumstatin lacking N-terminal 53 amino acids (tum-1) encompassing the pathogenic Goodpasture auto-epitopes (Fig. 5A). E. coli produced tum-1 at 6 mg/kg inhibited the growth of 786-O human renal cell carcinoma xenografts significantly from day 4 to day 10 as compared with control (day 10: tum-1 110.0 ± 29.0 mm³, control 345.0 ± 24.0 mm³) (Fig. 5B).

Expression of Tumstatin Deletion Mutants—To further characterize the antitumor activity of tumstatin, three different
deletion mutants of tumstatin and the one described above were expressed in E. coli using the pET28a system, and soluble protein was isolated (Table I and Fig. 6). 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. Tum-1 consists of 191 amino acids and is lacking the N-terminal 53 amino acids.

**FIG. 5.** Tum-1 inhibits growth of the 786-O tumor in vivo. The first 53 N-terminal amino acids were deleted in the tumstatin mutant, Tum-1 (A, closed circles represent deleted amino acids). The disulfide bonds, marked by short bars, are arranged as they occur in α1(IV) NC1 and α2(IV) NC1 (59). Tum-1 was engineered to remove the pathogenic Goodpasture auto-epitope from tumstatin, and hence, any chance of disease development. Tum-1 (6 mg/kg) greatly reduced 786-O renal cell carcinoma tumor volume compared with the PBS control in the mouse xenograft model (B). Each point represents the mean ± S.E. of five to six mice/group. *, p < 0.05 by a one-tailed Student’s t test.

**TABLE I**

| Protein     | Residue no. | Size (amino acids) |
|-------------|-------------|--------------------|
| Tumstatin   | 1           | 244                |
| tum-1       | 54          | 191                |
| tum-2       | 1           | 132                |
| tum-3       | 133         | 112                |
| tum-4       | 181         | 64                 |

**FIG. 6.** Recombinant production of tumstatin deletion mutants. Tumstatin deletion mutants were amplified from full-length tumstatin in pET-22b, and the polymerase chain reaction products were ligated into pET-28a. The recombinant proteins were expressed and purified as described under “Materials and Methods.” SDS-PAGE Coomassie Blue staining: MW; lane 1, tumstatin; lane 2, Tum-1; lane 3, Tum-2; lane 4, Tum-3; lane 5, Tum-4 (4 μg of each recombinant protein was loaded).

**FIG. 7.** Tum-1 and Tum-2 inhibit endothelial cell proliferation, and Tum-4 inhibits the growth of melanoma cells. C-PAE cells were treated with deletion mutants of tumstatin and proliferation was assessed by the methylene blue staining method (A). Tumstatin, tum-1, and tum-2, inhibited C-PAE proliferation in a dose-dependent manner. WM-164, a melanoma cell line, was not affected in a proliferation assay by any of the three proteins that demonstrate anti-angiogenic potential (B). Tum-4 shows antiproliferative activity in this tumor cell line but does not inhibit the proliferation of endothelial cells.

Tum-2 consists of 132 amino acids in the N-terminal half portion of tumstatin, and tum-3 is the C-terminal half portion (112 amino acids). Tum-4 consists of 64 amino acids in the C terminus of tumstatin, which includes the 185–203 peptide region (19).

**DISCUSSION**

The formation of new capillaries from pre-existing vessels, angiogenesis, is essential for the process of tumor growth and metastasis (2, 3, 48). The switch to an angiogenic phenotype requires both up-regulation of angiogenic stimulators and down-regulation of angiogenesis inhibitors (3). Vascular endothelial growth factor and basic fibroblast growth factor are abundantly expressed angiogenic factors in tumors. Vascularized tumors may overexpress one or more of these angiogenic factors, which can synergistically promote tumor growth. Inhibition of a single angiogenic factor such as the vascular endothelial growth factor with a receptor antagonist may not be enough to arrest tumor growth, because tumor subpopulations that produce angiogenic factors other than vascular endothelial...
growth factor can still influence tumor growth (49). A number of angiogenesis inhibitors have been recently identified, and certain factors such as interferon-\(\alpha\), platelet factor-4 (50), and PEX (51) are not endogenously associated with tumor cells. On the other hand, angiotatin (5) and endothasin (6) are tumor-associated angiogenesis inhibitors generated by the tumor tissue itself. In this report, we demonstrate the capacity of the NC1 domain of the \(\alpha3\) chain of human type IV collagen (tumstatin) (13, 26) to inhibit the proliferation of vascular endothelial cells and the formation of new blood vessels using in vitro and in vivo models of angiogenesis and tumor growth. Our results indicate that tumstatin may be exerting its effect at different stages in the process of tumor angiogenesis. The specific inhibition of proliferation of endothelial cells by tumstatin strongly suggests that it may function via a cell surface protein/receptor. Whether tumstatin functions by suppressing the activity of vascular endothelial growth factor and/or basic fibroblast growth factor remains to be elucidated. Induction of apoptosis in growth-stimulated endothelial cells by tumstatin was observed using annexin V-FITC. The induction of endothelial cell apoptosis by tumstatin was most pronounced when tumstatin was added to subconfluent monolayers, when cells were growing exponentially (data not shown). Conceivably, tumstatin is selective for tumor vasculature in which endothelial cells are activated. The pro-apoptotic effect of tumstatin was mediated by increased caspase-3 activity in endothelial cells. Additionally, tumstatin-treated tumors showed significantly increased apoptosis when compared with saline-injected control tumors.

Matrix metalloproteinases have been implicated as key enzymes that regulate the formation of new blood vessels in tumors (52). Recently, it was demonstrated that PEX, a domain of metalloproteinase-2, which can inhibit the interac-

tion of matrix metalloproteinase-2 and \(\alpha\beta_3\) integrin, suppressed tumor growth (51). Similarly, tumstatin may also function by inhibiting the activity of matrix metalloproteinases.

Recently, a synthetic peptide (19 amino acids) corresponding to the C-terminal portion of tumstatin was reported to bind to the \(\alpha\beta_3\) integrin (53). Because angiogenesis depends on specific endothelial cell adhesion events mediated by the \(\alpha\beta_3\) integrin (4, 54), it is possible that the anti-angiogenic effect of tumstatin is mediated by disrupting the interaction of proliferating endothelial cells to the matrix component, such as vitronectin and fibronectin, an event that is considered as an important anti-apoptotic signal (55). Interestingly, in these studies by Shahan et al. (53), only the synthetic peptide 185–203 was used and the full-length \(\alpha3(IV)\) NC1 (tumstatin) was not used. In the present study, we speculate if the anti-angiogenic activity of tumstatin is mediated by the peptide 185–203 and its \(\alpha\beta_3\) binding property. To address this issue, we employed deletion mutagenesis to generate truncated fragments of tumstatin. Our results indicate that although the isolated peptide 185–203 and tum-4 mutant containing this sequence inhibit melanoma cell proliferation and bind the \(\alpha\beta_3\) receptor, it is not responsible for the anti-angiogenic activity of tumstatin.

In contrast, the mutant tum-2, which contains the N-terminal half of the sequence of tumstatin, but not the peptide sequence 185–203, exhibited anti-angiogenic properties with no antitumor cell activity. Collectively, our studies with deletion mutants of tumstatin indicate that the exclusive anti-angiogenic activity is contained within amino acids 54–132. Additionally, our experiments indicate the anti-angiogenic activity residing within these amino acids is effective even when it is part of a full-length folded tumstatin. Interestingly, the antitumor cell activity residing within the peptide sequence 185–203 is not available when present as part of the full-length tumstatin. The activity imparted on the melanoma cells by the amino acid sequence 185–203 is only realized when this peptide is exposed either by truncation of the molecule (as in this study) or by synthesis of a representative peptide (19). To our knowledge, this is the first report of a molecule with such distinct antitumor activities. Of course, many molecules have been reported to carry peptide activities not associated with the full-length protein consisting of the peptides, and one such example is the heparin binding peptides of the \(\alpha1\) NC1 domain of type IV collagen (23), although the \(\alpha1(IV)\) NC1 domain itself does not exhibit a similar degree of binding capacity to heparin.

Because tumstatin possesses the pathogenic epitope for Goodpasture syndrome, it is possible that acute or chronic administration of tumstatin may induce this disease. We synthesized truncated tumstatin lacking the N-terminal 53 amino acids (tum-1) to remove this epitope, and this molecule continues to exhibit an inhibitory effect on the growth of 786-O xenografts. Additionally, this molecule did not bind autoantibodies from several patients with Goodpasture syndrome (data not shown). Tum-1 also potently decreased cell viability of the WM-164 melanoma cell line (54). Each point represents the mean ± S.E. of triplicate wells. Tum-1 and Tum-2 treatment increased viability in a dose-dependent manner. At dosages of 1 and 5 \(\mu\)g/ml, tum-1 was significantly more effective than tumstatin at decreasing cell survival. Tum-4 was the only deletion mutant that decreased the viability of the WM-164 melanoma cell line (B). Each point represents the mean ± S.E. of triplicate wells. Tum-1 and Tum-2 treatment increased the activity of caspase-3 in C-PAE cells as shown in C. TNF-\(\alpha\) was used as a positive control.

FIG. 8. Endothelial cells become less viable and undergo apoptosis when treated with Tum-1, Tum-2, and tumstatin. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide assay was used to evaluate cell viability in C-PAE cells after treatment with tumstatin and the deletion mutants (A). Tum-1 decreased the cell viability in a dose-dependent manner. At dosages of 1 and 5 \(\mu\)g/ml, tum-1 was significantly more effective than tumstatin at decreasing cell survival. Tum-4 was the only deletion mutant that decreased the viability of the WM-164 melanoma cell line (B). Each point represents the mean ± S.E. of triplicate wells. Tum-1 and Tum-2 treatment increased the activity of caspase-3 in C-PAE cells as shown in C. TNF-\(\alpha\) was used as a positive control.
make it a promising therapeutic candidate for inhibition of tumor growth in cancer patients.

Acknowledgments—We thank Dr. Mark Manfredi of Flex Oncology, Inc. for the help provided in making pET28a clones. We thank Dr. William Schnaper for help with the endothelial tube assay.

REFERENCES

1. Folkman, J. (1972) Ann. Surg. 175, 409–416
2. Folkman, J., and Shing, Y. (1992) J. Biol. Chem. 267, 10931–10934
3. Folkman, J. (1995) Nat. Med. 1, 27–31
4. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) Cell 79, 1157–1164
5. O’Reilly, M. S., Holmgren, L., Shing, Y., Wight, T. N., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (1994) Cell 79, 315–328
6. O’Reilly, M. S., Boehm, T., Shing, Y., Fukui, N., Vasiou, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) Cell 88, 277–285
7. Ramchandran, R., Dhanabal, M., Volk, R., Waterman, M. J., Segal, M., Lu, H., Knebelmann, B., and Sukhatme, V. P. (1999) Biochem. Biophys. Res. Commun. 255, 735–739
8. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouck, N. P. (1999) Science 285, 245–248
9. Paulsson, M. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 93–127
10. Timpl, R. (1996) J. Biol. Chem. 271, 50923–50927
11. Frojdman, K., Pelliniemi, L. J., and Virtanen, I. (1998) J. Biol. Chem. 273, 20333–20337
12. Prockop, D. J., and Kivirikko, K. I. (1995) Annu. Rev. Biochem. 64, 403–434
13. Tinney, E. C., Reger, L. A., Volk, R. M., Kormann, G. G., Anderson, S. S., Charron, A. S., Alegren, J. N., and Furcht, L. T. (1990) J. Cell Biol. 111, 1583–1591
14. Zhang, X., Brown, G. D., and Sarras, M. P., Jr. (1994) Dev. Biol. 164, 10–23
15. Kampp, G. D., Clark, R. A., Pili, R., Gunz, P., and Folkman, J. (1994) J. Clin. Invest. 94, 2520–2526
16. Turner, N., Mason, P. J., Brown, R., Fox, M., Povey, S., Rees, A., and Pusey, C. D. (1992) J. Clin. Inves. 89, 592–601
17. Colorado, P. C., Torre, A., Kamphaus, G., Maeshima, Y., Hopfer, H., Takahashi, K., Volk, R., Zamborsky, E. D., Herman, S., Sarkar, P. K., Ercken, M. B., Dhanabal, M., Simonis, M., Pili, R., Maeshima, Y., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. (2000) Cancer Res. 60, 2520–2526
18. Neilson, E. G., Kalluri, R., Sun, M. J., Gunwar, S., Danoff, T., Mariyama, M., Myers, J. C., Reeds, S. T., and Hudson, B. G. (1993) J. Biol. Chem. 268, 8402–8405
19. Dhanabal, M., Ramchandran, R., Volk, R., Stillman, I. E., Lombardo, M., Iruela-Arispe, M. L., Simonis, M., and Sukhatme, V. P. (1996) Cancer Res. 56, 189–197
20. Kalluri, R., Sun, M. J., Hudson, B. G., and Neilson, E. G. (1996) J. Biol. Chem. 271, 9062–9068
21. Herlyn, M., Kathan, W., Williams, N., Valyi-Nagy, I., and Rodeck, U. (1990) Adv. Cancer Res. 54, 213–234
22. Liu, S., Tobias, R., McElure, S., Styba, G., Shi, Q., and Jackowski, G. (1997) Clin. Biochem. 30, 455–463
23. Grant, D. S., Kibbey, M. C., Kinsella, J. L., Cid, M. C., and Kleinman, H. K. (1994) Pathol. Pract. 190, 853–863
24. van England, M., Niebeland, L. J., Ramaekers, C. F., Schutte, B., and Reutelingsperger, C. P. (1998) Cytometry 31, 1–9
25. Sugiyama, H., Kashiwara, N., Maeshima, Y., Okamoto, K., Kanao, K., Sekikawa, T., and Makino, H. (1998) Kidney Int. 54, 1188–1196
26. Wylie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D. (1984) J. Pathol. 142, 67–77
27. Haralabopoulos, G. C., Grant, D. S., Kleinman, H. K., Leukes, P. L., Papaiouannou, S. P., and Maragoudakis, M. E. (1994) Lab. Invest. 71, 575–582
28. Vernon, R. B., Lara, S. L., Drake, C. J., Iruela-Arispe, M. L., Angelo, J. C., Little, C. D., Wight, T. N., and Sage, E. H. (1995) In Vitro Cell Dev. Biol. Anim. 31, 120–131
29. Zhang, G., Gurtu, V., Kain, S. R., and Yan, G. (1997) BioTechniques 23, 523–531
30. Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keenhen, R. M., Pals, S. T., and van Oors, M. H. (1994) Blood 84, 1415–1420
31. Casciola-Rosen, L., Nicholson, D. W., Chong, T., Rowan, K. R., Thornberry, N. A., Miller, D. K., and Rosen, A. (1996) J. Exp. Med. 183, 1957–1964
32. Salvesen, G. S., and Dixi, V. M. (1997) Cell 91, 443–446
33. Passantini, A., Taylor, R. M., Pilu, R., Guo, Y., Lanz, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R. (1992) Lab. Invest. 67, 519–529
34. Wilson, C., and Dixon, F. (1996) In The Kidney (Berner, B., and Bector, F., eds) 5th Ed., pp. 1519–1524, W. B. Sanders Co., Philadelphia
35. Hellen, M., Segelmark, M., Unger, C., Burkhardt, H., Saus, J., and Wieslander, J. (1999) Kidney Int. 55, 936–944
36. Hellen, M., Burkhardt, H., and Wieslander, J. (1999) J. Biol. Chem. 274, 25862–25868
37. Netzer, K. O., Leinonen, A., Bourou, A., Borza, D. B., Todd, P., Gunwar, S., Hagele, J. P., and Hudson, B. G. (1999) J. Biol. Chem. 274, 12167–12174
38. Hanausak, D., and Folkman, J. (1996) Cell 86, 353–364
39. Yoshiji, H., Harris, S. R., and Thorpe, R. G., U. P. (1997) Cancer Res. 57, 3924–3928
40. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Denner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. (1990) Cancer Res. 50, 183–191
41. Brooks, P. C., Silleto, S., van Schaltsch, T. L., Friedlander, M., and Cheryse, D. A. (1998) Cell 92, 391–400
42. Ray, J. M., and Stetler-Stevenson, W. G. (1994) Eur. Respir. J. 7, 2062–2072
43. Shahab, T. A., Atia, Z., Pastso, S., Fawzi, A., Bellin, G., Monboisse, J. C., and Kefalides, N. A. (1999) Cancer Res. 59, 4584–4590
44. Brooks, P. C., Clark, R. A., and Cheryse, D. A. (1994) Science 264, 569–571
45. Isik, F. F., Gibran, N. S., Sekikawa, T., and Makino, H. (1998) J. Pathol. 178, 1479–1485
Distinct Antitumor Properties of a Type IV Collagen Domain Derived from Basement Membrane
Yohei Maeshima, Pablo C. Colorado, Adriana Torre, Kathryn A. Holthaus, James A. Grunkemeyer, Mark B. Ericksen, Helmut Hopfer, Yingwen Xiao, Isaac E. Stillman and Raghu Kalluri

J. Biol. Chem. 2000, 275:21340-21348.
doi: 10.1074/jbc.M001956200 originally published online April 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001956200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 20 of which can be accessed free at http://www.jbc.org/content/275/28/21340.full.html#ref-list-1