Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6788–6792, July 1995
Cell Biology

Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis

(angiogenesis/transforming growth factor β/polyoma middle-sized tumor antigen/plasminogen activator)

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Communicated by P. Roy Vagelos, Bedminster, NJ, April 20, 1995

ABSTRACT **Murine endothelial cells are readily transformed in a single step by the polyomavirus oncogene encoding middle-sized tumor antigen. These cells (bEND.3) form tumors (hemangiomas) in mice which are lethal in newborn animals. The bEND.3 cells rapidly proliferate in culture and express little or no thrombospondin 1 (TS1). To determine the role of TS1 in regulation of endothelial cell phenotype, we stably transfected bEND.3 cells with a human TS1 expression vector. The cells expressing human TS1 were readily identified by their altered morphology and exhibited a slower growth rate and lower saturation density than the parental bEND.3 cells. The TS1-expressing cells also formed aligned cords of cells instead of clumps or cysts in Matrigel. Moreover, while the bEND.3 cells formed large tumors in nude mice within 48 hr, the TS1-expressing cells failed to form tumors even after 1 month. The TS1-transfected cells expressed transforming growth factor β mRNA and bioactivity at levels similar to those of the parental or vector-transfected bEND.3 cells, indicating that the effects of TS1 expression are not due to the activation of transforming growth factor β by TS1. TS1 expression resulted in a >100-fold decrease in net fibrinolytic (urokinase-type plasminogen activator, uPA) activity due to more plasminogen-activator inhibitor 1 and less uPA secretion. TS1 thus appears to be an important regulator of endothelial cell phenotype required for maintaining the quiescent, differentiated state.**

The thrombospondins are a multigene family of modular glycoproteins involved in regulation of adhesion, migration, and proliferation of a number of normal and transformed cell types (1–4). Human thrombospondin 1 (TS1) and a fragment of hamster TS1 (gp140) are inhibitors of angiogenesis driven by many different stimuli (5, 6). Synthetic peptides from two adjacent domains of human TS1 (the procollagen-like segment and the type 1 repeats) block angiogenesis in several bioassays and inhibit the migration of endothelial cells (ECs) *in vitro* (5, 6). Furthermore, TS1 and its proteolytic fragments containing these domains inhibit proliferation of ECs *in vitro* (5–8). TS1 is expressed and secreted by a variety of cell types, including ECs proliferating in culture (9). Several findings suggest that TS1 is a physiological inhibitor of angiogenesis: TS1 is present in quiescent vessels and absent in actively forming EC sprouts (10); TS1 levels are low in EC involved in cord formation *in vitro* (10); TS1 expression in a human breast carcinoma line reduces tumor growth, metastasis, and angiogenesis (11); TS1 mRNA is decreased in cultures of ECs forming tubules (12); and TS1 expression is virtually shut off in rapidly growing ECs from hemangiomas (13).

The mechanisms by which TS1 regulates EC phenotype are not understood. We have used the polyoma virus middle-sized tumor antigen (mT)-transformed mouse brain capillary EC line (bEND.3) (13, 14) to examine the role of TS1 in the regulation of EC phenotype. These transformed cells express little or no TS1 (13), and perhaps as a result, they proliferate more rapidly and to higher saturation densities than normal ECs. However, their proliferation, like that of normal ECs, is inhibited by added TS1 (13). It is not clear whether the inhibition by TS1 is direct or is due to the presence of transforming growth factor β (TGF-β), a potent inhibitor of EC proliferation, which is associated with TS1 purified from platelets (15). To eliminate this ambiguity and determine the role of TS1 in regulation of EC phenotype, we have expressed human TS1 in bEND.3 cells. Expression of TS1 in these cells results in a reduced growth rate, lower saturation density, decreased fibrinolytic activity, and restored ability to form cords *in vitro*. Furthermore, TS1 expression blocks the ability of bEND.3 cells to form hemangiomas *in vivo*. All of these changes occur independently of increased TGF-β activity.

MATERIALS AND METHODS

Cell Culture and Transfection. bEND.3 cells (obtained from W. Risau, Max Planck Institute) and normal mouse brain ECs (MBECs) (obtained from R. A. Modzelewski, University of Pittsburgh) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The normal ECs were grown on gelatin-coated tissue culture dishes. DNA transfection was performed with Lipofectin (GIBCO/BRL). The bEND.3 cells already express the *neo* gene, conferring G418 resistance; thus transfectants were selected by cotransfection with a hygromycin-resistance plasmid, pMEP4 (Invitrogen). The cells were selected in medium containing hygromycin B (50 μg/ml; Sigma) for 2–3 weeks and individual clones were isolated.

Construction of TS1 Expression Vector. The expression plasmid (pcDNATS1) containing the full-length human TS1 cDNA was prepared by ligation of the 4.4-kbp *Sac* I–*Xba* I fragment of pBS6STXE (16) (blunt ended at the *Sac* I site) into pcDNAIneo (Invitrogen) cut with *Eco*RV and *Xba* I. This allows expression of TS1 from the cytomegalovirus promoter.

Cell Proliferation Assay. Cells (≈10,000) were plated in triplicate in multiple sets of 60-mm tissue culture plates. Cells were fed every other day, and the cell number in one set of plates was determined by counting on each indicated day.

Northern Analysis. Poly(A)$^+$ RNA (5 μg) prepared from logarithmically growing cells (17) was size fractionated in a 1.2% agarose/formaldehyde gel and transferred to a Zeta-Probe membrane (Bio-Rad). mRNA for human TS1 was detected with an internal 1.4-kbp *Bam*HI fragment of human TS1 cDNA (16). mRNA for urokinase-type plasminogen

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Abbreviations: TS1, thrombospondin 1; EC, endothelial cell; MBEC, mouse brain EC; TGF-β, transforming growth factor β; uPA, urokinase-type plasminogen activator; PAI, plasminogen-activator inhibitor; CM, conditioned medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mT, middle-sized tumor antigen.

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activator (uPA) was detected with a 1.5-kbp *Pst* I fragment of human uPA cDNA, obtained from the American Type Culture Collection. mRNA for PAI-1 was detected with a 2.0-kbp *Eco*RI fragment of human plasminogen-activator inhibitor 1 (PAI-1) cDNA, obtained from J. Billadello (Washington University, St. Louis). The blot was also probed with a 1.3-kbp *Pst* I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to control for loading. The exposure time varied with each probe.

Western Blot Analysis. Cells were plated at 20,000 per 60-mm tissue culture plate and allowed to reach near-confluency. Cells were fed the day before the experiment started. Near-confluent monolayers were washed three times with DMEM, and 2 ml of serum-free culture medium was added to each dish. Conditioned medium (CM) and cell extracts were prepared as described (18) 24 hr later. Cell number was determined in triplicate for each cell type incubated under identical conditions. Sample aliquots normalized for cell number were analyzed by SDS/PAGE and blotting. The nitrocellulose blots were incubated with blocking solution [20 mM Tris·HCl, pH 7.6/137 mM NaCl/0.05% Tween 20 (TBS-T) with 3% bovine serum albumin and 3% non-dairy creamer] for 1 hr. Blots were then incubated with a rabbit anti-mouse uPA IgG or rabbit anti-rat PAI-1 IgG (American Diagnostica, Greenwich, CT) at 2 μg/ml or with anti-TS1 monoclonal antibody A6.1 at 0.5 μg/ml for 1 hr. Blots were washed extensively with TBS-T, incubated with a solution of horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Pierce) for 1 hr, washed extensively as above, and developed with the ECL kit from Amersham. Purified recombinant mouse uPA and mouse PAI-1 were from American Diagnostica. uPA activity was assayed with a uPA-specific chromogenic substrate (Spectrozyme UK 244X) according to the manufacturer's instructions (American Diagnostica).

Three-Dimensional Culture. Matrigel (Collaborative Research) (0.5 ml per well of 12-well tissue culture plates) was allowed to gel at 37°C for 30 min, and cells (2×10^4 in 0.5 ml of growth medium) were gently added to duplicate wells. The plates were monitored for 6–24 hr and photographed with a Nikon microscope at ×100.

Tumorigenesis Assay. Cells ($\approx 10^7$) of the bEND.3 parental line, vector control, and TS1-transfected clones TS-11 and TS-26 were injected subcutaneously on each side into the rear flanks of 6-week-old male nude mice (Harlan–Sprague–Dawley) (two sites per mouse and three mice per cell line). The mice were maintained in a sterile environment, examined daily, and photographed with Kodak color print film. The care of the mice was provided by the Washington University veterinary staff and was according to institutional guidelines.

RESULTS

Expression of TS1 in bEND.3 Cells. bEND.3 cells grow rapidly in culture and reach much higher saturation densities than normal ECs. These cells express little or no TS1 (13). In addition, the proliferation of these cells, as well as other ECs, is inhibited when TS1 or its fragments are added to culture medium (7, 8, 13). To better understand the role of this natural inhibitor of angiogenesis in regulation of EC behavior, we transfected bEND.3 cells with an expression vector (pcDNATS1) containing the entire coding region of human TS1 cDNA (16). The TS1-transfected cells exhibited a strikingly different morphology than the parental bEND.3 cell line. Fig. 1*A* shows a population of TS1-transfected cells which display two different morphologies. Immunostaining of such cultures indicated that only the cells in colonies with a cobblestone morphology, a morphology very similar to that of normal ECs, expressed TS1. The other cells exhibited a morphology identical to that of the bEND.3 cells, which have an elongated spindle shape similar to the spindle cells of

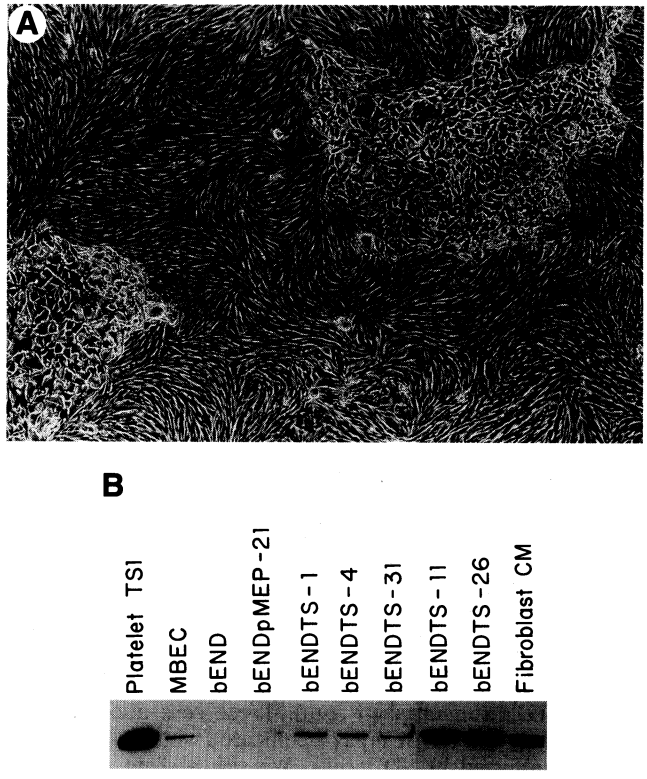

FIG. 1. (*A*) Phase-contrast micrograph of TS1-transfected bEND.3 cells growing in the presence of hygromycin. Two colonies of TS1-expressing cells are surrounded by cells identical in morphology to bEND.3 cells which grow faster and take over the culture. Immunostaining of parallel cultures revealed TS1 expression in only those colonies with a normal EC appearance. (×50.) (*B*) Western blot analysis of TS1. CM was prepared from the indicated cell lines and subjected to SDS/4–12% PAGE (nonreducing conditions) and Western blotting. A mouse anti-human TS1 monoclonal antibody (A6.1) was utilized. Human platelet TS1 and CM from human dermal fibroblasts were used as controls. All lanes shown are from the same blot (extraneous lanes were removed for brevity).

Kaposi sarcoma (19, 20). The spindle-shaped cells did not stain with anti-TS1, thus indicating that they had hygromycin resistance conferred by the pMEP4 vector but did not express human TS1. With time those cells which did not express TS1 overgrew the culture due to their more rapid growth rate (see below).

We isolated 50 hygromycin-resistant clones from the TS1 transfection and 20 control clones (transfected with pMEP4 alone) and screened them for expression of human TS1 by ELISA, indirect immunofluorescence, and Western blotting using anti-human TS1 polyclonal and monoclonal antibodies. We thus identified many clones expressing trimeric human TS1. Fig. 1*B* shows a Western blot of CM collected from normal MBECs, bEND.3, a vector-transfected clone (pMEP-21), and several TS1-transfected clones with either parental bEND.3-like morphology (TS-1, TS-4, and TS-31) or normal EC morphology (TS-11 and TS-26). A sample of human platelet TS1 and CM from human dermal fibroblasts (which express high levels of TS1) were used as controls. The TS1-transfected cells with normal EC morphology are those which express human TS1 at high levels, while cells with bEND.3-like morphology express much less TS1. Thus, the change from bEND.3 (transformed) to normal EC morphology correlates with higher levels of TS1 expression.

The TS1-transfected bEND.3 cells stained positive for factor VIII-related antigen (an EC-specific marker; data not shown), as previously shown for the parental bEND.3 cells (21). The

growth rate of several representative clones of transfected cells was determined. The TS1-expressing cells had a consistently reduced growth rate and reached a much lower saturation density, about one-third to one-sixth that of bEND.3 cells or vector-transfected clones (Fig. 2). This is consistent with our observation that when revertants appear in some cultures of TS1-transfected clones they rapidly crowd out the TS1-expressing cells, which grow more slowly.

The TS1 transfectants expressed readily detected levels of human TS1 mRNA, while parental or vector-transfected bEND.3 cells expressed very low levels of endogenous mouse TS1 mRNA (Fig. 3*A*), even when detected at lower stringency (data not shown). Except for clone TS-4, the mRNA levels for the clones shown were consistent with the estimates of TS1 protein obtained by ELISA and Western blotting. Clone TS-4 expressed a truncated TS1 mRNA (Fig. 3*A*) and a low level of TS1 (Fig. 1*B*) and exhibited a morphology similar to that of bEND.3 or vector-transfected cells.

To examine the role of TGF-β in mediating the effects of TS1 on EC proliferation, we examined the expression of TGF-β and determined the level of active TGF-β produced by these cells. CM was collected from two representative clones of TS1-transfected cells (TS-11 and TS-26) and the level of active TGF-β was assayed in the normal rat kidney cell colony-forming bioassay (15, 22). CM from the TS1-transfected clones had the same level of TGF-β activity as CM from the parental bEND.3 cells (unpublished data). These cells also expressed similar levels of TGF-β1 mRNA compared with the parental or vector-transfected bEND.3 cells. We saw a slight increase in TGF-β2 mRNA in some TS1-transfected clones which was not consistent (data not shown). Thus, TGF-β does not appear to mediate the effects of TS1 in these transfected cells.

TS1 Expression in bEND.3 Cells Restores Their Ability to Differentiate on Matrigel. A distinguishing characteristic of ECs is their ability to differentiate and form tubes or capillary-like structures when cultured on three-dimensional extracellular matrices such as collagen, fibrin, or Matrigel, a process which has been called *in vitro* angiogenesis (23–25). In contrast, the bEND.3 cells form cyst-like structures in fibrin gels which, over many days, grow into large EC-lined sacs instead of the regular network of tubules formed by normal ECs (14). These sacs histologically resemble cavernous hemangiomas seen in transgenic (26) and chimeric (21, 27) mice expressing the mT oncogene. We examined the ability of TS1-expressing cells to form tube-like structures when plated on Matrigel. Normal

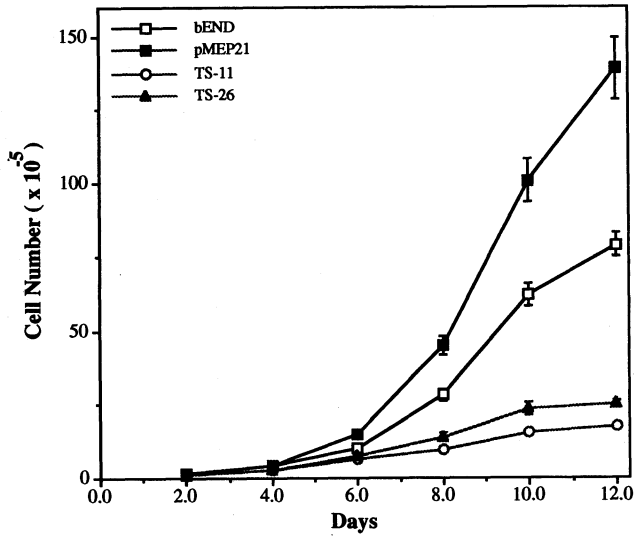

FIG. 2. Proliferation rate of the parental bEND.3 cells (□), a vector control clone (pMEP-21, ■) and two clones expressing human TS1 (TS-11, ○, and TS-26, ▲).

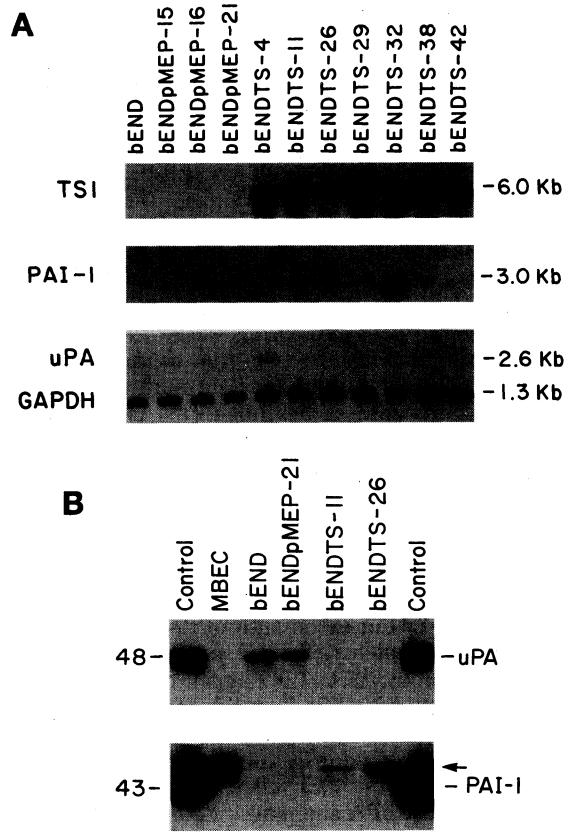

FIG. 3. (*A*) Northern blot analysis of mRNA isolated from clones of bEND.3 cells transfected with an expression vector containing human TS1 cDNA (bENDTS), vector control clones (bENDpMEP), and parental bEND cells. The steady-state mRNA levels for TS1, uPA, and PAI-1 were determined with specific cDNA probes. The blot was also probed with a cDNA for GAPDH to control for loading. (*B*) Western blot analysis of uPA and PAI-1. CM was prepared from indicated cell lines and subjected to SDS/12% PAGE (nonreducing conditions) and Western blotting. Recombinant mouse uPA (250 ng, 48 kDa) and PAI-1 (100 ng, 43 kDa) were run as controls. Arrow indicates the endogenous mouse PAI-1, which is slightly bigger than the recombinant PAI-1.

mouse brain ECs rapidly (6–12 hr) formed networks of tubes on Matrigel, while the bEND.3 cells initially formed undifferentiated balls of cells that appeared to expand into cavernous cysts after 6–7 days just as in fibrin gels (14). The few thin processes extended by the cells were retracted by day 2. In contrast, the TS1-expressing bEND.3 cells organized themselves into extensive networks on Matrigel (Fig. 4). These structures seem to be "cords" of cells that do not fully differentiate into capillary-like tubes and appear, in most cases, to lack a central lumen. Thus, some essential step or function required for tube formation is still lacking in the TS1 transfectants.

TS1 Expression Results in Altered Expression of uPA and PAI-1. The formation of the hollow cysts by bEND.3 cells in fibrin gels is attributed to the high levels of fibrinolytic activity produced by these cells (14). This in turn is the result of the enhanced expression of uPA and the apparent lack of PAI-1 in these cells (14). The alteration of uPA and PAI-1 expression is not a property unique to bEND.3, since it was noted in EC lines derived from various tissues of mT-expressing chimeric mice as well as EC lines derived from *in vitro* transfection of ECs with mT (14). To determine whether the improved ability of the TS1 transfectants to differentiate in three-dimensional culture was due to the restoration of a proteolytic balance more characteristic of normal ECs, we examined the mRNA levels of uPA and PAI-1 in the TS1-expressing clones by Northern

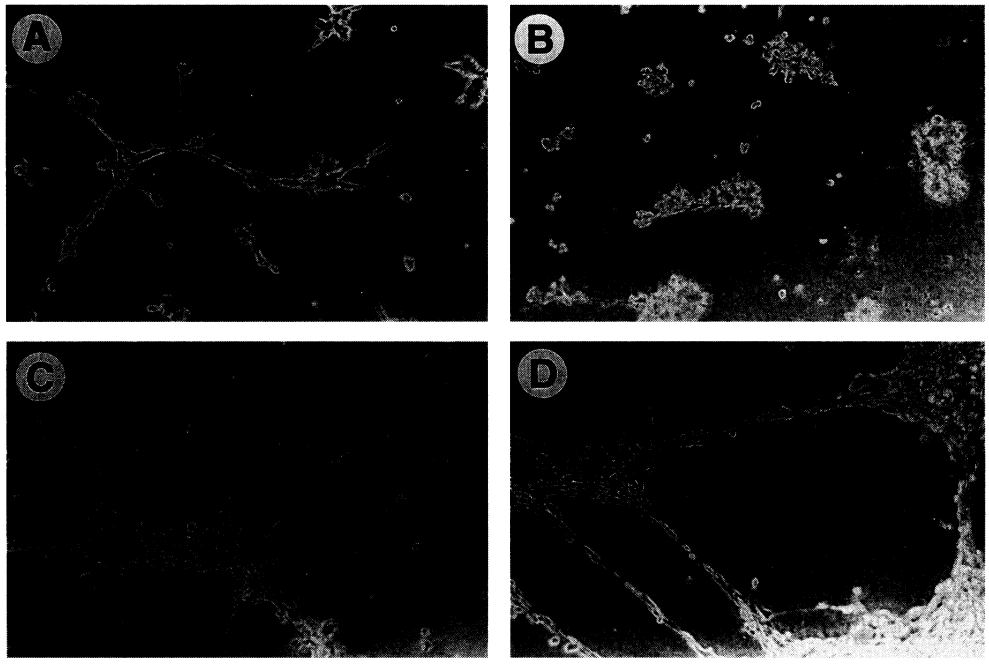

FIG. 4. Differentiation of ECs in three-dimensional Matrigel cultures. Phase-contrast micrograph of normal mouse brain ECs (*A*), a vector-transfected bEND.3 clone (*B*), and TS1-transfected clones TS-11 (*C*) and TS-26 (*D*). (×50.)

analysis. mRNA for uPA was decreased and mRNA for PAI-1 was increased in nearly all TS1-expressing clones (Fig. 3*A*). By Western blot analysis of CM, uPA was detected only in the medium conditioned by the parental bEND.3 cells or vector-transfected cells and not in the medium conditioned by the normal MBECs or the TS1-transfected clones (Fig. 3*B*). The opposite pattern was observed for PAI-1—that is, PAI-1 protein was detectable only in the medium conditioned by normal MBECs and TS1-transfected clones. Western analysis of uPA and PAI-1 in extracts of the control and transfected cells detected no cell-associated proteins (data not shown). A direct assay, utilizing a specific chromogenic substrate for uPA, detected activity only in CM from parental bEND.3 cells and vector-transfected cells. By comparison with purified uPA assayed in parallel, we estimated that medium conditioned for 24 hr by bEND.3 cells contained uPA activity at 20 units/ml. The level of active uPA secreted by TS1-transfected cells and normal MBECs was <0.2 unit/ml. These observations are consistent with a decrease in the fibrinolytic activity of the cells expressing human TS1. The levels of tissue-type PA (tPA) and PAI-2 mRNA were not affected (data not shown).

TS1 Expression in bEND.3 Cells Suppresses Their Ability to Form Hemangiomas *in Vivo*. Polyoma mT-expressing ECs were originally isolated from chimeric mice because of their propensity for hemangioma formation, the cause of lethality in chimeric embryos (27) and in adults expressing all three polyoma T antigens (26). bEND.3 cells rapidly form hemangiomas when injected into mice (Fig. 5*A*; refs. 14 and 21). To determine whether TS1 expression affects the ability of the bEND.3 cells to form tumors, nude mice were injected with cells from two representative TS1-transfected clones, a vector-transfected control clone, and the parental bEND.3 cell line. Both bEND.3 and the vector-transfected control cells rapidly formed large (1- to 2-cm-diameter) hemangiomas in all the animals at all sites injected (total of 12) within 2 days (Fig. 5*A*). However, neither of the two TS1-transfected clones (TS-11 and TS-26) formed any visible tumors after 1 week (Fig. 5 *B* and *C*) or 2 weeks. After 1 month, one of the three mice injected with clone TS-11 had bilateral hemangiomas while all of the mice injected with clone TS-26 cells remained free of tumors. Clone TS-11 cells carried *in vitro* for the same time period gave rise to revertant colonies as judged by morphology while TS-26 cells remained pure. This suggests that the tumors which formed with a long latency in one of the TS-11 mice were due to the appearance of revertants that lost TS1 expression *in vivo*. Histological examination of the tumors revealed the expected "blood lakes" surrounded by one to several layers of ECs (21, 26, 27). The injection sites of TS-11 (two negative mice) and TS-26 (all three negative) showed only necrotic cells surrounded by a fibrous capsule. As reported for bEND.3 (21, 27), there was no evidence of metastases in other tissues of any tumor-bearing mice.

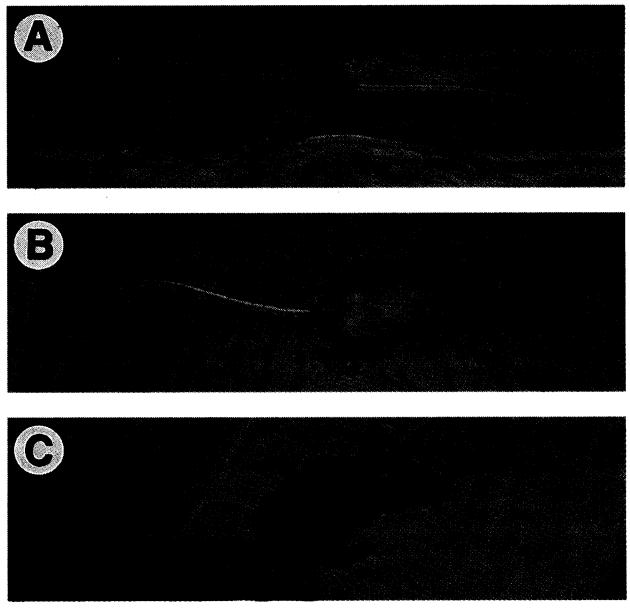

FIG. 5. Tumorigenesis assay of bEND.3 cells expressing human TS1. (*A*) Mice injected 2 days before with bEND.3 cells (front) or a clone of vector transfected control cells (pMEP-21) (back). (*B* and *C*) Mice injected 1 week before with TS1-transfected clones TS-11 and TS-26, respectively.

DISCUSSION

The expression of TS1 in bEND.3 cells is sufficient to suppress the tumorigenic phenotype of these cells *in vivo* and restore their ability to form cords, if not tubes, *in vitro*. The inhibition of tumor formation is not simply due to the slower growth rate of the TS1 transfectants, since tumor formation by the parental bEND.3 cells is too rapid to result from expansion of the injected cells. Hemangiomas form by the recruitment of host ECs through contact with the transformed ECs (21). The slower growth rate and lower saturation density of the TS1-transfected cells do, however, demonstrate a direct effect of TS1 on EC proliferation. Platelet TS1 inhibits EC growth *in vitro* (7, 8), but the contamination of TS1 with platelet TGF-β, a known inhibitor of EC proliferation *in vitro*, makes these experiments difficult to interpret (15). Our experiments rule out contamination with TGF-β as an explanation for TS1 growth inhibition. The ability of TS1 to bind and activate endogenous latent TGF-β (22) might have accounted for the growth inhibition in the TS1-transfected bEND.3 cells. However, CM from the TS1-transfected clones had the same level of TGF-β activity as the parental bEND.3 cells. Therefore, the effects of TS1 expression on bEND.3 cells do not appear to be mediated through changes in the levels of available TGF-β activity.

TS1 expression restores the ability of bEND.3 cells to differentiate on Matrigel in a fashion similar to normal ECs. However, they do not appear to form fully differentiated tubes. Thus, some step or function is still lacking in the TS1 transfectants, possibly due to the premature regulation of a component of EC differentiation by constitutive expression of TS1. This is likely to be a defect in regulation of cell adhesion which does not allow the cells to anastomose tightly (unpublished data). The behavior of the transfectants is clearly more similar to that of the normal ECs in that they no longer form the cysts characteristic of bEND.3 cells. A characteristic of bEND.3 cells is the high protease (fibrinolytic) activity which results in or contributes to vascular malformations (hemangiomas) *in vivo* and cavernous hemangiomas *in vitro* in fibrin gels (14). However, TS1 expression appears to reestablish a normal proteolytic balance by promoting enhanced expression of PAI-1 and decreased expression of uPA (Fig. 3), resulting in a >100-fold lower net uPA activity in the TS1 transfectants. This level of uPA activity is comparable to that of normal MBECs. Polyoma mT transformation of other cell types such as fibroblasts, mast cells, and embryonic stem cells does not result in altered uPA and PAI-1 expression (14). Thus, this effect in ECs may be a specific result of the lack of TS1 expression in mT-transformed ECs (13). Further, a decrease in net uPA activity in neovascularized tissue (28) and an increase in production of TS1 during late stages of EC differentiation (refs. 10 and 11; G. Jeong and W.A.F., unpublished data) suggest a direct role for TS1 in keeping the proteolytic balance in check.

The experiments reported here establish a primary role for TS1 as a regulator of EC phenotype and underscore the physiological relevance of TS1, a normal product of ECs, as an inhibitor of angiogenesis. TS1 should perhaps be viewed in a broader context as maintaining the differentiated phenotype of ECs in the face of angiogenic stimuli which convert ECs to a migratory, invasive phenotype (5, 6, 14, 23–25, 29, 30). The highly regulated expression of TS1 in a number of embryonic tissues suggests that this role may be of importance in development (31–33). The dramatic reduction in TS1 expression in bEND.3 cells is essential for the maintenance of their transformed phenotype, since replacement of TS1 expression in the transfectants results in a normal morphology and a slower growth rate, restores cord formation *in vitro*, and blocks tumor formation *in vivo*. The generality of TS1 regulation is supported by the finding that a similar reduction in TS1 expression also occurs in Py4-1 cells, a transformed endothelial line derived from hemangiomas of transgenic mice expressing all three polyoma T antigens (13, 34). In further support of the role of TS1 as a major regulator of EC phenotype, we have found that restoration of TS1 expression in the bEND.3 cells affects the expression of a number of components with roles in angiogenesis and EC differentiation.

We acknowledge Dr. W. Risau for the generous gift of the bEND.3 cell line, Dr. V. Bautch for Py4-1 cells, Dr. R. A. Modzelewski for normal MBECs, Dr. P. A. D'Amore for bringing the bEND.3 cells to our attention and for stimulating discussions and advice, and Dr. M. C. LaRegina for autopsies and histological examinations. M. B. Finn and J. M. Dimitry provided excellent technical support. We thank Drs. J. I. Gordon, S. Korsmeyer, P. Majerus, and L. Pike for critical reading of the manuscript. This work was supported by Public Health Service Grant HD27712 and a grant from Monsanto/Searle.

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