Tenascin-C Expression by Angiogenic Vessels in Human Astrocytomas and by Human Brain Endothelial Cells in Vitro

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ABSTRACT

The expression of the extracellular matrix glycoprotein tenascin-C (TN) is enhanced in human astrocytomas and correlates with angiogenesis. To determine whether vascular cells are able to synthesize TN, we investigated the expression of TN protein and mRNA in nine astrocytomas. Immunogold electron microscopy in two glioblastomas multiforme detected the presence of TN in an extracellular perivascular location and to a lesser extent among tumor cells, confirming light microscopy immunohistochemical findings. In situ hybridization of astrocytomas using a digoxigenin-labeled antisense riboprobe detected strong staining for TN mRNA in vascular cells, especially in hyperplastic vessels, including those at the invasive edge of the tumors but not in vessels of normal brains. We observed weaker staining in tumor cells indicating a higher level of TN mRNA in vascular than in tumor cells. No staining was detected with the sense probe. Moreover, we investigated the ability of human brain microvessel endothelial cells (HBMECs) in primary culture to synthesize TN in vitro. Western blot analysis of the culture supernatants from HBMECs detected large amounts of TN. Immunogold silver staining demonstrated the presence of TN on the surface of HBMECs and in the subendothelial matrix. The distribution of TN mRNA in vascular cells of astrocytomas and the ability of HBMECs to synthesize TN in vitro demonstrate that vascular cells, including endothelial cells, are a major source of TN associated with angiogenesis. Furthermore, our results suggest that TN expression may be associated with endothelial cell activation and may play an important role in angiogenesis.

INTRODUCTION

TN is a large complex protein of the extracellular matrix which is expressed in developing brain, cartilage, and mesenchyme and is re-expressed in tumors, wound healing, and inflammation (1). It is believed to be important for several cellular processes, including cell adhesion, migration, and proliferation (1). TN consists of an amino-terminal cysteine-rich region involved in oligomerization, followed by linear segments of EGF-like domains, fibronectin type III repeats, and a fibrinogen-like region at the carboxyl terminus (1). In contrast to the low levels of TN found in normal adult brain, enhanced expression occurs in human astrocytomas (2-5). Expression of TN is increased up to 4-fold in brain tumors such as glioblastomas compared to normal tissues (5), and TN immunostaining is consistently stronger in hyperplastic than in nonhyperplastic vessels of astrocytomas where its expression correlates with angiogenesis (5).

Although the source of TN in brain tumors has not been elucidated, several observations suggest that astrocytes could be the main source of TN in astrocytomas by secreting and depositing it around blood vessels by glial end feet. These include: (a) glial cell lines can secrete TN in vitro (2); (b) glial cells in primary cultures secrete TN and show punctate TN staining extracellularly and around the nucleus corresponding to the Golgi apparatus (6); (c) enhanced TN expression occurs in reactive astrocytes in cerebral injury (7) and in cerebellar degeneration (8); (d) cytoplasmic immunoactivity for TN can be seen in occasional tumor astrocytes (3, 5); and (e) by using electron microscopy and in situ hybridization, TN mRNA has been detected in free ribosomes and ribosomes of the rough endoplasmic reticulum of astrocytes (9). Despite these studies indicating that astrocytes may be a major source of TN in astrocytomas, the strong angiogenic TN immunoactivity (3-5) suggests that vascular cells may also contribute to its local deposition. Vascular cells that have been implicated in the neovascular proliferative phenomena associated with glioblastomas include endothelial cells (10-12) and vascular smooth muscle cells (13, 14). A number of studies indicate that these two types of vascular cells can synthesize TN: (a) TN immunoactivity has been detected in the subendothelial matrix of human brain endothelial cells (15); (b) murine cerebral (16) and hepatic (17), bovine aortic (18), and human arterial (19) endothelial cells express TN; (c) TN immunoactivity has been detected in the media of normal human cerebral arteries (5); (d) rat (20, 21) and human aortic (19) vascular smooth muscle cells synthesize TN; and (e) bovine retinal pericytes express TN (22).

To investigate whether human cerebral vascular cells are able to synthesize TN and to determine its cell of origin in astrocytomas, we have performed immunogold electron microscopy and in situ hybridization. To detect expression and secretion of TN by HBMECs in primary culture, we have used immunogold silver staining and analyzed their supernatants by immunoprecipitation and Western blotting. We now report that vascular cells of astrocytomas express high levels of TN mRNA, and that HBMECs are able to synthesize and release TN in vitro.

MATERIALS AND METHODS

Immunohistochemistry

We studied five GBMs, two AAs, and two JPs. Low-grade nonpilocytic astrocytomas were not available for this study. These tumors showed features similar to and were graded as those described previously (5). Three samples of histologically normal brain were removed in the course of surgical exposure and used as controls. Fresh surgical specimens were fixed with 4% paraformaldehyde in PBS overnight. For TN immunostaining, we used a mouse monoclonal antibody (Dako, Carpinteria, CA), directed against purified human TN from cultured U251MG glioma cells at 1:50 dilution, as described previously (5). For negative controls, the immunosubstrate was replaced by PBS or non-
immunofluorescence microscopy. Samples of about 1 mm³, were cut from two GBMs, fixed in 3% paraformaldehyde with 0.25% glutaraldehyde for 5 min, and incubated with 5% NGS in PBS containing 0.5% BSA for 60 min at room temperature. Tissues were then incubated overnight at 4°C with monoclonal (Dako) or polyclonal (Chemicon, Temecula, CA) antibodies to human TN at 1:4 dilution in 0.1% BSA and 1% NGS and washed 4 times with PBS for 10 min each. Control sections were incubated with nonimmune serum or were kept in 0.1% BSA and 1% NGS overnight at 4°C. To detect antibody binding, the samples were incubated overnight at 4°C with secondary antibodies (goat antimouse AuroProbe EM for the monoclonal anti-TN antibody and goat antirabbit AuroProbe EM for the polyclonal anti-TN antibody) conjugated to 10-nm colloidal gold particles (Amersham Life Science, Arlington Heights, IL) at 1:3 dilution in PBS containing 0.1% BSA. Tissues were washed with PBS (three times for 10 min), fixed with 3% glutaraldehyde for 3 h, and then washed in PBS (three times for 5 min). Immunostaining, fixation, and washings were carried out under continuous, mild rotation. The samples were postfixed with 1% OsO₄ in sodium cacodylate trihydrate at room temperature for 1 h, dehydrated in a graded series of ethanol, and embedded in Medcast resin (Ted Pella, Inc., Redding, CA). One-μm-thick sections were cut, and after selection of the most appropriate areas, ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 transmission electron microscope operated at 60 kV.

**In Situ Hybridization**

**Probe.** Oligonucleotide primers complementary to a 5’ region of exon 1 of the human TN gene (primer 1: 5’-CTA GAA TTC CAG CAC CCA GC-3’ and primer 2: 5’-CTC AAG CTT CAC ACA ATG G-3’) were designed based on the published sequence (23). With human genomic DNA as a template for these primers, PCR was used to amplify a 231-bp product. The PCR product was cloned into pBluescript II SK± (Stratagene Cloning Systems, La Jolla, CA). Two resulting clones each containing a 0.23-kb insert were sequenced and the Sanger method using Sequenase (United States Biochemicals, Cleveland, OH) and were found to match exactly a 231-bp fragment of the published sequence of exon 1 of the human TN gene (23).

**Antisense and sense riboprobes.** Oligonucleotide antisense and sense riboprobes were prepared by using a digoxigenin RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The specificity of the probes was verified by Northern hybridization of human fetal brain total RNA (Strategene). Using the antisense probe, we detected a band of 6–7 kb, which corresponds to the size of the human TN mRNA. No signals were generated by using the sense probe.

**Tissue Preparation.** We performed in situ hybridization on sections adjacent to those used for immunohistochemistry (five GBMs, two AAs, two JPAs, and three normal brain controls). The tissues were dehydrated with an increasing percentage of ethanol, soaked in xylene, and embedded in paraffin. Five-μm-thick sections were cut onto 6-μm-thick poly-L-lysine-coated slides. Slides were dried overnight at 60°C, deparaffinized in 100% xylene and absolute ethanol, rinsed in diethyglycolcarboxylate-treated sterile water, washed with 2× SSC, and treated with 0.3% Triton X-100-1× SSC for 10 min at room temperature. The slides were then transferred to 0.5% SDS in 1× SSC for 5 min at 37°C, rinsed in 2× SSC, and incubated in 200 μg/ml proteinase K (250 μg/ml) in 2× SSC for 10 min at 37°C, and rinsed three times in 2× SSC. The sections were then blocked with denatured/sheared salmon sperm DNA (1 mg/ml) and BSA (50 mg/ml) in 2× SSC at 37°C for 20 min and rinsed briefly in 2× SSC and in diethyglycolcarboxylate-treated sterile water. Finally, the slides were dehydrated with an increasing percentage of ethanol and air dried.

**In Situ Hybridization and Detection.** Ten ng of the digoxigenin-labeled probes (sense or antisense) were heated in 20 μl of hybridization buffer (4× SSC, 50% formamide, 1× Denhardt’s solution, 0.3% Triton X-100, 0.1% sarkosyl, 1 mg/ml sheared salmon sperm DNA, 1 μg/ml RNA, 10% Dextran sulfate, and 1:1000 β-mercaptoethanol) at 70°C for 5 min. Hybridization was achieved by adding 17 μl of probe to the treated slides, which were then covered with a glass coverslip, sealed with rubber cement, and incubated at 42°C overnight. This was followed by one brief wash in 2× SSC at 60°C and two washes in 0.1× SSC at 65°C for 7 min each and one brief wash in 2× SSC at room temperature. The sections were then treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA) at two units/slide for 5 min at 37°C and rinsed in maleate buffer containing 0.3% Triton X-100. The sections were blocked with 4% blocking buffer (boehringer)-0.3% Triton X-100 for 30 min at 37°C. Detection was performed by using antidigoxigenin antibody conjugated to alkaline phosphatase at 1:500 dilution in 3% blocking buffer-0.3% Triton X-100 for 30 min at 37°C, followed by an overnight incubation at 4°C. Sections were washed with maleate buffer-0.3% Triton X-100, three times for 3 min each, rinsed in 1× SSC, and then washed in alkaline substrate buffer and 100 μM Tris-150 mM NaCl-50 mM MgCl₂ for 5 min at room temperature. The slides were then incubated with the substrate containing 45 μl NBT salt (75 mg/ml) in dimethylformamide (70%) and 35 μl BCIP toluidinium salt (50 mg/ml) in dimethylformamide in 10 ml alkaline substrate buffer for 3 h at 37°C or for 72 h at room temperature. The sections were then washed three times for 9 min total time in maleate buffer-0.3% Triton X-100, rinsed in water, counterstained with nuclear fast red, rinsed in water again, mounted with aqueous-mounting medium, and examined. Staining was assessed as strong, weak, or not detectable.

**HBMECs**

**Culture.** HBMECs were isolated from human brains at autopsy and cultured as described previously (24). Isolated clumps of endothelial cells were plated onto fibronectin-coated plastic wells (Corning Plastics, Corning, NY) and maintained in medium 199 (M199; Gibco, Burlington, Ontario) supplemented with 10% horse serum (HyClone Labs, Logan, UT), 20 μg/ml endothelial cell growth supplement, 100 μg/ml heparin (both from Sigma Chemical Co., St. Louis, MO), and antibiotics (Gibco) at 37°C in 2.5% CO₂-97.5% air atmosphere. Confluent monolayers of Factor VIII-related antigen-positive cells were obtained by 6–10 days in culture.

**Immunocytochemistry.** Confluent monolayers of HBMECs grown in triplicate wells for 12 days were processed for localization of TN by the immunogold silver staining technique as described previously (25). To expose the subendothelial matrix to the antibodies, some of the cells were cultured without horse serum for 24 h before immunocytochemistry because in the absence of serum, endothelial cells foci retracted, thus allowing for the antibodies to reach the subendothelial region. The cultures were washed briefly with PBS containing 1% BSA and 1% NGS and incubated for 40 min at room temperature with the monoclonal antibody to TN (Dako) at 1:25 dilution (432 μg/ml) in PBS containing 5% BSA and 4% NGS. At the end of the incubation period, the monolayers were washed and then incubated with the secondary antibody (AuroProbe LMGAM IgG; Cedarlane, Hornby, Ontario) at 1:40 dilution for 1 h at room temperature. The cultures were then fixed in buffered formaldehyde-acetone, incubated in silver enhancing solution (IntenseSEM; Jansen/Cedarlane, Mississauga, Ontario), and counterstained with Giemsa. Controls included cultures incubated with normal mouse IgG (Cedarlane) at the same concentration as the primary antibody or carrier buffer instead of the primary antibody. Stained monolayers were viewed under a Nikon Labophot light microscope.

**Immunoprecipitation and Immunoblotting.** The presence of TN in supernatants of cultured HBMECs was analyzed by immunoprecipitation and immunoblotting. Supernatants (10 ml) from four individual cultures were collected on days 2, 4, and 8 after plating. Supernatants were analyzed for TN content and compared to control culture medium and purified human TN. Samples were incubated with the monoclonal antihuman TN (Dako) bound to protein G/agarose beads. Bound proteins were eluted by boiling in SDS sample buffer, resolved through SDS-PAGE, transferred to nitrocellulose, stained with amido-black, and probed with the polyclonal (Chemicon) antihuman TN (26, 27).

**RESULTS**

Expression of TN Protein and mRNA in Astrocytomas. To determine the cellular origin of TN in astrocytomas and especially that of the angiocentric TN, the expression of TN assessed by immunohistochemistry and immunogold electron microscopy was compared to the expression of TN mRNA. The results of TN immunohistochemistry...
TN expression was similar to those reported previously (5). Briefly, TN immunoreactivity was more pronounced in high-grade tumors and was found to be heterogeneous within individual tumors. TN immunostaining was consistently greater within and around the walls of hyperplastic than nonhyperplastic blood vessels of GBMs where it was also detected adjacent to vascular sprouts (Fig. 1). Although no vascular hyperplasia was observed in either the JPAs or the AAs, TN immunoreactivity was greater in the vasculature than among tumor cells where it was faint and focal. Enhanced TN expression was detected in GBMs as a fine fibrillary anastomotic network surrounding individual tumor cells, as described previously (5). In high-grade astrocytomas (AA and GBM), TN expression helped to delineate the tumor “margin” against the surrounding gliotic brain tissue, where it was mainly seen around hyperplastic blood vessels. In normal brain, TN immunoreactivity was observed in the media of arterial vessels, as described previously (5). No staining was detected with nonimmune serum or PBS.

To investigate the ultrastructural localization of TN, we performed immunoelectron microscopy. Anti-TN antibodies revealed colloidal gold particles associated with perivascular fine, filamentous, and granular structures (Fig. 2) and, in lesser amounts, among tumor cells in the intercellular space, confirming the light microscopy findings. No intracellular TN immunogold reactivity was detected in vascular or tumor cells. Polyclonal and monoclonal antibodies gave similar results. No immunoreactivity was seen in the control sections when the primary antibodies were omitted or when the immune serum was replaced by nonimmune serum (data not shown).

In situ hybridization experiments performed to determine the cellular distribution of TN mRNA, revealed specific staining in hyperplastic capillaries of astrocytomas (Fig. 3). There was no detectable staining in the three control normal brains (Fig. 4). After 3 h exposure to the NBT/BCIP substrate, in situ hybridization demonstrated strong staining in vascular cells especially at the invasive edge of the GBMs (Fig. 5). Staining was observed lining the vascular lumens, indicating the presence of TN mRNA in endothelial cells (Fig. 3c). However, staining was also noted within the walls of the vascular complexes, raising the possibility that other cell types such as vascular smooth muscle cells could also be labeled (Fig. 3c). TN mRNA was also detected in vessels beyond the tumor “margin” in the brain tissue adjacent to the tumor (Fig. 5). These findings may explain the variable levels of TN found in samples of macroscopically “normal” peritumoral brain submitted by the neurosurgeon, which we reported previously (5). In three GBMs and one AA where brain tissue more distant to the tumor was present, no detectable staining was seen in blood vessels. Tumor cell staining was weak in all five GBMs (Fig. 3, a and c). In the two AAs and the two JPAs that failed to show hyperplastic vessels, weak staining was observed in the tumor vasculature. Tumor cell staining was focal and weak in the two AAs and undetectable in the two JPAs. After incubation with the NBT/BCIP

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Fig. 1. Immunohistochemistry for TN in a GBM. The immunoreactivity is seen adjacent to a vascular sprout toward tumor cells (open arrows). No immunostaining is seen beyond the tip of the sprout (solid arrow). (Immunoperoxidase and hematoxylin counterstain, ×400).

Fig. 2. Immunoelectron microscopy for TN in a microvessel of a GBM. Labeling with monoclonal anti-TN antibody conjugated to colloidal gold is seen in the perivascular compartment (arrowheads) of a hyperplastic vessel with slit-like lumen (L); ×10,000. Inset, gold particles are heavily localized on a fine filamentous and granular matrix; ×50,000. (Uranyl acetate-lead citrate stain.)

Fig. 3. In situ hybridization for TN mRNA in astrocytomas (AA and GBM). Representative examples of in situ hybridization showing vascular localization of TN mRNA. The hybridization signal (black deposits) is more intense in hyperplastic vessels of astrocytomas (13). Sections from normal brain show no detectable staining (14). A. Low-power view of a GBM showing strong hybridization signal in the walls of hyperplastic vessels of the tumor (arrows). The adjacent normal brain shows no detectable signal. ×20. B. High-power view of the hybridization signal in the walls of a hyperplastic vessel of a GBM. The signal is also seen in tumor cells (arrows). ×500. C. In situ hybridization for TN mRNA in a normal brain shows no detectable signal in arteries and arterioles. ×500.
There was a tendency for the periphery of cells and the perinuclear region to stain most intensely (Fig. 6a). In cultures grown in the absence of horse serum for 24 h to expose the intercellular spaces, dense, granular deposits of TN were present between adjacent, retracted endothelial cells (Fig. 6b) indicating deposition of TN under the basal cell surface in the subendothelial matrix. No labeling was seen in the cultures used as controls (Fig. 6c).

Immunoprecipitation of supernatants from HBMECs by using specific antibodies against human TN indicated that these cells are able to synthesize and release TN for several days in vitro (Fig. 7). TN was detected in the culture supernatants of HBMECs taken at 2, 4, and 8 days in culture. HBMECs expressed different proportions of the two isoforms (Mr 320,000 and Mr 220,000–230,000) of TN (1), with the larger form being prevalent. No TN was immunoprecipitated from fresh culture medium, indicating that the TN in the supernatant was synthesized and released by the HBMECs.

The HBMECs were isolated from normal brain. It may seem contradictory that we could detect TN in the subendothelial matrix and in the supernatant of HBMECs in vitro, whereas no detectable staining for TN mRNA was seen in normal brain by in situ hybridization. We believe the explanation for this is that endothelial cells in the normal brain are quiescent and do not have or have undetectable low levels of TN mRNA. Moreover, these cells were grown in endothelial cell growth supplement (which contain acidic fibroblast growth factor, a potent endothelial cell mitogen) and horse serum, which may further activate and stimulate the endothelial cells.

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are able to synthesize and release TN. These results indicate that vascular cells contribute to the deposition of TN in astrocytomas in vivo and provide further support to implicate TN in angiogenesis. TN is expressed at high levels in hyperplastic vessels of astrocytomas (3–5). Evidence indicates that astrocytes synthesize TN (2–9), and given that many astrocytes have end feet on capillaries, the perivascular TN in astrocytomas could be deposited by the tumor cells. However, because of the strong angiocentric TN immunoreactivity, we wanted to test whether vascular cells might also be a source of TN and, therefore, we investigated the expression of TN by these cells in vivo and in vitro. Several experiments have suggested that endothelial cells (15–19) and pericytes/smooth muscle cells (5, 19–22), both cellular components of the hyperplastic vascular structures seen in high-grade gliomas (10–14), are able to synthesize TN. Bovine macrovessel (18) and murine (16, 17) endothelial cells and HBMECs synthesize TN, indicating that its expression is independent of the size of the vessel of origin in various mammalian species.

Our results suggest that hyperplastic vessels are a major source of TN. Mechanisms regulating TN expression have only been partially elucidated. Among possible modulators, growth factors may play an important role. Although TN expression can be stimulated by growth factors, including FGF, TGF-β, and platelet-derived growth factor in vitro (19, 21, 28–32), it is not known what leads to the increased expression of TN in brain neoplasia. These factors stimulate angiogenesis in brain tumors and are associated with neovascularization (33). Whether angiogenic factors up-regulate TN expression in endothelial cells is currently unclear. However, because TN mRNA and protein expression are up-regulated at sites of vascular hyperplasia in brain tumors, but not in vessels of control normal brains, it is possible that TN expression might be important for endothelial cell activation and may be enhanced by angiogenic factors that are up-regulated at sites of vascular hyperplasia. Moreover, the observation that vessels of the brain tissue adjacent to the tumor express TN mRNA, but those vessels distant to it do not, suggests that diffusible factors produced by the neoplasm may stimulate the up-regulation of TN expression in blood vessels beyond the tumor “margin.” For example, basic FGF, which up-regulates TN expression in rat cerebral cortical astrocytes.induce them to express TN. Indeed, recent evidence demonstrates that human endothelial cells can be induced to express TN in vitro (19).

**DISCUSSION**

This study demonstrates the presence of TN mRNA in vascular cells of astrocytomas and establishes that HBMECs in primary culture...
TN expression by endothelial cells

(31), in a mouse embryo fibroblast cell line (32) and in primitive neuroectodermal tumor cells (28) is overexpressed by tumor cells and endothelial cells in high-grade astrocytomas (34). However, the control mechanisms may be complex, especially for indirect angiogenic factors (33), such as TGF-β and tumor necrosis factor. Both factors up-regulate the expression of TN in vitro (19, 21, 28, 30) and inhibit endothelial cell growth in vitro (35, 36) but are angiogenic in vivo (36, 37). The effects of vascular endothelial growth factor/vascular permeability factor on TN expression are unknown and are of special interest because, in contrast to other angiogenic factors, it is a rather specific endothelial cell mitogen (38).

Several experiments have implicated TN in adhesion, migration, and proliferation of endothelial cells. Both adhesive and counteradhesive domains for a variety of cell types have been characterized in TN (39), which include binding sites for endothelial cells, such as the third fibronectin type III domain containing the Arg-Gly-Asp (RGD) sequence and the fibrinogen-like terminal knob (40, 41). Endothelial cell adhesion is mediated by receptors present on their plasma membrane, including the integrin α5β3 for the third fibronectin type III domain (40, 41) and probably the integrin αvβ3 for the fibrinogen-like terminal knob domain (40, 41). By contrast, soluble TN and the alternatively spliced region of TN bind with high affinity to bovine aortic endothelial cells via a Mr 35,000 nonintegrin receptor, annexin II (42). Endothelial cells elongate and extend interconnecting processes when plated on TN in vitro (41). Similar behavior is not seen when endothelial cells are plated on fibronectin, collagen, vitronectin, or laminin substrata, suggesting a specific role for TN in modulating endothelial cell phenotype (41).

The heightened expression of TN mRNA and TN immunoreactivity at the invasive edges of tumors, especially around and in hyperplastic vascular complexes (5) and in close association with vascular sprouts, suggests a role for TN in endothelial cell migration. TN expression also correlates with cell migration in the embryo (43). For example, TN is present at the site of migration of both neural crest cells and of developing embryonic vasculature (44, 45). During cornea development, cells derived from the neural crest and destined to become endothelia migrate exactly along the line of the TN-rich stroma (46). Moreover, anti-TN antibodies inhibit neural crest cell migration (47) and endothelial cell migration in vitro (18). Furthermore, TN-rich matrices are permissive for endothelial cell migration, by contrast to inhibitory thrombospondin-rich matrices (18). The alternatively spliced region of TN induces the loss of focal adhesion in well-spread endothelial cells (48), and this effect can be blocked by antibodies against annexin II (49). Neural crest cells assumed a rounded morphology when placed on TN substrates (50), indicating a weak adhesion to the substratum (50), a step that is likely to allow for cell migration to occur (48).

TN inhibits (51) or enhances (52) the proliferation of a variety of cell types, and the effect of TN on endothelial cell proliferation depends, in part, on the state of growth of the endothelial cells (49). For example, TN has no effect on subconfluent cells but induces proliferation of confluent cells. The mitogenic activity of TN is in part due to its alternatively spliced region and can be blocked by antibodies against annexin II (49). Alternatively, the proliferative activity of TN may be mediated through altered adhesion of cells to the substrate (43). It is also possible that the EGF-like repeats (1) promote endo-

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**Fig. 6. Immunocytochemistry for TN in vitro.** Immunogold silver staining of HBMEC monolayers for TN. Labeling appears denser at the periphery (arrow) of the cells and in the perinuclear region (arrowhead); dense deposits of TN occupy the exposed subendothelial areas (arrowheads) following endothelial cell retraction; absence of staining in control cultures incubated with normal mouse IgG instead of the primary antibody (a, b, and c; ×220).

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**Fig. 7. Detection of TN in vitro.** Supernatants of HBMECs collected on days 2, 4, and 8 of an individual culture were analyzed for TN content in Western blots using specific anti-TN antibodies and compared to fresh medium and purified human TN (as described in “Materials and Methods”). HBMECs expressed a characteristic Mr ~300,000 TN form. Bar indicates the migration of the 205,000 molecular weight marker.
TN EXPRESSION BY ENDOTHELIAL CELLS

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