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Biochemical and Biophysical Research Communications 334 (2005) 231–238
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TEM-8 and tubule formation in endothelial cells, its potential role of its vW/TM domains

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Received 13 June 2005
Available online 27 June 2005

Abstract

Background and aims: Tumour endothelial marker-8 (TEM-8) has been found to be selectively up regulated in tumour-associated endothelial cells, it is implicated in tumour specific angiogenesis, but its mechanism in angiogenesis is not defined.

Methods: A ribozyme transgene (TEM-8) was cloned into a suitable mammalian expression vector (pc DNA 3.1-GFP-NT) and transfected into HECV cells. Various domains of TEM-8 were designed and cloned into pEF6/V5-His TOPO TA vector and transfected into Chinese Hamster ovarian cells (CHO), which do not form tubules and do not express TEM-8 in general (CHO^{vW}, CHO^{TM}, $CHO^{vW/TM}$, CHO^{AE}, CHO^{AC}, CHO^{IC}, and CHO^{FL} domains, respectively). The effect of TEM-8 knocked out HECV cells was tested (by angiogenesis and migration assays), and the effect of each cleavage domain of TEM-8 was tested by microtubule formation assay.

Results: TEM-8 stable transfectants ($HECV^{\Delta TEM8a}$) manifested a complete loss of TEM-8 gene expression at mRNA and protein levels. In contrast, control GFP plasmid ($HECV^{pControl}$) and wild-type HECV cells ($HECV^{WT}$) had similar levels of TEM-8 expression. TEM-8 transfected cell ($HECV^{\Delta TEM8a}$) significantly decreased the micro-vessels formation compared with controls ($HECV^{pControl}$) (mean ± SE, 20.3 ± 4.03 μm; $p = 0.0086$ vs. control 39.5 ± 10.1 μm), and migration (38.52 ± 2.17; $p < 0.05$ vs. control 80.23 ± 3.19), and micro-vessel formation of $HECV^{\Delta TEM8a}$ cell was also reduced compared with wild-type ($HECV^{WT}$) (mean ± SE, 20.3 ± 4.03 μm; $p = 0.0078$ vs. wild-type 42.5 ± 9.1 μm) and migration (38.52 ± 2.17 μm; $p < 0.05$ vs. wild-type 82.4 ± 4.45 μm). vW together with transmembrane domains of TEM-8 ($CHO^{vW/TM}$) and full-length CHO^{FL} showed formation of tubule-like structure in CHO cells, whereas the other domains showed no effect.

Conclusion: Targeting the TEM-8 gene by way of a hammerhead ribozyme knocks out TEM-8 cells, and is an effective way to reduce the micro-vessel formation or migration potential in tumour-associated endothelial cell through its vW domain. These results suggest that the vW domain together with the transmembrane domain of TEM-8 may play an important biological role in TEM-8 related tubule formation.

Keywords: TEM-8; Anthrax toxin receptor; Human endothelial cells; Ribozyme transgene; Angiogenesis and migration

Angiogenesis is a crucial step in the metastatic cascade of many solid tumours, they become angiogenesis dependent when their need for nutrients and oxygen outstrips what diffusion can provide [1]. The process of angiogenesis consists of multiple sequential and interdependent steps involving numerous positive and negative angiogenesis regulators. The survival of tumours and their metastases is dependent on the balance of endogenous angiogenic and antiangiogenic factors such that the outcome favours increased angiogenesis. Targeting the endothelial cells that line tumour vessels is a promising anticancer strategy that has generated widespread interest among biologists and clinicians [2]. However, most angiogenic factors, angiogenic

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doi:10.1016/j.bbrc.2005.06.085

markers, and intended targets are not entirely specific to tumours, i.e., they exist in both normal and tumour tissues, although some may be more prevalent in the later. Targeting these molecules would undoubtedly have side effects.

Recently, a group of new endothelial molecules, tumour endothelial markers (TEMs), were identified in human endothelial cells of malignant colorectal tissues [3]. Discovered by comparing the differentially expressed genes in endothelial cells obtained from normal colon tissues and from colon cancer, the TEMs were initially thought to be highly specific to endothelial cells in tumour tissues, and not to exist in endothelial cells in normal tissues. However, subsequent studies have shown that the tumour specificity of these markers is dependent on the type of tissue of tumour and the methods used. Most of them are identifiable in normal tissues. However, among these tumour endothelial markers (TEMs), TEM-8 was one of the TEMs that showed superior specificity to tumour endothelial cells, compared with other markers. TEM-8 is also of particular interest because of its cell-surface localization, conservation in mice, and unique pattern of expression [4]. TEM-8 is a type I transmembrane protein 564 amino acids in length, and has been postulated to be involved in the interaction of cells with the surrounding extracellular matrix [5,6], and has been indicated to have a potential role in angiogenesis.

TEM-8 mRNA expression was readily detected in tumour endothelium by in situ hybridization but was absent or barely detectable in normal adult endothelium and the proliferative endothelium of the corpus luteum [3,4]. We recently found that TEM-8 protein expression was significantly higher in tumour tissues compared to normal tissues. TEM-8 antibody identified more micro-vessels in colon tumour tissue by immunohistochemistry, than in normal colon tissues [7].

We have reported that IL-1β significantly raised the level of TEM-8 at the protein level, as revealed by Western blotting. In vitro tubule forming assay revealed that IL-1β significantly induced the formation of capillary-like tubules from the HECV cells, accompanied by an increase in TEM-8 expression [8]. This indicates that TEM-8 is potentially involved in angiogenesis and that IL-1β is a powerful regulator of the expression of TEM-8 in vascular endothelial cells, suggesting an important pathway through which IL-1β regulates tumour-associated angiogenesis.

To date, it is not clear if TEM-8 is directly involved in angiogenesis, and if so, by what mechanisms. In the current study, we have shown that knock out of TEM-8 significantly decreased its tubule formation and that the vW domain together with the transmembrane domain of the TEM-8 is key to the tubule forming process.

Materials and methods

Materials. Human endothelial cell line HECV was obtained from Interlab Cell Line Collection (ICLI), Naples, Italy. Cells cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) (pH 7.3) containing: 2 mM L-glutamine, 15 mM Hepes, and 4.5 mM $NaHCO_3$. DMEM was supplemented with 10% heat inactivated foetal calf serum (PAA Laboratories, Yeovil, England, UK), 50 U/ml benzopenicillin (Britannia Pharmaceuticals), and 50 μg/ml streptomycin (Gibco-BRC, Paisley, Scotland). Rabbit polyclonal antibody to human TEM-8 was generated in our laboratory [8]. Preoxidase conjugated secondary antibody to rabbit was obtained from Sigma (Poole, Dorset, UK). The KPL chemiluminescence kit was purchased from Insight Biotechnology (Wembley, Middlesex, UK). Matrigel [extracted from Engelbreth–Hom–Swarm (EHS) sarcoma] was purchased from Collaborative Biochemical (Bedford, MA, USA). RNA extraction and reverse transcription kits and PCR mix were purchased from Abgene (Surrey, England, UK).

Generation of TEM-8 null endothelial cells via construction of expression vectors and ribozyme transgenes. TEM-8 ribozymes were designed based on the secondary structure of the TEM-8 RNA GenBank Accession No. NM-0322087, generated from Zuker's m-Fold program [9] and constructed as we recently reported [10,11] (Fig. 1). Three ribozymes were designed and engineered to target the AUC sites on positions 164,529 and 775 which are within its coding region of TEM-8 mRNA. Touch down PCR was used to generate the ribozymes, using the following primers: TEM-8rib1F, 5′-ctgcagagaccactggaagccctgatgag-3′ and TEM-8rib1R, 5′-actagttggagcggagaccctcggcatttcgtcctcacgca-3′;

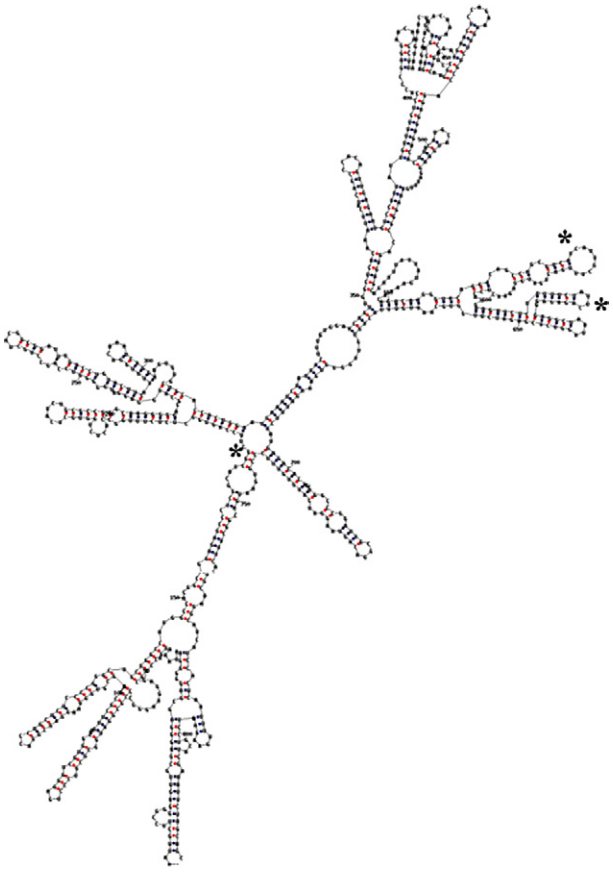

Fig. 1. The graph shows the predicted secondary structure of human TEM-8, generated using mFold program. *The suitable sites for the hammerhead ribozyme to target.

TEM-8rib2F, 5′-ctgcagtccatcagtcaaagcaatctgatg-3′ and TEM-8rib2R, 5′-actagtacaggacagccagcgtcatttcgtcctcacgga-3′; and TEM-8rib3F, 5′-ctgcagacttcttcaaaattgagtggatctgatgagtccgtga-3′ and TEM-8rib3R, 5′-actagttttcaggctctgcaaggcatttcgtcctcacgga-3′. Purified products were T-A cloned into GFP vector, followed by amplification with *Escherichia coli* and selection of positive strains.

Purified and verified plasmids were used for transfection of HECV cells by way of electroporation ($HECV^{\Delta TEM8a}$ for Rib 1, $HECV^{\Delta TEM8b}$ for Rib 2, and $HECV^{\Delta TEM8c}$ for Rib 3). This procedure was based on a modified version of our previously published method [12]. Briefly, 10 μg respective plasmid was mixed with 8×10^6 cells for 2 min and then electroporated at 310 V with an electroporator (Easyjet; Equibio, Kent, UK). Cells were immediately transferred to complete medium pre-warmed to 37 °C and plated into 25-cm^2 tissue culture flasks. After 24 h, selection began with G418 at 100 μg/ml, and the medium was changed every 3–4 days, until positive stable colonies were identified, which were changed to maintenance medium (with 25 μg/ml G418) and grown to sufficient numbers for experimental studies. The expression of TEM-8 variants and its effacing of ribozymes were examined using respective PCR primers. Cells were subsequently frozen for later use.

Construction of expression cassettes for various TEM-8 domains. As shown in Fig. 2, a series of primers were designed in order to generate various fragments (domains) and full-length cDNA for TEM-8, the vW domain, ED (the extracellular domain), ED-TM (extracellular domain plus transmembrane domain), ICD (intracellular domain), and FL (full length). Primer sequences are given in (Table 1) and were synthesized by Invitrogen, Pasley, Scotland, UK. A cDNA template from HUVEC cells was used for generating the domain, by using RT-PCR. Following verification of each product, each domain of TEM-8 was T-A cloned into a mammalian expression vector (pEF6/V5-His) and followed by transformation of *E. coli*. The colonies with their corrected inserts were selected using orientation specific PCR. Plasmid was extracted using the standard plasmid extraction kits. Following re-verification using PCR and restriction digestion, the purified plasmid, the ribozyme, or the respective control plasmids were used to transfect CHO cells, using an electroporator (V.340 v). Cells were immediately transferred to complete medium prewarmed to 37 °C and plated into 25-cm^2 tissue culture flasks. After 24 h, selection began with blasticidin S HCl at 100 μg/ml, and the medium was changed every 3–4 days. After 3 weeks of culture, the cells were changed to maintenance medium (with 25 μg/ml blasticidin) and grown to sufficient number for experimental studies.

Extraction of RNA and RT-PCR. Cellular RNA was extracted using an RNA extraction kit (AbGene, London, UK) and quantified using a spectrophotometer (Wolf Laboratories). cDNA was synthesized using a first-strand synthesis with an oligo(dT) primer (AbGene). The PCR was performed using a Perkin-Elmer thermocycler and PCR mastermix reaction mixture (Abgene, Surrey, UK): 5 min at 95 °C for 38 cycles and then 30 s at 94 °C, 40 s at 55 °C, 60 s at 72 °C, and finally 72 °C for 10 min. β-Actin was amplified simultaneously using primers 5′-gctgatttgatggagttgga-3′ and 5′-tcagctacttgttcttgagtgaa-3′. PCR products were then separated on an 0.8% agarose gel, visualized under UV light, photographed using a Unisave camera (Wolf Laboratories, York, UK), and documented with Photoshop software.

Western blotting analysis of TEM-8. Cells were lysed in HCMF buffer containing 1% Triton, 0.1% SDS, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min before clarification at 13,000*g* for 10 min. Protein concentrations were measured using fluorescamine (Sigma Chemical) at 200 μg/ml and quantified by using a multifluoroscanner (Denly, Sussex, UK) [13]. Equal amounts of protein from each cell sample (15 μg/lane) were added onto an 8% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min before probing with the anti-human TEM-8 or anti-human β-actin antibody and peroxidase-conjugated secondary antibodies. A molecular weight marker mixture (SDS-6H; Sigma Chemical) was used to determine the protein size.

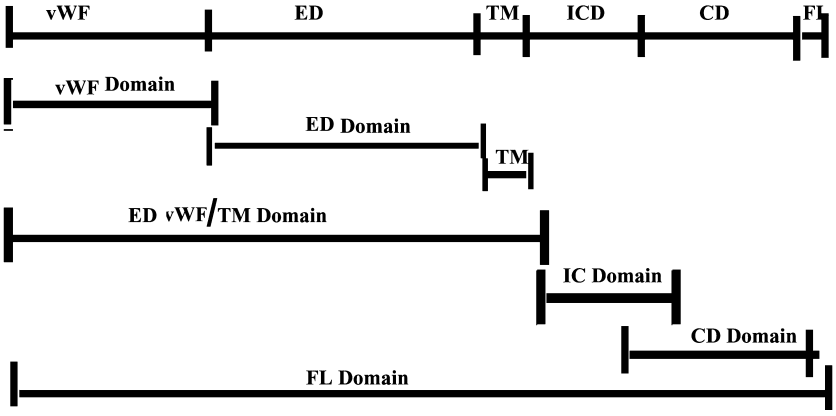

Fig. 2. The graph shows the main domains within the TEM-8 and design of PCR primers to amplify various domains of TEM-8.

Table 1
Primer sequences of the synthesized various TEM-8 domains

Primers	Sense primer (5′–3′)	Antisense primer (5′–3′)
TEM-8vW	attggccacggcggagggagagccctcgg	aattgagtggatgatgccttg
TEM-8TM	attggccacggcggagggagagccctcgg	agagtcccagatg
TEM-8vW/TM	attggccacggcggagggagagccctcgg	ccagaaccaccagaggagag
TEM-8ED	attggccacggcggagggagagccctcgg	accgtcagaacagtgtgt
TEM-8CD	attggccacggcggagggagagccctcgg	gttgttcttgaccctggtg
TEM-8ICD	attggccacggcggagggagagccctcgg	ctccattcttttaatgcct
TEM-8FL	attggccacggcggagggagagccctcgg	gacagaaggcctggaggag

Protein bands were visualized with a chemiluminescence system (Santa Cruz Biotechnology). Exposed films were scanned with a scanner and the density of protein bands was analyzed with the software Optimas (Optimas (UK), Milton Keynes, UK).

In vitro cellular migration assay. This assay was performed as previously reported [12]. Briefly, $HECV^{WT}$, $HECV^{pControl}$, and $HECV^{\Delta TEM8a}$ cells were seeded into a 24-well plate and grown to confluence. The cell monolayer was scraped using a fine gauge needle to create a wound approximately 200 μm wide. Mineral oil was used to overlay the medium (Hepes buffered environment for the cells and to prevent evaporation occurring from each well) [14]. The degree of migration across the wounded cell surface was then examined microscopically on a stage maintained at 37 °C for 90 min using a time-lapse video recording facility (Leitz DMIRB; 10× lens objective). Wound closure between the leading cell fronts was then assessed at 15 min intervals in real time, using Optimas image analysis software (Version 6, Optimas, UK).

Vascular endothelial tubule formation assay. This was based on a Matrigel-sandwich tubule forming assay developed in our laboratory [14–16]. Briefly, 200 μg of cold Matrigel solution dispersed in 100 μl (reconstituted basement membrane, Becton–Dickinson, Bristol, England) was added to a 96-well plate and allowed to air-dry at 37 °C. Following rehydration of Matrigel to allow formation of a thin bottom layer. Wild HECV ($HECV^{WT}$), GFP control plasmid ($HECV^{pControl}$) and anti-TEM-8 transgene transfected HECV ($HECV^{\Delta TEM8a}$) cells, and TEM-8 variants domains transfected CHO cells (CHO^{vW}, CHO^{TM}, $CHO^{vW/TM}$, CHO^{AE}, CHO^{AC}, CHO^{IC}, and CHO^{FL} domains, respectively) were seeded onto the Matrigel at 10,000 per well and allowed to attach for 2 h. The medium was carefully removed. The second solution of matrigel, which was mixed with the test agents (IL-1β or medium), was added to the cells, followed by incubation at 37 °C for 3 h when the second layer of Matrigel solidified. Medium with matched IL-1β, VEGF, IL-8, and HGF as a positive control (40 ng/ml) was added over the second layer of Matrigel and the cells were then incubated at 37 °C for 24 h. Microtubules were visualized microscopically and photographed using a digital camera.

The length of tubules in a fix-sized frame was quantified using the Optimas-6 software, as we previously described [14,16].

Results

TEM-8 ribozyme transgene eliminated expression of TEM-8

Transfection of HECV cells with ribozyme transgenes and control plasmids generated the following cells: $HECV^{\Delta TEM8a}$, $HECV^{\Delta TEM8b}$, $HECV^{\Delta TEM8c}$, $HECV^{pGFP}$, together with the wild-type $HECV^{WT}$. One of three cells transfected with the TEM-8 ribozyme transgene ($HECV^{\Delta TEM8a}$) exhibited a complete loss of TEM-8 expression at the mRNA level and other two showed partial knockout (Fig. 3). In contrast, both wild-type ($HECV^{WT}$) and control plasmid ($HECV^{pGFP}$) exhibited strong transcript signals for TEM-8 expression.

From the three cells electroporated with the TEM-8 ribozyme transgene only one cell ($HECV^{\Delta TEM8a}$) exhibited a dramatic reduction in the level of TEM-8 protein (Fig. 4), whereas in the other two, the TEM-8 was only partially extracted. This was in contrast to wild-type ($HECV^{pWT}$) and GFP control plasmid ($HECV^{pGFP}$),

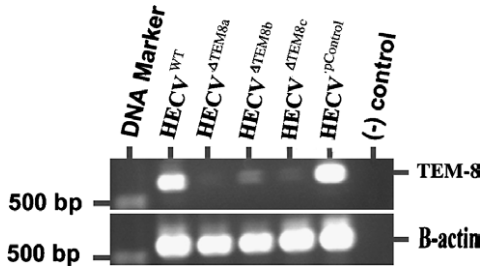

Fig. 3. Expression of TEM-8 in genetically modified HECV cells. HECV cells were transfected with anti-TEM-8 ribozymes (a, b, and c, respectively). The established cell lines, shown as $HECV^{\Delta TEM8a}$, $HECV^{\Delta TEM8b}$, and $HECV^{\Delta TEM8c}$, respectively, were tested for the presence of TEM-8 mRNA by RT-PCR. TEM-8 mRNA was completely knocked out from $HECV^{\Delta TEM8a}$ cells by TEM-8-rib-1 ribozyme and partially by TEM-rib-2 and -3 ribozymes ($HECV^{\Delta TEM8b}$ and $HECV^{\Delta TEM8c}$ cells, respectively). β-Actin was used as the housekeeping gene.

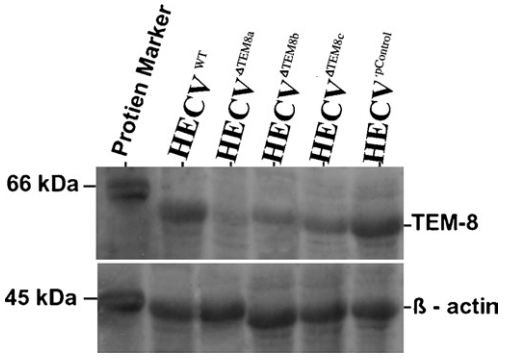

Fig. 4. Presence of TEM-8 protein in TEM-8 ribozyme modified cells. TEM-8 at protein level was completely knocked out from one of the three HECV cells, $HECV^{\Delta TEM8a}$, using TEM-8-rib-1. The other two cells ($HECV^{\Delta TEM8b}$ and $HECV^{\Delta TEM8c}$) had only partial loss of TEM-8 protein, as shown by the Western blotting. β-Actin was used as the housekeeping marker, using an anti-actin antibody.

which were both found to express high levels of TEM-8 protein as demonstrated by Western blotting.

Reduction of in vitro migration in TEM-8 transfected cells

Using the TEM-8 knockout cells, we conducted a cellular motion analysis, which showed that there were substantial changes of migration in HECV cells after being transfected with TEM-8 ribozyme. Fig. 5 showed a significant reduction of the migration distance of HECV cells that had been transfected with the anti-TEM-8 ribozyme transgene ($HECV^{\Delta TEM8a}$) compared with wild-type ($HECV^{WT}$) and control plasmid ($HECV^{pControl}$) cells (38.52 ± 2.17; $p < 0.05$ vs. wild-type 82.4 ± 4.45 and vs. GFP control plasmid 80.23 ± 3.19 μm), respectively, as described in detail in Table 2. Both wild-type cells ($HECV^{WT}$) and GFP control plasmid ($HECV^{pControl}$) showed a similar pattern of

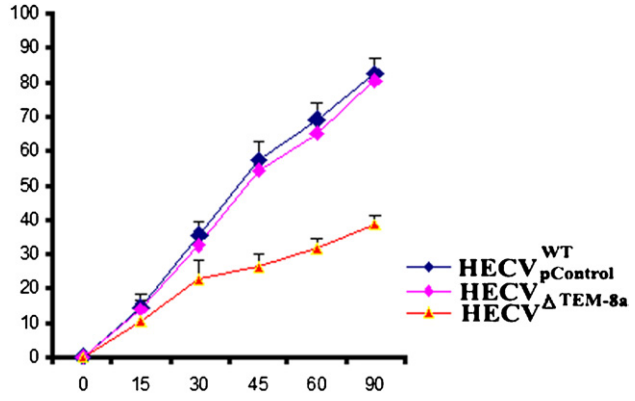

Fig. 5. In vitro HECV migration assay. TEM-8 knocked out cell significantly decreased HECV migration compared to the wild-type HECV and CFP control plasmid cells after 40 min incubation. *$p < 0.05$ by student's t test (p value is significant if less than 0.05).

Table 2
Numerical data from the migration assay

	Time (min)				
	15	30	45	60	90
$HECV^{WT}$					
Mean	14.4	35.3	57.3	69.2	82.4
SD	3.92	4.15	5.34	4.89	4.45
$HECV^{pControl}$					
Mean	13.7	32.4	54.3	65.2	80.23
SD	4.67	2.05	1.89	4.26	3.19
$HECV^{\Delta TEM8a}$					
Mean	10.34	22.42	26.12	31.43	38.52
SD	6.14	5.76	3.77	2.96	2.17

migration after 90 min (mean ± SE, 82.4 ± 4.45 μm; $p > 0.05$ vs. control plasmid 80.23 ± 3.19 μm), respectively.

Reduction of in vitro tubule formation in TEM-8 knocks out cells

The TEM-8 knockout cell ($HECV^{\Delta TEM8a}$) had significantly decreased the micro-vessel formation when compared with $HECV^{WT}$ and $HECV^{pGFP}$ (20.3 ± 4.03, 42.5 ± 9.1, and 39.5 ± 10.1 μm) ($p = 0.0078$ and $p = 0.0086$, respectively). Furthermore, HECV cells transfected with anti-TEM-8 transgene ($HECV^{\Delta TEM8a}$) exhibited an increase in tubule formation by the addition of angiogenic factors except for IL-1β and VEGF which had no significant effect, HGF, IL-8, IL-1β, and VEGF 56.321 ± 3.563, 26.385 ± 14.396, 21.434 ± 4.236, and 21.67 ± 3.55 μm ($p = 0.0095$, $p = 0.0581$, $p = 0.164$, and $p = 0.173$, respectively) (Fig. 5B). There was no difference in tubule formation between the $HECV^{WT}$ and $HECV^{pGFP}$ cells (42.5 ± 9.1, $p = 0.306$ vs. control plasmid 39.5 ± 10.1 μm), respectively (Figs. 6A and B).

vW domain with transmembrane domain portion of TEM-8 have the most powerful role in micro-vessels formation in CHO cells

Having established the impact of TEM-8 knockout on tubule formation and on cellular motility, we went onto transfect the CHO cells, which do not form tubules and do not express TEM-8, with expression plasmids. From all the seven expression cassettes (CHO^{vW}, CHO^{TM}, $CHO^{vW/TM}$, CHO^{AE}, CHO^{AC}, CHO^{IC}, and CHO^{FL} domains, respectively) tested, the vW domain with transmembrane domain portion of TEM-8 ($CHO^{vW/TM}$) showed a strong effect on micro-vessel formation (80.943 ± 7.43 μm), whereas, each domain (vW domain CHO^{vW} and transmembrane domain CHO^{TM}) separately showed less effect (65.78 ± 7.13 and 32.85 ± 3.63 μm), respectively. Other domains showed no effect on the angiogenesis process except the full-length domain of TEM-8 CHO^{FL} showed strong micro-vessel formation (82.943 ± 8.83 μm) (Figs. 7A and B).

Discussion

The current study has shown that TEM-8, a tumour specific endothelial marker, which is highly expressed in tumour-associated endothelial cells, is involved in the tubule formation of endothelial cells and has an implication in the angiogenic process. In addition, the current study has also shown that the vW domain with the TEM-8 protein is the key to the tubule forming process.

TEM-8 appears to be unique among the cell surface TEMs in that its expression has not been detected during other forms of physiological angiogenesis in the adult, although expression has been observed in endothelial cells of the developing mouse embryo [3,4]. For example, its mRNA was undetectable in healing wounds and corpus luteum tissue. Likewise, recently developed polyclonal antibodies against TEM-8 have been little detected or almost failed to detect the protein in normal colorectal tissues [7]. To date, TEM-8 has been one of the most extensively studied cell-surface TEMs. Interest in TEM-8, however, has increased substantially since TEM-8 was identified as a receptor for anthrax toxin [17].

Anthrax toxin, the major virulence factor produced by *Bacillus anthracis*, consists of three polypeptides called protective antigen, lethal factor, and oedema factor. Protective antigen mediates binding of the complex to TEM-8, whereas lethal factor and oedema factor are responsible for eliciting toxicity. Recently, capillary morphogenesis protein 2 (CMG2), the closest homologue to TEM-8, was identified as a second receptor for anthrax toxin [18]. The extracellular region of both receptors contains a von Willebrand factor type A

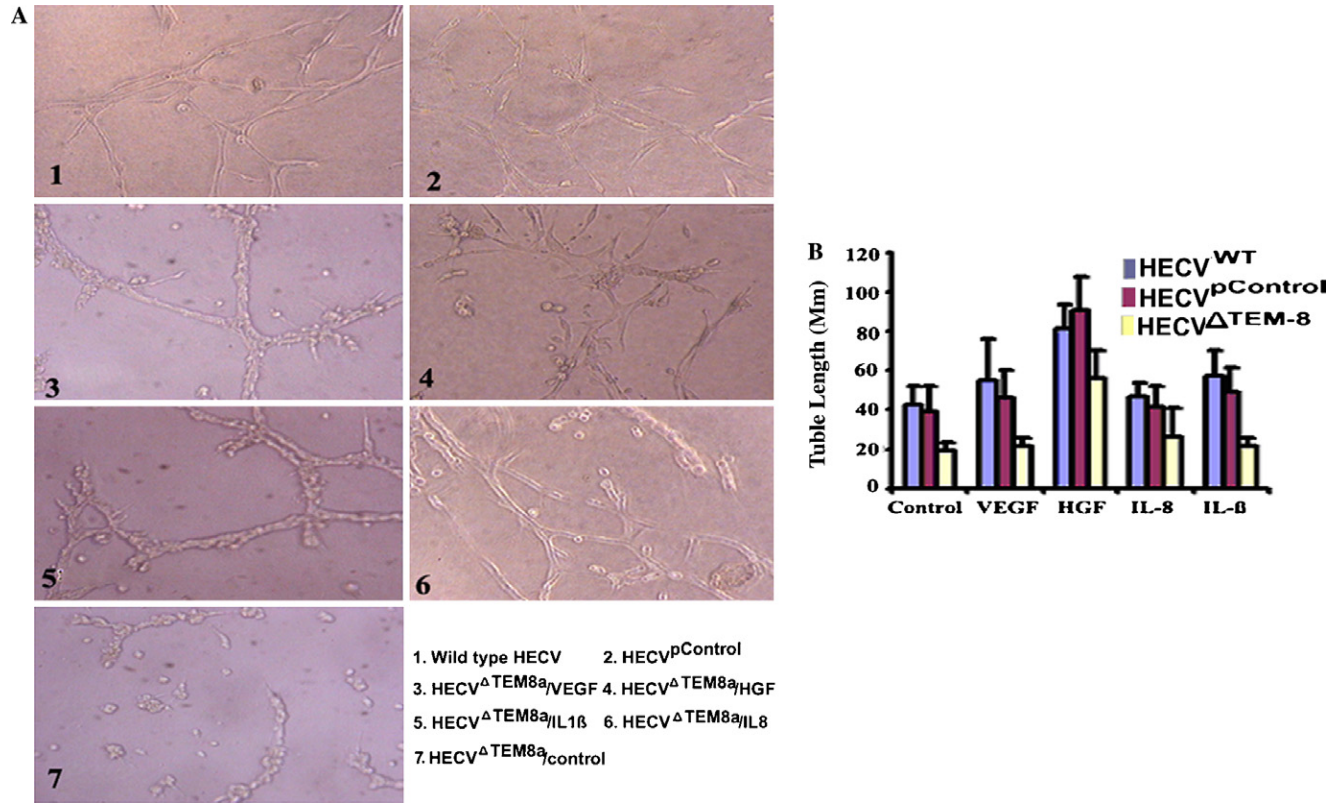

Fig. 6. In vitro tubule formation assay. (A) TEM-8 knocked out cell significantly decreased the ability of HECV cells to form tubules (7) compared to the wild HECV (1) and GFP control plasmid (2). HECV treated with HGF (4) and IL-8 (6) has shown increased in micro-tubule formation. In contrast, HECV cell treated with VEGF (3) and IL-1β (5) shows no significant change from TEM-8 knocked out cell (7). (B) The bar graph represents the means of tubule length in each condition.

domain. A metal ion-dependent adhesion site resides within the von Willebrand factor type A domain and appears to be necessary for interaction with protective antigen [19]. Despite these pivotal observations, it remains unclear if TEM-8 is directly involved in the angiogenic process.

In the current study, we first investigated the impact of genetically eliminating the expression of TEM-8 from endothelial cells on the formation of microtubules in vitro. Of the three ribozyme transgenes we constructed, one proved to be highly effective in that it has completely eliminated the expression of the transcript as revealed by RT-PCR, where as, other two showed partial elimination. Furthermore, it also substantially reduced the level of the protein, suggesting that the transgene is highly active and that the cell thus produced can serve as a good cell model in functionally based tests, such as in vitro tubule forming assays and cell migration assays. Indeed, using the TEM-8 knockout cells, we have shown that loss of TEM-8 from HECV cells has resulted in significant reduction of both tubule formation and in cell motility. This has indicated the following: first, TEM-8 directly contributes to the formation of microtubules from endothelial cells and second, TEM-8 is linked to the motile nature of endothelial cells, a cell function crucial for the angiogenic process. An additional point extracted from these tests was that TEM-8 may selectively regulate angiogenic factor regulated tubule formation. For example, it contributes to IL-1β induced tubule formation as shown here and in our previous study [8], but has little impact on that induced by VEGF.

TEM-8 has some very interesting features in its protein structure. It has a vW domain, which has been indicated in the vasculature integrity. It also shares homology with CMG-2 a protein known to regulate the morphology of capillaries. These properties, together with the observations in this study, have prompted us to further examine the potential protein domain that may contribute to the tubule forming activities of TEM-8. The selection of the domains was based on the following consideration: to include the vW domain without TM domain as vW may act as a secreted protein, construct the vW by inserting the TEM-TM domain in order to test the necessity of TM domain, the intracellular domain to test if intracellular part of the protein is a valid and full-length TEM-8.

von Willebrand factor (vWf), which is defined as a multimeric glycoprotein, is synthesized exclusively in endothelial cells and megakaryocytes and then stored

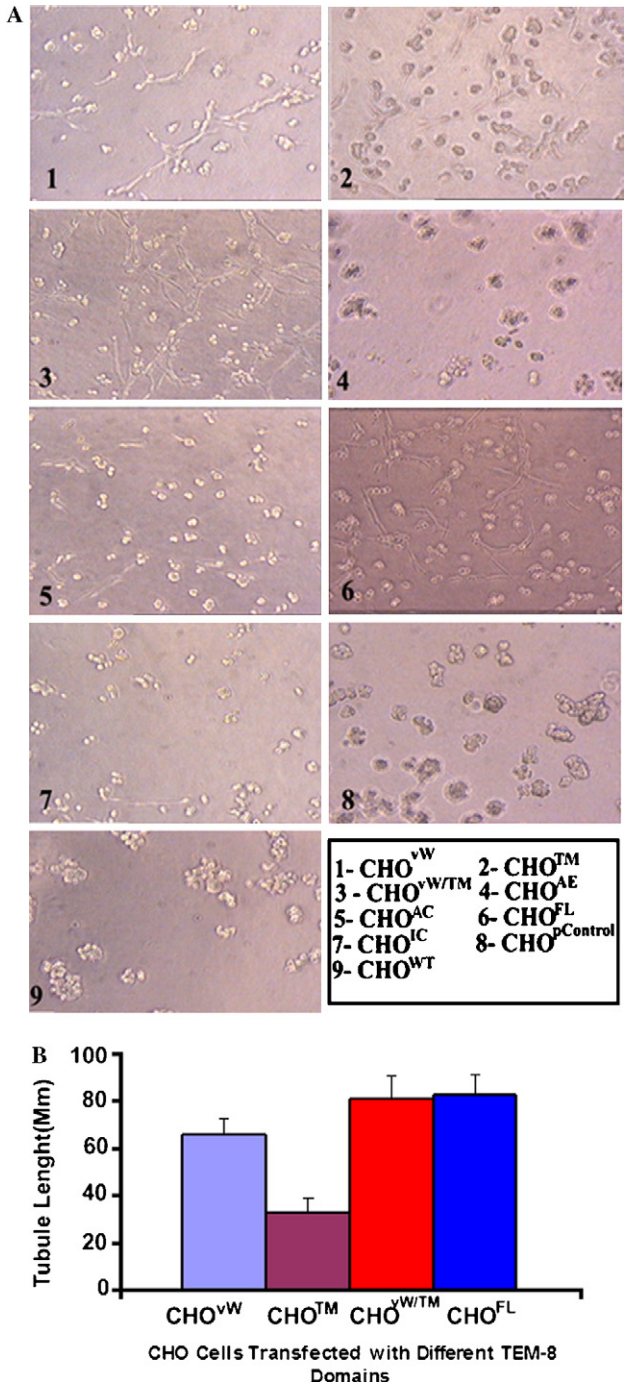

Fig. 7. In vitro tubule formation assay. (A) vW domain of TEM-8 induces the ability of CHO to form the micro-tubule (1). The expression constructs included the vW together with TM domains (2 and 3) and FL domain (6) they increased micro-tubule formation in CHO cell. On other hand, the wild CHO (9) and PEF6 control plasmid (8) together with other transacted TEM-8 domains (AE (4), AC (5), and IC (7)) have no effect in micro-tubule formation. (B) The bar graph represents the means of tubule length in each condition form tubule (CHO^{vW}, CHO^{TM}, $CHO^{vW/TM}$, and CHO^{FL}).

in Weibel–Palade bodies in endothelial cells and in platelet-granules [20], it is useful as a confirmatory marker, particularly in well-differentiated tumours [21]. The von Willebrand factor (vWF) is routinely used to identify vessels in tissue sections. Vessel density in tumour specimens, as determined by immunohistochemical staining for vWF or other endothelial cell markers, is a negative prognostic factor for many solid tumours. vWF is heterogeneously distributed throughout the vasculature, transcriptional control in response to the tissue microenvironment being responsible for local variations in endothelial cell levels of vWF [21].

For these expression cassettes, we chose CHO to test for the following reasons: first, CHO is superb in protein expression; second, CHO does not express TEMs; and third, CHO does not form tubules in the experimental condition as used here. The experiments have shown that expression of vW as well as the vW/TM domains in CHO cells, which otherwise do not form any tubules, has enabled the cells to form tubule-like structure. This observation has provided indirect evidence and may shed some light on the pro-tubule formation nature of TEM-8 in endothelial cells. However, CHO cell has no similarities to endothelial cells. Interpretation of this observation and its extrapolation on endothelial cells should be considered with care. The true angiogenic nature of these GM modified cells can only be verified using an in vivo model.

Anthrax toxin, when was injected into tumour-bearing mice at non-toxic doses, has been shown to induce a tumour inhibition, an effect thought to be mediated by the antineoplastic effects of anthrax toxin, because the treated tumours appeared "white" and were found to be deficient in CD31-positive blood vessels [22–24]. Given the TEM-8 protein expression patterns in tumour endothelium described here and in the literature, a possibility thus exists that anthrax toxin (TEM-8) may work together with the lethal factor, to interfere the tubule forming and other processes such as cell–matrix interaction that is involved in angiogenic process. However, more investigation is warranted in this area.

Thus, TEM-8 is an important tumour endothelial marker and contributes to the angiogenic process. This has raised important questions as to its candidacy in anti-angiogenic therapies in cancer. The highly specific expression of the molecular in cancer has indeed made it an ideal target as has recently been suggested [3,4,7,25]. Indeed, this current study has already shown that ribozyme transgene can be effective in this respect. Other factors, such as neutralizing antibodies, RNAi (similarities to the ribozyme approach in the current study), and indeed modified version of anthrax toxin, may prove to be highly valid options.

We conclude that TEM-8, a tumour specific endothelial marker, is a regulator of tubule formation in vitro, potentially via its vW/TM domain. Targeting the TEM-8 by way of hammerhead ribozyme encoding antisense to TEM-8 is an effective method to reduce the micro-vessel formation and migration potential of

endothelial cells, and may have important therapeutic implications. Together with solid clinical observations that TEM-8 is excessively expressed in tumour endothelial cells, but not in endothelial cells from the normal tissues, the validity of TEM-8 as a target in anti-tumour and anti-angiogenesis therapies should be explored.

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