Thrombomodulin Modulates Growth of Tumor Cells Independent of its Anticoagulant Activity

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Abstract

Thrombomodulin (TM), recognized as an essential vessel wall cofactor of the antithrombotic mechanism, is also expressed by a wide range of tumor cells. Tumor cell lines subcloned from four patients with malignant melanoma displayed a negative correlation between TM expression and cell proliferation in vitro and in vivo. Overexpression of wild-type TM decreased cell proliferation in vitro and tumor growth in vivo. TM mutants with altered protein C activation capacity lead to a similar effect. In contrast, transfection of melanoma cells with mutant TM constructs, in which a portion of the cytoplasmic or lectin domain was deleted, abrogated the antiproliferative effect associated with overexpression of wild-type TM. Experiments performed with either peptide agonists/antagonists of the thrombin receptor, with hirudin, or with inhibitors of thrombin–TM interaction did not alter the growth inhibitory effect of TM overexpression. These data suggest that TM exerts an effect on cell proliferation independent of thrombin and the thrombin receptor, possibly related to the binding of novel ligands to determinants in the lectin domain which might trigger signal transduction pathways dependent on the cytoplasmic domain. (*J. Clin. Invest.* 1998. 101:1301–1309.) Key words: coagulation • proliferation • thrombomodulin • tumor

Introduction

Thrombomodulin (TM),[1] an integral membrane glycoprotein, binds the final enzyme of the procoagulant pathway, thrombin, forming a 1:1 complex (1–7). The resulting thrombin-TM complex is the critical physiologic activator of protein C. Although initially TM was characterized on endothelium (8–10), where its antithrombotic properties were clearly associated with maintenance of blood fluidity, subsequent studies have shown broad expression including on syncytiotrophoblasts (10), platelets (11), megakaryocytes (12), monocytes (13), neutrophils (14), synovial lining cells (8), smooth muscle cells (15, 16), keratinocytes (17), meningeal cells (18), and tumor cells (19–24).[2] During mouse development, TM is expressed in extraembryonic placental tissues, in the developing cardiovascular system, airway epithelia, cartilage, and in restricted areas of the brain (26–30). The analysis of TM-deficient knockout mice has demonstrated that expression of TM in the developing placenta is necessary for embryonic survival (30, 31). Homozygous TM-deficient mice die before the cardiovascular system develops (30), at a time when thrombin, its recognized ligand, is not present. This raises the possibility that TM possesses functions distinct from those related to hemostatic regulation. Previous clinical findings in patients with hepatocellular carcinoma (22), ovarian cancer,[2] and esophageal squamous cell carcinoma (24) have indeed implicated a negative correlation between the expression of TM antigen and tumor cell proliferation.

These observations led us to postulate possible relationships between TM and cell proliferation in vitro and in vivo.

Subcloned human melanoma cells showed a negative correlation between TM expression and cell proliferation. The possible relationship between TM and cell proliferation is further supported by results with transfectants overexpressing wild-type TM. Transfection of melanoma cells with mutated TM constructs indicated that the effect of TM on proliferation requires intact cytoplasmic and extracellular NH_2-terminal lectin domains. The growth inhibitory function of TM was not mediated through the inactivation of thrombin or the generation of the potent anticoagulant, activated protein C. These data lead us to propose that TM regulates cellular functions, such as proliferation, through a previously unrecognized mechanism.

Methods

Human melanoma cells. Tumors were taken from four patients with histologically proven malignant melanoma. Tumor cells were cul-

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Received for publication 16 June 1997 and accepted in revised form 14 January 1998.

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; S, sense; SFM, serum-free medium; TM, thrombomodulin; V, vector.

2. Wilhelm, S., M. Schmitt, J. Parkinson, W. Kuhn, H. Graeff, and O. Wilhelm, manuscript submitted for publication.

J. Clin. Invest.

0021-9738/98/04/1301/09 $2.00
Volume 101, Number 7, April 1998, 1301–1309
http://www.jci.org

tured and subcloned by limiting dilution. Clones of tumor cells were cultured in RPMI 1640 medium containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2.

Determination of TM. Total human TM antigen expression in cultured human melanoma cells was determined by using an ELISA kit (Diagnostica Stago, Asnieres-Sur-Seine, France).

Total mouse TM antigen expression in mouse F9 teratocarcinoma cells was determined using an RIA as published previously (32).

The functional activity of TM expressed by stable transfectants was determined by a two-stage protein C activation assay.[2] Briefly, 2.5×10^5 transfected B16 melanoma cells in each well of 24-well plates were washed twice with HBSS (20 mM Tris/HCl, pH 7.4, 0.15 M NaCl, 3 mM $CaCl_2$, 0.5% BSA). 40 nM of human thrombin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) plus 3 μM protein C (Sigma-Aldrich Chemie GmbH) in 250 μl HBSS were added into each well and incubated at 37°C for 90 min. The reaction was stopped by adding 5 U of hirudin (Sigma-Aldrich Chemie GmbH). A standard curve using serial dilutions of activated protein C (range 0–0.8 μM) and controls omitting one of the components ($CaCl_2$, TM, thrombin, or protein C) were assayed identically. 150 μl of the reaction mixture from each well was transferred into a 96-well plate. 50 μl of 0.6 mM chromogenic substrate (Spectrozyme Pca, American Diagnostica, Greenwich, CT) (final concentration 0.15 mM) was added and the OD was recorded at 405 nm.

The TM antigen expressed by stable transfectants was also determined in Western blots using the methods described previously (33, 34). The polyclonal antibody against mouse TM was raised in rabbits using purified TM from mouse lungs.

Distribution of TM in stable transfectants was determined by immunofluorescent staining. Briefly, cells were cultured in slide chambers (Nalge Nunc International, Naperville, IL). After being washed with PBS, cells were fixed and permeabilized by acetone/methanol (1:1) at 4°C for 2 min. After washing three times with PBS, cells were blocked with 1% BSA (Sigma-Aldrich Chemie GmbH) and 0.02% saponin (Calbiochem Corp., San Diego, CA) at room temperature for 3 h. Cell samples were then incubated with monoclonal rat anti–mouse antibody (26) overnight at 4°C. Afterward, slides were washed with PBS and incubated with Cy3-conjugated donkey anti–rat (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:600 in blocking buffer. The reaction was incubated in darkness for 2 h at room temperature. After the final wash with PBS, slides were dried and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed with a confocal laser microscope (Leica Laser-technic, GmbH, Heidelberg, Germany).

Induction of TM in F9 cells. For induction of TM, F9 teratocarcinoma cells were cultured with 0.5 mM dibutyryl-cAMP (Sigma-Aldrich Chemie GmbH) and 0.5 mM theophylline (Sigma-Aldrich Chemie GmbH) (32) in DME supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. For TM RIA, 5×10^6 cells were seeded in T25 flasks 12 h before harvest. Cells were washed twice with PBS. Cells were then lysed in 0.7 ml of lysis buffer (0.5% NP-40, 1 mM NaN_3, and freshly added 7 μl of PMSF in PBS) and centrifuged at 10,000 rpm for 2 min at 4°C. The cell lysate was then homogenized for 30 s and pelleted by centrifugation at 10,000 rpm for 2 min at 4°C. The protein concentrations of the supernatants were determined and the supernatants were immediately frozen in liquid nitrogen until use.

Determination of [^{3}H]thymidine incorporation. To determine [^{3}H]thymidine incorporation 1×10^6 untransfected cells were seeded into 35-mm dishes or, in the case of stable transfectants, 2.5×10^5 cells were seeded into 24-well plates. Cells were seeded 12 h before addition of [^{3}H]thymidine (74.0 GBq/mmol, 2 Ci/mmol) (Amersham Buchler GmbH and Co. KG, Braunschweig, Germany) (3 μCi to 35-mm dishes, 0.5 μCi to 24-well plates). After 2 h cells were washed twice with 0.9% NaCl, scraped, and suspended in 10% TCA (35). The precipitate was washed twice with 10% TCA. After an overnight incubation in scintillation solution, radioactivity was determined in a β-counter (model LS6000; Beckman Instruments, Inc., Fullerton, CA).

Determination of cell proliferation. 4,000 cells (stable transfectants or subcloned human wild-type melanoma cells from four patients) were seeded into 24-well plates in RPMI 1640 supplemented with 10% FCS. Stable transfectants were cultured in the presence of 250 μg/ml of hygromycin B. At the indicated time points cells were trypsinized and counted.

For testing the role of thrombin in proliferation of stable transfectants 4,000 transfected tumor cells were seeded into 24-well plates in RPMI 1640, 10% FCS, and 250 μg/ml of hygromycin B. Either 0.5 μg/ml of basic fibroblast growth factor (FGF) (Sigma-Aldrich Chemie GmbH), 5 U/ml of hirudin (Sigma-Aldrich Chemie GmbH), 100 μg/ml of SFLLRN (thrombin receptor activating peptide) (Bachem Biochemica GmbH, Heidelberg, Germany) (36), 100 μg/ml of YFLLRNP peptide (Bachem Biochemica GmbH) (37), or 150 μg/ml of B 147–158 peptide (Bachem Biochemica GmbH) (38) was added. The number of cells were counted after 7 d.

SFLLRN (thrombin receptor activating peptide) (36) corresponding to residues 42–47 of the thrombin receptor has been shown to be a thrombin receptor activator. YFLLRNP is an antagonist to α-thrombin (37) and to the thrombin receptor agonist peptide SFLLRNP in human platelets. The thrombin B 147–158 peptide (38) corresponds to amino acids 147–158 of the B chain of human thrombin. It has been shown to inhibit the thrombin binding to TM with $K_i = 94$ μM (∼ 120 μg/ml).

Cloning and expression of TM and TM mutants. pREP4 (Invitrogen, Leek, The Netherlands) is an episomal mammalian expression vector (V) that uses the Rous sarcoma virus long terminal repeat enhancer and promoter for transcription of recombinant genes inserted into its multiple cloning site. The Epstein-Barr virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) are carried by these plasmids to permit extrachromosomal replication in mammalian cell lines. pREP4 also carries the hygromycin B resistance gene for stable selection of transfected cells (39–41). Full-length mouse TM cDNA (a gift from Dr. Dittman, Washington University School of Medicine, St. Louis, MO) (42) was subcloned into pREP4 V to form the sense (S) construct.

B16 melanoma cells were stably transfected with DNA constructs encoding mutated forms of the receptor. The mutant *Pro387* (= M1) contains a single amino acid substitution (Glu387 to Pro), that has been shown to dramatically diminish the receptor's cofactor activity in the activation of protein C (43). Replacement of methionine 388 by leucine in the mutant *Leu388* (= M2) has been shown to increase the TM cofactor activity by approximately twofold (43). In the mutant Δ*lectin* (= M3), an evolutionary conserved portion (46–147 aa) of the NH_2-terminal, putative lectin-like TM domain was deleted. Finally, to generate the mutant Δ*cyto* (= M4), the cytoplasmic domain and part of the membrane spanning region (508 aa to the end of protein sequence) were replaced by a short synthetic membrane anchor (Arg-Pro-Arg-Leu-Gly-Ser-Gly) (44).

Preparation of stable transfectants. Mouse B16 melanoma cells (Tumor Bank, German Cancer Research Center, Heidelberg, Germany) were cultured in RPMI 1640 supplemented with 10% FCS. In vitro transfection was performed as described previously (34, 45). After reaching 50–70% confluency, cells were washed three times with serum-free medium (SFM), and 3 ml of SFM was added to each T25 flask. 200 μl lipofectin/DNA complex containing 30 μg of lipofectin (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany) and 5 μg of DNA were dropped into T25 flasks while gently shaking. The cells were incubated at 37°C in 5% CO_2 in an incubator for 12 h and then 3 ml of medium with 20% FCS was added into flasks. After 2 d, the cells were seeded into T75 flasks and cultured in medium with 10% FCS and 0.25 mg/ml of hygromycin B (46). The cells were washed and incubated every 3 d with fresh medium containing 0.25 mg/ml of hygromycin B. About $3–5 \times 10^2$ colonies of transfected cells appeared after 7–10 d of selection with hygromycin B. The colonies were trypsinized and cultured until confluent in the presence of hygromycin B. The selected stable transfectants were characterized by the protein C activation assay (7)[2] and Western blots as described

previously (34). The anti–mouse TM antiserum used in Western blots was raised from a rabbit.

Tumor growth in vivo. For in vivo experiments 10^6 cells in 100 μl PBS, pH 7.4, were injected intracutaneously. Tumor sizes were recorded every 2 d by measuring the two largest diameters. Tumor weights were determined immediately after excision at day 14 (34). $C_{57}BL_6$ (2×24) mice were used for the study of B16 melanoma transfectants.

To study the in vivo growth of subclones of human melanomas, BALB/C/Nu female mice, 6 wk old, were used. Tumor inoculation began 10 d after the animals were delivered to our animal center as described for B16 melanoma transfectants.

Determination of blood flow. For the measurement of blood flow in mice, E-Z Trac Ultrasphere™ (E-Z Trac, Inc., West Los Angeles, CA) was used. Briefly, 5×10^5 colored microspheres were injected into the left ventricle of the anesthetized mouse over 10–20 s with 1 ml of PBS. The tumors were harvested and weighed. Then, 4 ml of 1:1 diluted tissue/blood digest reagent I (E-Z Trac, Inc.), a strong alkaline solution, was added to each sample. The tubes containing tumor tissues were placed in a heated water bath at 80°C to hydrolyze the tumors overnight. The next day, the tubes with tightened screw caps were vigorously vortexed for ~ 30–60 s until the tissue was completely homogenized with only small particles of fatty white debris visible. Diluted tissue/blood digest reagent II (E-Z Trac, Inc.) was added to the sample suspension to bring the total liquid volume to 14–15 ml. After mixing by inversion, the tubes were centrifuged for 30 min at 1,500 *g* and the supernatant was aspirated to a level slightly above each pellet. Each greenish-brown sediment was resuspended in 10 ml of diluted microsphere counting reagent (E-Z Trac, Inc.). After centrifugation for 15 min at 1,500 *g*, the supernatant was aspirated to a level slightly above the visible pellet. The pellet was suspended in 100 μl counting reagent, and 10 μl of thoroughly mixed solution was used for counting. The number of colored microspheres in the final tissue preparations was determined using a hemocytometer counting slide.

VEGF antigen levels were determined by using reagents from Tebu GmBH (Frankfurt, Germany).

Statistical analysis. All values are given as mean±SD. Means of groups were compared with ANOVA using the Newman-Keul's test to correct for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

TM expression correlates with cell proliferation. Tumor tissue was obtained from four patients with malignant melanoma. From these samples, 86 monoclonal tumor lines were established by limited dilution subcloning. For each tumor cell line, we then determined the rate of in vitro proliferation by measuring the increase in cell number over a given time period and by determining the amount of [^{3}H]thymidine incorporation in actively replicating cells. The level of TM expression in each line was simultaneously determined using a specific ELISA. A negative correlation was noted between cell proliferation, evaluated by either change in cell number (Fig. 1 *a*; $P < 0.0001$, $r = -0.445$) or incorporation of [^{3}H]thymidine (Fig. 1 *b*; $P < 0.0001$, $r = -0.541$), and the level of TM antigen. Cells expressing more TM displayed diminished proliferation. A similar result was obtained when cells from each tumor were analyzed separately, or when cells from all tumors were analyzed together.

To further analyze a possible relationship between TM and cell proliferation, F9 teratocarcinoma cells were studied after induction of TM by elevating intracellular cAMP. After addition of db-cAMP and theophylline, increased TM antigen was paralleled by a decrease in [^{3}H]thymidine incorporation (Fig. 1 *c*). These data were consistent with an inverse relationship between TM expression and cell proliferation. The experiments were repeated in SFM with similar results to exclude the possibility that soluble TM present in the FCS might affect the results.

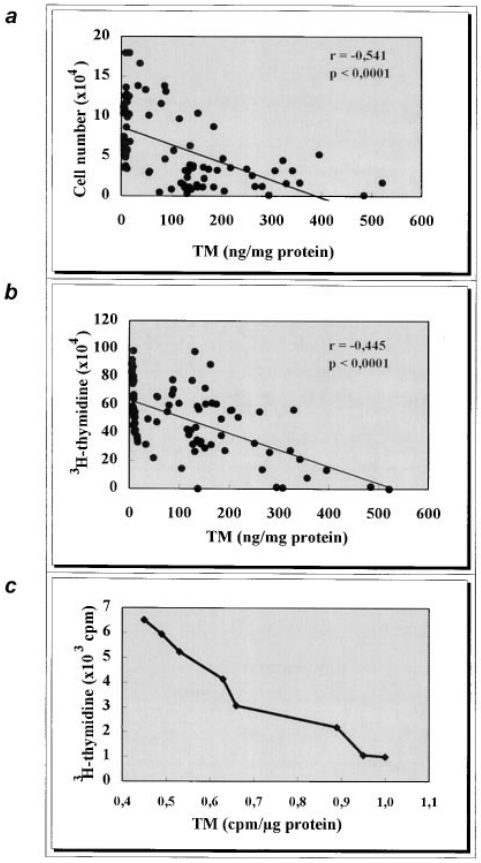

Figure 1. Correlation of TM antigen level and cell proliferation in melanoma subclones (*a* and *b*) and in teratocarcinoma cells treated with db-cAMP and theophylline (*c*). Human melanoma cell subclones (4,000 cells/well) were seeded in 24-well plates (*a* and *b*). Either the cell number after 5 d (*a*; $P < 0.001$, $r = -0.445$, $n = 86$) or the [^{3}H]thymidine incorporation 2 h after addition of [^{3}H]thymidine (0.5 μCi/ml) to each well (*b*; $P < 0.0001$, $r = -0.541$, $n = 86$) was determined. F9 teratocarcinoma cells were cultured in medium supplemented with db-cAMP (0.5 mM) and theophylline (0.5 mM); [^{3}H]thymidine incorporation and TM antigen (by ELISA) were measured at the same time (cell density was the same in each well, corresponding to ~ 70% confluence) (*c*). Each point represents the mean of six wells and the experiments were repeated twice with similar results (*a–c*).

Generation and characterization of stable B16 melanoma cell transfectants. The above data indicated a possible link between the amount of TM expression and in vitro tumor cell proliferation. Therefore, we investigated whether the reduction in tumor cell growth is caused by the increase in TM expression. To determine whether TM directly affects cell proliferation, stable transfected lines of B16 melanoma cells were prepared expressing wild-type or mutant forms of TM, using the V pREP4 to achieve overexpression. Six constructs were utilized: wild-type, full-length TM cDNA (S); V alone; TM into which point mutations were introduced in the thrombin

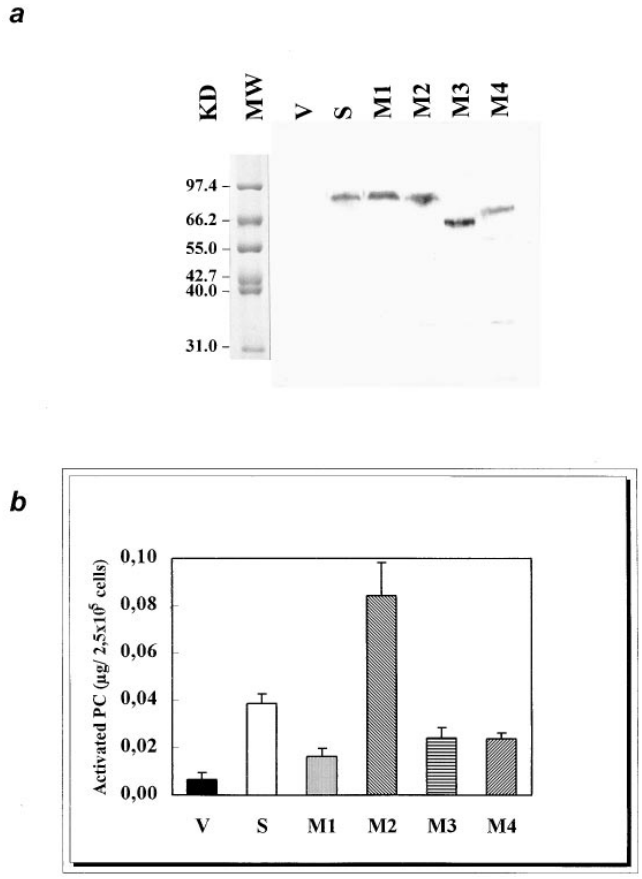

Figure 2. B16 melanoma cells stably transfected with TM constructs: expression of TM antigen (*a*) and thrombin-mediated protein C activation (*b*). Immunoblotting was performed on each of the B16 melanoma transfectants (V, S, M1, M2, M3, and M4) by preparing cell lysates and loading 10 μg/lane of total protein onto nonreduced SDS-PAGE (*a*). Migration of simultaneously run molecular weight standards (MW) is shown in kD on the left. V, vector, S, sense, M1, *Pro387*, M2, *Leu388*, M3, Δ*lectin,* and M4, Δ*cyto*. Stably transfected melanoma cells (2.5×10^5/well of a 24-well plate) were incubated with thrombin and protein C, to allow formation of activated protein C (*b*). Then, hirudin was added to inhibit thrombin and activated protein C was quantified by chromogenic assay. Each point is the mean±SD of 5 determinations.

binding site resulting in either diminished (*Pro387*-M1) (43) or enhanced (*Leu388*-M2) (43) thrombin-mediated protein C activation; TM with deletion of a portion of the lectin domain (aa 47–146; Δ*lectin*-M3); and, deletion of most of the cytoplasmic domain (aa 509 to the COOH-terminus; Δ*cyto*-M4) (44). Cells derived from more than 200 colonies and obtained from at least three independent transfections were used in the experiments.

TM expression in the parental untransfected cell line (data not shown) and stably transfected cells was analyzed by Western blot analysis (Fig. 2 *a*) and by determining the amount of cell surface associated TM cofactor activity in the thrombin-dependent protein C activation (Fig. 2 *b*). Whereas vector–transfected control melanoma cells demonstrated background levels of TM, the transfectants showed increased amounts of TM: a band with ~ 90,000 M_r was observed with S, M1, and M2; and more rapidly migrating bands were observed with the truncated TM forms M3 (~ 60 kD) and M4 (~ 70 kD) (Fig. 2 *a*). Thrombin-mediated protein C activation was minimal on V-transfected control melanoma cells (V) and greatest on mutant M2 which displays enhanced thrombin binding (Fig. 2 *b*). S-TM transfectants (S) showed enhanced protein C activation compared with other mutations, with diminished TM affinity for thrombin (M1), the lectin domain truncated (M3), or the cytoplasmic domain truncated (M4). These data are consistent with previous observations concerning the expressed mutant forms of TM, with respect to antigen expression and protein C activation (43, 44). Confocal laser microscopy demonstrated that in all transfectants studied TM is expressed in the cell membrane, while only minor staining was observed in the cytoplasm (Fig. 3). Thus there was no significant change in TM surface expression in the TM transfectants studied.

These data indicated that stably transfected B16 melanoma lines expressing different forms of TM were available to study the effect on cell proliferation in vitro, and tumor growth in vivo. TM transfectants were also made in murine methylcholanthrene A (Meth-A)-induced fibrosarcoma and F9 teratocarcinoma cells with similar properties (data not shown).

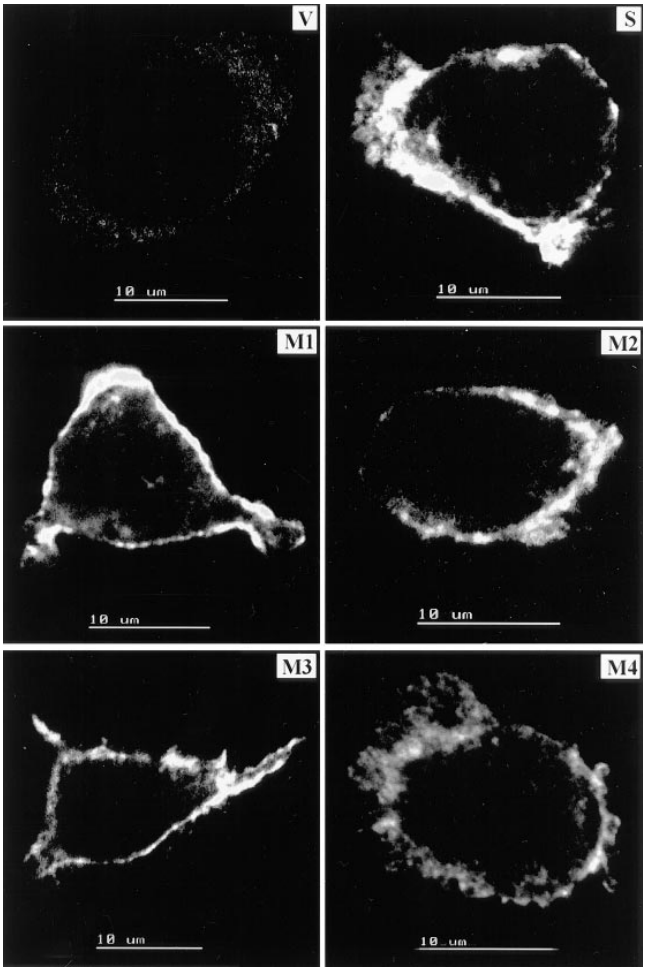

Figure 3. Distribution of TM in stable transfectants. The subcellular distribution of TM in the different stable transfectants was studied by confocal laser microscopy. Cells were stained and analyzed as described in Methods. Abbreviations are as in Fig. 2 *a.*

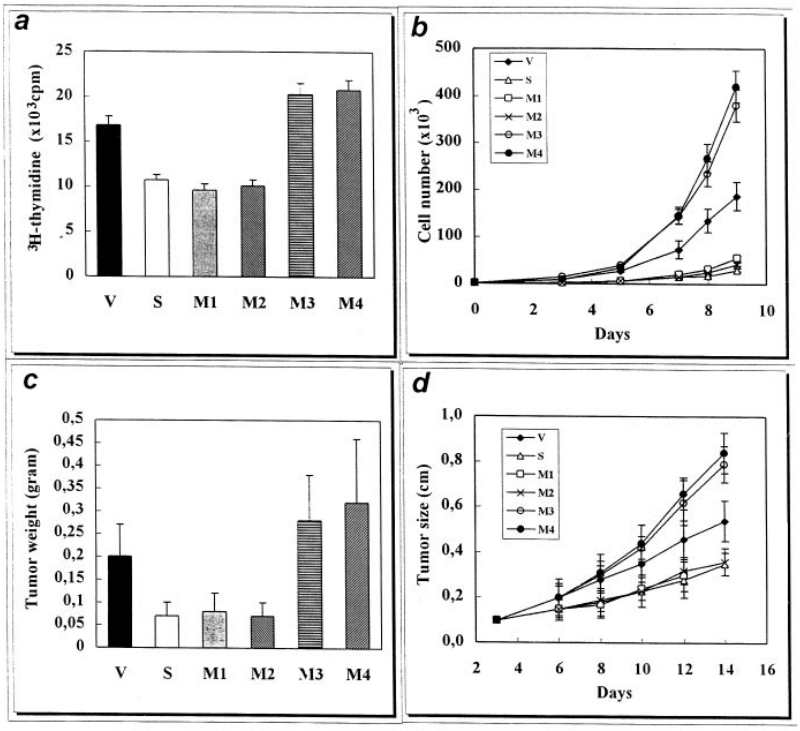

Figure 4. Characterization of TM-transfected B16 melanoma cells: cell proliferation in vitro (*a* and *b*) and tumor growth in vivo (*c* and *d*). Increase in [^{3}H]thymidine incorporation into DNA (*a*, 4,000 cells/well) or cell number (*b*, 2.5×10^5 cells/well with 0.5 μCi/ml of [^{3}H]thymidine) of B16 melanoma transfectants grown under selection conditions. Experiments were repeated three times and the mean±SD of six determinations is shown. S, M1, or M2 vs. V was $P <$ 0.001 in *a*, and $P < 0.01$ in *b*. M3 or M4 vs. V was $P <$ 0.05 in each figure. Tumors were grown in mice following subcutaneous inoculation of each of the indicated transfectants (10^6 cells/mouse) (*c* and *d*). Tumor weight (*c*) and size (*d*) were determined (weight was measured on day 14). In each case, data show mean±SD of eight tumors in each group, and the experiment was repeated twice. Statistical analysis: $P < 0.03$ after day 8 comparing size in S, M1, and M2 with V (*c*); $P < 0.05$ after day 10 comparing tumor size in M3 and M4 vs. V (*c*); $P < 0.001$ comparing weights in S, M1, and M2 with V (*d*); and $P < 0.005$ comparing tumor weight in M3 and M4 vs. V. Abbreviations are as in Fig. 2 *a*.

Overexpression of TM reduces murine tumor cell growth in vitro and in vivo independent of activated protein C-cofactor activity. Proliferation of stable TM-transfected B16 melanoma cell lines was studied by either directly assessing the incorporation of [^{3}H]thymidine (Fig. 4 *a*) or increase in cell number (Fig. 4 *b*). Both of these indices of cell division were markedly suppressed in M1, M2, and S transfectants (Fig. 4, *a* and *b*), which expressed the highest amounts of TM antigen (Fig. 2 *a*), though M1 showed the lowest, M2 the highest, and S intermediate levels of protein C activation (Fig. 2 *b*). In contrast, M4 and M3 displayed the greatest proliferation and V alone was intermediate, between the high- (M4, M3) and low- (S, M1, M2) growing transfectants. These data demonstrated a correlation between TM antigen and cell growth which was independent of thrombin-mediated protein C activation. However, the lectin domain and cytoplasmic domains were important in mediating TM effects on cell proliferation. To control for possible nonspecific effects of transfection/selection on cell proliferation, similar studies were performed with B16 melanoma cells stably overexpressing tissue factor; in the latter case, there was no effect on cell proliferation (data not shown). In addition, comparable results with respect to proliferative capacity of TM-transfected cell lines were obtained when Meth-A and F9 cells were used in place of B16 melanoma cells (data not shown).

The results strongly suggested that TM was a negative regulator of in vitro tumor cell proliferation. We then asked whether the observed effects of TM expression on the in vitro proliferation of B16 melanoma cells would also determine the growth of these tumor cells in vivo. Stably transfected TM melanoma lines were used to initiate tumors in mice. Tumor weight (Fig. 4 *c*) and size (Fig. 4 *d*) were greatest in tumors arising from M3 and M4 transfectants, intermediate in V transfectants, and lowest in S, M1, and M2 transfectants. Histologic examination demonstrated similar general morphologic features of each of the tumors except for differences in tumor size. These data indicate that changes in TM expression do not appear to cause overt tumor necrosis, hemorrhage, or thrombosis.

When high and low TM producer clones (Fig. 1, *a* and *b*) from the four patients were studied in nude mice, a larger tumor size (Fig. 5 *a*) and tumor weight (Fig. 5 *b*) were seen in low TM producers (*A* and *B*), while high TM producers (*C* and *D*) grew slower. Therefore, the effect was not only observed in stable transfectants but also in naturally occurring cells.

An effect of TM on angiogenesis is unlikely, since staining of vessels using indian ink revealed no differences between the various transfectants (data not shown). Furthermore, microbeads were used to quantitate vascularization and blood flow (34). In V transfectants 4,130±1,340 (mean±SD) microbeads and in S transfectants 4,210±1,260 microbeads per gram of tumor tissue were found. These data suggested that the growth of transfectants in vivo was independent of the vascularization of the tumor (Fig. 6 *a*). Consistently, we did not observe a correlation between TM expression and VEGF expression in naturally occurring high and low TM producers isolated from four patients (Fig. 6 *b*).

TM-mediated reduction of cell proliferation is independent of thrombin–thrombin receptor interactions. To further clarify the relationship between the TM-dependent suppression of tumor growth and other control mechanisms, the proliferation of tumor cell lines overproducing either normal TM or the receptor mutants was analyzed in response to growth factors and specific modulators of thrombin-dependent signaling pathways (Table I).

Addition of basic fibroblast growth factor (bFGF) increased cell growth of each transfectant, but the same relationship of proliferation of the TM transfectants to each other was

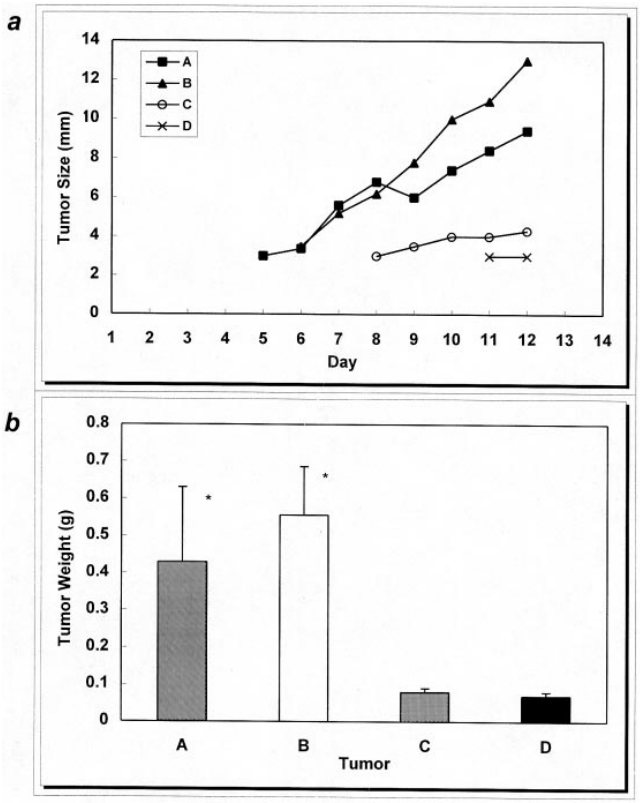

Figure 5. In vivo tumor growth of subcloned human melanoma cells. Subcloned human melanoma cells were cultured in RPMI medium supplemented with 10% FCS (*a* and *b*). Actively growing cells from four different cell lines with either low TM (*A* and *B*) or with high TM (*C* and *D*) were harvested. After being washed two times with PBS, cells were suspended in PBS at a concentration of 10^7 cells/ml. Subsequently, 10^6 cells from each cell line were injected intracutaneously into the flank regions of BALB/C/Nu mice. Tumor sizes (*a*) were measured daily and tumor weights (*b*) were determined 12 d after tumor innoculation. No tumor growth was observed in high TM-expressing cell lines after days 8–11 while low TM-expressing cell lines grew earlier (days 5–6) and faster. Statistical analysis: $P = 0.0045$ when tumor sizes were compared between low TM producers (*A* and *B*) and high TM producers (*C* and *D*) at day 5; $P < 0.001$ when tumor weights were compared between low and high TM producers at day 12.

maintained; M3 and M4 showed the greatest increase in cell number, V showed intermediate levels and S, M1, and M2 displayed the least growth (Table I).

The likelihood that thrombin interaction with TM was not mediating effects attributable to TM on proliferation was confirmed by the lack of any major changes in cell proliferation when this interaction was blocked; a peptide corresponding to the B chain thrombin binding site (aa 147–158) (38) for TM did not have a major effect on the growth of the TM transfectants (Table I).

The possible contribution of thrombin binding to the thrombin receptor was studied using blocking (YFLLRPN) (37) and stimulatory (SFLLRN) (36) peptides derived from the thrombin receptor. As expected, the thrombin receptor agonist peptide enhanced overall proliferation, and the inhibitory peptide had an inhibitory effect. However, the basic relationship between cell proliferation of the TM transfectants was unchanged (Table I).

Finally, the effect of the thrombin inhibitor hirudin was examined. In view of the known stimulatory effect of thrombin on cell growth, as well as the data above with YFLLRNP and SFLLRN, the observed inhibitory effect of hirudin on overall proliferation was expected. However, the relationship of different TM-transfectants to proliferation was again maintained (Table I).

These data indicated that the effect of TM on cell proliferation was not mediated by changes in thrombin binding to TM, or thrombin stimulation of the thrombin receptor. Furthermore, the pathway was likely to be independent of that triggered by a growth factor such as bFGF.

Discussion

Thrombin-independent actions of TM have been hypothesized to explain embryonic lethality in homozygous TM −/− mice (31), and the early expression of TM (previously termed fetomodulin [27–29]) during embryonic development. These data suggest the likely existence of functions of TM beyond that involved in homeostatic regulation of the coagulation mechanism. We show that high levels of TM expression are in fact associated with a reduced potential for proliferation in vitro. We

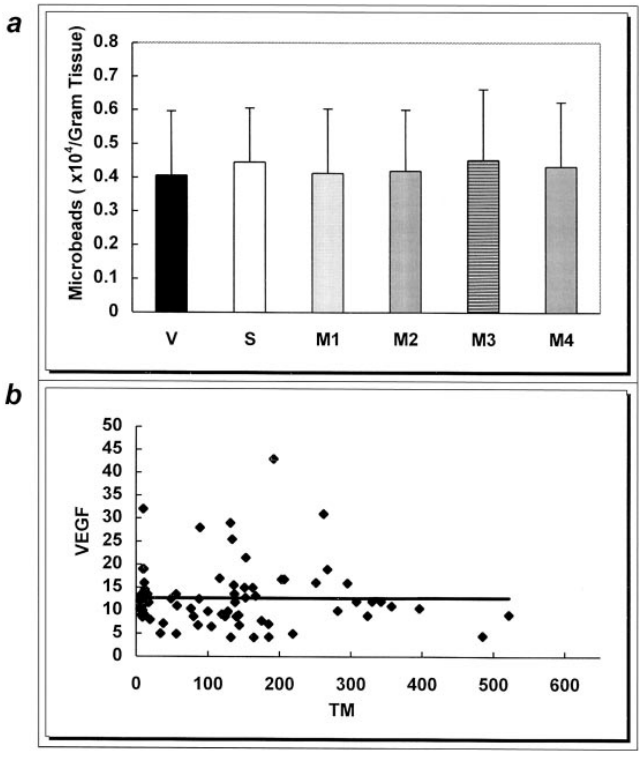

Figure 6. Determination of free blood flow. Free blood flow was estimated by using 10 μm E-Z TRAC ultraspheres (E-Z Trac, Inc.). 5×10^5 of the 10-μm microspheres were injected into the left ventricle of the mouse and tumor tissue was harvested after 5 min. After dissolving the tumor tissue, the beads present in the tumor were counted and presented as beads per gram of tumor tissue (*a*). The figure shows mean±SD of eight tumors in each group. Abbreviations are as in Fig. 2 *a.* Correlation of TM antigen level and VEGF antigen level in subcloned human tumor cells ($r \sim 0$) (*b*).

Table I. Proliferation of Stable Transfectants In the Presence of Different Conditions

	Proliferation of TM transfectants (cell number)					
	No addition	bFGF	B 147–158	YFLLRNP	SFLLRN	Hirudin
V	51300	66400	52200	41300	65900	40400
S	10300	14300	10500	7600	14100	7500
M1	11400	15000	11400	8100	14900	8300
M2	9800	14100	10200	7600	14300	7400
M3	73400	88700	72500	61800	87700	60900
M4	65400	79700	64400	55400	78600	54300

	Relative proliferation of TM transfectants					
	No addition	bFGF	B 147–158	YFLLRNP	SFLLRN	Hirudin
V	100%	100%	100%	100%	100%	100%
S	20%	22%	20%	18%	21%	19%
M1	22%	23%	21%	20%	23%	21%
M2	19%	21%	20%	18%	22%	18%
M3	143%	134%	139%	150%	133%	151%
M4	127%	120%	123%	134%	119%	134%

4,000 cells per well were seeded in a 24-well plate. Cells were cultured in the presence of hygromycin B with either bFGF (0.5 μg/ml), hirudin (5.0 U/ml), SFLLRN (100 μg/ml), YRLLRNP (100 μg/ml), or B147–158 peptide (150 μg/ml). The numbers shown represent the number of cells in a 24-well at day 5 after seeding. The experiment was repeated three times in duplicate ($n = 6$). Abbreviations are as in Fig. 2 *a*.

then altered the amount of surface-associated receptor expression in murine B16 melanoma cells, as well as in two additional murine tumor cell types, and monitored the ensuing effects on proliferation. In each case, we were able to document a growth suppressing effect of TM and thus established a cause–effect relationship between the increased production of TM and the reduced growth of tumor cells. More importantly, it is shown that increased expression of TM not only reduces proliferation in vitro, but also results in the diminished growth of tumor cells in vivo.

Three lines of evidence provide a foundation for our hypothesis that TM is a negative regulator of cell proliferation. First, subcloned melanoma cells from four patients showed a negative correlation between cell proliferation and expression of TM antigen. Second, enhanced expression of TM in F9 cells, induced by elevation of intracellular cAMP, also suppressed cell division. Finally, overexpression of functionally active TM in B16 melanoma cells inhibited cell proliferation in vitro, and suppressed tumor growth in vivo. Similar results were obtained with TM-transfected Meth-A and F9 cells (data not shown). These observations concerning the relationship between TM and cell growth are consistent with clinical findings in patients with hepatocellular carcinoma (22), ovarian cancer,[2] and esophageal squamous cell carcinoma (24). Further studies will be required to determine whether TM also has growth regulatory effects on nontumor cells, such as smooth muscle and other cells which express TM (15, 16).

To determine the precise molecular mechanism through which TM modulates tumor growth, we delineated the critically involved domains of TM by overexpressing TM variants carrying mutations in defined regions of the receptor. Surprisingly, our results showed that the tumor suppressive effect of TM apparently does not correlate with the receptor's anticoagulant cofactor activity. Overexpression of the cofactor activity–deficient mutant TM *Pro387* (= M1) had an almost identical inhibitory effect as the normal receptor. A detailed kinetic analysis of *Pro387*-mediated protein C activation indicated a 1.6-fold increase in the K_m for protein C, a 20-fold increase in the K_d for thrombin, and a 5.7-fold reduction in V_{max} compared to mouse wild-type TM (Weiler-Guettler, H., unpublished data). Together, these changes result in a 182-fold increase in cofactor activity in the presence of physiological concentrations of thrombin and protein C. The examination of receptor variants appears, therefore, consistent with the notion that the tumor growth regulatory effect of TM is not mediated through a mechanism involving the thrombin-dependent activation of protein C.

We subsequently investigated to what extent thrombin/thrombin receptor–dependent signaling pathways control the proliferation of B16 melanoma cells and how TM interferes with this mechanism. Our findings indicate that thrombin may indeed promote the growth of B16 cells through activation of the thrombin receptor. The growth stimulation achieved by thrombin was of comparable magnitude as observed with bFGF. However, neither the antiproliferative effect of TM overexpression nor the relative differences between B16 melanoma lines expressing receptor mutants could be abolished by the inhibition of thrombin by hirudin, direct activation or blocking of the thrombin receptor, or by inhibition of the thrombin–TM interaction. From these observations, it appears highly unlikely that the growth modulatory effect of TM depends on the binding and/or inactivation of thrombin. It is also evident that expression of TM or mutated receptor forms modulates tumor cell proliferation over a significantly wider range than could be achieved by bFGF and regulators of thrombin receptor–mediated signaling.

Interestingly, we have recently obtained evidence that replacement of the endogenous TM gene with the mutant *Pro387* receptor in transgenic mice was compatible with embryonic development and hence resulted in the generation of viable animals with a dramatically reduced ability to activate protein C (Weiler-Guettler, H., manuscript in preparation). It is tempting to speculate that the unsuspected activity of TM in tumor cells described above may also be involved in the embryonic lethality of TM knockout mice.

Enhanced growth of the M4 transfectant of TM, in which the cytoplasmic domain was deleted (and seven amino acids were added to the transmembrane domain), may be complex, as this mutation results in increased release of soluble TM into cultured supernatants. Previous studies have shown soluble TM to have mitogenic properties for certain tumor cells (35). Furthermore, the M4 mutation might exert a dominant negative effect on expression/function of endogenous TM. In this context, it may be relevant that phosphorylation of the cytoplasmic domain of TM has been linked to internalization and degradation (44).

The results of our study suggest that negative effects of TM involve the lectin and cytoplasmic domains, though other portions of the molecule may contribute as well. Previous studies in other systems have described involvement of lectin domains in cell proliferation (47, 48). The region deleted in our Δ*lectin* (M3) mutant is relatively small and highly conserved in evolu-

tion, though it is involved in secondary and tertiary structural assembly of the receptor. Despite possible changes in overall protein structure with the Δ*lectin* (M3) mutant, inherent in any mutational analysis, mutants M1 and M2, in which the affinity of thrombin binding was selectively altered with large changes in TM-dependent, thrombin-mediated protein C activation, retained growth inhibitory activity. These data lead us to hypothesize that determinants in the lectin domain of TM interact with a ubiquitous environmental factor/mediator resulting in ongoing inhibition of cell proliferation. Optimal function of this mechanism requires an intact TM molecule with a lectin domain involved in ligand recognition, and a cytoplasmic domain capable of activating intracellular pathways which exert a negative effect on cell growth. This hypothesis raises many questions for future studies concerning the identity of a putative thrombin-independent ligand(s) of TM, and the means through which it might trigger signal transduction mechanisms relevant to cell growth, and possibly, the modulation of other properties as well.

Acknowledgments

We thank W.A. Dittman (Washington University School of Medicine, St. Louis, MO) for providing the mouse TM cDNA. We thank Ms. Engstner and Dr. Tilgen (Department of Dermatology, Heidelberg, Germany) for providing the cultured melanoma cells from the patients. We also thank Dr. B. Isermann for assistance with the experiments.

Peter Nawroth was supported by grants from DFG and Schilling-Stiftung.

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