Two RGD-independent $\alpha_v\beta_3$ Integrin Binding Sites on Tumstatin Regulate Distinct Anti-tumor Properties*

Received for publication, March 21, 2000, and in revised form, May 30, 2000. Published, JBC Papers in Press, June 2, 2000. DOI 10.1074/jbc.C000186200

Yohei Maeshima‡, Pablo C. Colorado, and Raghu Kalluri§

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

Vascular basement membrane is an important regulator of angiogenesis and undergoes many alterations during angiogenesis and these changes are speculated to influence neovascularization. Recently, fragments of collagen molecules have been identified to possess anti-angiogenic activity. Tumstatin (a3(IV)NC1 domain) is one such novel molecule with distinct anti-tumor properties and possesses an N-terminal (amino acids 54–132) anti-angiogenic and a C-terminal (amino acids 185–203) anti-tumor cell activity (Maeshima, Y., et al. 2000 J. Biol. Chem. 275, 21340–21348). Previous studies have identified the 185–203 amino acid sequence as a ligand for $\alpha_v\beta_3$ integrin (Shahan, T. A., et al. 1999 Cancer Res. 59, 4584–4590). In the present study, we found distinct additional RGD-independent $\alpha_v\beta_3$ integrin binding site within 54–132 amino acids of tumstatin. This site is not essential for inhibition of tumor cell proliferation but necessary for the anti-angiogenic activity. A fragment of tumstatin containing 54–132 amino acid (tum-2) binds both endothelial cells and melanoma cells but only inhibited proliferation of endothelial cells, with no effect on tumor cell proliferation. A similar experiment with fragment of tumstatin containing the 185–203 amino acid (tum-4) demonstrates that it binds both endothelial cells and melanoma cells but only inhibits the proliferation of melanoma cells. The presence of cyclic RGD peptides did not affect the $\alpha_v\beta_3$ integrin-mediated activity of tumstatin, although significant inhibition of endothelial cell binding to vitronectin was observed. The two distinct RGD-independent binding sites on tumstatin suggest unique $\alpha_v\beta_3$ integrin-mediated mechanisms governing the two distinct anti-tumor properties of tumstatin.

Tumor growth and metastasis is associated with angiogenesis (1, 2). Integrin $\alpha_v\beta_3$ is associated with angiogenic vascular cells and plays a critical role in angiogenesis promoting endothelial cell survival (3, 4). Vascular basement membrane is speculated to play an important role in regulating angiogenesis (5, 6). Type IV collagen is expressed as six distinct $\alpha$-chains, namely $\alpha1$–$\alpha6$ (7), and assembled into triple helices and further forms a network to provide a scaffold for other macromolecules in basement membranes. These $\alpha$-chains are composed of three domains, N-terminal 7 S domain, the middle triple-helical domain, and C-terminal globular noncollagenous domain (NC1) (8). Type IV collagen is thought to play a crucial role in endothelial cell proliferation and behavior during the angiogenic process (5, 6, 9). Synthetic peptides derived from NC1 domain of $\alpha3$ chain of type IV collagen (a3(IV)NC1) has been shown to bind and inhibit the proliferation of melanoma, and other epithelial tumor cell lines, in vitro (10). Shahan et al. (11) localized the binding site for melanoma cells to amino acids 185–203 of a3(IV)NC1 and also found this site bound to $\alpha_v\beta_3$ integrin and CD47/IAP. Recently we identified that a3(IV)NC1 (termed “tumstatin” to include it in the family of endogenous inhibitor of angiogenesis derived from larger matrix protein with tumor “stasis” property) possessed a novel anti-angiogenic activity, and this activity was derived from amino acids 54–132, which does not contain the previously reported anti-tumor cell proliferation site (12). Recently, Petitclerc et al. (13) reported similar results further supporting the notion that recombinant a3(IV)NC1 inhibited angiogenesis and tumor growth. Although in these studies the issue of 185–203 $\alpha_v\beta_3$ binding site or RGD-independent sites within the NC1 domain were not addressed (13). In the present study, we demonstrate that amino acids 54–132 binds to $\alpha_v\beta_3$ integrin in an RGD-independent manner, and this binding is essential for the anti-angiogenic property.

MATERIALS AND METHODS

Production of Recombinant Tumstatin (a3(IV)NC1), Deletion Mutants and Endostatin, and Synthetic Peptides—The sequence encoding tumstatin or deletion mutants (tum-1–4) was amplified using polymerase chain reaction from the a3(IV)NCI/pDS vector (14). The resulting cDNA fragment was ligated into pET22b (+) or pET28a (+) (Novagen, Madison, WI). Expression of recombinant protein in Escherichia coli and purification using nickel-nitrioltriacetic acid-agarose column (Qiagen) was performed as described previously (9, 12). Recombinant mouse endostatin was expressed and purified as described previously (15). Only soluble protein was used in these experiments. Synthetic peptide CDCRGDCFC (RGD-4C), peptide CNGRC, and peptide PGLKGKRGDSSPATWTTRG, which consists of the N-terminal 20 amino acids of tumstatin, were kindly provided by Ilex oncology, Inc. (San Antonio, TX) as gifts. These peptides were synthesized and characterized as described previously (16, 17).

Immunoblotting—Recombinant tumstatin and deletion mutants

* This work was supported in part by Grants DK-51711 and DK-55001 from the National Institutes of Health (to R. K.), 1998 Prostate Cancer Research Award (to R. K.), 1998 American Society of Nephrology Carl Gottschalk Research 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.

§ To whom correspondence should be addressed: Nephrology Division, 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: rkaluri@caregroup.harvard.edu.

1 The abbreviations used are: NC1, noncollagenous 1; FCS, fetal calf serum; PBS, phosphate-buffered saline; C-PAE, bovine pulmonary arterial endothelial cells; HUVEC(s), human umbilical vein endothelial cells(s); BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.
were analyzed by SDS-PAGE and immunoblotting as described previously (18). Rabbit antibody raised against C-terminal 36 amino acids of tumstatin (tum-4 antibody) was prepared as described previously (19). Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Sigma.

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

Proliferation Assay—A suspension of C-PAE cells (7,000 cells/well, passage 2–4) in DMEM containing 1% FCS was added onto 96-well plates precoated with fibronectin. After 24 h, medium was replaced with DMEM containing 20% FCS and recombinant protein. Then, after 48 h, methylene blue staining was performed as described previously (9). Polymyxin B (Sigma, 5 μg/ml) was used to inactivate endotoxin (21). In separate experiments, T1 synthetic peptides were used for treatment.

Competition Proliferation Assay—C-PAE cells were plated onto 96-well plates and serum-depleted, as described above, and tumstatin (0.1 μg/ml, final concentration), tum-1 (2 μg/ml, final concentration), or tum-2 (50 μg/ml, final concentration) was incubated with varying concentration of human αβ3 or αβ1 protein (Chemicon) for 30 min at room temperature. Then the mixture was added onto wells and incubated for 48 h. Proliferation assays were performed using methylene blue staining method. In separate experiments, WM-164 cells were examined.

Cell Attachment Assay—This assay was performed as described previously (22). 96-Well plates were coated with 10 μg/ml recombinant protein, mouse laminin-1, or human type IV collagen (Collaborative Biomedical Products) and coated at a concentration of 0.5–2.5 μg/ml. Plates were blocked with 100 mg/ml of bovine serum albumin (BSA, Sigma) for 2 h. Synthetic peptides RGD-4C or CNGRC were coated at a concentration of 25 μg/ml, and plates were blocked with 30 mg/ml of BSA (Sigma) for an hour. T1 peptides were coated at a concentration of 40 μg/ml, and plates were blocked as described above. HUVECs or C-PAEs were incubated with 10 μg/ml of antibody for 15 min. In some assays, cells were incubated with synthetic peptides RGD-4C or CNGRC (1–5 μg/ml, final concentration). Then, 100 μl of the cell suspension was added onto plates and incubated for 45 min at 37 °C. After washing, the number of attached cells was determined with methylene blue staining. Control mouse IgG1 and mouse monoclonal antibody to the human β3 integrin (clone P4C10) were purchased from Life Technologies, Inc. Monoclonal antibody to the human α1–α6 (Alpha Integrin Blocking and IHC kit), αβ3 integrin (clone P1F6) were purchased from Chemicon. WM-164 cells were examined similarly in separate experiments.

Statistical Analysis—All values are expressed as mean ± 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 Human Tumstatin and Deletion Mutants—Recombinant human tumstatin was produced in E. coli using an expression plasmid, pET22b or pET28a, as a fusion protein with a C-terminal six-histidine tag (12). Four different deletion mutants of tumstatin were expressed in E. coli using pET28a system (Table I). Tumstatin consists of 244 amino acids, including 12 amino acids from triple-helical portion located in the N-terminal portion and 232 amino acids derived from NC1 domain. tum-1 consists of 191 amino acids and is lacking N-terminal 53 amino acids. tum-2 consists of 132 amino acids, including the N-terminal half portion of tumstatin, and tum-3 consists of the C-terminal half portion (125 amino acids). tum-4 consists of 64 amino acids in the C terminus of tumstatin (amino acids 181–244). The E. coli expressed deletion mutants were isolated predominantly as a soluble protein and SDS-PAGE analysis revealed a monomeric band corresponding to the size of each protein as described previously (12). Tumstatin, tum-1, tum-3, and tum-4 were immunodetectable using anti-tum-4 antibody in Western blotting (Fig. 1A panel A). tum-2 protein, which does not contain the tum-4 sequence and is anti-angiogenic, was not detectable by anti-tum-4 antibody (Fig. 1A, panel A).

Effect of Deletion Mutants of Tumstatin on Proliferation of C-PAEs and WM-164 Cells—tum-1 and tum-2 inhibited the proliferation of C-PAEs in a dose-dependent manner. At 15 μg/ml, tum-1 or tum-2 inhibited C-PAE proliferation by 65.6 and 73.3%, respectively (Table I). Tumstatin at 15 μg/ml inhibited the proliferation of C-PAE cells by 78.5%. In contrast, tum-3 and tum-4 did not inhibit the proliferation of C-PAE cells. tum-4 inhibited the proliferation of WM-164, and tum-1 or tum-2 did not (12). At 50 μg/ml, tum-4 inhibited the proliferation of WM-164 by 46.1% (Table I). In contrast, 50 μg/ml of tum-4 did not inhibit the proliferation of C-PAE cells (data not shown).

Tumstatin Binds to αβ3 and αβ6 Integrin on Endothelial Cells—We examined the attachment of HUVECs and C-PAEs to tumstatin-coated plates in the presence of α1–α6, β3, and αβ3 integrin blocking antibody. As shown in Fig. 1, panel B, αβ3 antibody inhibited the attachment of HUVECs by 80%, and α6 or β1 antibody blocked by 54% as compared with control IgG treatment. Although α5 antibody exhibited minor inhibition (20%), α1–α4 antibody did not block cell attachment. When αβ3 antibody and β3 antibody were used together, cell binding was blocked by more than 91%. Comparable inhibition was also observed using C-PAE cells instead of HUVECs (Fig. 1, panel F). The binding of C-PAEs to tumstatin was not inhibited by αβ6 antibody (Fig. 1, panel F). Collectively, these results suggest that αβ3 and αβ6 integrins may play roles in the anti-angiogenic property of tumstatin.

As controls, culture plates coated with type IV collagen, vitronectin, and laminin-1 were used to demonstrate the blocking activity of various antibodies used in this study (Fig. 1, panels C–E). The αβ3 integrins bind collagen IV (23, 24). Cell binding onto type IV collagen-coated plates was partially inhibited by α1 (20%), α5 (27%) antibody, and β1 antibody (53%), as compared with cells incubated with control IgG. αβ3 integrin is a major receptor for vitronectin (25). Cell binding onto vitronectin-coated plates was inhibited by αβ3 antibody by 61%. Previous studies have shown that α5β1 and α6β1 integrins bind laminin-1 (26, 27). Anti-α5 or α6 antibody blocked...
the binding of endothelial cells onto laminin-1-coated plates by 50 and 89%, respectively. These control experiments further support the functional integrin-blocking activities of these antibodies.

**Tumstatin Binds to Endothelial Cells via α₂β₃ Integrin, Independent of RGD Sequence—Within the sequence of tumstatin used in our study, an RGD sequence at amino acids 7–9 is present, and it is possible this RGD sequence binds to α₂β₃ integrin on endothelial cells. To address this issue, synthetic peptide RGD-4C, peptides CNGRC, which were previously reported to bind to vascular endothelial cells (16), and T1 peptide, which contains the N-terminal RGD containing sequence of tumstatin, were used in cell attachment assays. When plates were coated with RGD peptide, cell attachment of C-PAEs was increased by 144.25% as compared with noncoated (PBS) plates (Fig. 2, panel A). Cell attachment to plates coated with peptide CNGRC was also increased by 106.00%, as was expected from the previous studies (16). Next, plates were coated with vitronectin, and C-PAE cells were preincubated with RGD-4C or CNGRC peptides and plated on vitronectin. RGD peptide (5 μg/ml) significantly inhibited cell attachment onto vitronectin coated plates by 76.5%, whereas CNGRC peptides did not inhibit the binding of C-PAEs to vitronectin (Fig. 2, panel B). In contrast, attachment of C-PAEs to tumstatin-coated plates was not inhibited by preincubation with RGD-4C peptide (Fig. 2, panel C). When cells were plated on tumstatin, the presence of soluble α₂β₃ integrin protein to compete with endothelial cell surface binding to tumstatin, cell attachment was inhibited by 46.7%. Incubation of cells with RGD peptides in addition to α₂β₃ integrin protein did not alter the inhibitory effect of α₂β₃ integrin protein on cell attachment to tumstatin (Fig. 2, panel D). When HUVECs were plated on T1 peptide-coated plates was similar to the noncoated (PBS) plates. Panels E–G, attachment assay was performed with C-PAE using synthetic peptides RGD-4C (RGD) or CNGRC (CNGRC; 25 μg/ml) for coating plates. Cell attachment on RGD- or CNGRC-coated plates was increased as compared with noncoated (PBS) plates. Panel B, cell (C-PAE) attachment onto vitronectin-coated plates was significantly inhibited by RGD peptides (RGD, 5 μg/ml). Incubation of cells with CNGRC control peptides did not inhibit cell attachment. The matrix used for precoating plates is indicated on each graph (B–F). Panel C, cell attachment onto tumstatin-coated plates was significantly inhibited by α₂β₃ integrin protein. RGD or CNGRC peptides had no effect in inhibiting cell attachment. Cell attachment after addition of both α₂β₃ integrin protein and RGD peptides was not different from incubation with α₂β₃ integrin protein alone. Panel D, T1 peptides from N-terminal 20 amino acids of tumstatin containing RGD sequence were coated on plates. Attachment of HUVECs onto T1 peptide-coated plates was similar to the noncoated (PBS) plates. Panels E–G, attachment assay was performed with C-PAEs using tum-1, tum-2, and tum-4 for coating plates. Antibodies used for incubation with cells are described at the bottom (E–G). Panel F, cell attachment onto tumstatin-coated plates was significantly inhibited by anti-α₂β₃ integrin antibody. Panels F and G, cell attachment onto tum-2- or tum-4-coated plates were significantly inhibited by anti-α₂β₃ antibody. Anti-α₂β₃ integrin antibody did not inhibit cell attachment onto tum-1, tum-2, or tum-4-coated plates. Panel H, cell attachment onto WM-164 melanoma cells were significantly increased when cells were plated on tumstatin-, tum-1-, tum-2-, or tum-4 (10 μg/ml)-coated plates as compared with PBS control. Each column represents the mean ± S.E. of triplicate wells. These experiments were repeated three times. *, p < 0.05 by one-tailed Student’s t test.
**αβ3 and Tumstatin**

RGG and tum-2 by Soluble tumstatin, tum-1-, tum-2-, or tum-4-coated plates (8.1-, 8.4-, added to WM-164 cells. Anti-proliferative effect of tum-4 was tumstatin-induced anti-proliferative effect by 43.1%. Treat-
erative effect as compared with the recovery of tumstatin-
panel B

Tumstatin, tum-1, tum-2, and tum-4 Binds to WM-164 Mel-
anoma Cells—We next examined the attachment of WM-164 cells to tumstatin, tum-1-, tum-2-, or tum-4-coated plates. As shown in Fig. 2, panel H, melanoma cells bind specifically to tumstatin, tum-1-, tum-2-, or tum-4-coated plates (5.1-, 8.4-, 9.6-, and 8.2-fold increase as compared with PBS, respectively).

**Reversal of Anti-endothelial Cell Proliferative Effect of Tum-
statin and tum-2 by Soluble αβ3 Integrin Protein—**Tumstatin was incubated with αβ3 integrin protein for 30 min and then added to C-PAEs, which were then plated on culture plates and serum-depleted overnight. Anti-proliferative effect of tumstatin was reversed dose-dependently with increasing doses of αβ3-soluble protein (Fig. 3, panel A). The αβ3 protein at 2.4 μg/ml (3-fold molar excess of tumstatin) significantly reversed tumstatin-induced anti-proliferative effect by 43.1%. Treatment with αβ3 protein alone without tumstatin did not inhibit endothelial cell proliferation. When αβ3 integrin protein was incubated with tumstatin instead of αβ3 protein, anti-proliferative effect of tumstatin was not reversed (Fig. 3, panel B). When tum-2 was incubated with αβ3 protein for 30 min, and then added to C-PAEs as described above, the anti-proliferative effect of tum-2 was also reversed dose-dependently with increasing doses of αβ3-soluble protein (Fig. 3, panel C). The αβ3 protein at 2 μg/ml significantly recovered tum-2-induced anti-proliferative effect by nearly 74.1%. This increased recovery of activity, as compared with the recovery of tumstatin-induced anti-proliferative effect, may be partially mediated by the enhanced binding of αβ3 integrin protein to tum-2 compared with tumstatin, due to its significantly smaller size and thus potentially due to decreased steric hindrance for binding to αβ3 integrin.

**Reversal of Anti-proliferative Effect of tum-4 on Melanoma 
Cells by Soluble αβ3 Integrin Protein—**In our previous study we reported that tumstatin, tum-1, and tum-2 contained sequences that caused inhibition of endothelial cell proliferation (12). These proteins did not cause inhibition of melanoma cell proliferation. In contrast, tum-3 and tum-4 protein did not cause inhibition of endothelial cell proliferation, but tum-4 caused inhibition of melanoma cell proliferation. In this study, tum-4 was incubated with αβ3 protein for 30 min and then added to WM-164 cells. Anti-proliferative effect of tum-4 was reversed dose-dependently with increasing doses of αβ3-soluble protein (Fig. 3, panel D). The αβ3 protein at 2 μg/ml significantly recovered tum-4-induced anti-proliferative effect by 76.7%. Treatment with αβ3 protein alone without tum-4 did not inhibit melanoma cell proliferation.

**T1 Peptide (RGD Containing Peptide from Tumstatin) Does 
Not Inhibit Proliferation of C-PAE Cells—**T1 peptide, which contains N-terminal RGD sequence of tumstatin, was examined for its effect on endothelial cell proliferation. T1 peptides at 10 and 50 μg/ml did not inhibit proliferation of C-PAE cells (Fig. 3, panel E). This result further indicates that tumstatin’s activity to inhibit endothelial cell proliferation is RGD-independent.

**Tumstatin Inhibits Proliferation of Endothelial Cells in Con-
trast to Anti-αβ3 Integrin Antibody (LM609)—**To compare the anti-endothelial cell property of tumstatin and anti-αβ3 integrin antibody, an equimolar amount of human tumstatin and anti-αβ3 integrin antibody was added to C-PAE cells to compare their impact on endothelial cell proliferation. Increasing amount of anti-αβ3 integrin antibody did not inhibit proliferation, in contrast human tumstatin exhibited dose-dependent inhibition of proliferation. Mouse endostatin that was used as a positive control also inhibited endothelial cell proliferation. Control IgG did not inhibit proliferation of C-PAEs. Each column or points represents the mean ± S.E. of triplicate wells. These experiments were repeated three times, *p < 0.05 by one-tailed Student’s t test.

**DISCUSSION**

Recent reviews have suggested that a switch to an angiogenic phenotype requires both up-regulation of angiogenic...
stimulators and down-regulation of angiogenesis inhibitors (1, 28). In this regard, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are major inducers of angiogenesis. A number of angiogenesis inhibitors have been recently identified, and certain factors, such as angiostatin (29), endostatin (30), canstatin (9), and arresten (6), are tumor-associated angiogenesis inhibitors that are generated in vivo. Recently, tumstatin, recombinant NC1 domain of the α3 chain of human type IV collagen (8, 31), was identified as a protein with the dual anti-tumor activities. Tumstatin was identified as an inhibitor of vascular endothelial cell proliferation and formation of new blood vessels using in vitro and in vivo assays of angiogenesis and tumor growth (10, 12). Petitclerc et al. (13) recently suggested that NC1 domain of α2(IV), α3(IV), and α6(IV) may exert their anti-angiogenic activity via binding to αβ3 or β1 integrins. Although in these studies, functional experiments were not performed (13). According to our study utilizing cell adhesion assays, α3(IV)NC1 (tumstatin) was a strong RGD-dependent ligand for αβ3 and not for αβ2, integrin (13). Our results using cell adhesion method with two different endothelial cell types suggest that αβ3 and αβ6 integrin bind to tumstatin and that the αβ3 binding is RGD-independent. Recently, synthetic peptides (19 amino acids) corresponding to the C-terminal portion of tumstatin (amino acids 185–203) was reported to bind to αβ3 integrin and inhibit tumor cell proliferation including melanoma cell lines (11). In our previous study, we identified that anti-angiogenic activity of tumstatin is located within amino acids 54–124 by deletion mutagenesis and showed that the C-terminal fragment of tumstatin, including amino acids 185–203, did not possess anti-angiogenic activity (12).

In this study, we used tumstatin and deletion mutants of tumstatin in cell adhesion assays to analyze for integrin binding sites. Within the N-terminal portion of tumstatin used in this study, there is a RGD sequence (amino acids 7–9) derived from triple-helical noncollagenous portion. The RGD sequence is generally considered as an important binding site for αβ3 integrin receptor. In this study, we show that tum-1, lacking the N-terminal 53 amino acids (including this RGD site), still binds to αβ3 integrin. Also, a synthetic peptide T1 from 1–20 amino acids of tumstatin containing the RGD sequence neither bound to endothelial cells nor inhibited endothelial cell proliferation. These results suggest that RGD sequence in the N-terminal of tumstatin is not potentially a functional αβ3 integrin binding site. Therefore, the binding property of tumstatin to endothelial cells is likely RGD-independent as was previously shown for the 185–203 amino acid sequence of tumstatin (11). Furthermore, tum-2 (amino acids 1–132), which does not contain the C-terminal αβ3 binding site (amino acids 185–203), was shown to bind to αβ3 in cell adhesion assay and inhibit endothelial cell proliferation. When tumstatin or tum-2 were preincubated with αβ3 integrin protein to block their binding sites, anti-proliferative effect of tumstatin was significantly decreased (43–74%). This decrease can be considered impressive given that the affinity of soluble αβ3 receptor protein for tumstatin may be much weaker and the binding likely inefficient as compared with membrane-bound αβ3 integrin. This evidence strongly suggests that the αβ3 binding site is located within amino acids 54–132. When soluble αβ3 integrin protein was used instead of αβ3 protein, the recovery effect was not observed, strongly suggesting that the effect was specifically dependent on the blockade of tumstatin binding to αβ3 integrin. When endothelial cells were incubated with RGD-4C peptide, cell attachment to tumstatin-coated plates was not inhibited. Since the RGD-4C peptide binds to αβ3 integrin receptors and blocks vitronectin binding to endothelial cells, we speculate that tumstatin may bind to a site on the αβ3 integrin receptor, which is different from RGD binding site. Soluble αβ3 integrin was able to inhibit endothelial cell attachment to tumstatin, possibly by binding to tumstatin and masking it’s cell binding domain. These results again demonstrate the strong interaction of tumstatin with αβ3 integrin receptor. Also, neutralizing the RGD-dependent αβ3 integrin site by cyclic RGD peptide did not inhibit tumstatin binding to αβ3 integrin receptor on endothelial cells, again strongly suggesting that tumstatin binds to a RGD-independent and distinct site on αβ3 integrin receptor. Although, the possibility of an additional receptor for tumstatin cannot be ruled out due to the experimental strategy used in the current study.

Our results of αβ3 integrin involvement for tumstatin’s anti-angiogenic activity is consistent with the notion that VEGF up-regulates the expression of αβ3 on endothelial cells (32, 33). Since angiogenesis depends on specific endothelial cell adhesion events mediated by αβ3 integrin (3, 4), 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, events considered as important anti-apoptotic signal (34). Whether tumstatin functions by directly suppressing the activity of VEGF and/or bFGF remains to be elucidated. Collectively, our results reveal two RGD-independent sites on tumstatin. The previously identified site (11) and our discovery of a second site reveal no sequence homology, and presumably they may bind to distinct sites on αβ3 integrin receptor or bind to the same site by requiring different co-receptor protein for functionality. Interestingly, although the 185–203 sequence containing tum-4 binds to endothelial cells, and this binding can be inhibited by anti-αβ3 integrin neutralizing antibody, this interaction is not sufficient to input an anti-angiogenic property to this molecule. Hence, these results strongly suggest that although both tumstatin sites have the capacity to bind to αβ3 integrin on the cell surface of both cell types, additional cell-spectrum ligand-receptor interactions may be necessary for the specific effects on either endothelial or melanoma cells (35). In this regard, many recent studies suggest that αβ3-ligand interaction may need other cell surface proteins to facilitate appropriate downstream signaling into the cell interior (36). The cell-specific regulation of αβ3 integrin by different ligands (such as the two different sequence of tumstatin) may be facilitated by additional binding protein on the cell surface, such as CD47 (36–38). The presence of two novel RGD-independent αβ3 sites on tumstatin, facilitating two unique anti-tumor activities, makes it a valuable therapeutic agent for inhibition of tumor growth.

Acknowledgments—RGD, CNGRC, and T1 peptides were gifts from Ilex oncology, Inc. Also, we thank Dr. Meenhard Herlyn for providing us with WM-164 melanoma cells.

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doi: 10.1074/jbc.C000186200 originally published online June 2, 2000

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