Blood outgrowth endothelial cells from Hereditary Haemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions

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Abstract

Objective: Hereditary haemorrhagic telangiectasia (HHT) is originated by mutations in endoglin (HHT1) and ALK1 (HHT2) genes. The purpose of this work was to isolate and characterize circulating endothelial cells from HHT patients.

Methods: Pure primary cultures of blood outgrowth endothelial cells (BOECs) were obtained from 50 ml of peripheral blood by selection on collagen plates with endothelial medium.

Results: The amount of endoglin in HHT1–BOECs is half the controls, but HHT2–BOECs are also endoglin-deficient. Since the TGF-β/ALK1 pathway activates the endoglin promoter activity, these results suggest the involvement of ALK1 in endoglin gene expression. Endothelial TGF-β pathways, mediated by ALK1 and ALK5, are impaired in HHT cells. HHT–BOECs show disorganized and depolymerized actin fibers, as compared to the organized stress fibers of healthy–BOECs. Functionally, HHT–BOECs have impaired tube formation, in contrast with the cord-like structures derived from normal donors.

Conclusions: Decreased endoglin expression, impaired TGF-β signalling, disorganized and depolymerized actin fibers, and failure to form cord-like structures are common characteristics of endothelial cells from HHT patients. These features may lead to fragility of small vessels and bleeding characteristic of the HHT vascular dysplasia and to a disrupted and abnormal angiogenesis, which may explain the clinical symptoms associated with this disease.

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This article is referred to in the Editorial by R. Hirschberg (pages 180–182) in this issue.

1. Introduction

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant, multisystemic vascular dysplasia, characterized by recurrent haemorrhages [1,2]. Mutations in endoglin (ENG; chromosome 9) [3] and ALK1 (chromosome 12) [4] are responsible for HHT1 and HHT2, respectively. Endoglin and ALK1 play important roles in
cardiovascular development, angiogenesis and vascular remodelling [5–9].

Endoglin and ALK1 are components of the TGF-β receptor complex primarily expressed in endothelial cells (ECs) [9–11], where they collaborate with each other in TGF-β signalling [12,13]. TGF-β is a multifunctional cytokine member of a large superfamily of proteins including activins and bone morphogenetic proteins [14]. Cellular responses triggered by TGF-β are elicited via type I and type II serine/threonine kinase receptors and downstream by the Smad family of signal transducers [15]. Type I receptors act downstream from type II and determine the signalling specificity in the receptor complex. Since ECs express two different type I receptors, ALK1 and ALK5, TGF-β may activate two different pathways. ALK1 promotes Smad1 and Smad5 phosphorylation stimulating cell proliferation and migration, whereas activation of ALK5 promotes phosphorylation of Smad2 and Smad3, and stimulates inhibition of proliferation and extracellular matrix synthesis [16]. Endoglin interacts with TβRII, ALK1 and ALK5 [13,17] and cooperates with ALK1 in the endothelial TGF-β signal transduction favouring cell proliferation [12,13]. Endoglin binds different members of the TGF-β superfamily in the presence of the signalling receptors types I and II [18,19], and counteracts the TGF-β-induced growth inhibition of ECs [20].

The diagnosis of HHT remains at the clinical level, according to the consensus criteria of Curaçao [21]. These include epistaxis (spontaneous, recurrent nose bleeds), telangiectasias (multiple at the mucosa level), visceral lesions (in lung, brain, liver, or spinal cord) and family history. HHT diagnosis is definite if 3 criteria are present. Haploinsufficiency is generally accepted as the molecular basis for HHT, since many of the mutated endoglin and HHT penetrance [26], as endoglin deficiency in activated ECs, makes 2.1. Patient samples and DNA sequencing

Informed consent was obtained from individuals participating in the study. Controls were healthy donors, as certified by annual medical surveys. The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–4). HHT diagnoses were based on Curaçao criteria. Genomic DNA was isolated from peripheral blood lymphocytes using Qiagen Mini kit (Qiagen). The fifteen exons from endoglin and the nine exons from ALK1 were PCR-amplified using HotMaster Polymerase (Eppendorf), reported primers [22–24] and sequenced with a cycle sequencing protocol (Applied Biosystems, USA).

2.2. Plasmids and site directed mutagenesis

GAL4-Smad1 and GAL4-Smad3 plasmids were generously provided by Drs. Nakayama and Derynck, respectively. TGF-β-responsive constructs (CAGA)12-Luc, p(BRE)2-Luc, were kindly provided by Dr. ten Dijke. Endoglin promoter construct pCD105 (−450/+350) and vector encoding human endoglin have been described [17,31]. Vectors encoding wild type ALK1 (WT), kinase deficient ALK1 (K229R), and constitutively active ALK1 kinase (Q201D) in pcDNA3-HASL plasmid were generous gifts from Dr. Miyazono (University of Tokyo, Japan). The reporter vector for the ALK5 promoter, pTGFbeta type I receptor (−1422/−65)-Luc, was a kind gift of Dr. Koijima (RIKEN Wako, Saitama, Japan). Site directed mutagenesis of pcDNA3.1 ALK1 (WT) was made by PCR to substitute 1120 C by T yielding ALK1 (R374W).

2.3. Cell cultures and tube formation assay

BOECs were grown from 50 ml peripheral blood, cultivating buffy coat mononuclear cells on collagen I coated wells using EGM/EBM-2 medium (Clonetics), as described [28,29]. Briefly, cells from mononuclear layers were pelleted and resuspended in 5 ml of medium. Cells were centrifuged again and the pellet washed twice in culture medium, before resuspension in 5 ml of medium and plating on collagen coated P-6 wells. Cells were incubated in 5% CO2 at 37 °C and medium was replaced daily for the first week and thereafter medium was changed every two days. BOECs were established as pure endothelial cultures, being the only surviving cell type covering the wells after 30–45 days of growth. For characterization and functional studies, BOECs from 2nd to 4th passage were used. For tube formation assays, cells were plated in EBM/EGM-2 culture medium on matrigel (Becton Dickinson) and incubated at 37 °C, as indicated by the manufacturer. Human umbilical vein ECs (HUVECs) were cultured in EBM/EGM-2. Human primary
fibroblasts and 293 T cell line (kidney epithelium) were cultured in DMEM medium.

2.4. Western blot analysis

Cells were lysed on ice for 30 min in 1% SDS. Lysates were centrifuged at 14,000 ×g for 5 min. Aliquots of cleared cell lysates were boiled in SDS sample buffer and analyzed in 7.5% SDS-PAGE under non-reducing conditions. Proteins were electrotransferred to nitrocellulose membranes, followed by immunodetection with anti-endoglin (P4A4), anti-ALK1 (MAB370, R&D, sc-402 Santa Cruz), or anti-HLA class I (W6/32, Sigma). Secondary antibodies were horseradish peroxidase conjugates from Dako. Membranes were developed by chemiluminescence (ECL, Supersignal, Pierce).

2.5. Reverse transcription-PCR

RNA was extracted from 10^6 cells using RNAeasy kit (Qiagen). RNA was reverse-transcribed to cDNA using random hexamers and AMV reverse transcriptase. PCRs with oligonucleotides for ALK5, TβRII [32] or GAPDH were performed using the HotMaster Taq polymerase (Eppendorf).

2.6. Immunofluorescent microscopy

Cells grown onto glass coverslips coated with collagen type I were fixed with 3.5% formaldehyde in PBS, washed and blocked with 2% BSA in PBS for 1 h at 4°C. Cells were incubated for 1 h at 4°C with anti-endoglin (P4A4), anti-PECAM1 (Clone HC1/6; Chemicon) anti-ALK1 (MAB370, R&D), anti-von Willebrand Factor (vWF, sc-7154, Santa Cruz) and VE-cadherin (sc-9989, Santa Cruz) antibodies. For staining with vWF, cells were permeabilized with 100 μg/ml L-α-lysophosphatidylcholine, followed by incubation with Alexa-488 green-conjugated anti-rabbit/mouse IgG antibody (Molecular Probes). For staining actin filaments, cells were fixed, stained, and permeabilized in a single step by addition of 5 units/ml Alexa-546 phalloidin (Molecular Probes), 100 μg/ml L-α-lysophosphatidylcholine, and 3.5% formaldehyde in cold PBS. Coverslips were mounted with Mowiol 44-88 (Calbiochem) and visualized using an Axiosimpe microscope (Leica Microsystems). When required, BOECs were transfected with pEGFP, endoglin (pCMV5-fected, prior to actin staining, with expression vectors for (Leica Microsystems). When required, BOECs were trans

2.7. Flow cytometry

BOECs were incubated with mAb P4A4 (anti-endoglin), anti-vWF (permeabilized cells as above), anti-ALK1 (MAB370, R&D), anti-Flk-1/KDR (MAB3572, R and D), anti-Fli-1 (MAB321, R&D), anti-fibroblast surface protein (1B10, Sigma) or an isotype-matched control for 30 min at 4°C in cold PBS with human IgG, followed by secondary FITC-conjugated anti-IgG. Samples were analyzed on a Coulter Epics XL flow cytometer. Antigen measures are represented by the expression index, which is the result of multiplying the percentage of cells expressing antigen (positive cells) by the mean fluorescence intensity of the whole population (positive and negative).

2.8. Cell transfection and reporter assays

Transient transfections were made with Superfect (Qiagen) according to the manufacturer’s recommendations. For GAL4 transactivation assays, GAL4-Smad1 or GAL4-Smad3 plasmids were cotransfected with the GAL4-luciferase reporter pFr5-Luc (Stratagene). Reporter assays with TGF-β responsive promoter constructs (CAGA)_2-luc, p(BRE)_2, and pCD105(–450/+350), and with the ALK5 promoter construct (–1422/–65)-Luc were performed as described [13,32]. After transfection, cells were incubated with 10 ng/ml TGF-β1 (Preprotech) for 24 h (reporters (CAGA)_2-luc, GAL4-Smad3, and GAL4-Smad2) or with 1 ng/ml TGF-β1 for 3 h (reporters p(BRE)_2, GAL4-Smad1, and ALK5/–1422/–65-Luc). Relative luciferase units were measured in a TD20/20 luminometer (Promega, Madison, WI). Samples were cotransfected with the SV40-β-galactosidase expression vector to correct for transfection efficiency. Measures of β-galactosidase activity were performed using Galacto-light (Tropix). Transfections were made in duplicate and repeated at least in three independent experiments. Representative experiments are shown in the figures.

3. Results

3.1. Culture and characterization of ECs from peripheral blood

EC cultures were established using peripheral blood from either control donors or HHT patients. On average, it took between 30–45 days to get a confluent pure endothelial culture of about 10^5 cells since plating the mononuclear layer. Distinguishable clusters of ECs appeared after 9–20 days, depending on the sample. Five independent control BOECs and a total of 11 HHT–BOEC cultures (from 8 different families/mutations) were obtained. ECs were characterized by morphology and staining with endothelial markers (endoglin and vWF), and actin cytoskeleton (Fig. 1). HHT–BOECs showed a significant number of binucleate cells, in contrast with the predominant mononuclear controls, supporting the reported involvement of endoglin and ALK1 in cell proliferation [12,16]. Cultured BOECs were classified into four groups: healthy controls, HHT1, HHT2/ALK1 missense mutants (HHT2m), and HHT2/ALK1 nonsense mutants (HHT2n). As representative of these groups, we will report here data corresponding to controls and families HHT1(1), HHT2(2) and HHT2(10) (Figs. 2 and 3).
Healthy BOECs had the characteristic endothelial cobblestone shape at confluence (Fig. 1A). Five different cultures of BOECs were derived from HHT1 patients. Two of them, belonging to father and son of family #1 where DNA sequencing revealed a nonsense mutation in c.511C>T leading to a stop codon (R171X) (Fig. 2). The other three belonged to families #4 and #23 (Fig. 1A), each one with a new ENG mutation (Fernandez-L et al., in preparation). The HHT1 cells were bigger and rather round-shaped (Fig. 1A) compared to controls. Six HHT2 BOECs were derived from 4 different families and mutations. Two families, HHT2(2) and HHT2(10) (Fig. 2), are representative for the two types of HHT2–BOECs so far analyzed. HHT2(2) harboured a missense mutation already described [24] in exon 8 of ALK1, c.1120C>T (R374W) (Fig. 2). Two independent cultures from two members of this family showed the same elongated morphology (Fig. 1B). A similar morphology was observed in BOECs derived from families #14 and #16 (1 and 3 independent cultures, respectively), harbouring new missense mutations in exon 7 and 8 of ALK1 (Fig. 2). HHT2(10) family showed the deletion c.434delG in exon 4, leading to a frameshift and premature stop codon in ALK1 (R145fs) (Fig. 2). These HHT2 BOECs are morphologically intermediate between control and HHT1 ones, showing a bigger and a rounder shape than controls (Fig. 1B). To this same type belong cells of the family #13 (Fig. 1B).

BOECs were characterized as mature ECs by positive staining with specific antibodies against endoglin, PECAM-1, VE-cadherin, ALK1, anti-vWF, and anti-Flk-1/KDR (Figs. 1 and 3), and negative staining with the endothelial precursor marker CD133 and the monocytic marker CD14 [30; data not shown]. Quantification of vWF and Flk-1 demonstrated that BOECs showed a similar levels of expression as HUVECs (Fig. 3B). As a control, primary cultures of human fibroblasts were negative for vWF, Flk-1, Flt-1, PECAM-1 and VE-cadherin, but positive for the fibroblast marker 1B10 (Fig. 3C).

3.2. Decreased endoglin levels in HHT endothelial cells

Western blot analysis showed decreased amounts of endoglin in HHT1–BOECs (Fig. 4A). Surprisingly, endo-
Endoglin was also significantly reduced in HHT2m–BOECs. In addition, ALK1 protein levels showed no major differences among controls, HHT1, and HHT2m, whereas a reduction in the ALK1 levels was observed in HHT2n (Fig. 4A). Fig. 4B summarizes endoglin and ALK1 expression levels from eleven different types of HHT–BOECs. HHT2m showed similar amounts of ALK1 (between 75% and 90%) than controls, suggesting that missense ALK1 mutants may be expressed. ALK1 levels were reduced in HHT2n, but were not affected in HHT1 families. Endoglin levels showed a discrete reduction in HHT2n–BOECs, while they were decreased below 50% in HHT1 and HHT2m ECs (Fig. 4B). The reduction levels of endoglin and ALK1 among HHT–BOECs were confirmed by flow cytometry analysis, whereas expression levels of Flt-1 were unaffected (Fig. 4C). These results differ from studies in HHT2 HUVECs [23,25] where no decreased endoglin levels were found.

This discrepancy may be explained by the HHT age-dependent penetrance [26], the age-dependent endoglin deficiency in HHT patients [27], and the different pathological situations represented by HUVECs (newborns 0 age) and BOECs (adult patients).

3.3. Involvement of ALK1 in endoglin gene expression of HHT2 cells

The down-regulated expression of endoglin in HHT2 patients suggests the involvement of ALK1 in endoglin expression. ALK1 is a type I receptor which participates in TGF-β signalling. Since endoglin expression is increased by TGF-β [33], mutations of ALK1 receptor in HHT2 patients, may lead to an improper endoglin regulation in ECs. To test this hypothesis, cells were cotransfected with the reporter pCD105(−450/+350), containing the proximal part of...
Fig. 2. Mutation analysis of HHT patients. Chromatograms show partial sequence of mutant ENG or ALK1 genes compared to the corresponding normal sequence. The DNA mutation and the stop codon generated at the corresponding protein are shown on the left boxes.

Fig. 3. Expression analysis of endothelial markers in BOECs from HHT patients. BOECs from HHT1 (family #1) and HHT2 (families #2 and #10) patients and from control donors were immunostained with the indicated endothelial specific antibodies and analyzed by fluorescence microscopy (A) or flow cytometry (B). HUVECs (upper row in B) and primary fibroblasts (C) were included as controls in flow cytometry analysis. The percentage of positive cells and the mean fluorescence intensity (in parenthesis) are indicated in the upper right corner of each flow cytometry histogram.
endoglin promoter, and expression vectors for different versions of ALK1 kinase.

As shown in Fig. 5A, transfection of control BOECs with ALK1 increased endoglin promoter activity, a constitutively active kinase form of ALK1 (Q201D) doubled the transcriptional activity of endoglin promoter, while a dominant negative version (ALK1, K229R) decreased by half the activity of endoglin promoter. Interestingly, an ALK1 variant, carrying the mutation of the HHT2 (2) family was able to inhibit 50% the activity of the endoglin promoter.

Fig. 4. Expression of endoglin and ALK1 in ECs derived from control and HHT patients. A. Western blot analysis. Total protein extracts from control (C), or HHT–BOECs were analyzed by Western blot with either anti-endoglin or anti-ALK1 antibodies. Loading controls included a coomassie blue stained band (lowest left panel), and the HLA class I antigen (lowest right panel). Arrows indicate the positions of endoglin, ALK1 and molecular size markers. HHT1 stands for HHT1(1), HHT2m for HHT2(2), and HHT2n for HHT2 (10) families. B. Expression levels of endoglin and ALK1 in eleven HHT–BOECs. Protein extracts from control (n = 5), HHT1 (n = 5), HHT2m (n = 5), or HHT2n (n = 1) cultures were analyzed by Western blot as in panel A. Measures of densitometry of each endoglin or ALK1 band relative to loading controls were performed and relative values are represented. n represents the number of independent primary cultures from different families analyzed in each case. HHT2n histogram represents the mean of three different extracts. (*p < 0.05; Student’s “t” test). C. Flow cytometry analysis. BOECs were subjected to flow cytometry analysis of endoglin, ALK1 and Flt-1. Levels of antigens are represented as relative expression index (percentage of positive cells x fluorescence intensity). Absolute expression indexes in control BOECs were 1500, 120, and 34 for endoglin, ALK1, and Flt-1, respectively. HHT1 stands for HHT1(1), HHT2m for HHT2(2), and HHT2n for HHT2 (10) families. (*p < 0.05; Student’s “t” test).
TGF-β induced a significant increase of the endoglin promoter activity that was abolished upon ectopic expression of the ALK1 mutants. To exclude a possible interference of endogenous ALK1, transfections were also performed in the non-endothelial cell line 293 T, leading to similar results (Fig. 5 B). In this experiment, treatment with TGF-β further increased the stimulating effect of ALK1-WT over the endoglin promoter. These results suggest that ALK1 mutations in HHT2 may interfere with endoglin gene expression.

3.4. TGF-β signalling pathways in HHT endothelial cells

As endoglin expression is TGF-β-inducible, the response to this factor was analyzed in HHT–BOECs. Control ECs displayed a 2.5-fold induction of cell surface endoglin, while HHT1, HHT2(10) and HHT2(2) cells showed a marked reduction in endoglin induction levels (Fig. 6A), suggesting that HHT–BOECs have a
deficient TGF-β signalling. ECs coexpress two different TGF-β type-I receptors, the ubiquitous ALK5, and ALK1, which is more specific for ECs [7,11,16,32]. Since endoglin cooperates with TGF-β/ALK1 pathway, but interferes with TGF-β/ALK5 route [12,13], these pathways were studied in HHT BOECs. The reporters (CAGA)_{12}-Luc and (BRE)_{2}-Luc were used to monitor ALK5 and ALK1 dependent signalling (Fig. 6B). In addition, since ALK1 and ALK5 signal via Smad1/5 and Smad2/3, respectively, GAL4 fusion constructs of Smad1, Smad2 and Smad3 were used to assess TGF-β/receptor I (ALK1 or ALK5)-dependent signalling in these cells (Fig. 6C).

As shown in Fig. 6, the reporter activities of (CAGA)_{12}-Luc and (BRE)_{2}-Luc were stimulated from 4-to 5-fold upon TGF-β treatment in control BOECs. Similarly, GAL4-Smad1, GAL4-Smad2 and GAL4-Smad3 were transactivated in response to TGF-β, from 3-fold in GAL4-Smad1, to 7–8-fold in Gal4-Smad2 and GAL4-Smad3. Thus, both ALK1/TGF-β and ALK5/TGF-β pathways are active in ECs from normal donors. By contrast, TGF-β pathways were seriously affected in HHT–BOECs. As expected from the endoglin/ALK1 cooperation [12,13], the TGF-β response of the ALK1-dependent reporters was impaired in HHT cells. Thus, the transactivation activity of GAL4-Smad1 and the (BRE)_{2}-Luc reporter activity in the presence of TGF-β were severely reduced (from 4- to 1.5-fold) in HHT1- and HHT2–BOECs (Fig. 6). Surprisingly, the ALK5-dependent TGF-β induction levels of (CAGA)_{12}-Luc reporter or GAL4-Smad3 transactivation were significantly reduced in HHT1- and HHT2–BOECs (Fig. 6). Altogether the results show that the responses of HHT–BOECs to TGF-β are severely compromised.

![Fig. 7. ALK5 expression is downregulated in HHT–BOECs. A and B. Semiquantitative RT-PCR of ALK5 (A) or TβRII (B) versus GAPDH in control and HHT BOECs. RNA from control, HHT1(1), HHT2(2) and HHT2(10) BOECs, was retrotranscribed and the resulting cDNA was amplified using specific primers for ALK5, TβRII and GAPDH for 25 cycles. Bands were quantified by densitometry as shown in the histograms. Representative experiments out of three independent sets of experiments are shown. C and D. Effect of ALK1 and endoglin on ALK5 promoter activity. Control BOECs (C) or 293 T cells (D) were cotransfected with a luciferase reporter for ALK5 promoter, and expression vectors for wild type ALK1, constitutively active ALK1 (Q201D), dominant negative ALK1 mutant (K229R), endoglin (ENG), or empty vector (pcDNA3), as indicated. After transfection, cells were treated or not with TGF-β1, and the luciferase activity was normalized by β-galactosidase measures.](https://academic.oup.com/cardiovascres/article-abstract/68/2/235/299131)
Fig. 8. Analysis of actin cytoskeleton and formation of cord-like structures in HHT–BOECs. A. Control, HHT1 and HHT2(2) BOECs were transiently co-transfected with pSUPER-Endo-Ex4, pCMV5-Endoglin and pEGFP [34], as indicated. Cells were stained with phalloidin to visualize the F-actin cytoskeleton (red) and transfected cells were identified by the green fluorescence of EGFP. B. Control BOECs were transiently transfected with pSUPER-Endo-Ex4, pSUPER-C (control vector) and pEGFP [34], as indicated. Cells were stained with anti-endoglin or phalloidin (red) and transfected cells were identified by the green fluorescence of EGFP. C. Formation of cord-like structures on matrigel. BOECs and HUVECs were analyzed for their capacity to form tubes on matrigel at 37 °C for different times of incubation. Photographs corresponding to 20 h or 5 days are shown.
3.5. ALK5 expression is reduced in HHT ECs and regulated by ALK1 and endoglin

Since the ALK5 pathway is disrupted in HHT–BOECs and a previous report [12] have shown a significant reduction in the levels of ALK5 in eng null mouse embryonic ECs, we hypothesized that ALK5 might be downregulated as a cell adaptation to compensate for decreased endoglin/ALK1 expression. Therefore, we examined ALK5 expression by RT-PCR. Fig. 7A shows a substantial decrease (80%) of ALK5 expression in endoglin mutant HHT1 compared to control BOECs. Similarly, a reduction of ALK5 was observed when ALK1 was mutated in families HHT2(2) and HHT2(10). These results are ALK5 specific and are not due to a general decrease of the TGF-β signalling components since the TβRII transcript levels were not affected (Fig. 7B).

Next, we explored the mechanism leading to the downregulation of ALK5 expression in HHT–BOECs. The possibility of a transcriptional regulatory crosstalk between ALK1/endoglin and ALK5 pathways was assessed by transient cotransfections of control BOECs with an ALK5 promoter construct and different expression vectors for ALK1 and endoglin. As shown in Fig. 7C, cotransfection of wild type ALK1, constitutively active ALK1 (Q201D), or wild type endoglin, stimulate the ALK5 promoter activity by 3-, 5-, or 5.2-fold, respectively. By contrast, a dominant negative ALK1 (K229R) decreased ALK5 promoter activity almost to basal levels, even after TGF-β treatment. Moreover, TGF-β significantly enhanced the transactivation activity of cells transfected with wild type ALK1 or endoglin. Similar results were obtained with the non-endothelial cell line 293 T (Fig. 7D).

Altogether, these results suggest that ECs keep a fine tuning between ALK1/endoglin and ALK5 levels, and that mutations in either endoglin (HHT1), or ALK1 (HHT2) downregulate ALK5 gene expression to maintain a physiologically adaptative balance between ALK1 and ALK5 routes.

3.6. HHT BOECs show changes in cytoskeleton and tube formation

The abnormal shape of HHT–BOECs compared to controls (Figs. 1 and 3), suggests that actin cytoskeleton may be affected. While control ECs showed a highly organized cytoskeleton with stress fibers crossing the entire cell, HHT1- and HHT2–BOECs had a disorganized actin structure (Fig. 1). Most of the F-actin was disorganized and depolymerized at many points giving rise to very intrincated patterns with different foci of actin polymerization in HHT1–BOECs. HHT2(2) BOECs also showed a poor organization of the actin cytoskeleton, with extense areas of depolymerization. HHT2(10) BOECs showed a combined situation: some cells displayed a completely normal F-actin cytoskeleton, while others had patches of F-actin depolymerization. Since HHT1 and HHT2 BOECs have in common a significant decrease in the amount of endoglin, these results are compatible with the reported role of endoglin in the organization of actin cytoskeleton via its interaction with the zyxin family of proteins [34]. Supporting this view, endoglin suppression by siRNA in control BOECs leads to a disruption of the actin cytoskeleton (Fig. 8A). Conversely, overexpression of endoglin in HHT1 BOECs significantly restored the actin network. However, overexpression of endoglin in HHT2 BOECs resulted only in a slight recovery, visualized by some stress fibres, suggesting that endoglin expression was not sufficient to reverse the adaptation process induced by ALK1 mutations. As expected, control BOECs transfected with pSUPER-Endo-Ex4 showed a decreased expression of endoglin compared to untransfected cells or to cells transfected with an irrelevant vector (pSUPER-C); also, transfection with pSUPER-C did not affect endoglin F-actin cytoskeleton (Fig. 8B).

The TGF-β/ALK1/endoglin pathway induces proliferation, migration and tube formation of ECs [12,16]. To gain more insight into possible functional problems of HHT–BOECs, we investigated their behaviour when building up cord-like structures in vitro. HHT1 and HHT2 cells were plated on matrigel, using normal donor BOECs and HUVECs as controls (Fig. 8C). While normal donor HUVECs and BOECs formed robust cord-like structures in less than 24 h, HHT–BOECs displayed deficient tube formation, but the pattern varied depending on the type of mutation. HHT1–BOECs did not give rise to clear cord-like structures and only after 5 days, a few short threads were visible. HHT2n cells, represented by family 10, formed a weak and thin tube-network after 20 h, but involving much less surface than control BOECs. In the case of HHT2m, represented by family 2, no tubes were formed, but instead big clusters of cells showing visible sprouting were quite abundant and, occasionally, were interconnected by short tubes after long time of incubation. Similar results were obtained with BOECs from different members of HHT2m families #14 and #16 (data not shown). Interestingly, when comparing HHT2(2) vs. HHT2(10), the lower capacity to form tubes was associated with a lower endoglin expression levels suggesting that endoglin is involved in tube formation. As endoglin and ALK1 collaborate in the TGF-β/ALK1/endoglin pathway leading to the formation of cord-like structures, it could be hypothesized that levels of endoglin or ALK1 activity below a critical threshold might hamper the tube formation in HHT–BOECs.

4. Discussion

This study represents the first molecular characterization of ECs from adult HHT patients, primary targets of the vascular disorder. The development of pure EC cultures from patients with clinical symptoms is a direct attempt to understand the status of HHT endothelial function. We have characterized and studied 5 different cultures from healthy...
donors and 11 different cultures from HHT patients. HHT samples belonged to three types of mutations: ENG nonsense (5 independent cultures), ALK1 nonsense (one culture), and ALK1 missense (5 independent cultures). Common features to these HHT cells were positive staining for endothelial markers, endoglin deficiency, reduced ALK5 expression and TGF-β signalling, disorganization of the F-actin cytoskeleton, and abnormal formation of cord-like structures.

As expected, HHT1–BOECs were endoglin haploinsufficient but, surprisingly, HHT2m and HHT2n cells were also endoglin-deficient. This is in agreement with the upregulation of endoglin observed upon ALK1 overexpression [35]. In addition, we have previously reported endoglin deficient upregulation in activated monocytes of HHT1 patients [27], suggesting that ALK1 may modulate endoglin promoter activity during the monocyte–macrophage transition. The present work not only confirms this role for ALK1, but also demonstrates that ALK1 mutations have an impact on the endoglin promoter activity when transfected in control BOECs (Fig. 5).

HHT–BOECs showed impaired TGF-β signalling. The decreased TGF-β/ALK1 pathway could be predicted for HHT1 and HHT2 cells since endoglin cooperates with ALK1 in ECs [12,13]. On the other hand, HHT1 and HHT2– BOECs exhibited a downregulation of TGF-β/ALK5 signalling. In the case of HHT1, the ALK5 downregulated activities agree with the decreased expression of ALK5 observed in eng+/eng− mouse embryonic ECs [12] and in HHT–BOECs (Fig. 7). Since ALK1/endoglin is an antagonistic mediator of ALK5 signalling [32], Lebrin et al. postulate that this adaptation occurs in order for ECs to survive to the potentiation of ALK5-induced growth arrest that would otherwise occur [12]. Accordingly, this adaptation could occur in HHT2–BOECs, where ALK5 signalling is also downregulated (Fig. 6). The molecular basis for this adaptation is likely achieved through a transcriptional regulation of ALK5 promoter by ALK1 and endoglin, as shown in HHT–BOECs (Fig. 7).

The common clinical features for HHT1 and HHT2 may lie in the similar anomalies observed for the HHT cells compared to control BOECs, as hypothesized in Fig. 9. A shared consequence of mutations in ALK1 or ENG, is endoglin deficiency, as shown in this work. This raises the interesting possibility that endoglin deficiency below a critical threshold is the ultimate pathogenic trigger in HHT1 and HHT2. Supporting this view, endoglin and ALK1 are components of the same TGF-β receptor complex, and despite being structurally and functionally different, they cooperate in the TGF-β/ALK1 endothelial pathway. As in ECs, both ALK1 and ALK5-dependent TGF-β signalling pathways are active, an adequate balance must be attained among them. Since ALK1 signalling promotes EC proliferation and migration, and ALK5 inhibits proliferation, migration and induces extracellular matrix synthesis, both pathways must be tuned within ECs to coordinate the different types of responses adequate for each physiological

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**Fig. 9. Hypothetical model for HHT.** Schematic model representing TGF-β receptor complex in healthy (Non-HHT) compared to the situation in HHT1 and HHT2 endothelial cells. Under a normal situation (Non-HHT), ALK1 and endoglin are cooperating in the TGF-β/Smad pathway, and the endoglin levels are maintained to meet the physiological needs of the EC. However, in HHT1 and HHT2 cells, either endoglin or ALK1, fail as partners in the cooperative signalling. As a result, endoglin expression decreases below a critical threshold which leads to impaired TGF-β signalling, and abnormal cytoskeleton and tube formation in HHT ECs. These altered endothelial functions may explain the HHT phenotype.
situation. Since ALK1 pathway is decreased in HHT–BOECs, a correcting mechanism to downregulate ALK5 levels is probably induced to avoid the ALK5-dependent inhibition of cell proliferation. This regulation seems to be based on direct signals from ALK1 and endoglin to the ALK5 promoter. Further experiments in this line of investigation may help to elucidate the complicated network of regulatory interactions which ultimately lead to a fine tuning among the signalling components in the TGF-β pathway. In HHT, this tuning requires a physiological adaptation of ECs presumably reached during the differentiation from precursor to mature EC.

A direct consequence of ALK1/TGF-β deficient signalling in HHT–BOECs is the decreased capacity to form cord-like structures during angiogenesis [12], which may affect the organization of the capillary network. In addition, the abnormal actin cytoskeleton of HHT–BOECs may also be related to endoglin decreased levels. In fact, endoglin cytoplasmic domain interacts with ZRP1, present at the points of actin polymerization [34]. Accordingly, the decrease in endoglin levels would disrupt the actin polymerization sites. A disorganized cytoskeleton is prone to cell breaking with changes in shear stress and blood pressure. This might lead to vessel haemorrhages and eventual disappearance of the capillary network, as reported for the HHT vascular disorder. Finally, although our data suggest that BOECs constitute a novel and interesting cellular model to study the basis of HHT, it will be important to confirm these findings in mature vessel ECs from HHT patients.

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