Patients with hereditary hemorrhagic telangiectasia have increased plasma levels of vascular endothelial growth factor and transforming growth factor-β1 as well as high ALK1 tissue expression

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Background and Objectives. Hereditary hemorrhagic telangiectasia (HHT), an inherited vascular dysplasia, is caused by mutations in endoglin or activin receptor-like kinase (ALK)-1. Haploinsufficiency for these genes is thought to result in an imbalanced angiogenic activity. The aim of this study was to evaluate the plasma levels and the expression profiles of angiogenic and angiogenesis-related factors in the context of HHT.

Design and Methods. Vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 plasma concentrations were determined in 31 HHT patients and 40 healthy controls by ELISA. VEGF and TGF-β1 plasma concentrations were correlated with the patients' clinicopathological features. Tissue expression of angiogenic and angiogenesis related proteins was determined by immunostaining on nasal cryostat sections from 13 HHT patients and 5 healthy controls.

Results. Of the 31 patients, 29 had statistically significantly raised plasma concentrations of VEGF and TGF-β1 but there was no correlation with specific clinicopathological features. Increased VEGF, TGF-β1 and ALK1 immunostaining was seen in all 13 investigated patients. β-smooth muscle actinin immunostaining was increased in 12 patients. Increased endoglin immunostaining was seen in only 9 patients.

Conclusions and Interpretations. This study provides evidence of the role of VEGF and TGF-β1 in the pathogenesis of HHT. Plasma concentrations of these two factors may serve as further diagnostic criteria for HHT. For the first time, we report increased TGF-β1 plasma concentrations and increased TGF-β1 and ALK1 tissue expression in HHT, which appear not to be specifically associated with either endoglin or ALK1 mutations. The data suggest that HHT is an angiogenic disorder characterized by an over-expression of VEGF, TGF-β1 and ALK1

Key words: hereditary hemorrhagic telangiectasia (HHT), angiogenesis, VEGF, TGF-β1, ALK1.

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Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Rendu-Weber syndrome, is an autosomal-dominant, vascular disorder with a disease prevalence of approximately 1 case in 8,000 individuals.[1] This multisystemic angiogenic disorder is clinically characterized by cutaneous and muco-cutaneous telangiectases, severe and recurrent epistaxis and gastrointestinal hemorrhages caused by telangiectases of the nose and upper gastrointestinal tract. Patients also have arteriovenous shunts, most commonly in the lung (pulmonary arteriovenous malformation), brain and liver.[2-5] Electron microscopy of lesion biopsies revealed that dilated post-capillary venules connect directly to arterioles without an intervening capillary bed.[6] HHT is a heterogeneous disorder, both genetically and phenotypically. Genetic linkage studies mapped two loci and the genes involved in HHT have been identified as endoglin (HHT type 1) on chromosome 9q33-34, and activin receptor-like kinase 1 (*ALK1*) (HHT type 2) on chromosome 12q13, Haploinsufficiency for either endoglin or *ALK1* appears to be sufficient to cause HHT. Both genes are involved in the transforming growth factor (TGF)-β signaling pathway and expression of these genes is mainly restricted to endothelial and vascular smooth muscle cells. *Endoglin* and *ALK1* knock-out mice die between embryonic days 9 and 11 due to vascular defects,[9-13] demonstrating the importance of these proteins in angiogenesis. Recently, it was reported that mutations in the *SMAD4* gene are associated with an overlapping phenotype of HHT and juvenile polyposis.[14] *TGF-β1* is a multifunctional cytokine that belongs to the TGF-β superfamily. Members of this family play a pivotal role in cellular proliferation, differentiation, migration, and extracellular matrix production and deposition.[15,16] TGF-β family members initiate their activities on the cell by binding to a heteromeric complex of type I and type II serine/threonine kinase receptors at the cell surface. Binding of the ligand to the type II receptor recruits the type I receptor that

becomes phosphorylated and itself phosphorylates members of the Smad protein family. Once Smad proteins have been activated they are translocated into the nucleus and regulate gene expression as transcription factors. The signaling receptors for TGF-β1 are the type II receptor TβRII and the type I receptors ALK5/TβRI and ALK1.[17,18] Angiogenesis is the development of new capillary blood vessels by sprouting from pre-existing vessels and occurs in a variety of normal and pathological processes. It is a complex activity involving degradation of the capillary basement membrane, migration and proliferation of endothelial cells, and eventual formation of tubes.[19,20] The formation of new capillaries is induced and/or controlled by different cytokines such as vascular endothelial growth factor (VEGF) and TGF-β1. VEGF is a 32-45 kDa multifunctional cytokine that acts preferentially on vascular endothelium. It is produced by a number of different cell types such as endothelium, and monocytes. *In vitro*, TGF-β1 has been shown to be an extremely potent stimulator for the production of VEGF.[21] One of the main functions of VEGF is to induce mitotic activity in endothelial cells, and thus commencement of capillary sprouting, playing a key role in the angiogenic process. Four different isoforms of VEGF have been identified and are distinguished by their molecular weights: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. The larger forms, $VEGF_{189}$ and $VEGF_{206}$, are primarily responsible for vascular integrity and are tethered to the cell surface, whereas the smaller forms, $VEGF_{121}$ and $VEGF_{165}$, are soluble proteins.[22] $VEGF_{165}$ is the predominant isoform for induction of angiogeneses.[22,23] Several studies have focused on the functions of VEGF in a variety of pathologies such as tumor growth, metastasis, psoriasis, rheumatoid arthritis, retinopathies and vascular diseases, including ischemic heart disease and atherosclerosis.[24-27]

Reports on the angiogenic effect of TGF-β are conflicting. TGF-β1 inhibits endothelial cell replication and migration in cell culture and also reduces extracellular proteolysis *in vitro*.[28] In addition, it was reported that TGF-β1 promotes the formation of tube-like structures in collagen gels,[29,30] while other studies demonstrated that TGF-β1 inhibits endothelial cell invasion and tube formation.[31-33] Subsequently, it was shown that TGF-β1 has a biphasic effect on angiogenesis depending on its concentration.[34] At high concentrations TGF-β1 inhibits the VEGF-induced endothelial cell invasion and capillary lumen formation, whereas lower TGF-β1 concentrations potentiate VEGF activity. This biphasic effect is probably due to the two different TGF-β1 type I receptors, ALK1 and ALK5. Signaling at low TGF-β1 concentrations is mediated by ALK1 and at high TGF-β1 concentrations by ALK5.[18]

The aim of this study was to evaluate the plasma levels and the expression profiles of angiogenic factors in the context of HHT, as the pathogenesis of HHT is thought to result from an imbalance in the process of angiogenesis. It has been reported recently that the serum levels of VEGF are significantly higher in HHT patients than in healthy controls[35-37] and increased VEGF tissue expression has been found in a limited number of patients.[36,37] The fact that TGF-β1 induces VEGF expression led us to investigate whether TGF-β1 expression is also dysregulated in patients with HHT. As one of the major clinical manifestations of HHT is frequent epistaxis, nasal tissue samples from HHT patients and healthy controls were obtained to determine the expression profiles of TGF-β1, ALK1, endoglin, matrix metalloproteinase (MMP)-2, α-smooth muscle actinin (α-sma), and the VEGF receptors FMS-like tyrosine kinase (Flt)-1 and kinase insert domain receptor (KDR) by immunohistochemical staining. These results are presented in context with our previous VEGF data.[36,37]

Design and Methods

Patients and materials

Between May 2002 and April 2003, 31 patients (20 females, 11 males) with clinical features of HHT were enrolled in the study at the Department of Otolaryngology, Head and Neck Surgery, University Hospital of Mannheim, Germany. Patients were considered to be affected by HHT if they had at least three out of the four cardinal symptoms – epistaxis, telangiectasia, family history of the disorder and organ involvement – according to the current Curaçao criteria.[36] All patients were screened for asymptomatic organ involvement. The diagnostic screening examinations included magnetic resonance imaging of the brain, computerized tomography (CT) of the lungs and contrast echocardiography, to detect arteriovenous shunts in the pulmonary circulation, esophago-gastroduodenoscopy and colonoscopy as well as an ultrasound examination of the liver. Forty volunteers without any history of known neoplasms, without recent trauma or surgery, and who were not pregnant served as healthy controls. All studies were approved by the Ethics Committee of the Faculty for Clinical Medicine Mannheim, University of Heidelberg, Germany. Informed consent was obtained from all subjects prior to the study.

ELISA assay for VEGF and TGF-β1

Peripheral venous blood samples from the 31 HHT patients and 40 healthy controls were collected into sterile tubes containing heparin and immediately centrifuged at 2000 g for 10 minutes. The plasma was stored at -70°C until assayed collectively for VEGF and TGF-β1 levels by an investigator who was blind to the disease status of the participants. The VEGF and TGF-β1 plasma concentrations were determined using a

Human VEGF Quantikine Colorimetric Sandwich ELISA (Catalogue #DVE00) and a Human TGF-β1 Quantikine Colorimetric Sandwich ELISA (Catalogue # DB100), both from R&D System (Wiesbaden, Germany) according to the manufacturer´s instructions. The minimum detection sensitivity of the VEGF ELISA is 9 pg/mL and the minimum detection sensitivity of the TGF-β1 ELISA is <7 pg/mL. All analyses and calibrations were carried out in duplicate. The TGF-β1 ELISA used can only detect the active form of TGF-β1. Therefore, to determine the total amount of plasma TGF-β1, the plasma samples were acidified, according to the manufacturer's instruction, a process which converts the latent TGF-β1 form into the activated form. VEGF and TGF-β1 plasma concentrations were determined by researchers without knowledge of the main clinicopathological features of the patients studied.

Tissue expression analysis of VEGF, ALK1, endoglin, MMP-2, α-sma, Flt-1 and KDR by immunohistochemistry

Tissue samples of the nasal mucosa of 13 HHT patients and 5 healthy controls were collected and snap frozen in liquid nitrogen then stored at -70°C. The frozen tissue samples were cut in 6 μm sections and fixed in acetone. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide in methanol for 10 minutes at room temperature. The sections were then incubated separately in 10% normal sheep serum in phosphate-buffered saline (PBS) solution for 30 minutes, followed by incubation overnight at 4°C with either rabbit anti-human VEGF polyclonal antibody (1:50) (Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-human TGF-β1 monoclonal antibody (1:50) (R&D, Wiesbaden, Germany), rabbit anti-human ALK1 polyclonal antibody (1:200),[17] rabbit anti-human endoglin polyclonal antibody (1:200),[37] goat anti-human MMP-2 polyclonal antibody (1:50) (Santa Cruz Biotechnology), mouse anti-human α-sma monoclonal antibody (1:50) (Novostada, Dossenheim, Germany), rabbit anti-human Flt-1 polyclonal antibody (1:20) (Santa Cruz Biotechnology) or mouse anti-human KDR monoclonal antibody (1:10) (Santa Cruz Biotechnology).

Next, each slide was treated with the appropiate biotinylated secondary antibody for 10 minutes, and then incubated with streptavidin-peroxidase complex for 45 minutes. Aminoethylnorbazole (AEC) was used as a chromogen, and nuclear counterstaining was performed with Mayer´s hematoxylin solution.

Negative controls were performed using PBS instead of primary antibody, resulting in no detectable staining. Immunohistochemical sections were analyzed by light microscopy (Zeiss Axiophot) semi-quantitatively and assessed independently by two investigators who were unaware of the clinicopathological features of the patients studied.

Table 1. Clinical characteristics and VEGF and TGF-β1 levels in the 31 patients with HHT.

Patients	*Sex*	*Age (years)*	*Epistaxis (Grade 1-3)*	*Organ manifestation*	*VEGF Plasma levels (pg/mL)*	*TGF-β1 Plasma levels (pg/mL)*	*IHC*
#01	f	96	3	GI-tract, liver, lung, brain	336	56	
#02	f	64	3	none identified	171	42	
#03	f	82	3	none identified	359	58	X
#04	f	76	3	none identified	331	56	
#05	f	40	3	none identified	443	59	X
#06	m	66	3	GI-tract	156	48	X
#07	f	32	3	GI-tract	173	38	
#08	f	67	3	none identified	149	64	
#09	f	68	3	none identified	120	43	
#10	f	70	3	GI-tract, liver	120	58	X
#11	m	53	3	none identified	32	55	X
#12	m	55	3	GI-tract, liver	95	37	X
#13	m	63	3	GI-tract	41	7	
#14	f	71	3	none identified	308	44	X
#15	f	32	3	liver	186	60	
#16	f	79	3	GI-tract, lung	249	57	X
#17	f	62	3	GI-tract, lung, liver	58	34	X
#18	m	67	3	none identified	103	42	
#19	f	65	3	liver, brain	666	62	
#20	f	63	3	liver	715	76	
#21	m	56	3	none identified	216	72	X
#22	m	66	3	none identified	363	29	X
#23	f	78	3	GI-tract, liver	578	67	X
#24	m	46	3	none identified	378	70	
#25	m	79	3	none identified	1674	91	
#26	f	38	3	GI-tract	363	49	X
#27	f	47	3	GI-tract, liver	1116	60	
#28	f	67	3	none identified	101	27	
#29	m	38	3	none identified	88	7	
#30	f	55	3	GI-tract, liver, lung	293	60	
#31	m	74	3	none identified	279	18	

Grade 1: stains on handkerchief; Grade 2: blood-soaked handkerchief; Grade 3: bowl or similar utensil necessary; y: years; X: performed; f: female; m: male. Epistaxis graded according to Bergler et al.[38]

Statistical analysis

Data are expressed as means and standard deviations. For statistical analyses, VEGF and TGF-β1 plasma levels in HHT patients and healthy controls, as well as in correlation to the clinicopathological features of the patients were compared using the two-samples t-test. The ANOVA test was used to correlate the plasma levels of VEGF and TGF-β1 with the tissue expression of VEGF and TGF-β1, respectively. p values <0.05 were considered statistically significant. The SAS program (SAS for Windows, Version 8.2) was used for all statistical analyses.

Results

HHT patients

Thirty-one patients with clinical features of HHT and a positive family history were enrolled in this study. These patients are part of a larger previously published cohort.[36,37] Twenty patients were female and 11 were male, the mean age of the cohort was 62 years (range, 32 years to 96 years). The mean age of the control group was 57 years with a range from 19 years to 87 years. All

Table 2. Clinical findings and VEGF plasma levels in 31 HHT patients.

Patients	(n)	VEGF plasma levels (pg/mL) Mean±SD	Median	p value
Healthy controls	40	20±30	14	$p<0.0001$
HHT patients	31	331±335	249	
Organ manifestation				
without	16	320±370	247	$p=0.859$
with	15	344±292	249	
GI-tract	12	298±299	211	
Liver	10	416±326	315	
Lung	4	234±106	271	
Brain	2	501±165	501	

Table 3. Clinical findings and TGF-β1 plasma levels in 31 HHT patients.

Patients	(n)	TGF-β1 plasma levels (ng/mL) Mean ± SD	Median	p value
Healthy controls	40	6±3	4	$p< 0.0001$
HHT patients	31	50±19	56	
Organ manifestation				
without	16	49±22	50	$p=0.69$
with	15	51±17	57	
GI-tract	12	48±16	53	
Liver	10	57±13	60	
Lung	4	52±12	57	
Brain	2	59±5	59	

HHT patients had visible telangiectasias on the skin and showed symptoms of severe epistaxis (grade 3).[38] According to the diagnostic screening examinations, 16 patients did not have any visceral organ involvement. Two patients had hepatic arteriovenous malformations only and one patient had both hepatic and cerebral arteriovenous malformations. In 4 cases only the gastrointestinal tract was affected. Another eight patients showed HHT manifestations not only in the gastrointestinal tract but also in other organs (Table 1).

HHT patients have increased levels of plasma VEGF

An ELISA assay was performed to determine the VEGF plasma levels in HHT patients and healthy controls. The mean VEGF plasma concentration in HHT patients was higher than that in the healthy control group ($p<0.0001$). The VEGF levels of the 31 HHT patients ranged from 32 pg/mL to 1674 pg/mL with a mean value of 331 pg/mL (median 249 pg/mL). In contrast, the VEGF plasma levels in the healthy control group ranged from below the detection limit to 168 pg/mL (mean 20 pg/mL, median 14 pg/mL). Table 2 shows the mean VEGF plasma concentrations with the standard deviation. The VEGF plasma levels of the HHT patients were subsequently correlated to the patients' clinicopathological findings (Table 2). Statistically no significant correlation could be seen between the elevated VEGF plasma concentrations and any of the HHT-phenotypes, i.e. specific organ manifestations ($p=0.8592$, t-test).

HHT patients have increased levels of plasma TGF-β1

The total TGF-β1 plasma concentrations were determined in HHT patients and healthy controls by an ELISA assay. The design of the TGF-β1 ELISA does not distinguish between the biologically inactive and active TGF-β1 forms. In HHT patients, the plasma levels of TGF-β1 ranged from 7 ng/mL to 91 ng/mL with a mean value of 50 ng/mL (median 56 ng/mL). In contrast, the levels of TGF-β1 in plasma samples from the healthy controls ranged from 2 ng/mL to 14 ng/mL with a mean value of 6 ng/mL (median 4 ng/mL) (Table 3).

Overall, the TGF-β1 plasma concentrations were significantly higher in HHT patients than in healthy controls ($p<0.0001$). The TGF-β1 plasma levels of the HHT patients were compared to the patients' clinicopathological findings. Again, as with the VEGF plasma levels, no significant correlation ($p=0.69$, t-test) was observed between TGF-β1 plasma levels and specific clinical features (Table 3).

Increased VEGF and TGF-β1 plasma concentrations in 28 HHT patients

The VEGF and TGF-β1 plasma concentrations were compared in each of the 31 HHT patients. The mean plasma levels plus the standard deviations of the healthy controls for VEGF (50 pg/mL) and TGF-β1 (9 ng/mL) of the healthy controls were used to determine baseline VEGF and TGF-β1 plasma levels. One HHT patient had high TGF-β1 but low VEGF levels, whereas another HHT patient had high VEGF but low TGF-β1 levels. In another HHT patient, plasma levels of both VEGF and TGF-β1 were low. In the remaining 28 HHT patients, plasma levels of VEGF and TGF-β1 were above the levels of those in the control group. Half of these HHT patients did not have any organ manifestations of HHT. Five patients had only involvement of the liver and 9 patients showed HHT manifestations in more than one organ. Statistical correlations of the increased VEGF plasma levels with the increased TGF-β1 levels were not significant (Fisher´s exact test, $p> 0.05$) due to the limited number of patients for this calculation. Nevertheless, HHT patients clearly showed a trend towards elevated VEGF as well as TGF-β1 plasma levels.

High VEGF and TGF-β1 plasma levels correlate with strong VEGF and TGF-β1 tissue expression in HHT patients

Tissue samples from the nasal mucosa of 13 HHT patients and 5 healthy controls were immunostained.

Tissue samples from all HHT patients showed stronger VEGF expression than did samples from healthy controls. VEGF staining in HHT tissue sections was mainly detected in the cell layers around the vessels, presumably endothelial and/or smooth muscle cells, and in cells of the stroma. The immunoreactivity grading in HHT patients showed seven sections with medium staining, classified as $^{++}$ (54%) and six sections with strong staining, classified as $^{+++}$ (46%). In the control group, all sections revealed weak staining, graded as $^{+}$. Representative photomicrographs of VEGF staining are shown in Figure 1.

The VEGF plasma concentrations were then compared to the degree of VEGF staining in HHT patients and healthy controls. Two HHT patients had normal VEGF plasma concentrations (< 50 pg/mL) but showed medium VEGF staining ($^{++}$). Out of six patients with high VEGF plasma concentrations (between 51 pg/mL and 331 pg/mL), four had medium VEGF staining ($^{++}$) and two had a strong staining ($^{+++}$). Of five HHT patients with very high VEGF plasma levels (> 331 pg/mL) four showed strong VEGF expression ($^{+++}$) whereas one patient had medium expression ($^{++}$). In general, increased VEGF plasma levels could be correlated with strong VEGF tissue expression in HHT patients (Figure 2; ANOVA test, $p<0.05$). The healthy controls not only had lower VEGF plasma levels, but also showed weak VEGF tissue staining.

TGF-β1 immunostaining of the vessels of the control group was negative, graded $^{-}$ (Figure 3A). In contrast, all 13 HHT patients showed positive staining of a single cell layer lining the vessel lumina, presumably the endothelial cells (Figures 3B and 3C). No staining was observed in the stroma or of cells adjacent to the endothelial cells, either in patients or in controls. Four patients were graded as having $^{+}$ and 9 patients as having $^{++}$ immunostaining. Unlike for VEGF, it appears that TGF-β1 staining is restricted to the endothelial cells. However, we saw a strong staining of gland cells, which served as an internal positive control of the staining procedure, but there was no difference between controls and patients (Figures 3A and 3B). This suggests that the observed increased TGF-β1 expression in the endothelial cells is HHT-related and might also be the source of increased TGF-β1 plasma levels. As for VEGF, increased TGF-β1 plasma levels could be correlated in HHT patients with increased TGF-β1 tissue staining (ANOVA test, $p< 0.05$).

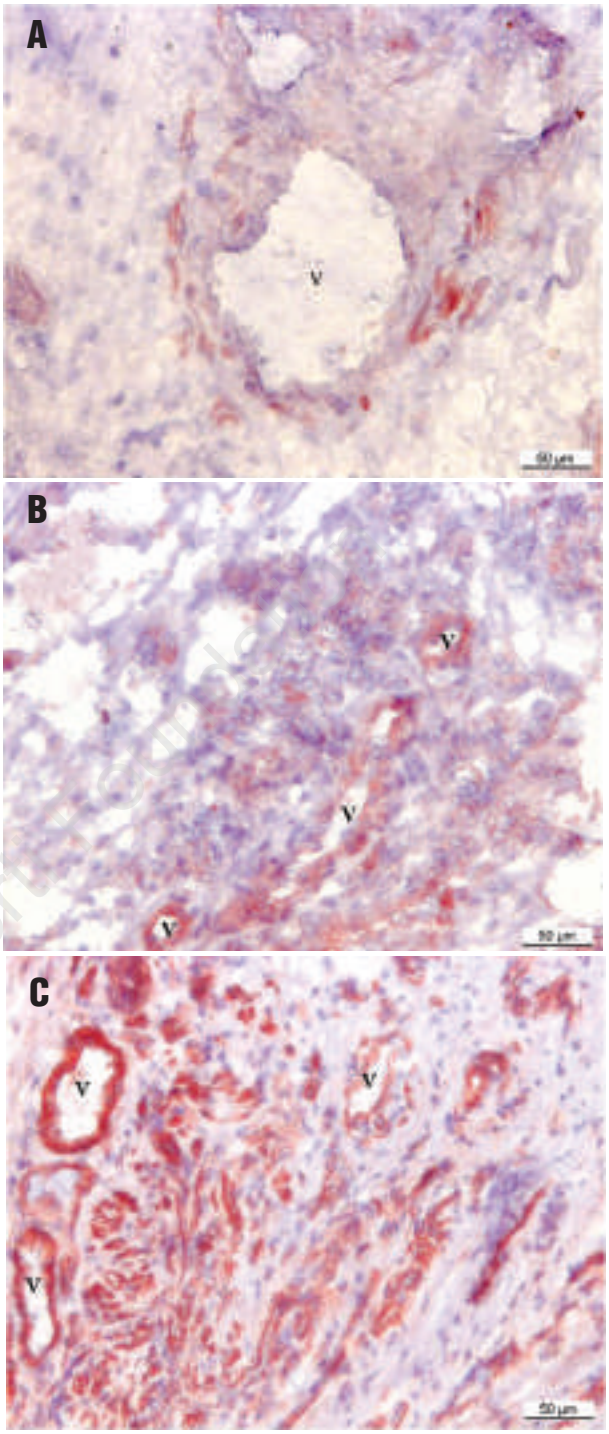

Figure 1. Immunohistochemical staining of nasal tissue samples for VEGF. (V) marks a vessel lumen. A. Immunostaining of a healthy control tissue, graded $^{+}$. B. Immunostaining for patient #12 with medium staining, graded $^{++}$. C. Immunostaining for patient #03 with strong staining, graded $^{+++}$. Light microscopy, magnification × 200.

High ALK1 tissue expression in all 13 HHT tissue samples

We also analyzed the expression of ALK1, endoglin, MMP-2, α-sma, Flt-1, and KDR in tissue samples of 13 HHT patients and 5 healthy controls by immunohistochemistry with the specific antibodies for the listed proteins. The results are summarized in Table 4. Strong ALK1 staining was noted within the HHT group. ALK1 expression was not restricted to the cells lining the vessels, but was also observed for cells in the stroma and for gland cells (*data not shown*). We do not know what

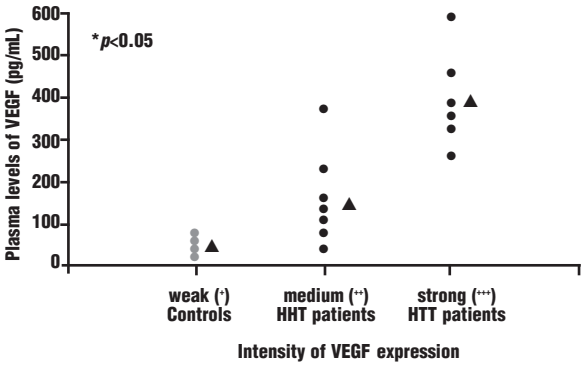

Figure 2. Intensity of VEGF nasal tissue staining in correlation with the corresponding patients' VEGF plasma concentration. The results are for 13 HHT patients and a healthy control group of 5 individuals. In each staining group, graded from "+" to "+++", the VEGF plasma concentrations of each individual is represented by a dot. The mean value of plasma concentrations in each grading group is represented by a triangle (▲).

cells in the stroma express ALK1, but it is possible that these cells are endothelial and smooth muscle cells of a vessel cut at an angle such that the lumen of the vessel is no longer seen. All HHT tissue samples showed higher ALK1 expression compared to the control tissue. The ALK1 staining ranged from medium $^{++}$ (62%) to strong $^{+++}$ (38%), whereas the control tissues only showed weak or no ALK1 staining classified as $^{+}$. Representative photomicrographs are shown in Figure 4.

Unlike the pattern seen for ALK1, endoglin expression was localized to the vessels, and showed only moderate staining or weak staining of equal intensity as that in the healthy control samples. Within the HHT group four patients showed weak endoglin staining $^{+}$ (31%), seven patients demonstrated medium staining $^{++}$ (54%) and only two HHT patients showed strong staining $^{+++}$ (15%). All of the tissue sections of the healthy controls showed weak staining, $^{+}$ (100%) Representative photomicrographs of endoglin staining are shown in Figure 5. MMP-2 is involved in activation of angiogenic factors and in degradation of the extracellular matrix, which is a prerequisite for endothelial cell migration. MMP-2 expression was not only seen in cells lining the vessels, but also in the stroma of the samples examined. Ten HHT patients showed the same medium staining intensity $^{++}$ as the controls. One HHT patient showed weak staining $^{+}$ and the remaining two HHT patients demonstrated strong staining $^{+++}$. Twelve HHT patients demonstrated increased α-sma staining; staining was graded as $^{++}$ in eleven patients and as $^{+++}$ in one patient. Only one HHT patient showed weak staining $^{+}$, which was similar to the level of staining seen in controls. The angiogenic activity of VEGF is mediated by its receptors Flt-1 and KDR and during angiogenesis VEGF upregulates the expression of these

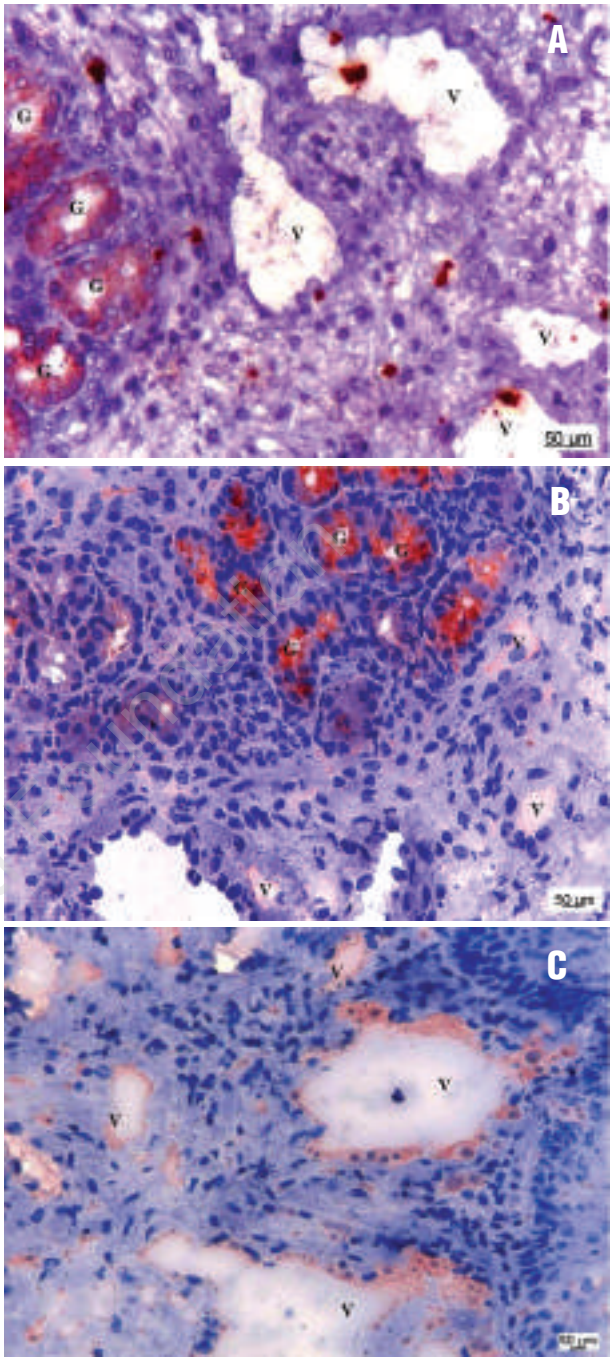

Figure 3. Immunohistochemical staining of nasal tissue samples for TGF-β1. (V) marks the vessel lumen and (G) marks a gland. A. Immunostaining of a healthy control tissue with negative TGF-β1 vessel staining, $^{-}$, but staining of gland cells. B. Immunostaining for patient #10 with positive vessel staining, graded $^{+}$, and staining of gland cells. C. Immunostaining for patient #12 with strong vessel staining, graded $^{++}$. Light microscopy, magnification × 200.

receptors. However, in tissue samples from both HHT patients and the healthy controls, Flt-1 and KDR staining was weak $^{+}$ to absent $^{-}$. Of six samples from HHT patients, only two showed medium Flt-1 expression and one demonstrated weak staining for KDR.

Table 4. Overview of the immunohistochemical staining results for different angiogenic factors in nasal tissue samples of HHT patients and healthy controls.

	Patients													
	Control	#03	#05	#06	#10	#11	#12	#14	#16	#17	#21	#22	#23	#26
Plasma levels (pg/mL)														
VEGF	30	359	443	156	120	32	95	308	249	58	216	363	578	363
Plasma levels (ng/mL)														
TGF-β1	5	58	59	48	58	55	37	44	57	34	72	29	67	49
Immunohistochemistry (IHC)														
VEGF	+	+ + +	+ + +	+ +	+ +	+ +	+ +	+ + +	+ + +	+ +	+ +	+ + +	+ + +	+ +
TGF-β1	?	+ +	+ +	+ +	+	+ +	+ +	+ +	+	+	+ +	+ +	+	+ +
ALK1	+/-	+ +	+ +	+ + +	+ +	+ +	+ +	+ + +	+ + +	+ +	+ +	+ +	+ + +	+ + +
Endoglin	+	+ +	+ + +	+ +	+	+ + +	+ +	+	+	+	+ +	+ +	+ +	+ +
MMP-2	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ + +	+ +	+ + +	+	+ +	+ +	+ +
α-sma	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ + +	+ +	+ +	+ +	+ +	+
Flt-1	+	n.d.	n.d.	+	+ +	n.d.	+	+	n.d.	n.d.	n.d.	n.d.	+ +	+
KDR	?	n.d.	n.d.	?	?	n.d.	?	+	n.d.	n.d.	n.d.	n.d.	?	?

Grading of VEGF, ALK1, endoglin, MMP-2, α-sma, Flt-1 and KDR immunostaining as well as plasma concentrations of VEGF and TGF-β1 in 13 HHT patients and 5 controls. The plasma concentrations in the control column are the mean values for 5 healthy controls.

Discussion

Angiogenesis is a process described as the formation of new blood vessels by capillary sprouting from pre-existing vascular endothelium, and includes vessel assembly and maturation, vessel maintenance, and vessel remodeling. Clearly then, angiogenesis requires a finely balanced series of events. Many of the angiogenic disorders result from unbalanced activity or loss of activity of different angiogenic factors. The vascular disorder HHT, caused by mutations in the TGF-β receptors endoglin or ALK1, or the TGF-β signaling mediator Smad4, represents an excellent example of an unbalanced angiogenic process. Studies of endoglin and *ALK1* knock-out mice have demonstrated the key roles of these proteins in early angiogenesis, thus adding these two proteins to the growing list of important angiogenic factors.

Of the 31 HHT patients examined in this study, 29 (90%) had statistically significant high to very high VEGF plasma levels which is in agreement with the findings of a previous study.[35] We extended these observations by measuring TGF-β1 plasma concentrations. Here we report for the first time statistically significant high to very high TGF-β1 plasma levels in 29 (90%) of 31 patients with HHT. Furthermore, these high TGF-β1 plasma levels correlated with positive TGF-β1 staining of a single layer of cells lining the vessel lumina in thirteen investigated patients; this staining was not observed in controls. In this study, the total amount of plasma TGF-β1 was determined, which does not allow a distinction to be made between the latent (inactive) or activated TGF-β1 forms. However, the overall increase of TGF-β1 plasma concentration suggests an increase in TGF-β1 expression. It is not yet clear which cells are responsible for the increased TGF-β1 expression, but our TGF-β1 tissue staining results suggest that endothelial cells might be a possible source of TGF-β1 secretion. Further studies are now necessary to address these questions. Zhang and colleagues recently reported three HHT type 2 patients who had no change in TGF-β1 plasma concentrations.[39] However, they examined only three patients (first degree relatives) and three individuals who served as the control group, whereas we examined 31 patients and 40 controls. Out of our 29 HHT patients with high VEGF plasma levels, 28 also had elevated levels of TGF-β1. This suggests that increased VEGF and TGF-β1 plasma levels might serve as diagnostic criteria for HHT. However, these levels were observed in symptomatic individuals with severe epistaxis and it would be of great interest to measure plasma levels in asymptomatic or mildly affected HHT patients.

VEGF expression is regulated by hypoxia and other growth factors such as TGF-β1, while TGF-β1 is able to induce its own expression.[23,24,40,41] Endoglin and ALK1 are inhibitors of Smad2/Smad3-mediated TGF-β1 signaling.[11,17,42,43] Thus, reduced amounts of endoglin or ALK1 could cause increased TGF-β1 signaling activity in endothelial and smooth muscle cells due to heightened Smad2/Smad3 phosphorylation, which would then induce VEGF expression in smooth muscle cells or other cell types.[41] This might explain the increased VEGF plasma levels and strong tissue staining in our HHT patients. Further supporting data come from *ALK1* knock-out mice embryos in which the levels of VEGF transcripts and angiogenesis-related genes such

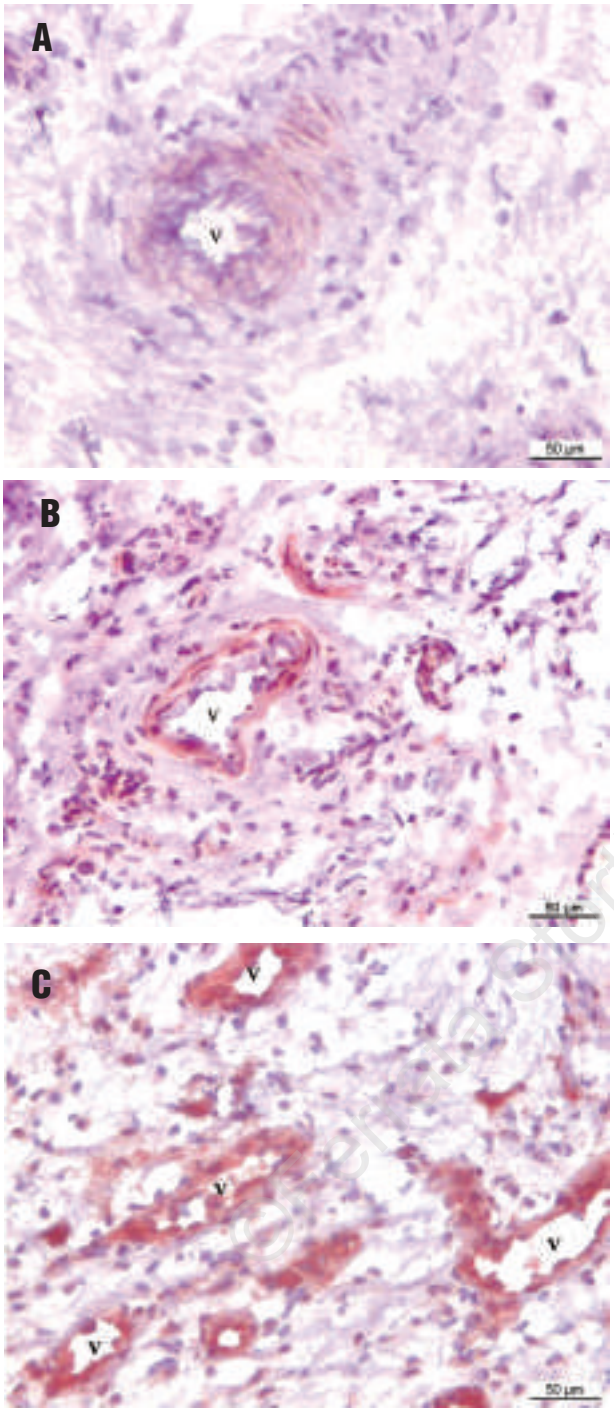

Figure 4. Immunohistochemical staining of nasal tissue samples for ALK1. (V) marks the vessel lumen. A. Immunostaining of a healthy control tissue with weak staining, graded $^{+}$. B. Immunostaining for patient #10 with medium staining, graded $^{++}$. C. Immunostaining for patient #06 with strong staining, graded $^{+++}$. Light microscopy, magnification × 200.

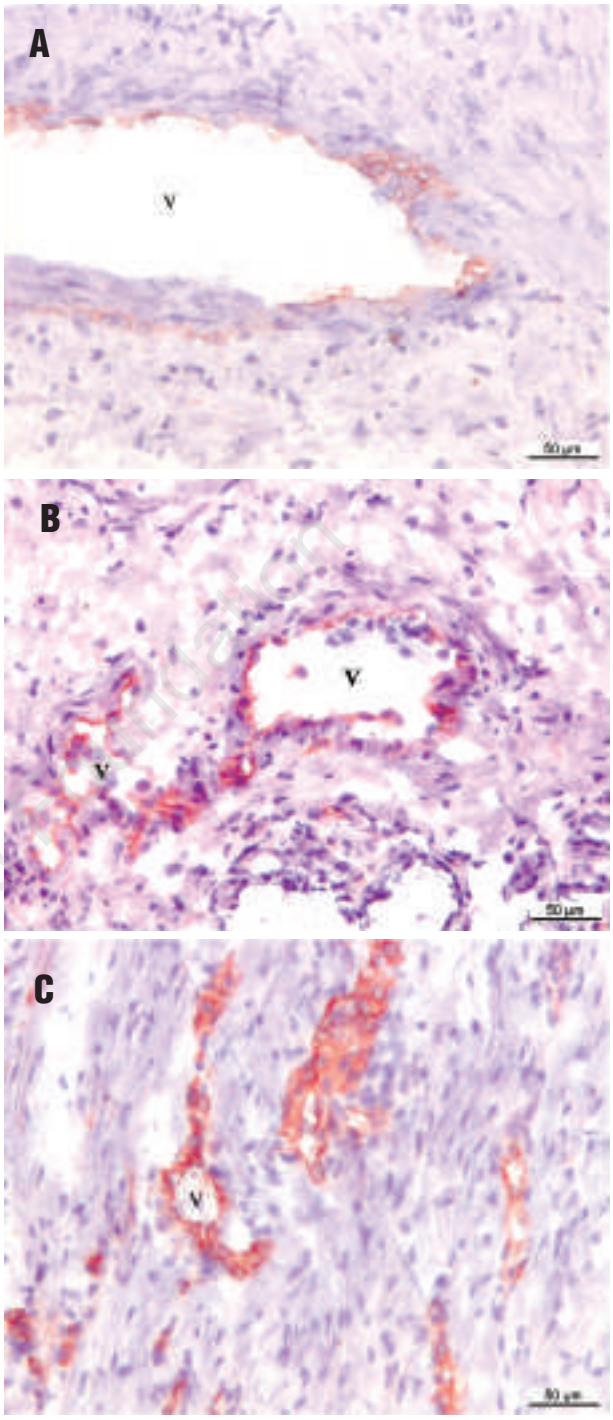

Figure 5. Immunohistochemical staining of nasal tissue samples for endoglin. (V) marks the vessel lumen. A. Immunostaining of a healthy control tissue, graded $^{+}$. B. Immunostaining for patient #06 with medium staining, graded as $^{++}$. C. Immunostaining for patient #11 with strong staining, graded $^{+++}$. Light microscopy, magnification × 200.

as tPA, uPA, PAI-1 and Ang-2 were significantly elevated.[11] VEGF is able to up-regulate the expression of its own receptors, Flt-1 and KDR.[22] Surprisingly however, although 90% of the HHT patients had increased VEGF plasma levels and tissue expression, only one patient showed upregulation of KDR and two patients showed moderate upregulation of Flt-1. It is unclear how Flt-1 is regulated in this context, although endothelial Flt-1

induction by TGF-β1 secreted from pericytes has been reported.[44] More patients need to be investigated to determine whether there is a correlation between TGF-β1 concentrations and Flt-1 expression in HHT patients.

The unchanged KDR expression might be due to the high TGF-β1 plasma concentration, as TGF-β1 inhibits KDR expression in endothelial cells.[45,46] A recent study reports that induced over-expression of $VEGF_{165}$ in the brain resulted in increased vessel density but reduced KDR expression in heterozygous endoglin ($^{+/-}$) mice as compared to in endoglin wild type ($^{+/+}$) mice,[47] supporting our observations. Furthermore, VEGF over-expression induced angiogenesis in both the heterozygous endoglin mice and wild type mice, with no difference in the number of microvessels. Abnormal vessel structure was, however, seen in the endoglin $^{+/-}$ mice. Other studies have shown that VEGF over-expression leads to an irregular, non-organized vascular architecture which is probably associated with poor function.[48] These results suggest that continuous VEGF hyperstimulation may lead to the development of abnormal microvessels due to unbalanced angiogenesis as seen in heterozygous endoglin ($^{+/-}$) mice and, possibly, in HHT patients. The elevated level of ALK1 tissue expression in our HHT patients is unexpected as HHT is thought to be caused by haploinsufficiency for either *ALK1* or endoglin. About 90% of our patients showed increases in TGF-β1 and VEGF plasma levels and strong tissue expression of VEGF, as well as of ALK1. These increased TGF-β1, VEGF and ALK1 protein levels do not appear to be associated specifically with either endoglin or *ALK1* mutations. The specific mutations of our patients are not known, but it is reasonable to assume that approximately half of our group will have endoglin mutations and the other half, *ALK1* mutations (see the HHT mutation database, available at URL *http://www.macs.hw.ac.uk/hht*).

The fact that over-expression of VEGF in heterozygous endoglin ($^{+/-}$) mice resulted in angiogenesis and therefore in increased vessel density[47] would be in agreement with our findings for α-sma. The elevated tissue expression of α-sma in the HHT patients suggests an angiogenesis-related increase in the number of microvessels and therefore also an increase in vessel-supporting smooth muscle cells. Indeed, we did demonstrate an increased number of microvessels in our HHT patients in another study by determining the microvessel density in nasal tissue samples by staining for von Willebrand factor.[49] All HHT tissue samples examined in this study showed a statistically significant higher microvessel density than did the control samples. We believe that the moderately enhanced endoglin staining of samples from most of the patients in this study is due to an increased number of microvessels. This suggests that the increased endoglin expression seen during the process of angiogenesis in HHT is related to increased vessel density and not to endoglin being specifically induced by angiogenic factors.

Several factors and mechanisms are known to activate TGF-β, such as proteolysis, thrombospondin-1, reactive oxygen species and a mildly acidic pH.[50] One of the factors that activate latent TGF-β by proteolytic cleavage is the extracellular matrix located protease MMP-2. VEGF induces both MMP-1 and MMP-2 expression. MMP-2 exists as an enzymatically inactive form, the pro-MMP-2 protein, which becomes activated by proteolytic cleavage, i.e. by MMP-1.[45,51] Although HHT patients show an increase in microvessel density, we can not conclude from our data whether MMP-2 contributes to the putative angiogenic process in HHT patients. Our immunohistochemical staining revealed increased MMP-2 in only two of 13 patients, whereas 10 patients showed no difference from the control tissues. Nevertheless, it is possible that there is an increased conversion of pro-MMP-2 into its active form in the HHT patients since the antibody we employed does not distinguish between the two forms of MMP-2.

How do our data fit with the current knowledge and understanding about the development of HHT? The initial event is most likely a reduced amount of functional ALK1 or endoglin protein at the cell surface. This may lead to aberrant TGF-β1 signaling through the reduced inhibitory or modulating activity of ALK1 or endoglin. TGF-β1 then induces its own expression and an increased VEGF expression. How can the increased amount of ALK1 be explained? Increased TGF-β1 levels may directly induce ALK1 expression or other TGF-β1 regulated factors may initiate ALK1 expression. Our own studies with primary human umbilical vein endothelial cells demonstrated that TGF-β1 is able to increase ALK1 expression (*A. Lux, unpublished data*). Furthermore, it was recently reported that the matrix GLA protein in concert with TGF-β1 activates ALK1 signaling in bovine aortic endothelial cells which induces VEGF expression.[52] However, the increased VEGF expression in *ALK1* knock-out mice implies that in the absence of ALK1 further pathways are involved in VEGF induction. Subsequently, increased amounts of VEGF induce angiogenesis-related factors starting the angiogenic process. In conclusion, the sequential and cumulative effect of elevated VEGF and TGF-β1 levels is accompanied by an increase of ALK1. ALK1 expression and signaling might foster the angiogenic process by angiogenesis-related endothelial cell proliferation and migration.[18] We hypothesize that HHT is a disorder of localized angiogenesis due to an unbalanced over-expression of VEGF, TGF-β1 and ALK1 and probably other factors that remain to be identified.

HS was responsible for the conception and design of the project, collection of clinical samples, data handling and writing of the manuscript. FR, RN and UG contributed to the collection of clinical samples and data handling. KH and MH gave final approval of the manuscript. AL was responsible for the overall conception and design of the project, data handling and writing the manuscript. All authors approved the version submitted for publication.The authors declare that they have no potential conflicts of interest.

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