Extracellular Matrix-derived Peptide Binds to αvβ3 Integrin and Inhibits Angiogenesis*

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Angiogenesis is associated with several pathological disorders as well as with normal physiological maintenance. Components of vascular basement membrane are speculated to regulate angiogenesis in both positive and negative manner. Recently, we reported that tumstatin (the NC1 domain of α3 chain of type IV collagen) and its deletion mutant tum-5 possess anti-angiogenic activity. In the present study, we confirm that the anti-angiogenic activity of tumstatin and tum-5 is independent of disulfide bond requirement. This property of tum-5 allowed us to use overlapping synthetic peptide strategy to identify peptide sequence(s) which possess anti-angiogenic activity. Among these peptides, only the T3 peptide (69–88 amino acids) and T7 peptide (74–88 amino acids) inhibited proliferation and induced apoptosis specifically in endothelial cells. The peptides, similar to tumstatin and the tum-5 domain, bind and function via αvβ3 in an RGD-independent manner. Restoration of a disulfide bond between two cysteines within the peptide did not alter the anti-angiogenic activity, Additionally, these studies show that tumstatin peptides can inhibit proliferation of endothelial cells in the presence of vitronectin, fibronectin, and collagen I. Anti-angiogenic effect of the peptides was further confirmed in vivo using a Matrigel plug assay in C57BL/6 mice. Collectively, these experiments suggest that the anti-angiogenic activity of tumstatin is localized to a 25-amino acid region of tumstatin and it is independent of disulfide bond linkage. Structural features and potency of the tumstatin peptide make it highly feasible as a potential anti-cancer drug.

Tumor growth and metastasis are dependent on angiogenesis, the formation of new blood vessels from pre-existing ones (1, 2). Growth factors and cytokines released from tumor cell components stimulate the endothelial cells of blood vessels leading to the expansion of tumor tissue (1). A theory of an “angiogenic switch” has been recently proposed, where the net balance of pro-angiogenic molecules and anti-angiogenic molecules are hypothesized to regulate the “on” and the “off,” respectively, of such a switch (1). Recent studies have identified several endogenous anti-angiogenic molecules produced de novo which regulate angiogenesis and tumor growth (3–8).

Basement membranes are composed of thin layers of specialized extracellular matrix that provide the scaffold for epithelial and endothelial cells (9). Basement membranes also influence differentiation, proliferation, and migration of various cell types. Vascular basement membranes are located as insoluble structural walls in capillaries and they interact with endothelial cells. They are speculated to play an important role in regulating angiogenesis (7, 10, 11). Type IV collagen is one of the major macromolecular constituent of basement membranes (12) and is expressed as six distinct α-chains, namely, α1–α6 (13). These α-chains are assembled into triple helices which further form a network to provide a scaffold for other macromolecules of the basement membrane. These α-chains are composed of three domains, the N-terminal 7S domain, the middle triple helical domain, and the C-terminal globular non-collagenous domain (NC1) (14). Type IV collagen is thought to be important in endothelial cell proliferation and behavior during the angiogenic process (6, 7, 10). 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 (10, 12, 15). Recently, we identified that α3(IV)NC1 (termed “tumstatin”) possessed a novel anti-angiogenic activity, and the activity was localized to amino acids 54–132 (tum-5) using deletion mutagenesis of tumstatin (8, 16). Integrin αvβ3 is involved in interactions between angiogenic vascular cells and extracellular matrix and it potentially plays a critical role in promoting angiogenesis and in endothelial cell survival (17, 18). In this regard, we recently showed that tumstatin and the active tum-5 deletion mutant bind to αvβ3 integrin (19, 20). The anti-angiogenic activity of tumstatin or tum-5 is not dependent on disulfide bond formation (20). This prompted us to examine further the anti-angiogenic site within tum-5 using a synthetic peptide strategy. We synthesized eight, overlapping peptides

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1 The abbreviations used are: NC1, non-collagenous 1; C-PAE, bovine pulmonary arterial endothelial cells; HUVEC, a human umbilical vein endothelial cell; PCS, fetal calf serum; PBS, phosphate-buffered saline; FAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; BrdUrd, bromodeoxyuridine; bFGF, basic fibroblast growth factor; MTU, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide; TNF-α, tumor necrosis factor-α; HPLC, high performance liquid chromatography; RA, reduced and alkylated.
covering the tum-5 domain, and tested these peptides in several anti-angiogenic assays. We demonstrate that the anti-angiogenic property is localized to a 25-amino acid region of tumstatin. This activity was not dependent on the disulfide bond linkage between cysteine residues. These results provide a novel peptide fragment as a possible drug candidate in the treatment of diseases dependent on angiogenesis.

MATERIALS AND METHODS

Production of Recombinant Tumstatin, Deletion Mutants of Tumstatin, and Synthetic Peptides—Recombinant tumstatin, tum-1, tum-5, and a deletion mutant of tumstatin (tum-1–5) with a 6-histidine tag were expressed in SF9 insect cells as previously described (8). Deletion mutants of tumstatin (tum-1–5) with a 6-histidine tag were expressed in Escherichia coli as previously described (8). Briefly, the sequence encoding the deletion mutants (tum-1–5) of tumstatin was amplified using polymerase chain reaction from the a3V/NcoI/pDs vector (8, 21). The resulting cDNA fragment was ligated into pET28a (Novagen, Madison, WI). Each recombinant protein was expressed in E. coli and purified using Ni-NTA-agarose column (Qiagen). The amino acids 45–132 of tumstatin were expressed as tum-5 which includes the N-terminal 9 amino acids in addition to the previously identified anti-angiogenic domain (54–132 amino acids) (8). The additional 9 amino acids were added to enhance the efficiency of protein expression. Only soluble protein with low endotoxin level (less than 50 enzyme units/mg) was used in the present study.

Synthetic peptides CDRGIDFC (RGD-4C) and control peptides CNGRC were kindly provided by Ilex Oncology, Inc. (San Antonio, TX). T1 peptide consisting of the N-terminal 20 amino acids of tumstatin, and T2-T7 peptides covering 54–132 amino acids of tumstatin were also kindly provided by Ilex Oncology, Inc. (Table I). T8 peptide (69–96 amino acids) was synthesized with mutation of Leu to Lys at position 69. These peptides were synthesized and characterized as previously described (22).

Reduction and Alkylation of Tumstatin and Tum-5—In some experiments, tumstatin and tum-5 were processed for reduction and alkylation as described elsewhere (23, 24). Briefly, 2.5 mg/ml tum-5 or tumstatin in 6 M guanidine-HCl, 20 mM Tris-HCl (pH 7.5) were incubated for 1 h at 50 °C in 10 mM dithiothreitol. The reaction mixture was then brought to room temperature, and iodoacetamide was added to make the final concentration 25 mM. Following incubation for 1 h at room temperature, the resulting solution was dialyzed against 5 mM HCl (two changes, 5 h each) and finally against 1 mM HCl. The absence of free thiol groups in the final product was confirmed by using Ellman reagent.

Cell Lines and Culture—Bovine pulmonary arterial endothelial cells (C-PAE), human umbilical vein endothelial cells (HUVEC), and the human prostate adenocarcinoma cell line (PC-3) were all obtained from American Type Culture Collection. These cell lines were maintained in DMEM containing 20% FCS and either recombinant protein or synthetic peptide. For the cell attachment assay, cells were seeded at 105 cells/ml, in M-199) in DMEM (2% FCS) containing T3 peptide (10 or 50 μg/ml). Controls received PBS buffer. TNF-α (80 ng/ml) was used as a positive control. After 24 h, both the supernatant and attached cells were combined and an equal number of cells (4 × 104 cells/ml) were processed following the manufacturers instruction (CLONTECH). A specific inhibitor of caspase-3 activity, Z-DEVD-fmk, was used for specificity. The absorbance was measured in a microplate reader (Bio-Rad) at 405 nm. Similarly, non-endothelial cells (PC-3) were used and analyzed. This assay was repeated three times.

Cell Attachment Assay—This assay was performed as previously described (19, 29). 96-well plates were coated with 10 μg/ml recombinant protein or synthetic peptide overnight. Vitronecin (Collaborative Research, Inc.) was used for coating plates. Plates were blocked with 100 mg/ml bovine serum albumin (Sigma) for 2 h. HUVECs or C-PAEs (1.5 × 104 cells/ml, in M-199) were incubated with either 10 μg/ml antibody or synthetic peptide for 15 min. Then, 100 μl of the cell suspension was added to each well 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 human β1 integrin (clone P4C10) were purchased from Life Technologies. Monoclonal antibody to human αv (clone NKK-M9), β3 (clone B3A), αvβ3 (clone LM609), and αvβ5 integrin (clone P1F6) were purchased from Chemicon.

Synthesis of T3-folded Peptide (S-S Bridge Formation)—T3 peptide was dissolved in 10 ml of 50% acetonitrile, 10 mM ammonium bicarbonate buffer (pH 7.3) at 0.25 mg/ml. Aliquots (30 μl) of 2 mM potassium ferricyanide dissolved in 10 mM ammonium bicarbonate buffer (pH 7.3) were added 5 times at room temperature to the peptide solution at 5-min intervals. The reaction mixture was shortly vortexed every time after the oxidizer was added, was allowed to stay for 2 h at room temperature. Then, the absence of free thiol groups in the peptide solution was confirmed using Ellman reagent. The abundance of the peptide fragments and higher dimers in a final reaction mixture was confirmed by SDS-PAGE (16.5%) and silver staining.

HPLC was used for the final purification of the T3 peptide. T3 peptide was applied and run on a C-18 300-A Jupiter column (Phenomenex, CA) using an acetonitrile (CH3CN) gradient (20–60% buffer B, 30 min). Buffer A, 0.1% trifluoroacetic acid or buffer B, 0.1% trifluoroacetic acid in acetonitrile.
Recombinant tumstatin was reduced and alkylated (RA) to remove the disulfide bond linkages (Fig. 1, panel A). As expected, by SDS-PAGE the RA tumstatin migrated slower than non-reduced tumstatin. Similarly, deletion mutant tum-5 was also reduced and alkylated. Non-reduced tum-5 can exist as a monomer, dimer, and other multimers (Fig. 1, panel B). RA tum-5 migrates as a single band corresponding to a monomeric protein with molecular mass of ~12 kDa, with retarded mobility compared with unreduced tum-5 monomer (Fig. 1, panel B). RA tumstatin and RA tum-5 were used in an endothelial cell proliferation assay and compared with the non-reduced protein. Reduction and alklylation of tumstatin and tum-5, did not influence their anti-endothelial cell proliferative activity (Fig. 1, panels C and D).

Effect of Reduced / Alkylated Human Tum-5 on the Growth of Human Xenograft Orthotopic Breast Tumors (MDA MB435) in Nude Mice—We examined the effect of soluble human tum-5 and RA tum-5 on established primary human orthotopic breast tumors in nude mice. No evident toxicity was observed in any groups, as judged by weight change and terminal autopsy. Both human tum-5 and human tum-5/RA significantly inhibited the growth of MDA MB435 human breast carcinoma xenografts (Fig. 1, panel E). Human tum-5 at 1 mg/kg had a tumor growth inhibition of 37.1% (p = 0.03) and human tum-5/RA at 0.2 and 5 mg/kg had a tumor growth inhibition of 45.6% (p = 0.001) and 45.3% (p = 0.002), respectively, as compared with the vehicle injected control group. These experiments suggest that reduction and alkyllylation of tum-5 does not alter the in vivo effects of tum-5 in inhibition of tumor growth. These results strongly suggest tum-5 activity is independent of disulfide bond linkage.

Synthesis of 20 Amino Acid Peptides Encompassing the Entire Tum-5 Region and Anti-endothelial Cell Proliferation Assay—Our results show that disulfide linkage is not critical for the anti-angiogenic activity of tumstatin. This very interesting structural feature of tumstatin and its active deletion mutant tum-5, allowed us to pursue a synthetic peptide strategy to identify the amino acid sequence responsible for the anti-angiogenic activity of tumstatin. We synthesized a library of 20 peptides encompassing the entire tum-5 region. The peptides were synthesized using solid phase chemistry and purified by HPLC. The peptides were 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 H & E stained. Sections were examined by light microscopy, and the number of blood vessels from 4 to 7 high power fields per group was counted and averaged. All sections were coded and observed by an investigator who was blinded for study protocols. Each group consists of three or four Matrigel plugs.

In Vivo Tumor Studies—Female nude NCRNU mice, 5–6 weeks old, weighing ~20 g were implanted with 2 × 10^6 MDAMB-435 cells into the subcutaneous mammary fat pad. The tumors were measured using Vernier calipers and the volume was calculated using the standard formula (width^2 × length × 0.52) (4, 5). When the tumors were 100 mm^3, the animals were pair-matched into treatment and control groups (7 mice per group). Initial doses of tum-5 or vehicle control were given on the day of pair-matching (day 1), and were administered via intraperitoneal injection bi-daily at the doses indicated. Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on day 1. Mean fractional tumor volume ratios (VV_m) were plotted against time. Mice were euthanized at the end of the treatment period. Upon termination, the mice were weighed, sacrificed, and their tumors were excised. The mean tumor weight per group was calculated, and the mean treated tumor ratio/mean control tumor ratio was subtracted from 100% to give the tumor growth inhibition for each group. Statistical Analysis—All values are expressed as mean ± S.E. ANOVA 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

Anti-endothelial Cell Proliferation Property of Tumstatin and Tum-5 Is Not Dependent on Disulfide Bonds Linkage—

Matrigel Plug Assay—An in vivo Matrigel plug assay was performed as previously described (8). Five to six-week old male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) were obtained. All 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 heparin (Pierce), 50 ng/ml vascular endothelial growth factor (R&D), and 10 μg/ml tum-5 or 10 μg/ml synthetic peptide. The control group received neither the proteins nor the peptides. 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 H & E stained. Sections were examined by light microscopy, and the number of blood vessels from 4 to 7 high power fields per group. Initial doses of tum-5 or vehicle control were given on the day of pair-matching (day 1), and were administered via intraperitoneal injection bi-daily at the doses indicated. Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on day 1. Mean fractional tumor volume ratios (VV_m) were plotted against time. Mice were euthanized at the end of the treatment period. Upon termination, the mice were weighed, sacrificed, and their tumors were excised. The mean tumor weight per group was calculated, and the mean treated tumor ratio/mean control tumor ratio was subtracted from 100% to give the tumor growth inhibition for each group.

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Statistical Analysis—All values are expressed as mean ± S.E. ANOVA 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.
giogenic function of tumstatin. Since the entire anti-angiogenic activity of tumstatin is contained within deletion mutant tum-5 (20), our initial strategy was to synthesize 5 overlapping 20 amino acid peptides covering the entire tum-5 domain (Table I).

In addition, we also made tumstatin N-terminal sequence peptide (T1 peptide, Table I), since it contained a RGD sequence, although in former studies we had already shown that this region does not contribute to the anti-angiogenic activity of tumstatin (19). Among these peptides (T1-T6), only T3 peptide significantly inhibited proliferation of endothelial cells (Fig. 2, panel A). This anti-proliferative effect by T3 was dose-dependent (Fig. 2, panel B), and was not observed with the other peptides, and nor was it seen when αβ integrin expressing WM-164 tumor cells were used in these assays (Fig. 2, panel C).

**Endothelial Cell Cycle Analysis**—To further evaluate the mechanism of T3 peptide on endothelial cell proliferation, the effect of T3 peptide on cell cycle progression was analyzed. In the contact inhibited and growth arrested cells (0 h), 4.1% of cells were in S phase (Fig. 2, panel D). When the cells were stimulated with bFGF for 24 h, there was a 5.4-fold increase in the percentage of cells in S phase (22.1%). Treatment with T3 peptide, at a maximal dosage of 50 μg/ml, decreased the percentage of cells in S phase to 13.8%. In contrast, T1 or T6 peptide treatment exhibited no significant decrease of cells in S phase (T1, 22.3%; T6, 21.1%), even at a maximal dose of 100 μg/ml. This effect of T3 was dose-dependent (10 μg/ml, 21.4%; 25 μg/ml, 20.5%). The percentage of cells in G0/G1 was, non-significant shift of peak intensity was not observed when PC-3 cells were treated with T1, T6 (both 100 μg/ml), or T3 (10–50 μg/ml) peptide in the presence of 1% FCS and 3 ng/ml bFGF for 24 h. The cells were harvested and processed as described under “Materials and Methods.” The percentage of cells in G0/G1 phase in growth arrested cells was considered as 0-h time point. This experiment was repeated three times, and the representative data are shown.

**Endothelial Cell Apoptosis**—The effect of synthetic peptides derived from tum-5 on cell viability (C-PAE) was evaluated using the MTT assay as previously described (8). T3 peptide decreased endothelial cell viability as compared with the other peptides (Fig. 3, panel A). TNF-α treatment was used as a control (Fig. 3, panel A). Furthermore, the induction of apoptosis in endothelial cells by these peptides was examined using annexin V-fluorescein isothiocyanate as previously described (8). This assay detects the externalization of membrane phosphatidylserine which occurs in the early stages of apoptosis, as a fluorescein isothiocyanate conjugate of annexin V binds naturally to phosphatidylserine (28). T3 peptide at 25–50 μg/ml revealed a distinct shift in the annexin fluorescence peak after 18 h (Fig. 3, panel C). The shift in fluorescence intensity was similar for both T3 at 50 μg/ml and the positive control TNF-α (Fig. 3, panel B). T3 peptide at 10 μg/ml also showed a mild shift in annexin fluorescence intensity, but concentrations below 10 μg/ml did not demonstrate this shift (Fig. 3, panel C). T1 or T6 peptide treatment at the highest concentration (50 μg/ml) revealed insignificant shift in annexin fluorescence intensity (Fig. 3, panel B). T1 or T6 at lower concentrations (less than 50 μg/ml), as expected, also caused no shift (data not shown). This shift of peak intensity was not observed when PC-3 cells were treated with T3 peptide (Fig. 3, panel D).

**T3 Peptide Increases the Activity of Caspase-3—Caspase-3 (CPP32) is an intracellular protease activated during early stages of apoptosis, and initiates cellular breakdown by degrading structural and DNA repair proteins (30, 31). The protease activity of caspase-3 was measured spectrophotometrically by detection of the chromophore (p-nitroanilide) cleaved from the labeled substrate (DEVD-pNA). T3 peptide-treated (50 μg/ml) peptide in the presence of 1% FCS and 3 ng/ml bFGF for 24 h. The cells were harvested and processed as described under “Materials and Methods.” The percentage of cells in G0/G1 phase in growth arrested cells was considered as 0-h time point. This experiment was repeated three times, and the representative data are shown.

**Tumstatin and Tum-5 Binds to Endothelial Cells via T3**

| TABLE I | Sequence of peptides derived from tumstatin (amino acid) |
|---------|--------------------------------------------------------|
| T1 (1-20) | PGKLKRGDSGSPATWRGTFTRHOSOTAIPSCPGTVRLYSGFSFLVQGNQRA |
| T2 (54-73) | HGQDLTGLSGCRCFTTMCPCVVHCVNDCNFSRNDYSLWILPCMKPIAGEL |
| T3 (69-88) | RALEPSRHTVCVCEGPSAIAGVSSSTTPTCPPCHGWSLWKGFSFIMFSTAGSEQGQA |
| T4 (84-103) | LASPGCLEEPASFLHELVRGCTNYNSNYSFPLASNLPERMFIPSTVKAGEEL |
| T5 (98-117) | EKISKSCQVCMKKR |
| T6 (114-133) | *T8 peptide: Leu at position 69 mutated to Lys. |
Sequence—Tumstatin produced in 293 human embryonic kidney cells (tumstatin-293) was used to coat tissue culture plates in these experiments. Attachment of C-PAEs to tumstatin 293-coated plates in the presence of T1-T6 peptide (10 μg/ml) or tum-4 (amino acids 181–244 in the C terminus of tumstatin) (10 μg/ml) (8), was examined. T3 peptide at 10 μg/ml inhibited cell attachment to tumstatin-coated plates by 46.4% (Fig. 4, panel A). Inhibition of cell attachment to tumstatin-coated plates by T3 was dose-dependent (Fig. 4, panel B). Other peptides or tum-4 did not inhibit cell attachment (Fig. 4, panel A). Similarly, T3 peptide at 10 μg/ml inhibited cell attachment by 44.0% to tum-5-coated plates (Fig. 4, panel C). Inhibition of cell attachment to tum-5-coated plates by T3 was also dose-dependent (Fig. 4, panel D). The other peptides also did not inhibit endothelial cell attachment to tum-5 (Fig. 4, panel C). When tum-4 (a non-angiogenic deletion mutant of tumstatin (19)) was used for coating plates, the inhibitory effect of T3 was not observed (Fig. 4, panel C). These results suggest that endothelial cells bind to the T3 sequence within tumstatin and tum-5 and this binding is potentially responsible for the anti-angiogenic property of this molecule. Tumstatin and tum-5 may contain other endothelial cell-binding sites (19, 20), which explains the lack of complete inhibition of endothelial cell binding by the T3 peptide, as T3 potentially only inhibits αvβ3 integrin binding (see below).

T3 Peptide Binds to αvβ3 Integrin on Endothelial Cells—The capacity of tum-5 peptides in facilitating the adhesion of endothelial cells was examined initially by direct binding experiment. Our results show that T3 peptide facilitated a significant increase in binding of endothelial cells followed by weaker binding to T4 peptide (Fig. 4, panel E). Interestingly, this effect by T4 peptide was not observed in inhibition experiments in Fig. 4, panels A-D. Presumably, the effects of T4 peptide are weak, thus not detectable in all assays due to sensitivity parameters. We next examined the attachment of C-PAEs to peptide (T2-T6)-coated plates in the presence of αv, β1, β3, αvβ5, and αvβ3 integrin blocking antibodies. As shown in Fig. 4, panel F, αvβ3 antibody inhibited the attachment of C-PAEs to T3-coated plates by 50.5%. This antibody known as LM609, is potentially not a direct blocking antibody for interactions between C-PAE and T3 peptide, hence may be due to steric hindrance only achieving partial inhibition. Cell attachment of C-PAE cells to T4 peptide-coated plates was partially (14.5%) blocked by αvβ3 antibody. As expected, the baseline adhesion of endothelial cells to other peptides was not inhibited by αvβ3 antibody (Fig. 4, panel F). Cell adhesion to T3-peptide was not inhibited by αv integrin antibody, but β3 antibody significantly inhibited adhesion (Fig. 4, panel H). In this regard, no other integrin blocking antibody available was able to inhibit C-PAE binding to T3 peptide significantly (data not shown). Comparable inhibition was also observed using HUVECs instead of C-PAEs (data not shown). Collectively, these results suggest that αvβ3 integrin may play a role in the anti-angiogenic activ-

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**Fig. 3.** T3 peptide induces endothelial cell apoptosis. The MTT assay was used to evaluate viability of C-PAE cells after treatment with synthetic peptides (panel A). T3 (20 μg/ml) significantly decreased the cell viability as compared with the control receiving PBS. TNF-α (80 ng/ml) was used as a positive control. Each column represents the mean ± S.E. of triplicate wells. Annexin V-fluorescein isothiocyanate staining was performed on C-PAE cells treated with T1, T3, or T6 for 18 h (panels B and C). Control cells received vehicle buffer instead of synthetic peptides. FACS analysis was done to quantitate the percentage of cells undergoing apoptosis (annexin-V positive cells). Cells were stained with propidium iodide, and gating was performed to analyze only annexin V positive and propidium iodide negative cells. FL-1 height represents the annexin fluorescence intensity as a log scale. Panel B, T3 (green) at 50 μg/ml induced a distinct shift of fluorescence intensity peak as compared with the control (black) (C-PAE). T1 or T6 treatment (50 μg/ml) did not cause any shift of peak fluorescence. Panel C, T3 induced dose-dependent shift of peak fluorescence (C-PAE). Also, 10 μg/ml T3 (green) induced a mild shift. Panel D, when PC-3 cells were used, T3 at 50 μg/ml (pink) did not induce any shift of fluorescence intensity peak as compared with the control (black). As for positive control for PC-3 cells, 95% ethanol was used. This experiment was repeated three times, and the representative data are shown. Caspase-3 activity was examined as described under “Materials and Methods.” Increased caspase-3 activity was observed by treating C-PAEs with 50 μg/ml T3 (panel E). DEVD-fmk, a specific caspase-3 inhibitor, was used to show the specificity. TNF-α (80 ng/ml) was used as a positive control. This increased activity of caspase-3 was not observed by treating PC-3 cells with T3 (panel F). These experiments were repeated three times, and the representative data are shown.
ity associated with T3 peptide. Interestingly, although cell attachment to tum-5 is inhibited by β1 integrin antibody (20), cell attachment to tum-4 coated plates was not significantly inhibited by T3 peptide (20 μg/ml). Other peptides derived from tumstatin or tum-4 (181–244 amino acid) did not affect cell binding to tumstatin-coated plates. Attachment of C-PAE cells to tum-4 coated plates was not inhibited by T3 peptide.

Panel A, cell (C-PAE) attachment onto tumstatin (293 human embryonic kidney cells expressed)-coated plates was significantly inhibited by T3 peptide (10 μg/ml). Other peptides derived from tumstatin or tum-4 (181–244 amino acid) did not affect cell binding to tumstatin-coated plates. Attachment of C-PAE cells to tum-4 coated plates was not inhibited by T3 peptide. T3 peptide decreased cell attachment to tumstatin-coated plates in a dose-dependent manner (panel B). Panel C, cell (C-PAE) attachment onto tum-5 coated plates was significantly inhibited by T3 peptide (2.5 μg/ml). Other peptides derived from tum-5 did not affect cell binding to tum-5 coated plates. Attachment of C-PAE cells to tum-5 coated plates was not inhibited by T3 peptide. T3 peptide decreased cell attachment to tum-5 coated plates in a dose-dependent manner (panel D). Panel E, plates were coated with peptides and cell attachment assay was performed. Peptide used for coating plates are shown at the bottom. As for the PBS group, the plate was incubated with PBS without peptide overnight. C-PAE cell binding was significantly elevated when plated on T3 coated plates. Panel F, Cell (C-PAE) attachment onto T3 peptide coated plates was significantly inhibited by T3 peptide (20 μg/ml, final concentration) was significantly decreased by αvβ3 integrin protein (Chemicon) was performed as described under “Materials and Methods.” The anti-proliferative effect of T3 peptide (20 μg/ml, final concentration) was significantly decreased by αvβ3 integrin protein (0.1–1 μg/ml, final concentration). Panel G, competition proliferation assay using T3 peptide (20 μg/ml) and αvβ3 integrin protein (Chemicon) was performed as described under “Materials and Methods.” The anti-proliferative effect of T3 peptide (20 μg/ml, final concentration) was significantly decreased by αvβ3 integrin protein (0.1–1 μg/ml, final concentration). Panel H, attachment of C-PAE cells to T3 peptide-coated plates was inhibited by incubating with β1 integrin antibody. αv or β1 integrin antibody did not significantly inhibit cell attachment onto T3 peptide-coated plates. Each column represents the mean ± S.E. of triplicate wells. These experiments were repeated three times.

Reversal of Anti-proliferative Effect of T3 Peptide by Soluble αvβ3 Integrin Protein—A competition proliferation assay was performed as previously described (19). T3 was incubated with αvβ3 integrin protein for 30 min, and then added to C-PAEs with 20% FCS. After 48 h, cell proliferation was examined by methylene blue staining. The anti-proliferative effect of T3 peptide was reversed dose-dependently with increasing doses of αvβ3 soluble protein (Fig. 4, panel G). The αvβ3 protein at 1 μg/ml significantly reversed the T3-induced anti-proliferative effect.
Effect by 66.2%. Treatment with αβ3 protein alone, without T3, did not inhibit endothelial cell proliferation. These results suggest that the anti-angiogenic activity of T3 is mediated via αβ3 integrin on the endothelial cells. These results are consistent with similar experiments using tum-5, soluble αβ3 integrin protein, and anti-αβ3 antibody (LM 609) (20).

Disulfide Linkage-associated Folding of T3 Peptide Does Not Alter the Anti-angiogenic Activity—S-S bridge formation between two cysteine residues of T3 peptide was performed by oxidation as described under “Materials and Methods” (panel A). Panel B, HPLC analysis of T3 peptide before and after oxidation. Upper panel, unoxidized (unfolded) T3 peptide; lower panel, oxidized (folded) T3 peptide. The position of the major peak of each peptide appears at different time-points. A proliferation assay using C-PAE cells was performed as described under “Materials and Methods.” Anti-proliferative effect of T3 was not altered by oxidation (panel C). Each column represents the mean ± S.E. of triplicate wells.

Comparison of Anti-angiogenic Activity of Tumstatin and Deletion Mutants—Our experiments with T3 peptide suggest that at molar equivalent concentrations, T3 peptide is 2.5-fold less active in inhibiting proliferation of endothelial cells when compared with tumstatin or tum-5 (Figs. 1 and 2). In the present study, we also show that this loss in activity cannot be attributed to the lack of proper or any disulfide linkage in the T3 peptide (Fig. 5). Therefore, we hypothesized that the most likely explanation for the loss of activity may be the lack of additional amino acid sequence around the T3 peptide. Since the T2 peptide neither inhibited αβ3 binding to tumstatin, nor caused an inhibition of endothelial cell proliferation, we assumed this region to be unimportant for T3 peptide activity. Although T4 peptide revealed no inhibitory activity on endothelial cell proliferation, it exhibited weak binding to αβ3 integrin and facilitated weak binding of endothelial cells. Thus, we extended the T3 peptide by 10 amino acids of the T4 peptide which are not contained within the T5 peptide. The new peptide was named T7 peptide. An endothelial cell proliferation assay was used to determine comparative activity of tumstatin, deletion mutants, and peptides. At 1, 2.5, and 5 μM, tumstatin, tum-5, and the T3 peptide revealed anti-proliferative activity; however, the T3 peptide (folded or unfolded) was 2.5-fold less active in comparison with tumstatin and tum-5 at equimolar concentrations (Fig. 6, panel B). When T7 peptide was used in proliferation assays, it exhibited similar activity to tumstatin and tum-5, at equimolar concentration (Fig. 6, panel B). These results suggest that, although the 10 amino acids in the T4 peptide do not exhibit anti-angiogenic activity, they are potentially important for optimal binding of tumstatin to αβ3 integrin, possibly by facilitating better interactions and, hence, helping to attain maximal anti-angiogenic activity at limiting concentrations. The effect of T7 peptide to inhibit proliferation of endothelial cells was further confirmed by BrdUrd incorporation assay (Fig. 6, panel C). T7 peptide exhibited dose-dependent decrease of BrdUrd incorporation (Fig. 6, panel C). Although T7 peptide was able to exhibit the same activity as tumstatin and tum-5 at molar equivalent concentrations, it had

![Image](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322208/bin/31965-fig6.jpg)

**Fig. 6.** Potent anti-angiogenic effect of T7 and T8 peptide equivalent to the parent molecule tumstatin and tum-5. T7 peptide and T8 peptide covering parts of T3 and T4 peptide sequence were synthesized (panel A). Recombinant tumstatin (28 kDa), tum-5 (12 kDa), T7 peptide, and T3 peptide were used for a proliferation assay (methylene blue staining) with equimolar concentrations. Tumstatin, tum-5, and T7 peptide showed anti-proliferative effect with ED50 of 1 μM. ED50 of T3 peptide was 2.5 μM (panel B). Panel C, BrdUrd incorporation assay. T7 peptide decreased incorporation of BrdUrd in a dose-dependent manner in C-PAEs. T1 peptide with the same vehicle buffer did not show any effect. Panel D, MTT assay. T7 and T8 peptide decrease cell viability of C-PAEs in a dose-dependent manner. Each column represents the mean ± S.E. of triplicate wells.
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![Image](http://www.jbc.org/)

**Fig. 7. The anti-angiogenic effect of T3 and T7 peptide is not altered by the presence of provisional matrix.** Panel A, attachment of C-PAEs on vitronectin-coated plates was not inhibited by incubating with T3 peptide. Incubation of the cells with T6 peptide did not inhibit cell attachment. Panels B and C, plates were coated with vitronectin or provisional matrix (type I collagen, vitronectin, and fibronectin). When T3 or T7 treatment was started, matrix components were further added at the same time onto C-PAEs as described under "Materials and Methods." Proliferation assay was performed by methylene blue staining. Each column represents the mean ± S.E. of triplicate wells.

the solubility challenges in physiological buffers due to its high content of hydrophobic amino acids. In an effort to make a more soluble peptide with full anti-angiogenic activity, we synthesized a peptide that lacks 3 amino acids in the C terminus, YWL, and contains five more amino acids in the N terminus, in addition, leucine was switched to lysine (Fig. 6, panel A). This peptide was named T8 peptide. T8 peptide showed comparable effect in decreasing the cell viability of C-PAEs as compared with T7 peptide at the dosage of 6 μg/ml or higher, as assessed by MTT assay (Fig. 6, panel D).

**T3 Peptide Does Not Block Cell Attachment to Vitronectin—α3β3 Integrin on Endothelial Cells Bind to Vitronectin via the RGD Sequence (32).** Cell attachment of C-PAEs on vitronectin-coated plates was not inhibited in the presence of T3 peptide (Fig. 7, panel A). Incubation of cells with control T6 peptide also did not inhibit cell attachment to vitronectin-coated plates (Fig. 7, panel A). These results suggest that T3 may bind to a distinct site on α3β3 integrin which is independent of its RGD recognition site for vitronectin. These results support the notion that anti-angiogenic activity of tumstatin and its active deletion mutant is independent of vitronectin binding to α3β3 integrin, suggesting a possible role for integrin α3β3 as a negative regulator of angiogenesis, independent of its RGD dependent responses.

**T3 and T7 Peptide Inhibit Proliferation of Endothelial Cells in the Presence of Vitronectin and Provisional Matrix Constituents—**As reported earlier with tumstatin and tum-5 (19, 20), T3 peptide does not compete for vitronectin binding to α3β3 integrin on endothelial cells (Fig. 7, panel A). In order to assess the influence of vitronectin, fibronectin, and collagen I binding to the α3β3 integrin on the anti-proliferative effect of T3 and T7 peptides, plates were coated with vitronectin or provisional matrix mixture (type I collagen, fibronectin and vitronectin) and proliferation assays were performed. The effect of T3 (10 μg/ml), T7 (5 μg/ml), and tumstatin (50 μg/ml) in inhibiting C-PAE proliferation was not affected by binding to vitronectin or provisional matrix (Fig. 7, panels B and C). Soluble vitronectin was additionally added at a concentration of 2.5 μg/ml at the same time as the T3 or T7 peptide to further saturate any α3β3 integrin that is unengaged to vitronectin on the apical side of the cells. These results suggest the binding site of T3 and T7 peptide on α3β3 integrin is distinct from binding domains for vitronectin and sufficient to induce inhibition of proliferation of endothelial cells.

**Effect of Tum-5 and Synthetic Peptides on Angiogenesis in Matrigel Plugs in C57BL/6Mice—**To evaluate the in vivo effect of synthetic peptide, T3, on the formation of new capillaries, we performed a Matrigel plug assay in mice as previously described (8). Tum-1, an active deletion mutant (8), inhibits neovascularization by 95% (Fig. 8, panel C). T3 peptide at 10 μg/ml (Fig. 8, panel E) reduced the number of blood vessels by 96% as compared with untreated controls (Fig. 8,
DISCUSSION

Angiogenesis is involved in various pathological disorders including diabetic retinopathy, rheumatoid arthritis, as well as tumor growth and metastasis (1). The on of the angiogenic switch requires both up-regulation of angiogenic stimulators and down-regulation of angiogenesis inhibitors (1, 3). Vascular endothelial growth factor and bFGF are among the major inducers of angiogenesis. To date, a number of angiogenesis inhibitors have been identified, and certain factors such as angiotatin (4), endostatin (5), canstatin (6), arresten (7), and tumstatin (8) are tumor-associated angiogenesis inhibitors which are potentially generated in vivo. We recently identified a novel anti-angiogenic protein domain derived from the α3 chain of type IV collagen and associated with vascular basement membrane (8). 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 (8). Tumstatin (α3(IV)NC1) binds to endothelial cells via αβ3 integrin, and this binding is speculated to influence its activity (19). The binding to αβ3 integrin is pivotal for the anti-angiogenic activity of tumstatin, and this activity is restricted to amino acids 54–132 of tumstatin (19). The αβ3 binding to tumstatin is mediated via a mechanism independent of the RGD-containing amino acid sequence and the binding of vitronectin and fibronectin (19). Recently, we recombinantly produced a 45–132-amino acid fragment of tumstatin (tum-5) in bacteria and yeast and this domain contained all of the anti-angiogenic activity of tumstatin in vitro and in vivo (19). In these studies, we show that the anti-angiogenic activity of tumstatin and tum-5 is not dependent on disulfide linkage (bonds), and we further confirm this observation in the present study in vivo using a human xenograft orthotopic breast cancer model. These in vivo experiments show that reduction and alkylation of tum-5 does not alter the potency of tum-5 to inhibit tumor growth in comparison to folded tum-5. MDA/MB435 tumors are one of the most resistant orthotopic tumors in mice to treatment and thus might explain the reduced potency of tum-5 in comparison to PC-3 (human prostate carcinoma) xenograft experiments reported earlier (20). Human endostatin at 20 mg/kg has no effect on MDA/MB435 xenograft orthotopic breast tumor in nude mice (data not shown) (20). This feature of tum-5, provided us with a synthetic peptide strategy to narrow the anti-angiogenic site within this 88-amino acid domain. Initially, we synthesized six different overlapping peptides and identified one peptide, T3 peptide, which had both the anti-angiogenic properties and the capacity to bind to αβ3 integrin. T3 peptide was able to inhibit the binding of endothelial cells to tumstatin. In addition, as recently shown for tumstatin and tum-5, αβ3 soluble protein was able to compete with cell surface αβ3 integrin and reverse T3 induced anti-proliferative effect. These findings strongly suggest that T3 binds to αβ3 and this interaction is pivotal for its anti-angiogenic activity. Although T3 peptide contained all of the activity of tumstatin, on a molar basis it was 2–5-fold less active than tumstatin and tum-5. To further investigate this issue, we induced a disulfide bond between the two cysteines present in this peptide. This did not improve the activity, as predicted by earlier experiments showing that disulfide bonds are not important for the anti-angiogenic property of tumstatin.

It is interesting to note that T3 peptide, while retaining the anti-angiogenic activity of tumstatin requires 10 amino acids from the T4 region to make it equivalent in potency to tumstatin at molar equivalent concentration. Although T4 peptide did not inhibit proliferation of endothelial cells, it exhibited weak binding to αβ3 integrin in these cells and facilitated adhesion of endothelial cells. This lead us to hypothesize that T4 may contain additional sequences that may facilitate optimal binding of T3 peptide to αβ3 integrin. It is possible that although we have been able to identify a 20-amino acid region which has the same anti-angiogenic activity as tumstatin, truncation may have resulted in the loss of external sequences which potentially dictate optimal binding criteria for this molecule to functionally engage cell surface αβ3 integrin on endothelial cells. Conceivably, a lack of such optimal docking specificity results in the need for many more molecules of T3 peptide to sufficiently engage the αβ3 integrin on endothelial cells, operating through just random collision and potentially less affinity of interaction.

Synthetic peptides (amino acids 185–203) derived from the NC1 domain of the α3 chain of type IV collagen (α3(IV)NC1), have been shown to inhibit the proliferation of melanoma cells in vitro (33) and have been found to bind to αβ3 integrin and CD47/CDAP (34). This peptide domain was further identified to bind to a β3 integrin subunit site distinct from the RGD recognition site using HT-144 melanoma cells (35). Comparative analysis of this sequence with our peptide does not reveal any significant homology. Their studies coupled with our findings suggest that αβ3 integrin may bind to these peptides on distinct sites (20, 35).

In vivo experiments in C57BL/6 mice using Matrigel plugs show that peptide T3 is effective in inhibiting growth factor-induced neovascularization. Our studies also show that T3 is equally effective as tumstatin or tum-5 (8, 19). Future experiments with xenograft and syngeneic mouse tumor models will address issues of half-life and pharmacokinetics of the T3/T7 peptides. In comparison to proteins, peptides may have a less favorable half-life, but are very easy to manufacture. In the context of drug development, these issues need further exploration. Our experiments to understand the mechanism of action of T3 peptide are consistent with previous studies with tumstatin which document endothelial cell-specific apoptosis, associated with G1 arrest of the endothelial cell cycle. Whether cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, and transcription factors such as E2F are involved (36), needs further investigation. Identification of a 20-amino acid peptide from tumstatin with a capacity to regulate angiogenesis will potentially make future translational and mechanistic experiments highly feasible.

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