Alternative Splicing of Vasohibin-1 Generates an Inhibitor of Endothelial Cell Proliferation, Migration, and Capillary Tube Formation

Johann Kern, Monika Bauer, Kathrin Rychli, Johann Wojta, Andreas Ritsch, Günther Gastl, Eberhard Gunsilius, Gerold Untergasser

Objective—In this study, the alternative splicing product of vasohibin 1 (VASH1B) was analyzed in direct comparison to the major isoform (VASH1A) for antiangiogenic effects on endothelial colony forming cells (ECFCs) from peripheral blood and on human umbilical vein endothelial cells (HUVECs).

Methods and Results—Expression studies in primary human endothelial cells revealed that both vasohibin proteins, hVASH1A and hVASH1B, localized in the nucleus and cytoplasm. Adenoviruses carrying the cDNA for VASH1A/B and purified recombinant proteins were used to study the function of both molecules in ECFCs and HUVECs. Recombinant VASH1A protein did not inhibit cell proliferation, tube formation, or vessel growth in vivo in the chick chorioallantoic membrane (CAM) assay, but promoted endothelial cell migration in vitro. The VASH1B protein had an inhibitory effect on cell proliferation, migration, tube formation, and inhibited blood vessel formation in the CAM assay. Adenoviral overexpression of VASH1B, but not of VASH1A, resulted in inhibition of endothelial cell growth, migration, and capillary formation. Interestingly, overexpression of VASH1A and B induced apoptosis in proliferating human fibroblasts, but did not affect cell growth of keratinocytes.

Conclusion—Our data point out that alternative splicing of the VASH1 pre-mRNA transcript generates a potent antiangiogenic protein. (Arterioscler Thromb Vasc Biol. 2008;28:478-484)

Key Words: adenovirus ■ endothelial cells ■ vasohibin ■ alternative splicing

V asohibin-1 (KIAA1036, VASH1) has been identified in the human brain and is a vascular endothelial growth factor (VEGF)-regulated gene in human umbilical vein endothelial cells (HUVECs). The intrinsic antiangiogenic protein VASH1 has been shown to be a negative feedback-regulator of VEGF and bFGF signaling in HUVECs. In conjunction with the primary splicing product vasohibin1A, (hVASH1A) consisting of 7 exons, an alternative splicing product vasohibin1B (hVASH1B), generated by using an alternative splice acceptor site in exon 4, has been observed in HUVECs. Bioinformatic data analysis revealed that both proteins share the same 176 aa N terminus, but completely differ within the C-terminal protein region. Moreover both isoforms, hVASH1A (365 aa) and hVASH1B (204 aa), harbor a putative nuclear localization sequence (NLS) in their N terminus and lack a classical signal peptide sequence. Despite this fact, shorter proteolytical fragments of hVASH1A have been detected in supernatants of endothelial cells and have shown to exert antiangiogenic activities on HUVECs in vitro and on murine blood vessels formation in vivo. hVASH1 gene expression has been shown to be high in endothelial cells and low in fibroblasts and keratinocytes.

Based on these observations VASH1 proteins might be potential candidates for antiangiogenic therapies. Firstly, vasohibins should act specifically on endothelial cells, and secondly, proteolytical fragments systemically delivered into the blood stream could block VEGF induced permeability and destabilization of tumor vessels. In the present project we analyzed the alternative splicing isoform VASH1B for antiangiogenic activities in direct comparison with the major VASH1 isoform VASH1A. Therefore, we used 2 types of endothelial cells, ie, HUVECs isolated from umbilical veins and endothelial colony forming cells (ECFCs) propagated from peripheral blood mononuclear cells. In contrast to mature endothelial cells from the umbilical vein wall, ECFCs originate from circulating endothelial cells, a cell type that has been shown to contribute significantly to neovascularization in ischemia, inflammation, wound healing and tumor progression.
Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs, n=8 healthy volunteers, age: 30 to 60 years) were isolated by Ficoll density gradient centrifugation (Lymphoprep). Written informed consent was obtained from all donors. ECFCs were established as described elsewhere.6,9 ECFCs were characterized by fluorescence-activated cell sorter (FACS) analysis for endothelial, hematopoietic stem cell, and leukocyte surface marker expression (supplementary materials, available online at http://atvb.ahajournals.org). Human primary epithelial cells (HFK, human foreskin keratinocytes), were purchased from Clonetics. HFK were cultivated in keratinocyte cell growth medium with recommended supplements (KGM2, Clonetics). Human adult cardiac fibroblasts (HACF) were isolated and maintained in routinely cultivated in EGM-2 medium on collagen-type-I coated plastic flasks. Human adult cardiac myocytes (HACM) and human adult cardiac fibroblasts (HACF) were isolated and maintained in culture as previously described.10 Monocyte derived macrophages (MDM) were isolated and differentiated as described recently.13 Murine VEGF, was purchased from Sigma Biochemicals and human VEGF, bFGF and transforming growth factor (TGF)-β1 from Immunotools.

Cloning of cDNAs

The human VASH1A cDNA (pBluescript II KIAA0386) was a generous gift of Dr Mayumi Abe (Department of Vascular Biology, Tohoku University, Japan). The human VASH1B cDNA was cloned from ECFCs by specific primers to exon 1 and exon 4 of the genomic reference sequence on chromosome 14q24.3 (NC_000014.7) (for: 5-GTGTGGGAGGCCCTCTTGTGAAGC; rev: 5-GTGGGGTGTCTCTCCCATCTTGG). All cDNAs were subcloned in the pCRScript vector (Stratagene), and the nucleotide sequence was further verified by sequencing. The cloned VASH1B cDNA corresponded to the clone BC009031 of the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/). For subcellular localization studies, all cDNAs were cloned in frame with a FLAG-tag (p3xFLAG CMS-14 vector, w.ncbi.nlm.nih.gov/). For subcellular localization studies, all cDNAs were cloned and the nucleotide sequence was further verified by sequencing. The cloned VASH1B cDNA corresponded to the clone BC009031 of the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/). For subcellular localization studies, all cDNAs were cloned in frame with a FLAG-tag (p3xFLAG CMS-14 vector, Sigma Biochemicals, mouse monoclonal), tubulin-α (0.1 μg/mL, Sigma Biochemicals, mouse monoclonal), bak-1 (1 μg/mL, rabbit polyclonal, Sigma Biochemicals), or mcl-1 (1 μg/mL, rabbit polyclonal, BD-Pharmingen) for 1 to 2 hours and further incubated with a 1:2500 dilution of an HRP-conjugated goat-anti-mouse IgG or a 1:2500 dilution of an HRP conjugated goat-anti-rabbit IgG (Promega) for 1 hour. After washing, a chemoluminescent substrate (Super Signal West dura, Pierce) was added to the membrane, which was then exposed to the ECL Hyperfilm (Amersham Biosciences). Films were scanned by the Imaging Doc 2000 and the band intensity was analyzed by the Quantity One software (BioRad).

Western Blot Analysis

Cytosolic and nuclear extracts were prepared using NE-PER cytoplasmic and nuclear extraction reagents, respectively (Pierce). Twenty μg of total protein were denaturated and transferred on an Immuno-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After blocking of the membrane in 3% skim milk powder dissolved in PBS, it was probed with primary antibodies directed against human vasohibin (0.5 μg/mL, R&D systems, goat-polyclonal), Flag-TAG (0.1 μg/mL, Sigma Biochemicals, mouse monoclonal), tubulin-α (0.1 μg/mL, Sigma Biochemicals, mouse monoclonal), bak-1 (1 μg/mL, rabbit polyclonal, Sigma Biochemicals), or mcl-1 (1 μg/mL, rabbit polyclonal, BD-Pharmingen) for 1 to 2 hours and further incubated with a 1:2500 dilution of an HRP-conjugated goat-anti-mouse IgG or a 1:2500 dilution of an HRP conjugated goat-anti-rabbit IgG (Promega) for 1 hour. After washing, a chemoluminescent substrate (Super Signal West dura extended, Pierce) was added to the membrane, which was then exposed to the ECL Hyperfilm (Amersham Biosciences). Films were scanned by the Imaging Doc 2000 and the band intensity was analyzed by the Quantity One software (BioRad).

Immunofluorescence and Confocal Microscopy

Cells were fixed in acetone/methanol (1:1), permeabilized with 0.2% Triton-X-100 and then blocked with PBS containing 3% BSA for 45 minutes at room temperature (RT). An anti-Flag-TAG (1 μg/mL mouse monoclonal anti-FLAG2, SIGMA Biochemicals) antibody was applied for 2 hours at RT. After washing with PBS, cells were incubated with a secondary, fluorochrome-labeled antibody (polyclonal rabbit anti-mouse fluorescein isothiocyanate (FITC), Dako-Cytomation), and the nuclei were counterstained with TOP-RO-3 iodide (Molecular Probes). Cells were embedded in fluorescent mounting medium (DakoCytomation) and viewed by the CLSM (Zeiss Axioshot, µ-Radiance scanning system, Carl Zeiss Laser Optics/Laser Sharp Software, Bio-Rad Laboratories).

Adenoviruses

Replication-defective adenoviruses were generated by the AdEasy Adenoviral vector system (Strategene) according to the manufacturer’s instructions. In brief, VASH1A and VASH1B and GFP cDNAs were subcloned into the pShuttle CMV vector. Recombinant adenoviral DNA was generated in BJ5183 bacterial cells by a double-recombination event between cotransformed adenoviral backbone plasmid vector, pAdEasy-1, and a shuttle vector carrying the gene of interest. Replication-defective adenoviruses were generated by transfecting DNA in HEK293 cells with Lipofectamn 2000 (Invitrogen), and subsequently cytosolic extracts were prepared. All viral titers were determined by qPCR for the gene coding for the encapsulation signal (for:5-cagacaggttgccagaaatg; rev: 5-ctaaacccgcegaagaa) and the respective viral plasmid DNA standards.

Cell Proliferation Assays

Twenty-thousand cells were seeded into each well of a 6-well plate (Nunc) in culture medium in triplicates and were allowed to adhere overnight before the culture medium was changed. Thereafter, cells were incubated with the respective purified proteins or were adenovirally transfected. Cells were detached and cell numbers determined after 5 days using a Buerker counting chamber.

DNA synthesis (proliferation) was assayed by a BrDU assay (Amersham, GE-Healthcare) by labeling cells overnight after 4 days of growth. Incorporated BrDU was measured by an ELISA reader at 450 nm.

Capillary Tube Formation Assay

To analyze capillary tube-formation, 24-well-plates were coated with 200 mL growth factor reduced Matrigel (BD Biosciences). ECFCs were resuspended in 200 mL EGM-2 medium (1×10⁵ cells) and placed over the polymerized matrix and tube formation was observed after 6 hours. Tubes were viewed in an inverted transmission-microscope (Zeiss Axioline 200 μL) and documented by a digital imaging system (Axiovision Software, Zeiss). Statistical analysis was performed after calculating capillaries per mm².

Cell Migration Assay: Scratch Assay

ECFCS were seeded onto 6-well cell culture plates. Once at confluence, cells were serum-starved in medium containing 0.5% FBS overnight, and then scratch injury was applied using a disposable pipette tip or rubber cell scraper (1-mm width). After injury, the monolayer was gently washed with PBS, and the medium was replaced to growth factor reduced medium. Endothelial cell migration from the edge of the injured monolayer was examined and photographed 6 hours after scratching. Migrated endothelial cells were counted in 10 randomly selected high-power fields (HPF) adjacent to the scratch injury and were expressed as cells/mm².

Quantitative Polymerase Chain Reaction

RNA was purified by cell lysis and nucleic acid extraction by the use of the RNeasy Kit (Qiagen). Extracted total RNA was transcribed into cDNA by oligo-dT- and hexanucleotide-random-primers and the AMV-Reverse Transcriptase (all Promega). For analysis in the quantitative polymerase chain reaction (PCR), 20 ng of each cDNA were used. 5 μL Sybr-Green Mix (Bio-Rad), and 10 pMol of each primer were mixed to the cDNA sample. Elongation factor-1α (EF1α, Unigene: Hs.686423; forward 5'-caccagctgcactattgaag; reverse 5'-tgaggtcagcaagcaag) served as an internal housekeeping gene. VASH1A amplifies all VASH1 transcripts (Unigene: Hs.525479;
forward 5-AGATCCCCATACCGAAGTGG, reverse 5-GGGCCTC-TTTGCTTATTTCC) and VASH1B (5-AAGCTGTCACGGCTACATC, 5-ACCTTTACAGCAGGAAACGTGA) amplifies selectively the alternative splicing variant of exon 4. Analysis occurred within 50 cycles in the Bio-Rad iCycler (Bio-Rad). Data were collected and analyzed by the iCycler Software. Specificity of the amplified PCR product was checked by melting curve analyses, gel electrophoresis, and sequencing.

FACS Analysis

Cell phenotypes were determined by staining for surface markers specific for hematopoietic stem cells, such as CD34 (Beckton Dickinson) and PE-labeled anti-human CD133 (Milteny), or leukocytes, such as PE-labeled anti-human CD14 and PerCP-labeled anti-human CD45 (both Beckton Dickinson), or endothelial cells such as PE-labeled anti-human CD31, PE-labeled anti-human CD141 (both Beckton Dickinson), PE-labeled anti-human CD105 (Sero tec), and PE-labeled anti-human KDR (R&D Systems).

Analysis of late and early apoptosis was performed by incubation of cells with Annexin V FITC (Alexis) and 7-AAD (Beckman Coul ter) in a binding buffer consisting of 2.5 mmol/L HEPES pH 7.4; 40 mmol/L NaCl; 0.8 mmol/L CaCl2. Stained cells were analyzed in the Cytomics-FC-500 by the use of the Cytomics RXP-Software (Beckman Coul ter).

Purification of Recombinant Proteins

The generated cDNA-FLAG fusion protein sequences of the p3xFLAG-CMV14 vector (Sigma Biochemicals) were subcloned into the pT2 neo CIMS vector. Murine B16F10 cells were transfected either with the pT2 neo CMV VASH1A or the vector containing VASH1B. Cotransfection was performed with the pCMV SB11 vector encoding the sleeping beauty (SB) transposase. Clones resistant to neomycin were selected and propagated from the ClonaCell medium (Stem Cell Technologies Inc) and then analyzed for high protein expression by Western Blot (anti-FLAG M2 antibody, Sigma Biochemicals).

Recombinant Flag-tagged proteins were purified from cytosolic extracts according to the manufacturer’s instructions by the use of liquid chromatography columns and anti-FLAG M2 agarose beads (all SIGMA-Biochemicals). The purified proteins were quantified by the use of a standardized FLAG-BAP fusion protein (SIGMA-Biochemicals). Purity of the used preparations was checked by SDS-Page and Coomassie staining.

CAM-Assay

Fertilized white leghorn chicken eggs (SPF eggs) were purchased from Charles River (Kiesse l) and incubated in an egg incubator at 37°C, and 70% humidity (Compact S84, Grumbach) for 4 days. Subsequently, a window was incised into each eggshell and the underlying membrane. The eggs were incubated again for 4 hours with the windows sealed (Durapor tape). Then, a Ther manox Ring (Nunc) was added on the CAM and a 10 mmol/L Tris-Glycine solution (pH 7.4) containing the purified recombinant protein (in total 20 ng) or control extracts were applied on each egg. The eggs were sealed and incubated for further period of 3 days, after which they were opened, and the CAM with the Ther manox ring was observed and documented by a stereomicroscope with connected digital camera and flexible cold light (Olympus SZ51, Olympus E410). Each protein was assessed using 6 incubated eggs and thereafter, blood vessels were counted inside the ring area (20 mm²).

Statistical Analyses

Statistical analyses were performed with the GraphPad Prism software for Windows. All tests for statistical significance were 2-sided. Student t test and the Mann-Whitney U test were used to study differences between 2 groups. The Kruskal-Wallis H test was applied to analyze differences between 3 groups. Statistical analyses of quantitative PCR data were performed according to the delta Ct method described by Pfaffl et al14 and probability values were calculated with Student t test.

Results

VASH1 Is Induced by VEGF and Is Localized in the Nucleus and Cytoplasm of Endothelial Cells

Different concentrations of angiogenic cytokines (1 to 100 ng/mL) were tested for their effects on VASH1 gene expression in ECFCs. Only human and murine VEGF induced gene expression of hVASH1A and hVASH1B (Figure 1a). Interestingly, analysis of supernatants, cytosolic, and nuclear extracts revealed a strong presence of hVASH1A protein in nuclear extracts and in the cytoplasm of endothelial cells (Figure 1b and 1c). Immuno reactive protein increases on VEGF stimulation in cytoplasmatic and nuclear extracts (Figure 1b). Parallel to endothelial cells, VASH1 protein was also detected in nuclear and cytoplasmatic fractions of in vitro propagated human aortic cardiac myocytes (HACM), fibroblasts (HACF), monocyte-derived macrophages (MDM), and PBMCs (Figure 1c).

VASH1A and VASH1B Harbor an N-Terminal Nuclear Localization Sequence

Bioinformatic protein analysis (http://cubic.bioc.columbia.edu/predictNLS/) of human VASH1 revealed the presence of a nuclear localization sequence (NLS) motif in the N terminus of human VASH1A and VASH1B (Figure 2a). Both VASH1 proteins have no classical signal peptide sequence for efficient secretion. Bioinformatic findings were con-
confirmed by transfection of ECFCs and HUVECs with FLAG-tagged vasohibins, clearly demonstrating a cytoplasmic and a nuclear localization of hVASH1A/B (Figure 2b). Analysis of concentrated supernatants of endothelial cells cultured under standard culture conditions revealed no presence of secreted vasohibin protein or fragments still containing the C-terminal FLAG-tag (Figure 2c). The used HUVECs and ECFCs were cultivated under the same conditions, displayed a similar endothelial phenotype (KDR+/CD146+,CD31+,CD105+,CD141+) and did not express stem cell markers (CD34/CD133) or leukocyte antigens (supplemental Figures I and II, available online at http://atvb.ahajournals.org).

Effects of Recombinant hVASH1A and hVASH1B on In Vivo Blood Vessel Growth in the Chicken Chorioallantoic Membrane

Both vasohibin variants were purified for application in an in vivo angiogenesis assay, the CAM assay (Figure 3a). In comparison to the FLAG-tagged control protein purified from extracts of the same cell-type, deposition of recombinant VASH1A (20 ng, n=6) caused no inhibition of vessel growth, but blood vessels appeared more irregular and destabilized. Application of recombinant VASH1B (20 ng, n=6) strongly inhibited in vivo vessel growth within 3 days of observation. In comparison to the controls blood vessel number in the exposed area decreased from 30±3.9 to 13±4.1 vessels/20 mm² (P<0.05).

In Vitro Effects of Recombinant hVASH1A and hVASH1B on Human Endothelial Cells

Both recombinant proteins were tested at a concentration of 10 nmol/L on ECFCs and HUVECs (Figure 3b) in comparison to purified FLAG-tag control protein and unstimulated cells. Cell growth was inhibited only by the VASH1B protein. Also, cell migration in the scratch assay (Figure 3c) and tube formation in a matrigel assay (Figure 3d) were inhibited only by VASH1B. Interestingly, recombinant VASH1A significantly increased the migration of human endothelial cells in the scratch assay.
Adenoviral Overexpression of VASH1B Inhibits In Vitro Cell Growth, Migration, and Tube Formation in Endothelial Cells

Human ECFCs and HUVECs were transfected with an adenovirus encoding VASH1A, VASH1B, or green fluorescent protein (GFP, MOI = 100 viruses/cell). This ratio was chosen based on a strong protein expression with no detectable cytopathic effects caused by the adenovirus (supplemental Figures I and II). Both vasohibin proteins were found in total cell extracts but were not detectable in the respective supernatants (Figure 4a). Cells were incubated for 5 days, and thereafter cell numbers were determined. In comparison to GFP transfected cells, hVASH1A overexpression did not change cell number (Figure 4b). Interestingly, overexpression of the alternative splicing variant hVASH1B significantly inhibited cell growth and reduced cell number to 52.7 ± 7.4% (n = 4, P < 0.05). ECFCs and HUVECs were further analyzed for effects on in vitro cell migration and capillary formation in matrigel. In comparison to cells transfected with a GFP encoding adenovirus, those infected with the hVASH1B encoding AV were strongly inhibited in their migration (Figure 4c) and capillary formation (Figure 4d). Cell migration (cells/mm²) decreased to 53.2 ± 4.9% (n = 4, P < 0.05) and capillary formation capacity (capillaries/mm²) to 57.1 ± 8.9% (n = 4, P < 0.05). This was not observed in cells transfected with the hVASH1A encoding AV.

Adenoviral Overexpression of VASH1B Inhibits DNA Synthesis and Induces Apoptosis in Endothelial Cells

Adenoviral overexpression of VASH1B in ECFCs and HUVECs strongly inhibited DNA synthesis as determined by the incorporation of BrDU in newly synthesized DNA 96 hours after transfection (Figure 5a, n = 4, P < 0.05). Moreover, adenoviral overexpression induced apoptosis in HUVECs and ECFCs. Loss of membrane integrity was observed 3 days after transfection by externalization of phosphatidylserin and thus binding of Annexin (data not shown). In comparison to untreated cells (2.33 ± 0.87%) and hVASH1A transfected cells (3.73 ± 0.98) overexpression of hVASH1B increased the percentage of early apoptotic cells to 8.45 ± 1.48% (n = 3, P < 0.05). On the molecular level VASH1A was responsible for a strong upregulation of the antiapoptotic Mcl-1 protein (Figure 5b). In contrast, the hVASH1B overexpressing cells showed only an upregulation of the proapoptotic Bak-1 protein without simultaneous increase of Mcl-1. Thus, VASH1B overexpressing cells have an altered cellular Mcl-1/Bak-1 ratio (Figure 5c).

Figure 4. Adenoviral overexpression of vasohibin isoforms. A, Protein expression was analyzed after adenoviral transfection of VASH1A (1A) and VASH1B (1B) in total cell extracts (CE) and in supernatants (SN). Cell numbers of endothelial cells, keratinocytes (HFK), and fibroblasts (HFF) were determined after 5 days (B). Effects on cell migration of endothelial cells were analyzed in a scratch assay (C) or capillary tube formation assay (D). *Probability values < 0.05.

Figure 5. VASH1B overexpression inhibits proliferation and induces apoptosis. BrDU incorporation of ECFCs after adenoviral transfection (A). Western blot analysis of apoptosis regulating proteins of adenovirally transfected ECFCs (B). Tubulin-α served as internal loading control. Densitometric analysis of Mcl-1 to Bak-1 protein ratio (C). *Probability values < 0.05.
Overexpression of VASH1A Inhibits In Vitro Cell Growth and Induces Apoptosis in Human Fibroblasts

Adenoviral overexpression of VASH1A strongly inhibited cell growth of human fibroblasts, but not those of keratinocytes (Figure 4b). In fibroblasts we observed a significant induction of apoptosis as determined by loss of cell membrane symmetry (Annexin/7AAD) and activation of caspase-3 (data not shown). Apoptosis was also characterized by downregulation of Mcl-1 and Bcl-2, which was not observed in epithelial or endothelial cells (supplemental Figures I and II). Interestingly, exogenous stimulation with recombinant VASH1A resulted in fibroblast growth inhibition (supplemental Figures I and II).

Discussion

Angiogenesis is characterized by sprouting of new blood vessels into avascular, ischemic, or inflamed tissue and expanding tumors.15–17 This process is mainly induced by angiogenic factors, such as VEGF and angiopoietin-2, secreted from tissue or tumors.18,19 Angiogenesis is inhibited by a variety of extracellular matrix derived protein fragments, such as naturally occurring antiangiogenic factors.20,21 Recently, hVASH1, an endothelial cell specific factor has been created from tissue or tumors.18,19 Angiogenesis is inhibited by exogenous stimulation with recombinant VASH1A resulted in fibroblast growth inhibition (supplemental Figures I and II).

In accordance with Abe et al22 and Watanabe et al2 we found a significant upregulation of the hVASH1 gene and protein expression on VEGF stimulation in endothelial cells. In addition, we were able to demonstrate that ECFCs express the recently discovered alternative splicing variant VASH1B,4 which was also upregulated by VEGF treatment. We could show that both human VASH1 variants localized to the cytoplasm and nucleus and were not secreted by endothelial cells under standard culture conditions. In contrast to the data of Watanabe et al,2 we could not detect any processed VASH1 fragments in the respective supernatants, even after adenoviral transfection of untagged VASH1 in endothelial cells. However, we cannot rule out that vasohibin or fragments of the protein might be released by endothelial cells after a specific stimulus, such as inflammation, apoptosis, or mechanical damage. In vitro application of purified vasohibin proteins on human endothelial cells revealed a strong antiangiogenic effect of VASH1B, which was also confirmed in vivo in the CAM assay. Interestingly, recombinant VASH1A did not inhibit blood vessel growth in the CAM assay, but increased endothelial cell migration in vitro. In contrast to the published inhibition of in vitro cell migration and capillary formation of exogenously added VASH1A in HUVECs,2 endothelial cells in our study were not inhibited in proliferation, migration, and capillary formation by exogenous stimulation with VASH1A. Our data neither support the concept that VASH1A might act as a “chemokine” supporting endothelial cell migration. Interestingly, both purified recombinant vasohibin proteins did not affect cell growth of epithelial cells, but reduced cell numbers of human primary fibroblasts.

Because of the intracellular localization of FLAG-tagged vasohibins, we tested the effects of viral overexpression in ECFCs and HUVECs. Overexpression of VASH1B resulted in inhibition of cell growth, proliferation, migration, and tube formation. This was not observed with VASH1A. The shorter VASH1B isoform, with a unique C terminus of 28 aa, has an acidic isoelectric point (pl) of 5.0, in contrast to hVASH1A, with a basic pl of 9.5. These differences might be one reason for the distinct molecular actions and the growth-inhibitory effect of hVASH1B on endothelial cells.

Cell-type specific effects of both vasohibin isoforms were observed after overexpression in human fibroblasts and epithelial cells. Whereas fibroblasts were strongly inhibited in cell proliferation by both isoforms, the human epithelial cells were not inhibited by either VASH1A or VASH1B. These data implicate that hVASH1A requires specific intracellular interaction partners for growth inhibition/apoptosis, that are not present in primary epithelial and endothelial cells, but highly expressed in fibroblasts.

Watanabe et al reported that vasohibin is predominantly expressed in endothelial cells, and to a lesser extend in fibroblasts and keratinocytes.2 In addition to endothelial cells, we observed a strong expression also in freshly prepared PBMC samples and in vitro cultivated cardiac myocytes. To exclude the possibility of an uptake from the circulation or serum, vasohibin gene expression was also detected in freshly isolated PBMCs (unpublished data, 2007). These data fit to the EST profile analysis of the NCBI server for hVASH1 (Hs.525479) in human tissues, showing high expression in blood, vascular tissue, spleen, heart, and brain, and to data obtained by in situ hybridization in a variety of organs of the developing chicken.23 These observations could indicate that conserved vasohibins might have further functions beyond angiogenesis, e.g., in organ development or in immunobiology.

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Disclosures

None.

References

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**Figure I**

A) FACS analysis of marker expression on human endothelial cells.

B) Stimulation of human cell types with recombinant vasohibin isoforms. Endothelial cell growth (HUVEC, ECFC) was inhibited by VASH1B and epithelial cells (HFK) not. Fibroblasts (HFF) were inhibited by recombinant VASH1A and B. *p values < 0.05.

**Figure II**

A) Western blot analysis of vasohibin protein expression two days after adenoviral transfection with different multiplicities of infection (MOI).

B) Western Blot analysis of apoptosis regulating proteins in fibroblasts (HFF) and keratinocytes (HFK) two days after adenoviral overexpression of GFP, hVASH1A and hVASH1B.
### A

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