Proteolytically Processed Soluble Tumor Endothelial Marker (TEM) 5 Mediates Endothelial Cell Survival during Angiogenesis by Linking Integrin $\alpha_v\beta_3$ to Glycosaminoglycans

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Tumor endothelial marker (TEM) 5 is a member of the adhesion family of G-protein-coupled receptors and up-regulated in endothelial cells during tumor and physiologic angiogenesis. Here, we report that TEM5 is expressed on the surface of endothelial cells. A soluble TEM5 (sTEM5) fragment is shed by endothelial cells during capillary-like network formation and upon growth factor stimulation. We found that sTEM5 binds to several glycosaminoglycans. Furthermore, sequence analysis and functional and biochemical studies revealed that sTEM5 contains a cryptic RGD-binding site for integrin $\alpha_v\beta_3$. Matrix metalloprotease 9-processed, but not full-length, sTEM5 mediated endothelial cell adhesion by direct interaction with integrin $\alpha_v\beta_3$. Adhesion to proteolytically processed sTEM5 (ppsTEM5) or glycosaminoglycan-bound ppsTEM5 promoted survival of growth factor deprived endothelial cells. ppsTEM5-mediated cell survival was inhibited by a function blocking integrin $\alpha_v\beta_3$ antibody. Based on our results we conclude that sTEM5 is shed by endothelial cells during angiogenesis and binds to glycosaminoglycans present on extracellular matrix and cell surface proteoglycans. Further proteolytic processing of sTEM5 leads to exposure of its RGD motif mediating endothelial cell survival by linking integrin $\alpha_v\beta_3$ to glycosaminoglycans.

**Tumor angiogenesis is crucial for the growth of solid tumors. Angiogenic factors released by the tumor activate endothelial cells to form new blood vessels that supply the tumor with oxygen and nutrients and thereby promote tumor growth. Therefore, the directed destruction of the tumor endothelium for the treatment of cancer has been an issue for the last 15 years. Tumor vessels differ morphologically from vessels of healthy tissue and are characterized by the expression of molecules specific for angiogenesis. These molecules could serve as targets for the specific delivery of drugs into tumors (1, 2). However, the role of most of these molecules in angiogenesis is still unknown and needs further investigation.**

**Tumor endothelial markers (TEMs)3 1–8 were identified as previously unknown genes that displayed elevated expression in endothelial cells isolated from colorectal carcinoma (3). Further studies showed that TEMs are also up-regulated in endothelial cells undergoing physiologic angiogenesis in humans and mice (4). Recently, the extracellular matrix (ECM) proteins nidogen and collagen VI have been described as ligands of the transmembrane proteins TEM7 and TEM8, respectively (5, 6). By homology TEM5 belongs to the group III of adhesion G-protein-coupled receptors (GPCRs), which contain a seven-pass transmembrane domain and a long extracellular N-terminus bearing conserved domains involved in cell-cell and cell-matrix interactions (7). It remains to be verified by experimental evidence if TEM5 serves as a GPCR in endothelial cells. The TEM5 extracellular domain contains several conserved subdomains: a leucine-rich repeat (LRR) domain with four LRRs and an LRR C-terminal (LRRCT) motif, an immunoglobulin (IG) domain, a hormone receptor (H/HormR) domain, and a membrane proximal GPCR proteolysis site (Fig. 1A). The LRR domain shares homology with the membrane protein LIG-1 and the secreted SLIT proteins (8). It has been reported that proteolytic cleavage at the GPCR proteolysis site of the GPCRs brain angiogenesis inhibitor 1 and CD97 leads to shedding of their extracellular domains (10, 11). The TEM5 intracellular domain contains a conserved PDZ-binding motif at the C terminus. It has been shown that the tumor suppressor protein human disc large interacts with the TEM5-intracellular domain via this motif (12).

This study was designed to elucidate the role of TEM5 in angiogenesis. We found that TEM5 is localized on the surface of endothelial cells. Soluble TEM5 (sTEM5) is shed by capillary-like network forming or growth factor stimulated endothelial cells and binds to glycosaminoglycans. After proteolytic processing a binding site for integrin $\alpha_v\beta_3$ is exposed on sTEM5. Adhesion of endothelial cells to immobilized proteolytically processed TEM5 (ppsTEM5) promotes their survival.**

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3 The abbreviations used are: TEM, tumor endothelial marker; GPCR, G-protein-coupled receptor; LRR, leucine-rich repeat; H/HormR, hormone receptor; LRRCT, LRR C-terminal; s, soluble; pps, proteolytically processed soluble; HUVEC, human umbilical vein endothelial cell; HEK293, human embryonic kidney 293; eEND, embryonic endothelioma; MMP, matrix metalloprotease; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ECM, extracellular matrix; HRP, horseradish peroxidase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.
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MATERIALS AND METHODS

Reagents and Antibodies—Vascular endothelial growth factor (VEGF) was purchased from PeproTech (Rocky Hill, NJ). Basic fibroblast growth factor (bFGF) was from ImmunoTools (Friesoythe, Germany). Monoclonal anti-FLAG (clone M2) horse radish peroxidase (HRP) and agaroose-conjugated antibodies, laminin from Engelbreth-Holm-Swarm sarcoma, heparin-biotin, avidin, heparan sulfate, chondroitin sulfated A, and dermatan sulfate were from Sigma. Polyclonal TEM5 antibody (GB-TEM5–2) was from Genesis Biotech (Hsientien, Taiwan). Monoclonal anti-His tag antibody (clone ADL1.10) and AlamarBlue were from Serotech (Oxford, England). Monoclonal anti-myc HRP-conjugated antibody (clone 4A6) was from Upstate Biotechnology (Lake Placid, NY). Growth factor-reduced Matrigel and MatriSperse were from BD Biosciences. Arg-Gly-Asp (RGD) peptide was from Bachem (King of Prussia, PA). Cell function blocking integrin antibodies (anti-α1, clone FB12, anti-α2, clone P1E6, anti-α5, clone P1D6, anti-αv, clone P3G8, anti-αvβ3, clone LM609, anti-αvβ3, clone P1F6), monoclonal actin antibody (clone C4), and purified integrin α5β3 were from Chemicon. Monoclonal vitronectin antibody (clone VN58–1) was from Abcam (Cambridge, UK). Monoclonal capsase-3 antibody (clone 84803) was from R&D Systems (Minneapolis, MN). EDTA-free protease inhibitor mixture was from Roche Applied Science. Geneticin was from Invitrogen. Collagen from bovine calf skin was from Biochrom (Berlin, Germany). Vitronectin was from Promega (Manheim, Germany). Endothelial cell basal medium was from PromoCell (Heidelberg, Germany).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cords as described previously (17) and maintained in endothelial cell growth medium (PromoCell). Culture flasks, plates, and coverslips were coated with 0.1 mg/ml collagen in PBS for 30 min prior to seeding HUVECs unless indicated otherwise. HUVECs from passages 0–4 were used for experiments. Human embryonic kidney (HEK) 293 and embryonic endothelioma cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. M21, M21L, and TD2 cells were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum. Cells were cultured at 37 °C and 5% CO2.

Cloning, Expression, and Purification of TEM5 Constructs—Full-length TEM5 was amplified by PCR from the cDNA clone FLJ00170. sTEM5 was amplified from the plasmid pIVEX2.3-MCS-sTEM5. Amplified cDNAs were cloned into the p3xFLAG-CMV-9 expression vector (Sigma). The sTEM5 protein is proteolytically processed by matrix metalloprotease (MMP)-9 resulting in an N-terminal fragment of ~80 kDa (pp5TEM5, Fig. 4B). This fragment was cloned estimating the size of the nucleotide sequence from the apparent molecular weight of the protein. pp5TEM5, TEM5-HG, and TEM5-G were derived from the sTEM5 expression construct by deleting the concerned regions using the QuikChange site-directed mutagenesis kit (Stratagene). HEK293 cells were stably transfected with the soluble expression constructs using calcium phosphate coprecipitation and Geneticin (0.6 mg/ml) as a selective antibiotic. Culture medium from confluent stably transfected cells was replaced with Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and 0.6 mg/ml Geneticin. Supernatants were harvested every 72 h and centrifuged to remove dead cells and debris. Cleared supernatants were concentrated 500-fold using Centricron Plus-20 centrifuge filters (Millipore). Concentrated supernatants were diluted in 50 volumes of PBHST (10 mM sodium phosphate, pH 7.2, 300 mM NaCl, 0.05% Tween 20) and incubated with anti-FLAG-agarose for 1 h under constant rotation. Agarose beads were washed with 30 volumes of PBHST followed by 10 volumes of PBS. Elution was performed as described below. Because of weak secretion pp5TEM5 was also purified from cell lysates. Cells were detached from the culture flasks by rinsing with PBS and washed once with PBS. Cells were lysed in 10 mM sodium phosphate, pH 7.2, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (lysis buffer) and incubated on ice for 30-min vortexing occasionally. Lysate was cleared by centrifugation and incubated with anti-FLAG-agarose for 1 h under constant rotation. Agarose beads were washed with 30 volumes of lysis buffer, 10 volumes of PBHST, and 20 volumes of PBS. Bound proteins were eluted in fractions using 0.1 M glycine-HCl, pH 3.5. Eluates were neutralized, and proteins containing fractions were pooled and concentrated using Centricron Plus-20 centrifuge filters. Protein concentrations were determined using the Protein dotMetric Assay (Geno Technology, St. Louis, MO).

Transfection and Microinjection—HUVECs were transfected with plasmid DNA using Lipofectin (Invitrogen) according to the manufacturer’s protocol. For microinjections plasmid DNA (0.1 μg/μl in Tris-buffered saline containing 5 mM MgCl2) was injected into the cell nuclei using a micromanipulator.

SDS-PAGE and Immunoblot Analysis—Cells were lysed using reducing Laemmli sample buffer. Protein samples in Laemmli sample buffer were incubated for 5 min at 95 °C and separated by SDS-PAGE using 8, 10, or 14% gels. A full-range Rainbow molecular weight marker (Amersham Biosciences) served as the molecular weight standard. For immunoblots analysis separated proteins were blotted on polyvinylidene difluoride membranes (Amersham Biosciences), and blots were blocked with 5% nonfat skimmed milk in PBST (PBS containing 0.05% Tween 20). Blots were incubated overnight at 4 °C with primary antibody diluted in blocking buffer and washed three times with PBST. If unconjugated primary antibody was used membranes were incubated for 1 h with HRP-conjugated secondary antibody (Amersham Biosciences) directed against appropriate species diluted in blocking buffer. Membranes were washed three times with PBST and incubated with ECL substrate (Amersham Biosciences). Luminescence was detected using Kodak BioMax XAR films (Sigma).

Immunofluorescence Staining—CELLocate coverslips (Eppendorf, Hamburg, Germany) were placed in 6-well plates, and 5 × 105 HUVECs per well were seeded and incubated for 24 h. Patches of ~50 cells were microinjected with an equimolar mixture of full-length TEM5 and a GFP expression construct (pTurboGFP-C, Evrogen, Moscow, Russia). 24 h after microinjection cells were fixed with 3.7% formaldehyde in PBS for 10 min. Cells were washed three times with PBS and permeabi-
lized with 0.5% Triton X-100 in PBS for 10 min or left non-
permeabilized. After washing with PBS cells were incubated for
1 h with a polyclonal anti-FLAG antibody (Sigma) diluted 1:200
in PBS. Cells were washed with PBS and incubated for 1 h with
a TRITC-conjugated anti-rabbit IgG antibody (Invitrogen)
diluted 1:200 in PBS. Cells were washed with PBS, and cover-
slips were mounted on microscopic slides using Vectashield
hard set mounting medium (Vector Laboratories, Burlingham,
CA). Fluorescence microscopic images were taken at 400-fold
magnification using a digital camera.

**TEM5 Shedding Assay**—HUVECs were transfected with the
indicated expression constructs. 24 h after transfection cells
were trypsinized, and 2.5 × 10⁵ cells per well were seeded on
Matrigel-layered (1.5 ml/well) or collagen-coated 6-well plates.
Cells were stimulated with 100 ng/ml VEGF or bFGF or left
unstimulated. Phase contrast microscopic images were taken at
the indicated time points at 100-fold magnification using a dig-
ital camera. Supernatants from cells in collagen-coated wells
were centrifuged to remove dead cells and debris. Medium
from cells in Matrigel was aspirated, and cells were recovered
from Matrigel using MatriSperse according to the manufactur-
er’s protocol saving the solubilized Matrigel (supernatants).
Cells were lysed using 40 μl of reducing Laemmli sample buffer.
Cleared supernatants were incubated with 10 μl of anti-FLAG
agarose overnight at 4 °C under constant rotation. Agarose
beads were washed three times with PBS. For immunoblot anal-
ysis bound proteins were eluted with 1 volume of 2× reducing Laemmli sample buffer for 5 min at 95 °C. For the enzyme-linked immunosorbent assay (ELISA) bound proteins were eluted with 5 volumes of 0.1M glycine-HCl, pH 3.5. 96-well polystyrene plates were coated overnight at 4 °C with the neutralized eluates.

Cell Adhesion Assay—96-Well polystyrene plates were coated overnight at 4 °C with different proteins diluted in PBS at indicated concentrations. Wells were blocked for 1 h with 1% BSA in PBS (PBS/BSA). Cells were trypsinized and resuspended in culture medium. Cell suspensions were incubated for 2 h at room temperature under gentle agitation for cell recovery. Recovered HUVECs were resuspended in endothelial cell basal medium containing 0.1% BSA and recovered M21/M21L cells were resuspended in RPMI medium containing 0.1% BSA. HUVECs were treated with different inhibitors or left untreated for 30 min. 3 × 10^4 cells were seeded per well and allowed to adhere for 30 min at 37 °C. Soluble inhibitors were present where indicated. Cells were washed once with PBS and culture medium containing 0.1 volume AlamarBlue dye was added to each well. Cells were incubated for 3–16 h at 37 °C. Supernatants were transferred to new 96-well plates, and absorbance at 570 nm and 600 nm was measured. Relative adhesion was equal to relative reduction of the dye which was calculated according to the supplier’s manual.

MMP-9 Cleavage of TEM5 Constructs—Purified TEM5 constructs were dialyzed overnight against MMP-9 buffer (Tris-buffered saline containing 5 mM CaCl2 and 0.005% Brij35). 0.2 mg/ml protein was incubated with 10 μg/ml recombinant human MMP-9 (Merck, Darmstadt, Germany) at 37 °C for 20 h.

Affinity Chromatography—Vitronectin and TEM5 constructs were dialyzed overnight against PBS. 25 μg of protein was coupled to 25 μl of AminoLink Plus-agarose (Pierce) at pH 7.2 according to the manufacturer’s protocol. Coupled agarose was equilibrated with buffer A (Tris-buffered saline containing 0.1 mM CaCl2, 1 mM MgCl2, and 0.5 mM MnCl2) supplemented with 0.1% BSA and and 0.2% Triton X-100 (binding buffer) and incubated with 50 μg/ml integrin αvβ3 in binding buffer overnight at 4 °C under constant rotation. Beads were washed four times with 10 volumes of buffer A containing 0.2% Triton X-100. Bound integrin was eluted by incubating beads for 30 min at room temperature with 1 volume of 2× non-reducing Laemmli sample buffer. Eluates were subjected to SDS-PAGE, and proteins were visualized by silver staining.

Integrin αvβ3 Binding Assay—96-Well polystyrene plates were coated overnight at 4 °C with 10 μg/ml protein G-purified polyclonal integrin αv antibody (Chemicon). Wells were washed three times with buffer A containing 0.05% Tween 20 and incubated for 1 h with 3.5 μg/ml integrin αvβ3 in buffer A containing 0.2% Triton X-100. Wells were washed three times and blocked with 1% BSA in buffer A for 1 h. Immobilization of integrin αvβ3 was confirmed by ELISA. Immobilized integrin was incubated overnight at 4 °C with different proteins at indicated concentrations.

FIGURE 2. Expression and localization of TEM5 in endothelial cells. A, immunoblot analysis of HUVECs, embryonic kidney cells (HEK293), pancreatic tumor cells (TD2), and embryonic endothelioma cells (eEND) using a TEM5 antibody. B, HUVECs were transfected with full-length TEM5 or empty vector for 48 h. Cell lysates were analyzed by immunoblotting using anti-FLAG and anti-myc antibodies. A and B, blots were reprobed with an actin antibody to verify equal loading. C, HUVECs were microinjected with an equimolar mixture of the full-length TEM5 and a GFP expression construct. 24 h after microinjection cells were fixed and permeabilized or left non-permeabilized. Cells were stained for TEM5 by incubation with an anti-FLAG antibody followed by a TRITC-conjugated anti-rabbit IgG antibody. Fluorescence microscopic images were taken at 400-fold magnification. Scale bar: 55 μm. IB, immunoblot analysis.
centrations in blocking buffer. Bound proteins were detected by ELISA.

**Glycosaminoglycan Binding Assay**—96-Well polystyrene plates were coated overnight at 4 °C with 10 μg/ml collagen, laminin, or avidin diluted in PBS. Avidin-coated wells were incubated for 30 min with 100 μg/ml heparin-biotin. Wells were washed three times with PBST and blocked for 1 h with PBS/BSA. Wells were incubated for 1 h with different TEM5 constructs (100 nm) in PBS/BSA containing inhibitors where indicated. Bound TEM5 constructs were detected by immuno blotting using anti-FLAG and actin antibodies, respectively.

**HUVEC Survival Assay**—Plates and cells were prepared as outlined for the cell adhesion assay. After blocking heparan sulfate-coated wells were incubated for 2 h with different TEM5 constructs (200 nm) in PBS/BSA. 3 × 10^4 cells per well were seeded in 96-well plates and 1.5 × 10^5 cells per well were seeded in 24-well plates. Soluble inhibitors were present where indicated. 24 h after seeding phase contrast microscopic images were taken at 320-fold magnification using a digital camera.

48 h after seeding 0.1 volume of AlamarBlue was added to the cell supernatants in the 96-well plates and incubated for 4 h at 37 °C. Supernatants were transferred to new 96-well plates, and absorbance at 570 and 600 nm was measured. Relative cell viability was equal to relative reduction of the dye, which was calculated according to the supplier’s manual. Cells in the 24-well plates were lysed 48 h after seeding using 20 μl of reducing Laemml sample buffer. Cell lysates were subjected to immunoblot analysis.

**ELISA**—Depending on the preceding assay wells were washed with PBST or buffer A containing 0.05% Tween 20 and antibodies were diluted in PBS/BSA or 1% BSA in buffer A. Wells were washed three times and incubated for 1 h with a vitronectin antibody (1:3000) or an anti-FLAG-HRP antibody (1:10,000). Wells incubated with the vitronectin antibody were washed three times and incubated for 1 h with an HRP-conjugated antimouse IgG antibody (1:5000, Amer sham Biosciences). Wells were washed three times and incubated with TMB substrate (Sigma) for up to 30 min. Reaction was stopped by adding 0.5 volumes of 0.5 M H_2SO_4. Absorbance was measured at 450 nm and blanked with substrate or zero values.

**RESULTS**

**Expression and Purification of Soluble TEM5 Constructs in HEK293 Cells**—To investigate the function of TEM5 we cloned a full-length and several soluble deletion constructs of TEM5 containing N- and C-terminal epitope tags (Fig. 1A). Soluble TEM5 constructs were expressed in HEK293 cells and purified from cell supernatants or lysates. The purified proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining or immuno blotting to determine purity and integrity. All soluble constructs were highly pure and stably expressed bearing the N-terminal 3× FLAG tag and the C-terminal hexahistidine tag. Only ppSTEM5 showed slight degradation from the C-terminus (Fig. 1B).

**Expression and Localization of TEM5 in Endothelial Cells**—To confirm endothelial specific expression of TEM5 we performed immunoblot analysis of HUVEC and different cell lines using a TEM5 antibody. TEM5 was detected in HUVEC and embryonic endothelioma cells but not in HEK293 (embryonic kidney) and TD2 (pancreatic tumor) cells (Fig. 1A). TEM5 expression in HUVEC was not up-regulated by VEGF, bFGF, or

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interleukin-8 (data not shown). To further investigate TEM5 expression in endothelial cells we transfected HUVEC with a full-length TEM5 expression construct. Immunoblot analysis using antibodies directed against the N- and C-terminal epitope tags revealed that TEM5 is expressed as a single polypeptide chain migrating as a double band at 200 and 250 kDa in SDS-PAGE containing N and C terminus. The double band likely represents alternatively glycosylated forms of the protein (Fig. 2B). To determine the localization of TEM5 in endothelial cells we performed immunofluorescence staining of HUVEC microinjected with the full-length TEM5 expression construct. The cells were co-injected with a GFP expression construct as control. To discriminate between surface and intracellular expression permeabilized and non-permeabilized cells were stained for TEM5 using an anti-FLAG antibody. Permeabilized as well as non-permeabilized cells were positive for TEM5 indicating that TEM5 is localized on the surface of endothelial cells (Fig. 2C).

Shedding of a Soluble TEM5 Fragment by HUVEC—Since proteolytic processing at the GPCR proteolysis site of some GPCRs leads to shedding of their extracellular domains (10, 11) we hypothesized that this might also be the case for TEM5. To test our hypothesis we transfected HUVEC with full-length TEM5 and seeded them on Matrigel to induce differentiation into capillary-like networks (Fig. 3A) or stimulated them with VEGF or bFGF. Immunoblot analysis of the cell supernatants indeed revealed that a soluble TEM5 fragment is shed by HUVEC seeded on Matrigel or stimulated with VEGF or bFGF. The fragment could not be detected in supernatants of unstimulated cells. Immunoblot analysis of supernatants from HUVEC transfected with the TEM5 extracellular domain (sTEM5) showed that the shed TEM5 fragment corresponds in size to the complete extracellular domain. Interestingly, secreted sTEM5 is processed proteolytically resulting in an N-terminal fragment of ~80 kDa. Based on this finding we hypothesized that further proteolytic processing of shed TEM5 may play a role in the regulation of its function. Supernatants from HUVECs transfected with ppsTEM5 showed that this construct represents the N-terminal cleavage product of sTEM5 (Fig. 3B). The cell supernatants were also analyzed by ELISA using an anti-FLAG antibody. The higher sensitivity of this assay revealed a basal level of TEM5 shedding in unstimulated endothelial cells and a significant increase of shedding during capillary-like network formation (Matrigel) and upon stimulation with bFGF (Fig. 3C).

Proteolytically Processed Soluble TEM5 Mediates HUVEC Adhesion—By sequence analysis we found that the extracellularly located HormR domain of TEM5 contains an RGD motif which serves as a binding site for integrins in several proteins (9). Therefore, we hypothesized that TEM5 interacts with integrins via its RGD motif. To test this hypothesis we transfected HUVEC with TEM5 constructs immobilized on polystyrene. Binding to polystyrene was equal for the different TEM5 constructs as determined by ELISA (data not shown). Interestingly, cell adhesion only occurred on ppsTEM5 in a protein density dependent manner (Fig. 4A). This finding supported our hypothesis that proteolytic processing of sTEM5 is important for the regu-
We next treated the TEM5 constructs that did not mediate cell adhesion with MMP-9 and analyzed the digests by immunoblotting using an anti-FLAG antibody. Proteolytic processing of sTEM5 resulted in two N-terminal cleavage products corresponding in size to the full-length ppsTEM5 construct and a smaller degradation product of ppsTEM5. Cleavage of TEM5-HG resulted in a fragment corresponding in size to the TEM5 HormR domain (TEM5-H). Treatment of TEM5-G resulted in no detectable cleavage products (Fig. 4B). We performed HUVEC adhesion assays with the different digests and found that adhesion to MMP-9-treated sTEM5 and TEM5-HG was increased 11-fold as compared with the untreated proteins. The RGD motif lacking TEM5-G construct did not mediate adhesion upon MMP-9 treatment. These results indicate that sTEM5 contains a cryptic binding site for a receptor on endothelial cells.

Cell Adhesion to ppsTEM5 Is Mediated by Integrin \( \alpha_\nu \beta_3 \) —To investigate whether adhesion of endothelial cells to ppsTEM5 is mediated by integrins we used RGD peptide and different function blocking integrin antibodies to inhibit cell adhesion. We found that adhesion to MMP-9-treated sTEM5 is completely blocked with an integrin \( \alpha_\nu \beta_3 \) antibody (Fig. 4C). Adhesion to ppsTEM5 could be completely blocked with RGD peptide and antibodies directed against \( \alpha_\nu \beta_3 \). Adhesion was reduced to \( \sim 50\% \) with an antibody directed against \( \alpha_5 \) integrins but could not be blocked with antibodies directed against integrins \( \alpha_\nu, \alpha_2, \text{ or } \alpha_\beta_5 \) (Fig. 5A). These findings suggest that ppsTEM5 specifically interacts with integrin \( \alpha_\nu \beta_3 \) and to a lesser extent with \( \alpha_5 \) integrins. We also performed adhesion assays with the \( \alpha_5 \) integrin expressing melanoma cell line M21 and the M21 cell subpopulation M21L that lacks \( \alpha_5 \) integrins. Consistent with our inhibition experiments M21 cells adhered well to ppsTEM5 whereas M21L cells only weakly adhered to ppsTEM5. sTEM5 did not mediate adhesion of either cell line. The weak adhesion of M21L cells to ppsTEM5 can probably be attributed to the expres-

**FIGURE 5. Integrin \( \alpha_\nu \beta_3 \) mediates cell adhesion to ppsTEM5.** A, HUVEC adhesion to ppsTEM5 immobilized at 200 nM. Cells were pretreated and seeded in the presence of RGD peptide (600 \( \mu \)M) or function-blocking integrin antibodies (25 \( \mu \)g/ml). Mean values (columns) and standard deviations (bars) represent data from triplicates (*, \( p < 0.001; **, p < 0.01 \) versus untreated (ppsTEM5)). B, adhesion of M21 (\( \alpha_\nu \) integrin-positive) and M21L (\( \alpha_5 \) integrin-negative) cells to sTEM5 and ppsTEM5 immobilized at 200 nM and collagen immobilized at 1 \( \mu \)g/ml. Mean values (columns) and standard deviations (bars) represent data from triplicates (*, \( p < 0.001 \) versus M21 cells).

**FIGURE 6. Direct interaction of ppsTEM5 with integrin \( \alpha_\nu \beta_3 \).** A, indicated proteins were covalently coupled to agarose beads. Beads were incubated with purified integrin \( \alpha_\nu \beta_3 \) (probe). After extensive washing bound integrin was eluted using non-reducing Laemmli sample buffer. Probe and eluates were separated by SDS-PAGE, and proteins were visualized by silver staining. B, immobilized integrin \( \alpha_\nu \beta_3 \) was incubated with vitronectin or different TEM5 constructs at indicated concentrations. Bound proteins were detected by ELISA using anti-vitronectin and anti-FLAG antibodies. Mean values (data points) and standard deviations (bars) represent data from triplicates (*, \( p < 0.001; **, p < 0.01 \) versus TEM5-G).
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FIGURE 7. Direct interaction of sTEM5 and ppsTEM5 with glycosaminoglycans. A, collagen and laminin were immobilized at 10 $\mu$g/ml and heparin-biotin bound to avidin was immobilized at 10 $\mu$g/ml and incubated with the indicated TEM5 constructs (100 nM) for 1 h. Bound proteins were detected by ELISA using an anti-FLAG antibody. Mean values (columns) and standard deviations (bars) represent data from triplicates (*, p < 0.001 versus BSA). B, heparin-biotin bound to avidin and avidin alone were immobilized at 10 $\mu$g/ml and incubated for 1 h with 100 nM sTEM5 and indicated inhibitors. EDTA concentration was 1 mM. Heparin, heparan sulfate, chondroitin sulfate A, and dermatan sulfate concentrations were 1, 10, and 100 $\mu$g/ml. Bound sTEM5 was detected by ELISA using an anti-FLAG antibody. Mean values (columns) and standard deviations (bars) represent data from triplicates (*, p < 0.001 versus untreated (heparin)).

Vitronectin with integrin $\alpha_v\beta_3$ we performed an ELISA-based binding assay using immobilized integrin $\alpha_v\beta_3$, ppsTEM5 bound to integrin $\alpha_v\beta_3$ in a concentration-dependent manner with an intermediate affinity as compared with vitronectin. sTEM5 only weakly bound to integrin $\alpha_v\beta_3$ and the RGD motif lacking the TEM5-G construct did not bind to integrin $\alpha_v\beta_3$ at all (Fig. 6B).

sTEM5 and ppsTEM5 Directly Interact with Glycosaminoglycans—Direct interactions with ECM molecules have been reported recently for TEM7 and TEM8 (5, 6). Therefore, we tested whether soluble TEM5 constructs also bind to components of the ECM. Different ECM molecules were immobilized and incubated with soluble TEM5 constructs. Bound TEM5 constructs were detected by ELISA using an anti-FLAG antibody. Our experiments demonstrate that soluble TEM5 constructs do not interact with the ECM proteins collagen or laminin. However, sTEM5 and ppsTEM5 but not TEM5-HG interacted with the glycosaminoglycan heparin (Fig. 7A). Inhibition experiments revealed that the interaction of sTEM5 with heparin is not dependent on bivalent cations (EDTA) but depends on the ionic strength of the environment (1 M NaCl). Furthermore, by competitive inhibition of the sTEM5 heparin interaction we found that sTEM5 also interacts with several other glycosaminoglycans. The relative affinity for the glycosaminoglycans we tested is heparin > chondroitin sulfate > dermatan sulfate > heparan sulfate (Fig. 7B).

**ppsTEM5 Mediates HUVEC Survival by Interacting with Integrin $\alpha_v\beta_3$**—Endothelial cells that are not incorporated into differentiating vessels during angiogenesis eventually become apoptotic. Since sTEM5 is shed by endothelial cells during capillary-like network formation and ppsTEM5 interacts with integrin $\alpha_v\beta_3$, we hypothesized a role in endothelial cell survival during angiogenesis. To investigate this potential effect we seeded HUVEC on plates coated with different TEM5 constructs or vitronectin and incubated them for 48 h in the absence of serum and growth factors to induce cell death. We found that vitronectin and ppsTEM5 equally mediated protection from cell death as compared with sTEM5 and BSA supporting our hypothesis that ppsTEM5 plays a role in survival of endothelial cells. The presence of other RGD-dependent integrins such as $\alpha_5$ integrins. Adhesion to collagen, which is independent of $\alpha_v$ integrins, was equal for both cell lines (Fig. 5B).

**ppsTEM5 Directly Interacts with Integrin $\alpha_v\beta_3$**—To further confirm the interaction of ppsTEM5 with integrin $\alpha_v\beta_3$ we performed affinity chromatography. Different purified TEM5 constructs or the known $\alpha_v\beta_3$ ligand vitronectin were coupled to agarose beads and incubated with purified integrin $\alpha_v\beta_3$. After extensive washing of the beads bound integrin was eluted. Eluates were analyzed by SDS-PAGE followed by silver staining. Significant binding of integrin $\alpha_v\beta_3$ was only observed on vitronectin and ppsTEM5 (Fig. 6A). To quantify the interaction of the TEM5 constructs and
Soluble TEM5 Links Integrin \(\alpha_v\beta_3\) to Glysosaminoglycans

**FIGURE 8.** ppsTEM5 mediates endothelial cell survival. HUVECs were seeded on indicated supports and incubated in the absence of growth factors. A–C, indicated proteins were immobilized at 100 nM. A, HUVECs were pretreated and seeded in the presence of a function blocking integrin \(\alpha_v\beta_3\) antibody (25 \(\mu\)g/ml) or heparin (100 \(\mu\)g/ml) where indicated. Cell viability was measured after 48 h using Alamar Blue. Mean values (data points) and standard deviations (bars) represent data from triplicates (*, \(p < 0.001\) versus BSA; **, \(p < 0.01\) versus ppsTEM5). B, 48 h after seeding cells were analyzed by immunoblotting using a caspase-3 antibody. Blot was reprobed with an actin antibody to verify equal loading. C, phase contrast microscopic images taken at 320-fold magnification 24 h after seeding HUVEC. Scale bar: 55 \(\mu\)m. D, heparan sulfate (HS) was immobilized at 100 \(\mu\)g/ml, and the remaining protein-binding sites were blocked. Immobilized HS was incubated with indicated TEM5 constructs (200 nM) for 2 h or left untreated prior to seeding HUVECs. Cell viability was measured after 48 h using Alamar Blue. Mean values (data points) and standard deviations (bars) represent data from triplicates (*, \(p < 0.01\) versus heparan sulfate). IB, immunoblot analysis.

In this study we investigated the role of TEM5 in angiogenesis. TEM5 belongs to the group III of adhesion GPCRs (7). We found that TEM5 is expressed on the surface of endothelial cells. A soluble TEM5 fragment encompassing the complete extracellular domain (sTEM5) is shed by endothelial cells during capillary-like network formation and upon growth factor stimulation. The extracellularly located HormR domain of TEM5 contains an RGD motif which is a binding site for certain integrins (9). RGD-binding integrins such as \(\alpha_v\beta_3\) play an important role in angiogenesis. Integrin \(\alpha_v\beta_3\) is up-regulated in the endothelium during angiogenesis and mediates endothelial cell migration, invasion, proliferation, as well as survival. It interacts with a variety of ECM proteins such as fibronectin, vitronectin, and fibrinogen (13). Interactions with several non-ECM proteins such as CD97, prothrombin, and MMP-2 have been reported as well (14–16). Therefore, we hypothesized that integrins also interact with TEM5 via its RGD motif during angiogenesis. Indeed, proteolytically processed TEM5 (ppsTEM5) mediated adhesion of endothelial cells by interacting with RGD-dependent integrins. Interestingly, full-length sTEM5 did not mediate cell adhesion indicating that its RGD-binding site is cryptic. Proteolytic processing of TEM5 by proteases such as MMP-9 led to exposure of its RGD motif.

Adhesion of endothelial cells to ppsTEM5 is mediated by integrin \(\alpha_v\beta_3\) and to a lesser extent by \(\alpha_5\) integrins. It is interesting to observe that a function blocking integrin \(\alpha_v\beta_3\) antibody does not inhibit endothelial cell adhesion to ppsTEM5. Other \(\alpha_v\beta_3\) ligands such as vitronectin also interact with integrin \(\alpha_v\beta_3\), yet ppsTEM5 seems to be more specific for integrin \(\alpha_v\beta_3\). Interestingly, the N-terminally truncated sTEM5 construct TEM5-HG also mediated cell adhesion upon treatment with MMP-9 demonstrating that the LRR and IG domains are not involved in the interaction with integrin \(\alpha_v\beta_3\). Binding studies with immobilized ECM molecules revealed that sTEM5 and ppsTEM5 but not TEM5-HG interact with glycosaminoglycans. This finding suggests that the LRR and/or IG domains of TEM5, which are deleted in TEM5-HG, mediate this interaction. Therefore, it is possible that membrane-bound...
TEM5 present on the cell surface acts as a receptor for glycosaminoglycans. Glycosaminoglycans are linear heteropolysaccharides composed of repeating disaccharide blocks that are N-acetylated and sulfated. They are covalently and non-covalently linked to the core proteins of proteoglycans, which are present in the ECM and on cell surfaces.

Our data suggest that ppsTEM5 links integrin $\alpha_5\beta_3$ to the glycosaminoglycan side chains of proteoglycans. Adhesion to ppsTEM5 or heparan sulfate-bound ppsTEM5 promoted survival of endothelial cells in the absence of growth factors indicating that TEM5 plays a role in cell-matrix and cell-cell contact-induced survival. ppsTEM5-mediated cell survival was significantly inhibited by an integrin $\alpha_5\beta_3$ antibody suggesting that integrin $\alpha_5\beta_3$ plays a major role in mediating this effect. An excess of heparin did not inhibit ppsTEM5-mediated endothelial cell survival indicating that ligation of cell surface glycosaminoglycans by sTEM5 or ppsTEM5 does not contribute to cell survival.

Based on our results we conclude that sTEM5 is shed by endothelial cells during angiogenesis and binds to glycosaminoglycans present in the ECM and on cell surfaces. After proteolytic processing an RGD-binding site for integrin $\alpha_5\beta_3$ is exposed on sTEM5. Interaction of integrin $\alpha_5\beta_3$ with proteoglycan-bound ppsTEM5 mediates endothelial cell survival.

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