SPANDIDOS PUBLICATIONS

Endothelial cells from human cerebral aneurysm and arteriovenous malformation release ET-1 in response to vessel rupture

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Abstract. Cerebral aneurysms and arteriovenous malformations (AVM) are a common cause of stroke and cerebral hemorrage. Both are often discovered when they rupture, causing subarachnoid hemorrhage (SAH). SAH-induced vasospasm is mediated by enhanced vasoconstriction due to endothelin-1 (ET-1). We investigated whether endothelial cells (ECs) obtained from aneurysm and AVM express phenotypic and genotypic alterations contributing to the development of vasospasm after SAH. We isolated ECs from human AVM and aneurysm and then confirmed their EC origin by polymerase chain reaction and immunocytochemistry with endothelial markers. Experiments were also carried out with human cerebral microvascular and umbilical vein ECs (HCECs and HUVECs respectively) for comparison. We tested EC proliferation ability and microtubule formation in Matrigel at different cell passages. Five aneurysm (3 ruptured, 2 unruptured) and 3 AVM (2 ruptured, 1 unruptured) ECs were tested for ET-1 release in the culture medium. Aneurysm and AVM ECs expressed von Willebrand factor, Adrenomedullin, and exhibited a progressive reduction of proliferation and *in vitro* angiogenic ability after the V passage. Significantly higher levels of ET-1 have been detected in ECs from ruptured aneurysms and AVMs. We report the first successful isolation and characterization of primary EC lines from human cerebral vascular lesions. Augmented release of ET-1 is correlated with the rupture of the abnormal vessel confirming its role in vasospasm after SAH. Furthermore, ECs obtained from these vascular malformations can be used as an experimental model to study SAH-induced vasoconstriction.

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Introduction

Endothelin (ET), one of the most potent vasoconstrictors, was first isolated from porcine aortic endothelial cells in 1988. Four isoforms are known (ET-1, -2, -3 and -4) (1). ET-1 is a small peptide of 21 amino acids with a molecular weight of 2492 Da. Its transcription can be induced within minutes from a precursor peptide, prepro-endothelin-1, in several steps via Big endothelin to mature ET-1 (2). ET-1 is generated mainly in endothelial cells and neurons, but also in glial cells, choroid plexus cells, hypothalamus, and macrophages invading the brain under pathological conditions (3-7). In cerebral vasculature, ET-1 evokes a dose-dependent and extremely long-lasting constrictive effect *in vivo* and *in vitro* (8-11) by acting on ET-A and ET-B receptors present on the surface of vascular smooth muscle cells and endothelial cells. Various stimuli can increase the synthesis of ET-1: thrombin, transforming growth factor (TGF)-ß, angiotensin II (1), oxyhemoglobin (12,13), and interleukin 1 (14). Increased plasma endothelin levels have been demonstrated in patients after subarachnoid hemorrhage (SAH) (15), in patients undergoing nonruptured abdominal aortic aneurysm (AAA) repair with infrarenal (16,17) and supraceliac aortic cross-clamping (18), as well as in one animal model of infrarenal aortic clamping and subsequent exsanguinations.

To date, however, there are no reports of the endothelin response in ECs from patients undergoing repair of ruptured or unruptured cerebral aneurysm and arteriovenous malformation (AVM), vascular anomalies that can lead to SAH.

We report here for the first time the successful isolation and growth *in vitro* of primary EC lines from excised human cerebrovascular lesions. Then, we investigated whether there was an increase of ET-1 released by EC lines from either ruptured or unruptured vascular anomalies.

Materials and methods

Patients findings. This study has been approved by the local ethics committee. Samples were derived from surgical specimens of aneurysmal dome, AVM nidus obtained from patients operated on for aneurysm clipping or AVM excision. Eight

Table I. Patient and lesion characteristics.

Sample	Age	Sex	Description
AN 2	38	Female	Ruptured middle cerebral artery aneurysm
AVM 3	23	Female	Ruptured arteriovenous malformation
AN 4	56	Female	Unruptured internal carotid artery aneurysm
AN 5	71	Male	Ruptured middle cerebral artery aneurysm
AN 7	33	Female	Ruptured internal carotid artery aneurysm
AN 8	69	Female	Unruptured anterior cerebral aneurysm
AVM 9	26	Female	Unruptured arteriovenous malformation
AVM 11	37	Female	Ruptured arteriovenous malformation

Table II. Oligonucleotide primers used for reverse transcription polymerase chain reaction.

Gene	Primer	Length (bp)	Reference sequence
Endothelin-1	F: aagacaaaccaggtcggaga R: tggaggctatggcttcagac	154	XM_043856
Endothelin receptor-a	F: tctccatctggatcctgtcc R: atgaagagggaaccagcaaa	350	XM_003485
Endothelin receptor-b	F: ccctgaagccataggttttg R: cagagggcaaagacaaggac	303	XM_007108
Calcitonin receptor-like receptor	F: agaaggcgtttactgcaacag R: tccctgtccaattatggtcag	271	XM_043055
Glyceraldehyde-3-phosphate dehydrogenase	F: tggggaaggtgaaggtcgga R: gaggtcaatgaaggggtcat	100	BC004109
Adrenomedullin	F: acttcggagttttgccattgc R: catccggactgctgtcttc	358	BC015961

F, forward; R, reverse.

specimens were collected (7 female, 1 male; age range 23-71 years). Ruptured aneurysms and AVMs were operated on within 48 h after hemorrhage. Normal brain microvasculature specimens were obtained from normal frontal lobe tissue of 2 males and 2 females. Table I gives a description of samples and summarizes the clinical characteristics.

Isolation and culture of endothelial cells from human brain vascolature, brain aneurysm and arteriovenous malformation. Tissue was sectioned into 3-mm^2 fragments, washed with PBS, and incubated in 0.1% collagenase B/0.1% dispase (Roche, Basel, Switzerland) at 37˚C for 25 min. In order to isolate ECs, the pre-digested tissue was triturated by a 2-ml pipette for 2 min and then filtered through a 100-µm strainer (BD Biosciences, NJ, USA). Cell suspension was centrifuged and re-suspended in culture medium MV2 supplemented with growth factors and 20% foetal bovine serum (FBS) (PromoCell, Heidelberg, Germany). Cells were then seeded at a density of 104 cells/cm^2 on fibronectin (1 $\mu g/cm^2$) (Sigma Aldrich Inc., St. Louis, MO, USA)-coated dishes and grown for 24 h at 37˚C with 5% CO_2. The day after seeding, cells were washed with PBS to remove unattached cells and fed with fresh medium. To obtain pure ECs, cultures grown to 80-100% confluence were exposed to immunoseparation using *Ulex europaeus* Agglutinin I (UEA)-coated (Vector Laboratories, Ltd., Peterborough, UK) beads (Dynabeads M-450 Tosylactivated, Oxoid, Hampshire, UK) as described by Jackson *et al* (19). ECs bound to the lectin-coated beads were collected with a magnetic particle concentrator and unbound cells were removed with 2 washes with basal medium. UEA-positive cells were re-suspended in culture medium and seeded on fibronectin-coated dishes to improve their adhesion and growth. Cultures became confluent within 4-6 days.

Characterization of endothelial cells. Immunocytochemistry to characterize the isolated cells was performed with rabbit polyclonal anti-human vWF (diluted 1/300; Dako Corp., Carpinteria, California, USA). For immunostaining, cells were

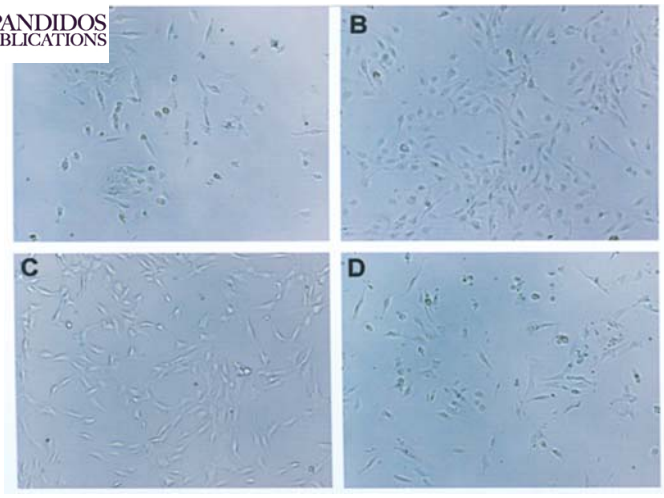

Figure 1. Endothelial cells isolated from different specimens. EC cultures established from human normal brain vascolature (A), cerebral aneurysm (C) and cerebral AVM (D) consist mostly of fusiform cells compared to HUVE cells (B). All the EC lines have been cultured under identical conditions, as revealed by phase-contrast microscopy. Magnification, x100.

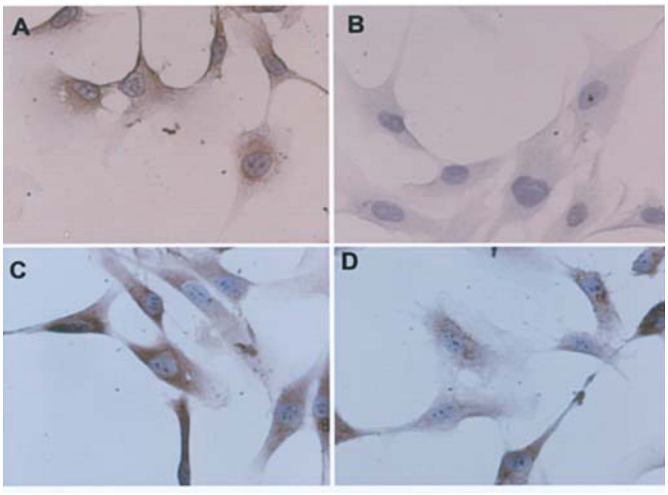

Figure 2. Immunocytochemical staining of endothelial cells. Purified HCECs stained positively for von Willebrand Factor (vWF) antibody showing a typical punctate brown (DAB) immunoreaction (A), which is not detectable if primary antibody is omitted (B). ECs from brain AVM and aneurysm also stained positively for vWf (C and D respectively). Nuclei are counter-stained with hematoxylin. Magnification, x10.

grown in MV2 on fibronectin-coated chamber slides, washed in PBS, and fixed in ice-cold acetone for 10 min at 4˚C. Immunostaining was performed at room temperature and cells were washed 3 times in PBS after each step. PBS with 1% BSA was used for antibody dilution. Cells were treated with 1% Triton in PBS for 5 min at room temperature and then incubated at room temperature with 0.5% BSA in PBS for 15-20 min to block nonspecific binding sites. The primary antibody rabbit anti-human von Willebrand factor (1:300) was added, followed by secondary peroxidase-conjugated anti-rabbit immunoglobulin G (1:150) incubation. The reaction was developed with avidin-biotin amplified immunoperoxidase method using the Large Volume Dako LSAB Peroxidase kit (Dako, Glostrup, Denmark). Finally, nuclei were counter-stained with hematoxylin. Negative controls were performed by similarly treating cultures and omitting the primary antibody.

Endothelial cell proliferation assay. ECs ($5x10^4$) at different passage numbers were re-suspended in MV2/20% FBS, seeded in each well of 24-multiwell plates and incubated in a 5% CO_2 incubator at 37˚C for 24 h. Proliferation was measured after 1 wash in PBS and trypsinization. Cells were collected and counted using Trypan blue staining. Results are means of 4 experiments.

Cell proliferation was also measured after 24 h using BrdU incorporation (Cell Proliferation ELISA, BrdU, Roche Applied Science, Monza, Italy). Briefly, BrdU labeling solution was added to the medium and incubated in a 5% CO_2 incubator at 37˚C for 18 h. The culture medium was removed after the labeling step and the FixDenat (cell fixation and DNA denaturation) reagent was added. After 30-min incubation at room temperature, the FixDenat was removed. Subsequently, cells were incubated with anti-BrdU-POD antibody conjugate for 1 h. Wells were then washed before substrate was added. The absorbance was determined at a 405-nm wavelength, with a reference wavelength of 490-nm, with a microplate autoreader EL 13. The results were means of 4 experiments.

PCR. Total RNA was extracted from $5x10^6$ cultured endothelial cells according to the Trizol method (Life Technologies, Carlsbad, CA, USA). mRNA was reverse-transcribed with the use of MultiScribe RT (Applied Biosystems, Foster City, CA, USA) and oligo-dT as primers for the first strand cDNA synthesis. The amplification of the resulting cDNA was carried out using specific primers (Table II). Briefly, in a thermal cycler (I-Cycler; Bio-Rad, Milan, Italy), after an initial denaturation step at 95˚C for 10 min, we used a denaturation step at 95˚C for 30 sec, an annealing step at 60˚C for 45 sec, and an extension step at 72˚C for 45 sec for a total of 35 cycles. To rule out the possibility of amplifying genomic DNA, RNA samples were treated with DNase (Ambion, Inc., Austin, TX, USA). Detection of the PCR amplification products was performed through electrophoresis on 2% agarose gel.

Capillary tube formation on Matrigel. We also checked purified control ECs, aneurysm and AVM ECs for their ability to form capillary-like structures at different passages. Briefly, growth factor reduced-Matrigel (BD Biosciences) was thawed on ice overnight and spread evenly over each well (50 µl) of a 24-well plate. The plates were incubated for 30 min at 37˚C to allow the Matrigel to gel. ECs ($2.5x10^2$ cells/mm^2) were seeded into each well in 500 µl of MV2/20% FBS medium and incubated for 24 h at 37˚C. Cultures were fixed with 10% paraformaldehyde and photographed (5 fields per well) at a magnification of x5. Phase contrast images were recorded using a digital camera (DG 200; Leica Imaging System, Cambridge, UK) connected to a Laborlux-S microscope. Image analysis was carried out using the Qwin image analysis software (Leica Imaging System), and the dimensional (percent area covered by cells and total length of cell network per field) and topological (mesh number and branching points per field) parameters were estimated.

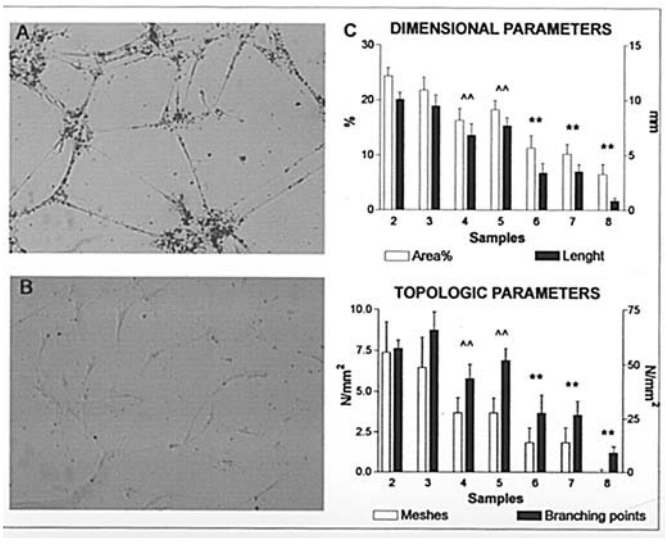

Figure 3. Tube formation by ECs on Matrigel. Representative photographs of HCECs at passage IX cultured on Matrigel after incubation for 24 h (A), and ECs from aneurysm 2 at passage VIII (B). Original magnification, x5. Quantitative analysis of tube formation (C). Each column represents mean ± S.D. of 5 different fields for each of 2 independent experiments carried out with aneurysm cells. Results are compared with every other passage. Statistical significance: $^{**}p<0.05$ comparing results with passages II, III, IV, V; $^{\wedge\wedge}p<0.05$ comparing results with passage II. Statistical analysis was performed by ANOVA followed by Bonferroni test.

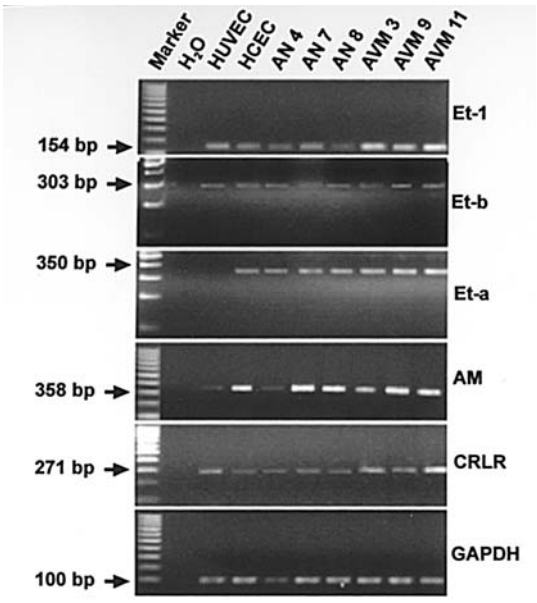

Figure 4. mRNA expression of EC markers. Agarose gel electrophoresis of different mRNA. Endothelin-1 (Et-1) and its receptors Endothelin-a (Et-a) and Endothelin-b (Et-b) have been detected in 3 out of 3 aneurysm (AN), and in 3 out of 3 arteriovenous malformation (AVM) ECs. Adrenomedullin (AM) and its receptor (CRLR) mRNAs have been detected in ECs from different brain vascular anomalies and for comparison in HCECs and in HUVECs. Bands are normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of ET-1. Purified ECs on II passage were seeded in MV2/20% FBS at 10^2 cells/mm^2 in 10^3-mm^2 plates and cultured. The medium was removed after 24 h and stored at -80˚C until assayed. Frozen samples were thawed on ice and centrifuged for 5 min at 1500 rpm to remove any residual cell. The supernatant was then assayed for ET-1 by using the Endothelin-1 EIA kit (Cayman, Ann Arbor, MI, USA) that consists of a 96-well plate coated with a monoclonal antibody specific for endothelin. Experiments were run in triplicate. After adding the standards, controls, and samples, an Acetylcholinesterase: Fab' conjugate was added as the detection antibody to form a sandwich, and the plate was incubated overnight at 4˚C. After several washes, the wells were incubated with Ellman's reagent at room temperature until colour was detectable, the plate was read in a plate reader at 405-nm. The standard curve was plotted and the samples read off the curve, since the amount of colour developed is directly proportional to the amount of ET-1 immunoreactivity (IR) present in the sample. The assay is sensitive enough to detect ≥1.5 pg/ml of endothelin.

Statistical analysis. Values are presented as mean ± S.D. Data within each group were compared by ANOVA for repeated measurements and, if significant, a post hoc comparison by Bonferroni test was performed. Significance was defined as $p<0.05$. Statistical analysis was performed using Primer software.

Results

Characterization of isolated ECs. To verify that the isolated *Ulex europaeus* I bead-bound cells, shown in Fig. 1, were indeed endothelial and free from contaminating non-ECs, we analyzed expression of endothelial-specific marker vWF by immunocytochemistry. ECs exhibited punctate cytoplasmic staining with anti-vWF (Fig. 2A), consistent with localization of vWF in Weibel-Palade bodies, a definitive feature of ECs. No background staining was observed omitting the primary antibody (Fig. 2B). Some smooth muscle α-actin (1-2%) positive cells were seen from passage V, with detectable increase by increased passage number (data not shown). In our experiments we used cells at passage II that stained 95% positive for vWF.

AM expression. ECs that we isolated had been analyzed for the expression of Adrenomedullin (AM) and its receptor Calcitonin receptor-like receptor (CRLR). AM was established as an important secretory product of vascular endothelium, together with nitric oxide, endothelins and prostanoids (20,21). In our results gel electrophoresis showed that AM and its receptor CRLR mRNAs are expressed in ECs isolated from both cerebral vascular malformations, at the same level as in HCECs (Fig. 4).

Endothelial cell growth and microtubule network formation. Cell proliferation assay, developed by counting the cells, has shown that the number of the cells collected after 24 h increased slightly from the II-V generation. From the V passage the number of cells collected decreased. Cell growth conditions were the same for all experiments. Immunologic assay, based on BrdU incorporation, confirmed these results showing a bell-shaped curve for proliferation at different passage numbers. We obtained these results with endothelial

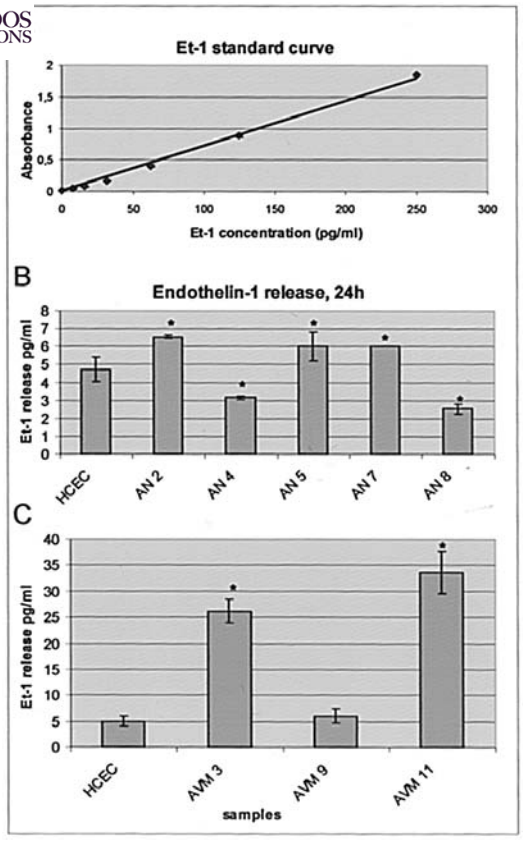

Figure 5. Endothelin-1 release. Representative standard curve for Et-1 (A) which has been used to plot absorbance for the samples in B. Et-1 concentration level in the medium of ECs from aneurysm (AN) and from brain microvasculature (HCECs). (C) The graph shows Et-1 release after 24 h from arteriovenous malformation ECs (AVM) and HCECs as control, standard curve for this experiment has not been shown. Error bars represent S.D. (n=3 experiments). Each group has been compared with control values (HCECs), *p<0.05 versus control. Statistical analysis was performed by ANOVA followed by Bonferroni test.

cells from every kind of vascular malformation analyzed. The proliferation ability of HCECs and HUVEC did not present any significant variation at different passage numbers until passage XII (data not shown).

To determine whether ECs from brain vascular malformation retain angiogenic potential, we also examined an *in vitro* angiogenesis model. Capillary-like tube formation was assessed in ECs from aneurysm, AVM and from normal brain microvascolature on growth factor-reduced Matrigel (22). Cells were seeded onto a Matrigel thin layer and, after 24 h, the formation of capillary-like structures was evaluated. Control cells, HCECs until IX passage showed a great ability to form capillary structures. After quantification, we did not detect any significant changes in microtubule network formation ability between different passages analyzed, from II-IX (data not shown). Fig. 3A shows a picture of HCECs at passage IX seeded in Matrigel after 24 h. For comparison, ECs from aneurysm 4 at passage VIII after 24 h are shown in Fig. 3B. Capillary-formation ability of ECs from aneurysms and AVM exhibited a slow decrease correlated with the increase of cell passage. Quantitative results indicate that ECs from vascular anomalies studied retain angiogenic ability from the II-V passage (Fig. 3C). Significant decrease of angiogenic properties has been detected from passage VI ($p<0.05$). Angiogenic ability almost disappeared from the VIII cell passage as confirmed with analysis of parametric and topologic parameters.

Endothelin-1. Several authors have demonstrated increase of Et-1 concentration in plasma and cerebral spinal fluid (CSF) after subarachnoid hemorrhage. Our interest was to determine if endothelial cells from cerebral vascular anomalies as aneurysm and AVM showed an increase of the basal release of ET-1. A total of 8 patients were examined, 5 aneurysm and 3 AVM. Endothelial cells were cultured and after 24 h the medium was collected and subjected to ET-1 measurement. In aneurysm ECs and HCECs ET-1 release is in the range of 2.5-6.9 pg/ml. Results obtained showed, compared to control, a significant increase ($p<0.05$) of ET-1 release in ECs from ruptured aneurysms and a significant decrease ($p<0.05$) in unruptured aneurysms (Fig. 5B). We found the same correlation between ET-1 release and brain vascular anomaly rupture in ECs isolated from AVM (Fig. 5C). Media collected from ruptured AVM ECs showed a significant increase ($p<0.05$) in ET-1 concentration compared to HCECs (approximately 4-7 fold higher). Furthermore AVM 9, which is unruptured, showed ET-1 concentration levels comparable to HCECs. Et-1 expression has also been detected by PCR (Fig. 4). Comparing Et-1 gel bands with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) we can confirm that ECs that release high levels of ET-1 also have high Et-1 mRNA concentration, suggesting that the increase of ET-1 is also at the transcriptional level. Furthermore, ECs from cerebral vessels show expression of both ET-1 receptor mRNAs, Et-a and Et-b (23,24), whereas HUVECs express only Et-b (Fig. 4).

Discussion

Reports about culturing of endothelial cells (ECs) from human intracranial vascular malformations are scarce. We describe for the first time a simple protocol for the isolation and growth *in vitro* of human cerebral aneurysm and arteriovenous malformation ECs. We obtained successful characterization of these ECs by means of such typical endothelial markers as von Willebrand factor, Adrenomedullin (AM) and Calcitonin receptor-like receptor (CRLR). We found that until passage V cerebral malformation ECs possess a good proliferation rate and tube formation abilty in Matrigel.

Intracranial aneurysms usually develop at the bifurcation of the conductive cerebral arteries in the subarachnoid space at the base of the brain (25,26). An inherited maldevelopment of the supportive structure of cerebral arterial walls with a lack or disruption of the internal elastic lamina, (25-29) together with hemodynamic stress (30), may facilitate formation of aneurysms. A rupture of an aneurysm causes subarachnoid bleeding or hemorrhage (SAH).

AVMs are high-flow lesions consisting of arteries and veins with direct fistulous communications and lacking normal intervening capillary beds (31). The molecular mechanism inherent to the genesis and maintenance of these abnormal vascular phenotypes has not been elucidated. The most morbid and frequent presentation of AVMs is cerebral hemorrhage (32) and less frequently can lead to hemorrhage in the subarachnoid space (SAH) (33-35).

SAH is a serious neurosurgical emergency generally associated with a consistent morbidity and mortality due to vasospasm (36). SAH-induced vasospasm can affect nearly 30% of patients, leading to further delayed ischemic consequences. For this reason many studies have attempted to identify the risk factors contributing to aneurysm and AVM formation and rupture and to investigate the pathogenesis of SAH-induced vasospasm.

ET-1 is a potent vasoconstrictor that has been studied extensively as a mediator of vasospasm (37-42). The evidence that ET-1 is important in the vasoconstriction seen in SAH-induced vasospasm is based on 3 findings: i) levels of ET-1 are increased in CSF and plasma of patients with vasospam (43); ii) experimental vasospasm can be evoked by administration of ET-1; and iii) antagonists of ET-1 attenuate the vasoconstriction in experimental vasospasm (44).

In isolated canine mammary veins and in cultured bovine arterial cells, oxyhemoglobin caused a significant increase of ET-1 in the incubation medium. Methemoglobin also increased ET-1 levels (45,13). The effect of oxyhemoglobin on the stimulation of ET-1 production was much higher than that induced by other stimulators, such as thrombin and phorbol-12-myrisate-13-acetate. Therefore, it is suggested that the oxyhemoglobin-induced ET-1 production in endothelial and smooth muscle cells is regulated by protein kinase C and a cyclic adenosine monophosphate-dependent pathway. Cisternal administration of ET-1 caused a delayed and prolonged spasm of intracranial vasculature in animal models (9,10,39) and isolated cerebral vessels reacted with a strong and long-lasting vasoconstriction after ET-1 exposure from the adventitial side (46).

Our observations have shown increased ET-1 release *in vitro* from ECs of ruptured aneurysm and AVM. We propose the following pathophysiological sequence: The blood clot formed after the vessel rupture releases oxyhemoglobin (OxyHb) in increasing concentrations into the subarachnoid space. OxyHb, interacting with the abluminal side of the endothelium, enhances the release of ET-1 and its vasoconstrictive effect (9,12).

In contrast, Pluta and colleagues did not support the hypothesis that ET-1 formation is stimulated by OxyHb (47). An *in vitro* examination has demonstrated that ET-1 formation in cultured endothelial cells did not increase 6 h after exposure to hemoglobin, whereas the production of ET-1 was significantly elevated in cultured astrocytes. In a primate model of SAH there was no correlation between the development of vasospasm and the perivascular ET-1 levels. Some authors have suggested that ET-1 in CSF is unlikely to be derived only from the systemic vasculature and have postulated that ET-1 is released by mononuclear leucocytes in the CSF stimulated by aging blood (48,49). Another study has shown that the ppET-1 gene is locally repressed in AVM lesions (50).

We show that ECs obtained from ruptured aneurysms and AVMs participate in ET-1 release following SAH. However, we cannot yet demonstrate if OxyHb is responsible for this. Some authors suggest that OxyHb by itself is insufficient to elicit the degree of vasoconstriction that they observed with the whole hemolysate (51). Kastner *et al* showed that only ET-1 levels in cerebrospinal fluid (CSF) correlate with cerebral vasospasm after SAH and not ET-1 levels in plasma (43). In accordance with these findings we demonstrate here that ET-1 release is a localized event that follows vessel rupture and hemorrhage. Further studies are needed in order to clarify if injured ECs are solely responsible for the ET-1 augmented release observed by some authors in patient plasma (52) and by other authors in CSF (43), or if other cells participate in this process. We suggest using damaged ECs from aneurysm and AVM as an experimental model to elucidate ET-1 receptor antagonist actions (53,54) on human cells that have already been subjected to *in vivo* mechanical stresses and hemorrhage induction.

In summary, we report here for the first time the successful isolation and growth *in vitro* of primary EC lines from excised human cerebral aneurysm and AVM. We show that augmented release of ET-1 is correlated with the rupture of the abnormal vessel suggesting a specific role of ECs during vasoconstriction. As ECs grown *in vitro* maintain, after a few passages, the *in vivo* ability to release ET-1, we suggest that these cells should be used as an experimental model to study SAH-induced vasoconstriction.

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