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Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells

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ABSTRACT **Tumor-derived adhesion factor (TAF) was previously identified as a cell adhesion molecule secreted by human bladder carcinoma cell line EJ-1. To elucidate the physiological function of TAF, we examined its distribution in human normal and tumor tissues. Immunochemical staining with an anti-TAF monoclonal antibody showed that TAF was specifically accumulated in small blood vessels and capillaries within and adjacent to tumor nests, but not in those in normal tissues. Tumor blood vessel-specific staining of TAF was observed in various human cancers, such as esophagus, brain, lung, and stomach cancers. Double immunofluorescent staining showed apparent colocalization of TAF and type IV collagen in the vascular basement membrane. *In vitro* experiments demonstrated that TAF preferentially bound to type IV collagen among various extracellular matrix components tested. In cell culture experiments, TAF promoted adhesion of human umbilical vein endothelial cells to type IV collagen substrate and induced their morphological change. Furthermore, when the endothelial cells were induced to form capillary tube-like structures by type I collagen, TAF and type IV collagen were exclusively detected on the tubular structures. The capillary tube formation *in vitro* was prevented by heparin, which inhibited the binding of TAF to the endothelial cells. These results strongly suggest that TAF contributes to the organization of new capillary vessels in tumor tissues by modulating the interaction of endothelial cells with type IV collagen.**

Angiogenesis, or neovascularization, is critical for normal physiological processes such as embryonic development and wound repair. Tumor growth and metastasis are also dependent on angiogenesis (1–3). Tumor cells must continuously stimulate the formation of new capillary blood vessels to receive sufficient supply of nutrients and oxygen from blood. In addition, the new blood vessels embedded in the tumor mass provide a gateway for tumor cells to enter the blood circulation and to metastasize to distant sites. To induce angiogenesis, tumor cells produce various angiogenic factors, such as vascular endothelial cell growth factor (4, 5) and basic fibroblast growth factor (6), both of which stimulate the growth and migration of capillary endothelial cells. Endothelial cell behavior during angiogensis is not only mediated by some growth factors, but also dependent on the precise regulation of the synthesis and degradation of the extracellular matrix (ECM) components (7, 8). Although there are many reports showing the effects of ECM components and other extracellular proteins on the proliferation, migration, and phenotypic organization of endothelial cells (9–13), their exact roles in angiogenesis are unknown.

We previously purified a cell adhesion protein with a molecular size of about 30 kDa, tentatively named tumor-derived adhesion factor (TAF), from conditioned medium of human bladder carcinoma cell line EJ-1 (14). *In vitro* TAF stimulated direct adhesion of various types of cells, such as rat liver cells and human vascular endothelial cells, to plastic plates. Structural analysis of purified TAF indicated that this protein was closely related to a putative protein encoded by the mac25 gene. The mac25 gene was previously identified by Murphy *et al.* (15) as a gene whose expression is decreased in meningioma cells and tumors relative to normal leptomeningeal cells. The deduced mac25 protein has a characteristic sequence containing 11 cysteine residues, which is conserved in insulin-like growth factor (IGF) binding proteins. The expression of the mac25 gene in the brain, lung, heart, skeletal muscle, testis, ovary, and pregnant uterus of normal mice has been shown by Northern blot analysis. Recently, Swisshelm *et al.* (16) has reported that the expression of the mac25 gene is increased during replicative senescence of human mammary epithelial cells and up-regulated by retinoids. On the other hand, Yamauchi *et al.* (17) recently identified a protein that stimulates prostacyclin production in vascular endothelial cells from conditioned medium of human fibroblasts, and cloned its cDNA. The deduced sequence of the prostacyclin-stimulating factor (PSF) is essentially identical to that of mac25, with the exception of several amino acid residues in its N-terminal and C-terminal amino acid sequences. Our cDNA cloning and amino acid sequence analysis of TAF have shown that the deduced sequence of TAF is identical to that of PSF (unpublished data).

In this report, we demonstrate that TAF is specifically accumulated in new blood vessels in various human cancer tissues and in capillary tube-like structures of cultured vascular endothelial cells. TAF has specific affinity to type IV collagen and appears to be colocalized with the collagen in the vascular basement membrane of tumor tissues. These and other results suggest that TAF is involved in the formation of new capillary vessels by vascular endothelial cells. The new, unified name "angiomodulin" is proposed for TAF/mac 25/PSF.

MATERIALS AND METHODS

TAF and Anti-TAF Antibody. TAF was purified to homogeneity from the serum-free conditioned medium of human bladder carcinoma cell line EJ-1 according to the procedures described in refs. 14 and 18. A monoclonal antibody (#88) against the purified TAF was prepared in a mouse.

Immunohistochemistry. All tissue sections were obtained by cancer surgery. Immunohistochemistry was performed with

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Abbreviations: ECM, extracellular matrix; TAF, tumor-derived adhesion factor; IGF, insulin-like growth factor; FCS, fetal calf serum; PBS, Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline; PSF, prostacyclin-stimulating factor.

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the affinity purified monoclonal antibody against TAF (#88) and rabbit antiserum against bovine type IV collagen (LSL, Tokyo). The anti-TAF antibody (2 mg/ml) was diluted 100-fold with PBS (Dulbecco's phosphate-buffered saline) containing 3% (vol/vol) normal rabbit serum. Paraffin-embedded sections were treated with 0.05% protease type XXIV (Sigma) in PBS at room temperature for 15 min, exposed to 3% (vol/vol) hydrogen peroxide (H_2O_2) for 15 min to inactivate endogenous peroxidase and then incubated with PBS containing 10% (vol/vol) normal rabbit serum at room temperature for 1 h for blocking. Incubation with primary antibody was performed overnight at 4°C in a humidified chamber. TAF staining was performed with a Histofine SAB-PO (M) kit (Nichirei, Tokyo). Briefly, the sections were incubated sequentially with biotinylated rabbit anti-mouse IgG at room temperature for 1 h and with a horseradish peroxidase-labeled streptavidin solution for 1 h. The color was developed with 0.6 mg/ml 3,3-diaminobenzidine (Dojin, Tokyo) in 50 mM Tris·HCl buffer (pH 7.5) containing 0.1% (vol/vol) hydrogen peroxide. The sections were counterstained with hematoxylin. Negative controls included replacement of the primary antibody with preimmune mouse IgG. For double immunofluorescent staining, fluorescein isothiocyanate-conjugated horse anti-mouse IgG (Vector Laboratories) and Texas red-

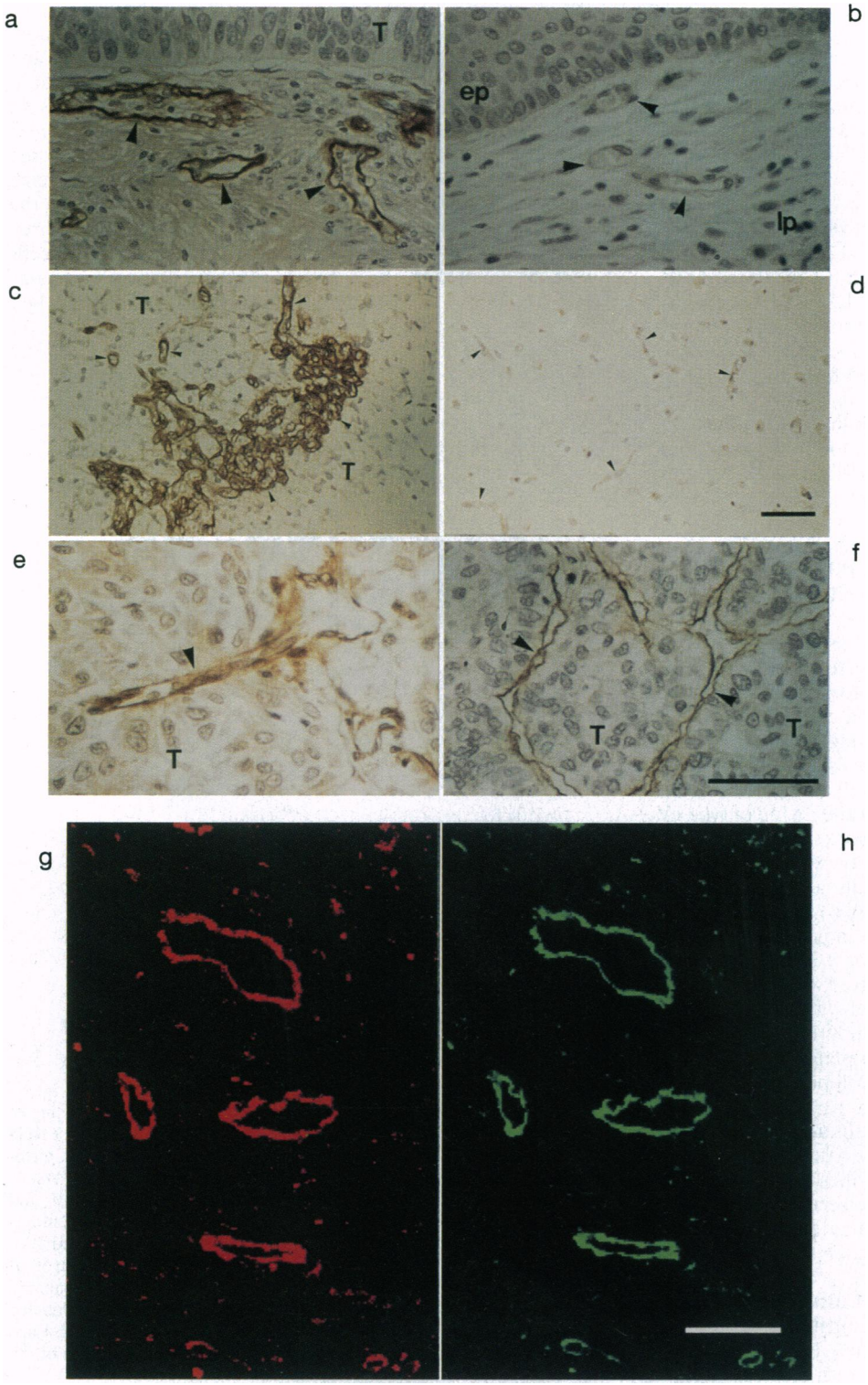

FIG. 1. Distribution of TAF in normal and tumor parts of various human cancers. (*a*) Squamous cell carcinoma of the esophagus, (*b*) normal esophagus tissue, (*c*) glioblastoma, (*d*) normal brain tissue, (*e*) squamous cell carcinoma of the lung, (*f*) hepatoma. T, tumor cells; ep, epithelium (normal nonkeratinized stratified squame); lp, lamina proprium. The magnification of *a*, *b*, *e*, and *f* is indicated by the scale bar (50 μm) in *f*, whereas that of *c* and *d* is by the scale bar (50 μm) in *d*. In the four tumor tissues (*a*, *c*, *e*, and *f*), intense immunoreactivity to TAF, shown by brownish staining, is exclusively seen in blood vessels (arrowheads) including capillaries and hemoceloms (*f*) near or within tumor nests. In contrast, capillaries (arrowheads) in the normal tissues (*b* and *d*) hardly show the immunoreactivity. (*g* and *h*) Double immunostaining of a gastric adenocarcinoma with antibodies against type IV collagen (*g*) and TAF (*h*). (Bar = 50 μm.)

conjugated goat anti-rabbit IgG (Vector Laboratories) were used as second antibodies at a dilution of 1:100. Mixtures of 3% each and 10% each of horse serum and goat serum in PBS were used for dilution of antibodies and for blocking of the sections, respectively. Fluorescent images were obtained using a laser scanning confocal microscope (Bio-Rad). To examine the extracellular localization of endogenous TAF or type IV collagen in the endothelial cell culture containing capillary tube-like structure, the anti-TAF monoclonal antibody and anti-type IV collagen antiserum were directly added to the confluent culture of ECV-304 cells, followed by incubation for 2 h. The cultures were washed with PBS, fixed with 10% (vol/vol) formalin in PBS. Double immunofluorescent staining was performed as described above. Negative controls included replacement of the primary antibody with preimmune mouse IgG for anti-TAF monoclonal antibody or with normal rabbit serum for anti-type IV collagen antiserum.

Cells and Cell Culture Conditions. Human umbilical vein endothelial cell line ECV-304 (19) was a kind gift from K. Takahashi (National Defense Medical College, Tokovozawa, Japan). Human bladder carcinoma cell line EJ-1 was provided by the Japanese Cancer Resource Bank (Tokyo). Both cell lines were routinely cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DME/F12) (GIBCO-BRL), supplemented with 0.1 mg/ml streptomycin sulfate, 100 units/ml penicillin G, 1.2 mg/ml $NaHCO_3$, and 10% (vol/vol) fetal calf serum (Moregate, Melbourne, Australia), (DME/F12/10% FCS), at 37°C in 5% CO_2/95% air. Plastic culture dishes were purchased from Sumibe Medical (Tokyo).

Assay of Binding of TAF to Extracellular Matrix Proteins Immobilized on Plastic Plates. TAF was iodinated according to the Bolton–Hunter method as described (20). Specific activity of labeled TAF was 3.9×10^6 cpm/μg. For the binding assay, 50 μl (0.5 μg) each of various ECM protein solutions was dried up in each well of microtiter plates at room temperature overnight. The plates were washed with PBS-0.05% (vol/vol) Tween 20 (PBS–Tween), and then blocked with 1% (wt/vol) casein at 37°C for 90 min. After washing, ^{125}I-TAF (3.5×10^4 cpm/9 ng in PBS–Tween) was added into each well and incubated overnight at room temperature, followed by washing five times with PBS–Tween. The radioactivity of TAF bound to the plates was determined in a gamma counter (Packard).

Cell Attachment Assay to Type IV Collagen Substrate. The cell attachment assay was carried out by essentially the same method as described in ref. 21. Each well of 96-well ELISA plates (Sumibe Medical) was coated with 100 μg/ml of type IV collagen in PBS at 37°C for 2 h and blocked with 1% BSA (wt/vol) in PBS. ECV-304 cells were trypsinized and suspended in serum-free DME/F12 medium, and then 3×10^4 cells were plated in each well of the BSA-coated or collagen-coated plate in the presence of TAF at indicated concentrations, followed by incubation at 37°C for 2 h. After washing twice with PBS, the attached cells were fixed with 5% (vol/vol) glutaraldehyde for 20 min and stained with 10 μg/ml of Hoechst 33342 in 0.001% (vol/vol) Triton X-100 for 1.5 h. The fluorescent intensity of each well of the plates was measured using a CytoFluor 2350 fluorometer (Millipore) with excitation at 360 nm and emission at 530 nm. To examine the morphological change of endothelial cells induced by TAF, Lab-Tek chamber slides (Nunc) were coated with type IV collagen and blocked with BSA, as described above. The cells were plated in each well containing the serum-free medium without or with 10 μg/ml of TAF and incubated at 37°C for 2 h. The cell morphology was examined under a phase-contrast microscope.

Formation of Tubular Structures by Cultured Endothelial Cells. ECV-304 cells were grown to confluence in 90-mm dishes containing 10 ml of DME/F12/10% FCS. The culture medium was replaced with 10 ml of the fresh medium supplemented with 100 μg/ml type I collagen and further incubated. The next day, the formation of capillary tube-like structures was assessed by phase-contrast microscopy. For immunohistochemical analysis, the cells were plated on Lab-Tek chamber slides (Nunc) and treated similarly. To examine the effects of heparin on the formation of capillary tube-like structures, 20 μg/ml of porcine intestinal heparin (Wako Biochemicals, Tokyo), in addition to type I collagen, were added into the culture of ECV-304. The next day, the culture was fixed with methanol, stained with 2.5% Giemsa solution, and then photographed.

Assay of Binding of TAF to Cultured Endothelial Cells. ECV-304 cells were plated at 1×10^4 cells per well on 96-well culture plates, followed by incubation at 37°C for 3 days. The culture was rinsed three times with PBS and then fixed with 3.75% (vol/vol) glutaraldehyde in PBS for 15 min at room temperature. The fixed cells were washed with PBS and incubated with 50 mM Tris·HCl (pH 7.5)/0.1 M glycine to stop the fixation. The wells were blocked with 1% (wt/vol) casein at 37°C for 90 min. After washing, TAF in PBS was added into each well and incubated at 37°C for 90 min. To examine the effect of heparin on binding of TAF to fixed cells, porcine intestinal heparin, as well as TAF, were incubated with the cells. Unbound TAF molecules were removed by washing three times with PBS–Tween. Amounts of TAF bound to the cells were determined by the enzyme-linked immunosorbent assay with the anti-TAF antibody, biotinylated anti-mouse IgG

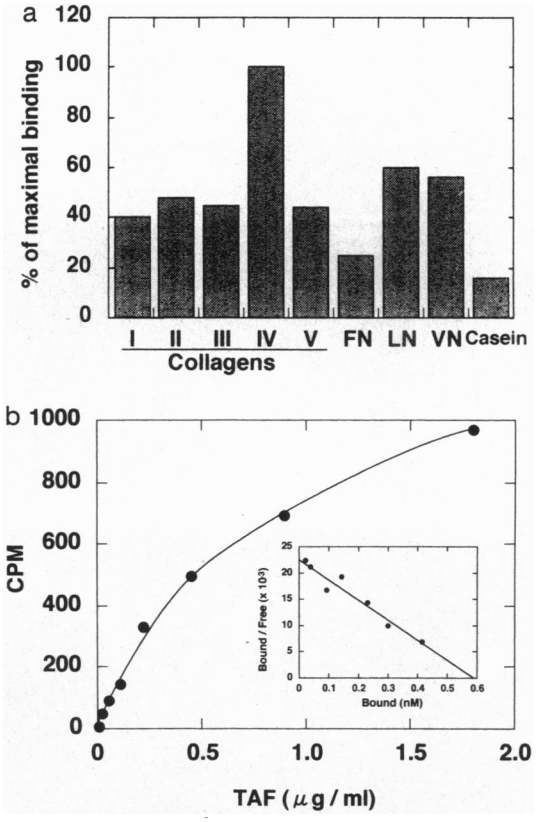

FIG. 2. Specific binding of TAF to type IV collagen. (*a*) Binding of ^{125}I-labeled TAF to various extracellular matrix components and casein immobilized on plastic plates. FN, fibronectin; LN, laminin; VN, vitronectin. Experimental conditions are described in *Materials and Methods*. Radioactivity in wells coated with type IV collagen was set at 100%, and binding of TAF to the other ECM proteins was expressed as a percentage. Results represent the mean of duplicates. (*b*) Binding of ^{125}I-labeled TAF to type IV collagen. Each well of microtiter plates was coated with 50 μl of 2 μg/ml type IV collagen, blocked with 1% casein, and then incubated with ^{125}I-labeled TAF at indicated concentrations. Binding of ^{125}I-labeled TAF to casein was subtracted from each point as a background. Each point represents the mean of duplicate. (*Inset*) Scatchard analysis of the binding of TAF to type IV collagen.

antibody (Vector Laboratories), avidin–alkaline phosphatase (Vector Laboratories), and *p*-nitrophenylphosphate sodium salt (Wako Biochemicals) as substrate. The color development was measured at 405 nm in Immunoreader NJ-2,000 (Inter Med, Tokyo).

Determination of Protein Concentrations. Protein concentration of TAF was determined by the method of Lowry (22). Other protein concentrations were determined by the dye method with a Bio-Rad protein assay kit (23). In both assays, BSA was used as a standard.

Reagents. Bovine plasma fibronectin and pepsin-treated bovine lens type IV collagen were purchased from Iwaki Glass (Tokyo); pepsin-treated bovine skin type I collagen, bovine cartilage type II collagen, and pepsin-treated bovine placenta types III and V collagens were from Koken (Tokyo); the BSA was from Sigma.

RESULTS

Distribution of TAF in Normal and Tumor Tissues. To elucidate the physiological function of TAF, its distribution in various human cancer tissues was examined by immunohistochemical staining with an anti-TAF monoclonal antibody. In esophagus carcinomas, TAF antibody densely stained small blood vessels proximal to tumor cells (Fig. 1*a*), whereas it hardly stained blood vessels in the normal part of the tissue (Fig. 1*b*). New blood vessels in tumor tissues often have a tortuous architecture compared with normal vessels. In a malignant glioma tissue intense staining for TAF was observed in the irregular blood vessels and capillary tubes formed within the tumor lump (Fig. 1*c*). However, TAF was little detected in the vessels present in the normal part of the tissue (Fig. 1*d*). TAF immunoreactivity in the hyperplastic vessels was observed in most gliomas tested, and it was more pronounced in high grade tumors than low grade ones (data not shown). TAF was also detected in capillaries surrounded by lung squamous carcinoma cells (Fig. 1*e*) and hemoceloms formed in a hepatoma tissue (Fig. 1*f*).

As shown in Fig. 1 *a* and *e*, TAF appeared to locate just under the endothelial cell layer of the tumor blood vessels, possibly in the basal lamina. To test the association of TAF with the vascular basement membrane, double immunofluorescent staining for TAF and type IV basement membrane collagen was carried out with a gastric carcinoma tissue. Anti-type IV collagen and anti-TAF antibodies preferentially stained blood vessels (Fig. 1 *g* and *h*). The two staining patterns completely overlapped, suggesting that they were colocalized in the vascular basement membrane. On the other hand, TAF was not detected in the nonvascular basement membrane underlying carcinoma cells or normal epithelial cells (Fig. 1 *a* and *b*). Tumor blood vessel-specific staining of TAF was also observed in other types of human cancers such as colon cancer, ovarian cancer, osteosarcoma, and Wilms tumor.

Binding of TAF to Type IV Collagen. The immunohistochemical study suggested that TAF might be accumulated in tumor blood vessels in association with some ECM component. Therefore, the affinity of TAF to various ECM proteins was determined with ^{125}I-labeled TAF. TAF most efficiently bound to type IV collagen, though it also showed some affinity to laminin, vitronectin, and four other types of collagens (Fig. 2*a*). In a quantitative analysis, ^{125}I-labeled TAF bound to type IV collagen immobilized on a plastic plate in a concentration-dependent manner (Fig. 2*b*). The binding of ^{125}I-labeled TAF to type IV collagen was inhibited by 100-fold excess of unlabeled TAF to the background level (data not shown). The dissociation constant of TAF for type IV collagen was determined to be approximately 2.6×10^{-8} M by the Scatchard plot analysis (Fig. 2*b*, *inset*). Furthermore, the specific binding of TAF to type IV collagen was confirmed with a type IV collagen-conjugated affinity column. When the serum-free conditioned medium of EJ-1 bladder carcinoma cells was applied to the column, TAF was specifically bound to the column and eluted with 7.5% dimethyl sulfoxide (data not shown). The interaction of TAF with denatured type IV collagen was also shown by the ligand blotting analysis, in which TAF bound to not only the triple-helix form of the collagen but also its subunit peptides on a nitrocellulose membrane (data not shown).

Effect of TAF on Adhesion of Endothelial Cells to Type IV Collagen. To understand the biological implication of the interaction of TAF with type IV collagen, their synergistic effect was examined with human umbilical vein endothelial cell line ECV-304. The addition of TAF into the culture medium promoted the attachment of ECV-304 cells to type IV collagen in a dose-dependent manner (Fig. 3*a*). The endothelial cells loosely attached to the type IV collagen substrate in the absence of TAF (Fig. 3*b*, left). In the presence of TAF, the cells well spread on the substrate and extended prominent pseudopodia-like processes (Fig. 3*b*, right). TAF also promoted the adhesion of primary cultures of human umbilical vein endo-

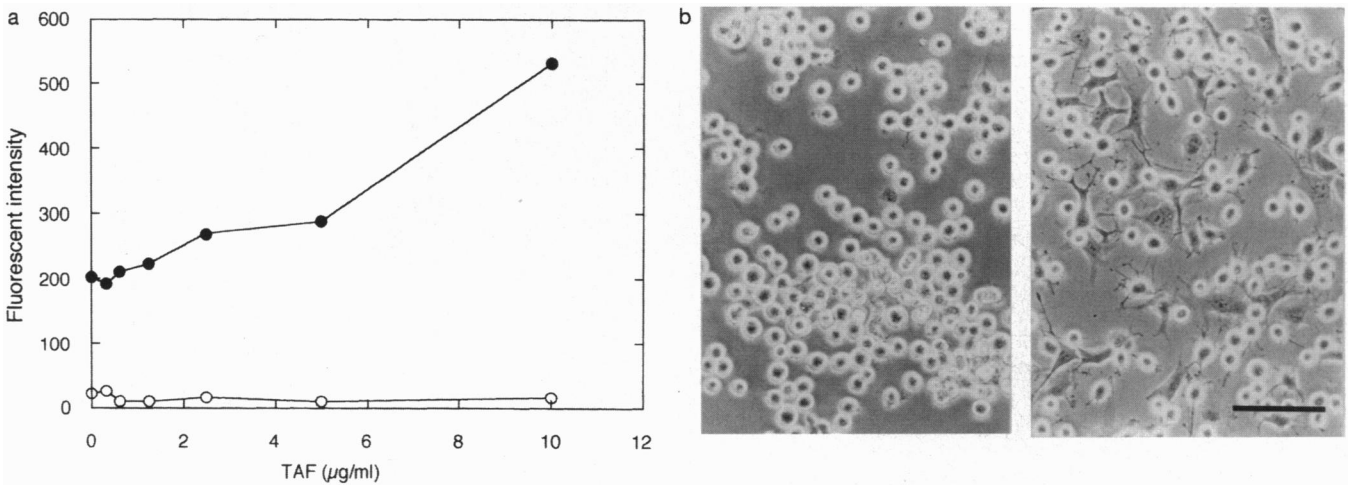

FIG. 3. Adhesion of ECV-304 endothelial cells to type IV collagen in the absence or presence of TAF. (*a*) ECV-304 cells were incubated with indicated concentrations of TAF on 96-well ELISA plates coated with 100 μg/ml type IV collagen (●) and BSA (○) as negative control, at 37°C for 2 h. Cells that attached to the substrates were quantitated by the Hoechst 33342 staining methods as described. (*b*) ECV-304 cells were incubated in the absence (*left*) and presence (*right*) of 10 μg/ml TAF for 2 h on Lab-Tek chamber slides pretreated with 100 μg/ml type IV collagen. (Bar = 50 μm.)

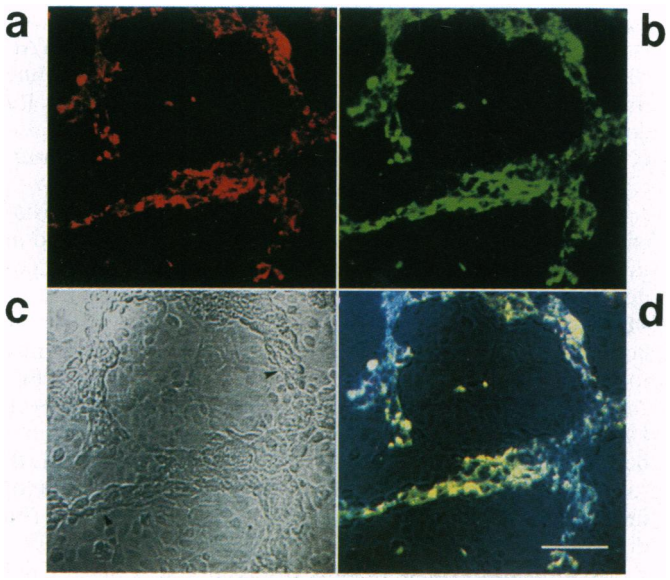

FIG. 4. Colocalization of type IV collagen and TAF in tube-like structures formed on monolayer of ECV-304 endothelial cells. ECV-304 cells were cultured on Lab-Tek chamber slides. The confluent monolayer of ECV-304 was incubated overnight in DME/F12+10%FCS containing 100 μg/ml type I collagen. Double immunofluorescent staining for type IV collagen (*a*) and TAF (*b*) present in capillary tube-like structures (arrowheads) formed on the monolayer culture was carried out as described. (*c*) Phase contrast image. (*d*) Overlay image of *a–c*. (Bar = 100 μm.)

thelial cells and human dermal microvascular endothelial cells to type IV collagen (data not shown).

Accumulation of TAF in Capillary Tube-Like Structures Formed by Cultured Endothelial Cells. The assembly of cultured endothelial cells into capillary-like tubes has been extensively used as a model system of angiogenesis *in vitro* (24–28). The tube formation of endothelial cells is promoted by type I collagen (28). ECV-304 cells also formed the tubular structures when type I collagen was added to the confluent culture (Fig. 4*c*). Extracellular localization of endogenous type IV collagen and TAF was immunochemically examined in the endothelial cell culture by adding the respective antibodies to the culture without fixation and permeabilization. Both type IV collagen and TAF were intensely immuno-stained along the fibrillar array of the endothelial cells with the tubular structures (Fig. 4 *a* and *b*), and their staining patterns were completely overlapped with each other (Fig. 4*d*). In contrast, both proteins were little detected on the surface of the basal cells. When type IV collagen instead of type I collagen was added to the endothelial cell culture, the tubular structures were not formed but some elongated cells piled up on the basal cell monolayer. TAF was also associated with these sprouting cells (data not shown).

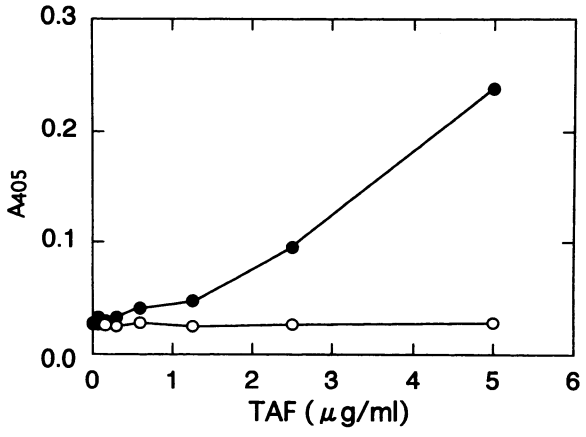

FIG. 5. Inhibition of TAF binding to glutaraldehyde-fixed ECV-304 cells by heparin. Glutaraldehyde-fixed ECV-304 cells were incubated with indicated concentrations of TAF in the presence (○) or absence (●) of 10 μg/ml of porcine intestinal heparin. After incubation for 1.5 h at 37°C, bound TAF was detected by anti-TAF monoclonal antibody as described. Binding of TAF to casein was subtracted from each point as a background. Each point represents the mean of triplicate.

Inhibition of TAF Binding to Cell Surface by Heparin. TAF is a heparin-binding protein. It bound to a heparin-Sepharose column and eluted from the column with 0.3 M NaCl (data not shown). Therefore, effect of heparin on the binding of TAF to cell surface was examined with glutaraldehyde-fixed ECV-304 cells. As shown in Fig. 5, TAF bound to the fixed endothelial cells in a dose-dependent manner, but it was almost completely inhibited by the presence of 10 μg/ml heparin. This suggested that TAF might recognize a heparin-like molecule on cell surface. Next, effect of heparin on the formation of capillary tube-like structures on the monolayer culture of ECV-304 endothelial cells was examined. When the confluent culture of ECV-304 cells was added with 20 μg/ml of heparin in addition to type I collagen, the formation of the fibrillar array of the endothelial cells was almost completely prevented though some cells still sprouted (Fig. 6). Heparin did not significantly affect the growth and morphology of the endothelial cells in monolayer culture under sparse conditions. The results shown in Figs. 4–6 strongly suggest that TAF supports the organization of endothelial cells into the tubular structures.

DISCUSSION

The present immunohistochemical analysis of human normal and tumor tissues with an anti-TAF antibody demonstrated that TAF was specifically accumulated in blood vessels of tumor tissues but not in those of normal tissues. This suggested a role of TAF in tumor angiogenesis and prompted us to investigate the interaction of TAF with vascular endothelial cells in more detail. Angiogenesis requires migration, proliferation, and morphogenesis (tube formation) of vascular endothelial cells (2, 7, 8). In the past, most studies were focused on angiogenic factors, which regulate two earlier steps of angiogenesis, i.e., the migration and proliferation of the endothelial cells (4–6, 29–32). On the other hand, the morphogenetic process of endothelial cells into capillaries, the last step

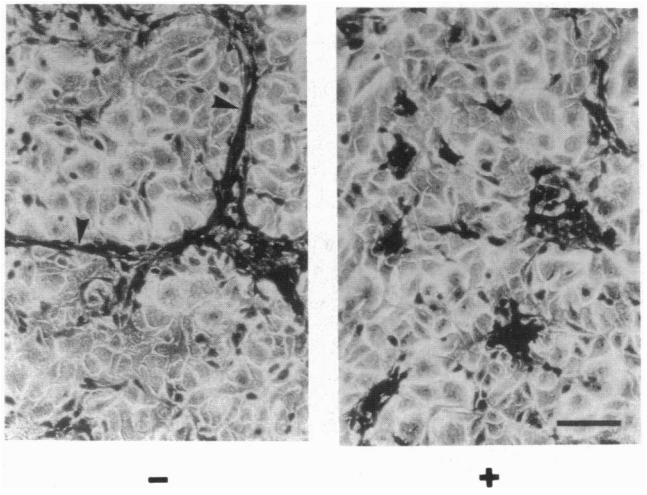

FIG. 6. Effect of heparin on capillary tube formation of ECV-304 endothelial cells. The confluent monolayer of ECV-304 cells was incubated overnight in the absence (−) or presence (+) of 20 μg/ml of porcine intestinal heparin in DME/F12+10%FCS containing 100 μg/ml of type I collagen. The cultures were stained with Giemsa and photographed. Arrowhead, capillary tube-like structures. (Bar = 100 μm.)

of angiogenesis, seems more complex and remains mostly unknown. It is expected that this step is regulated by complex interactions between endothelial cells and ECM, in particular the basement membrane components. It has been reported that laminin (10) and type IV collagen (11) promote the tube formation of endothelial cells *in vitro*. In addition, immunostaining with anti-type IV antibody has shown the matrix-associated labeling of the collagen especially in areas of the tube formation of cultured endothelial cells (11). Ingber and Folkman (33) showed that the inhibition of type IV collagen biosynthesis prevented angiogenesis *in vivo*.

In this study, TAF showed specific affinity for type IV collagen and appeared to be colocalized with the collagen in the vascular basement membrane in many human cancer tissues and in capillary tube-like structures formed by cultured endothelial cells *in vitro*. In addition, TAF promoted adhesion of the endothelial cells to type IV collagen and changed their morphology from a round shape to an elongated shape with prominent pseudopodia. These results give rise to the possibility that TAF is involved in the step of capillary tube formation of endothelial cells. The cell-adhesive activity and the morphological effect of TAF on endothelial cells strongly suggest that TAF may stabilize the 3D architecture of capillary tubes, in cooperation with type IV collagen. This hypothesis is strongly supported by the finding that heparin, which inhibited the binding of TAF to endothelial cells, prevented the organization of endothelial cells into capillary tube-like structures. Heparin did not inhibit the sprouting of endothelial cells but did inhibit their fibrillar array. These results suggest that the binding of TAF to the cells through cell surface heparin-like molecules is critical to the fibrillar array of the sprouting endothelial cells. It has been established that heparin-like molecules, most likely heparan sulfate proteoglycans, are closely associated with microvascular endothelial cells (34, 35).

A tortuous architecture and resultant irregular flow pattern are characteristic to tumor blood vessels. Tumor endothelium is also more permeable, has less basement membrane, and is made up of cells that divide at a much higher rate than normal. Dense accumulation of TAF in the vascular basement membrane may be responsible for these vascular anomalies in tumor vessels, in addition to the angiogenic effect. TAF (14), the putative mac25 protein (15), and PSF (17) are probably an identical protein. It has a unique N-terminal structure that is highly conserved among IGF binding proteins (15, 16). We have recently found that TAF has a relatively low but significant activity to bind insulin and IGF-I and -II, and that TAF strongly potentiates the mitogenic activities of IGF-I and insulin toward mouse fibroblasts on a type IV collagen substrate (unpublished work). In this study, TAF by itself affected neither chemotactic migration nor growth of endothelial cells (data not shown). However, it seems possible that TAF deposited on the vascular basement membrane accumulates these growth factors, which in turn stimulate the migration and growth of endothelial cells in the tumor angiogenesis. On the other hand, PSF has been reported to stimulate the production of prostacyclin (PGI_2) in vascular endothelial cells (17). PGI_2 is a potent vasodilative substance and inhibits platelet aggregation. The high permeability of tumor blood vessels and frequent invasion of metastatic tumor cells into the vessels may also be related to the dense deposition of TAF and resultant production of PGI_2 by endothelial cells.

It remains unclear whether TAF in tumor vessels is derived from endothelial cells or tumor cells. In immunohistochemical analysis, TAF immunolabeling was almost exclusively observed in blood vessels proximal to tumor cells, whereas tumor cells themselves were weakly stained only in rare cases. *In vitro* studies showed that vascular endothelial cells, fibroblasts, epithelial cells, and various kinds of human cancer cell lines secreted TAF into their culture media (unpublished work). In particular, endothelial cells such as ECV-304 cells and primary cultures of human umbilical vein endothelial cells and human dermal microvascular endothelial cells secreted high levels of TAF, suggesting the production of TAF by vascular endothelial cells *in vivo*. In preliminary *in situ* hybridization study we did not detect TAF mRNA in the endothelial cells of human glioma tissues, though the apparent lack of the message might be due to the technical difficulty. Relatively low levels of the TAF message was detected on tumor cells, indicating that tumor cells might be a part of the source of TAF. More extensive analysis is required to determine the origin of TAF in tumor blood vessels.

In summary, this study showed that TAF was accumulated in tumor blood vessels and in capillary tube-like structures of cultured endothelial cells. Such distribution and biological activities of TAF indicate that TAF is a new angiogenic modulator, which is probably responsible for angiogenesis and vascular abnormalities in tumor tissues. Therefore, we propose a more functional name "angiomodulin" for the newly identified vascular protein TAF/mac25/PSF.

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