Therapeutic action of tranexamic acid in hereditary haemorrhagic telangiectasia (HHT): Regulation of ALK-1/endoglin pathway in endothelial cells

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Summary

Recurrent epistaxis is the most frequent clinical manifestation of hereditary haemorrhagic telangiectasia (HHT). Its treatment is difficult. Our objective was to assess the use of tranexamic acid (TA), an antifibrinolytic drug, for the treatment of epistaxis in HHT patients and to investigate *in vitro* the effects of TA over endoglin and ALK-1 expression and activity in endothelial cells. A prospective study was carried out on patients with epistaxis treated with oral TA in the HHT Unit of Sierrallana Hospital (Cantabria, Spain). Primary cultures of endothelial cells were treated with TA to measure the levels of endoglin and ALK-1 at the cell surface by flow cytometry. RNA levels were also measured by real-time PCR, and the transcriptional effects of TA on reporters for endoglin, ALK-1 and the endoglin/ALK-1 TGF-beta pathway were assessed. The results showed that the fourteen HHT patients treated orally with TA improved, and the frequency and severity of their epistaxis were decreased. No complications derived from the treatment were observed. Cultured endothelial cells incubated with TA exhibited increased levels of endoglin and ALK-1 at the protein and mRNA levels, enhanced TGF-β signaling, and improved endothelial cell functions like tubulogenesis and migration. In summary, oral administration of TA proved beneficial for epistaxis treatment in selected patients with HHT. In addition to its already reported antifibrinolytic effects, TA stimulates the expression of ALK-1 and endoglin, as well as the activity of the ALK-1/endoglin pathway.

Keywords

Endothelial cells, HHT, endoglin, ALK-1, tranexamic acid, fibrinolysis

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Introduction

Hereditary haemorrhagic telangiectasia (HHT), or Rendu-Osler-Weber syndrome, is an autosomal dominant vascular disease with incomplete penetrance characterized by localized angiodysplasia. This is manifested as epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations in the pulmonary, cerebral or hepatic circulation (1). The prevalence is on average between one in 5,000 to one in 8,000, although it is higher in some regions, such as the Jura region in France, Funen island in Denmark and certain Caribbean islands in the Netherland Antilles (2, 3). Its prevalence in Cantabria (Northern Spain) was calculated to be one in 12,000 in 1997 (4). However, according to more recent HHT Spanish population studies, in progress since 2003, the prevalence may be around one in 8,000 (Zarrabeitia et al., data not shown).

There are two main HHT types, type 1 and type 2 which are caused by mutations in *endoglin* and *ALK-1* genes respectively (5, 6). In a few cases of the all HHT patients, around 2%, the origin of the disease is a mutation in Smad4 gene leading to the combined syndrome of Juvenile Polyposis and HHT (7). Recently a third locus for an unknown HHT gene has been described in chromosome 5 (HHT3, [8]). All these genes are coding for proteins involved in the TGFβ-signalling pathway, which is critical for the proper development of the blood vessels.

The most frequent clinical manifestation of HHT is epistaxis (nose bleeds), normally from light to moderate (9–11). However, some patients show severe epistaxis which notably interfere with

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their quality of life (12). The origin of this epistaxis is due to the existence of telangiectases on the nasal mucosa. These are focally dilated postcapilar venules, which in advanced phases show many layers of smooth muscle cells without elastic fibers, and very frequently directly connect with dilated arterioles (13). As a consequence of these vascular alterations, telangiectases are very sensitive to slight traumata and even to the friction with the air when breathing, which gives rise to nose bleeds.

There is no optimal treatment for the epistaxis in HHT patients. Many different therapies have been assayed, but none of them with conclusive results (14–17). The use of antifibrinolytic agents for the treatment of HHT patients, systemically administered (intravenously, intraparenteral, orally) was described for the first time by Saba et al. (18), using oral administration of aminocaproic acid to two patients. The result was satisfactory with an improvement in epistaxis and the associated anemia. More recently, other authors have also reported good results using tranexamic acid (TA), both orally and topically (20, 21). TA may also be administered endovenously in cases of very acute bleeding episodes (10). TA is a derivative of the amino acid lysine, 4 (aminomethyl cyclohexanecarboxyl) (22). It binds reversibly to plasminogen, avoiding fibrin degradation by plasmin (23, 24). TA enters into the extravascular space and accumulates in the tissues (25). The basis for its efficiency relies on the inhibition of the fibrinolytic activity in the tissues that leads to clot stabilization. TA or other antifibrinolytic lysine-analogues are indicated in hereditary deficiencies of the plasmin/PAI-1 system, in acute episodes of bleeding in von Willebrand disease or acute hemophilia, but in the latter cases the diseases can be treated supplying the corresponding deficient coagulation factor. TA is also indicated in severe bleedings like after surgery and in gynecologic bleedings like menorrhagia and in hyperfibrinolysis. The latter is exactly the case of HHT bleeding showing hyperfibrinolysis secondary to intravascular coagulation (22). The presence of local hyperfibrinolysis with increase in t-PA justifies the use of an antifibrinolytic agent, such as TA for the management of HHT epistaxis. By contrast, other hemostatic drugs increasing the release of coagulant factors, as desmopresin, are more indicated in the management of other hemorrhagic hereditary diseases as hemophilia or von Willebrand disease.

TA is not yet broadly used to treat HHT since there are few reports on the benefits of its oral, topical or endovenous administration in HHT. The scarce studies are either case reports, or include at the most three patients. In the present study we report the efficiency of orally administered TA in 14 HHT patients with moderate or severe epistaxis, according to the experience of the Spanish HHT reference unit. The aim of this study was to assess TA in in-vitro experiments with cultured endothelial cells, quantifying endoglin and ALK-1, deficient in HHT patients, to understand at molecular level why TA may be especially useful in HHT. These results are implemented with clinical data showing an improvement in epistaxis. These data do not represent a clinical trial because it is almost impossible to do a clinical trial in a rare disease, where the treatment is required to stop bleeding, and hence is hard to leave patients without treatment (controls). However, the comparison of bleeding parameters before and after the treatment is in itself a kind of control. Both, the in-vitro results and the success with patients makes TA a encouraging drug to improve the quality of life in HHT.

Material and methods

Clinical study of HHT patients

A prospective study of HHT patients attending the HHT unit of Sierrallana hospital between 2001 and the first semester of 2006, was conducted. HHT patients were diagnosed according to Curaçao criteria (26). Those patients with moderate or severe epistaxis which interfered with their quality of life were treated with TA when they did not show any contraindication. The treatment normally consisted of 500 mg oral TA (Amchafibrin, Fides Ecopharma, Rota-Research group) every eight hours, with corrections according to renal function. Doses of TA in some cases may reach up to 1 g every eight hours. Before the study, all patients were subjected to blood tests including hemogram, plasma biochemical studies, coagulation studies and genetic studies to look for the mutation origin of the disease. Moreover, a complete screening of head and neck was made by an ear-nose-throat specialist involved in the screening of HHT patients. The efficiency of the TA treatment was evaluated according to the transfusion needs, and the frequency and intensity of epistaxes following (27). To measure the degree of satisfaction a scale from I (very satisfied) to IV (very unsatisfied) was used.

Cell culture

BOECs (blood outgrowth endothelial cells) and HUVECs (human umbilical vein endothelial cells) were obtained as described (28). HMECs (human microvasculature endothelial cells) were incubated in the same endothelial enriched medium used for BOECs and HUVECs as described (29). Cells were incubated in the presence or absence of 2 mM TA diluted directly into the culture medium. ε-amino caproic acid (AC), another antifibrinolytic drug, was used for comparison purposes with TA (both chemicals from Fides Ecopharma, Rotta-Research group). The plasmin protease inhibitor, Aprotinin (AP) from Sigma-Aldrich, was also used as an agent mimicking the action of TA and AC. All the assays were made in the presence of 10% FBS supplemented medium, likely to contain around 1.4 and 1.5 U/ml of plasminogen biological activity measured by radioimmunodiffusion (The Binding Site LTD, Birmingham, UK)

Flow cytometry analysis

Measurements of endoglin, ALK-1 and PECAM (CD31) levels on the cell surface were carried out by flow-cytometric analysis from HUVECs and BOECs using monoclonal antibodies P4A4, anti-ALK-1 (R&D Systems) and HC1/16, respectively, according to the procedure described by Fernandez-L. et al. (28) and Sanz-Rodriguez et al. (29).

Real-time PCR

Endoglin and ALK-1 oligonucleotides were purchased from Sigma and chosen according to Roche's software for real-time PCR. Total RNA was extracted from endothelial cells (HUVECs, BOECs, and HMECs) using the RNAeasy kit (Qiagen), retrotranscribed using the AMV RT kit from Roche, and amplified in a real time PCR using the Universal Human Probe Roche library and the Real-time PCR kit from Roche.

The assays were performed in triplicates, compared with two different types of endogenous controls (18S rRNA and GAPDH)

and repeated at least twice. The oligonucleotide sequences and probe numbers for endoglin, ALK-1, GAPDH and 18S RNA used in the PCR were:

- *Endoglin* left oligo: 5' AGCCTCAGCCCCACAAGT 3′; right oligo: 5' GTCACCTCGTCCCTCTCG 3'
- Human 41 Probe of Roche library for e*ndoglin*
- *ALK-1*/*ACVRL1* left oligo: 5' ATCTGAGCAGGGCGACAC 3'; right oligo 5′ ACTCCCTGTGGTGCAGTCA 3'
- Human 79 probe of Roche library for *ALK-1/ACVRL1*
- *GAPDH* left oligo: 5' AGCCACATCGCTCAGACAC 3'; right oligo: 5' GCCAATACGACCAAATCC 3'
- Human 60 probe of Roche library for *GAPDH*
- 18S rRNA left oligo: 5' CTCAACACGGGAAACCTCAC 3'; right oligo: 5' CGCTCCACCAACTAAGAACG 3'
- Human 77 probe of Roche library for 18S rRNA.

Wound healing and tube formation assays

In vitro scratched wounds were created by scraping confluent HMEC monolayers in P-24 plate wells with sterile disposable pipet tips. The remaining cells were washed with Hanks Buffered Salt Solutions (HBSS) buffer (Hanks, Gibco) and incubated with Endolthelial Basic Medium (EBM)-2/Endothelial Growth Medium (EGM)-2 medium (Clonetics, Cambrex) in the absence or presence of antifibrinolytic drugs, TA, AC and AP, up to 24 hours (h). Endothelial cell migration into denuded area was monitored by photography of the plates at different times.

For tube formation assays, HMECs were plated in EBM/EGM-2 culture medium in the absence or presence of antifibrinolytic drugs, TA, AC and AP on P-6 well matrigel plates (Becton Dickinson) and incubated at 37ºC, as indicated by the manufacturer. Tube formation was monitored for 24 h.

Cell transfection and reporter assays

Transient transfections of BOECs were made using Superfect (Qiagen) with reporters for *endoglin* promoter: pCD105 (-450/+350) in pXP2 (30, 31), here denoted as pENG/pXP2 and ALK-1 promoter: pALK-1/pGL2, a construction encompassing the first exon and around 1.3-kb upstream of the ALK-1 genomic DNA inserted in *Sac* I and *Xho* I sites of pGL2 polylinker. The TGFβ pathway reporters were 3TP-lux, ALK-5 responsive $(CAGA)_{12}$ Luc, or ALK-1 responsive $(BRE)_2$-Luc (32). Reporter vectors were co-transfected or not with expression vectors coding for a dominant negative mutant (ALK-1-K229R), or a constitutively active mutant (ALK-1-Q201D) of ALK-1. Relative luciferase units (RLU) were measured in a TD20/20 luminometer (Promega, Madison, WI, USA).

Samples were co-transfected with the SV40-β galactosidase expression vector to correct for transfection efficiency. Measures of β-galactosidase activity were performed using Galacto-light (Tropix). The transfections were made in triplicates and repeated at least in three independent experiments. Representative experiments are shown in the figures.

Table 1: Characteristics of the HHT patients.

No/age/sex	Mutation	Previous treatments
1/47/M	Not found	None
2/80/M	ENG/HHT1	Cauterization, A/P package
3/69/M	ALK1/HHT2	Cauterization, A/P package
4/42/M	ALK1/HHT2	None
5/49/F	ALK1/HHT2	Cauterization, A/P package
6/47/F	ALK1/HHT2	None
7/59/M	ENG/HHT1	Cauterization, A/P package
8/45/F	ALK1/HHT2	None
9/78/F	ALK1/HHT2	None
10/41/F	ALK1/HHT2	None
11/49/F	ALK1/HHT2	None
12/48/F	ENG/HHT1	Cauterization, A/P package
13/43/M	Not found	A/P package
14/50/F	ALK1/HHT2	None

M, male; F, female; A/P, anterior/posterior.

Statistics

All data presented represent mean ± SD. Differences in mean values were analyzed using Student's t-test. P-values<0.05 were considered to be statistically significant. In the figures, the statistically significant values are marked with asterisks as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Clinical treatment with TA of a group of Spanish HHT patients

Previous reports on the successful use of TA for nose bleed management are based on one or, at the most, three patients (20, 21, 33). This fact led us to follow up the clinical use of oral TA treatment in a larger series of Spanish HHT patients. In parallel, in-vitro research was conducted in the laboratory to assess TA effects at cellular and molecular levels.

The HHT unit of Sierrallana Hospital (Cantabria, Spain) has screened from 2003 up to now, more than 150 patients belonging to around 40 different families. Of these, a total of 14 patients which had severe epistaxis interfering with their quality of life volunteered for a treatment with oral TA. In all these cases side effect risks of thrombosis were absent. The type of patient, mutation, sex, and management of the epistaxis before the treatment are recorded in Table 1. The TA treatment and the severity of epistaxis before and after the treatment are shown in Table 2. All patients showed a decrease in the intensity and frequency of nose bleeds after the first week of treatment, and when the treatment was discontinued the amount of haemorrhages increased immediately. At this moment, all of them continue with the TA treatment and none has presented adverse side effects derived from it until now.

Effect of TA on the amount of ALK-1 and endoglin at the surface of endothelial cells

As HHT1 and 2 are caused by endoglin and ALK-1 haploinsufficiency, respectively, we have explored the in-vitro TA effects on these two genes at the cellular level.

First, we decided to investigate if TA in a range of concentrations (from 0 to 10 mM) was having any effect on the amount

Table 2: Tranexamic acid treatment and its efficiency.

No	Dose/type	Period of treatment	Previous transfusions	Posttreatment transfusions	Pretreatment epistaxis (I/F)*	Posttreatment epistaxis (I/F)*	Satisfaction
1	500 mg/12 h	2 m	No	No	III/II	I/I	II
2	500 mg/8 h	24 m	>5	<5	III/III	II/II	II
3	500 mg/8 h	25 m	<5	No	III/III	I/I	II
4	500 mg/8 h	2 m	No	No	II/II	I/I	II
5	1000 mg/8 h	16 m	<5	<5	III/II	II/II	III
6	500 mg/8 h	11 m	<5	No	III/II	I/I	II
7	500 mg/8 h	12 m	<5	<5	III/III	II/II	III
8	500 mg/12 h	4 m	No	No	II/II	I/I	I
9	500 mg/12 h	6 m	No	No	II/II	I/I	II
10	500 mg/8 h	4 m	No	No	II/II	I/I	II
11	500 mg/8 h	4 m	No	No	II/II	I/I	II
12	500 mg/8 h	8 m	No	No	III/III	II/II	II
13	1000 mg/8 h	5 m	No	No	III/II	II/I	II
14	1000 mg/8 h	12 m	No	No	II/II	I/I	II

m, months; h, hours. (I/F), intensity/frequency.

of ALK-1 and endoglin at the surface of endothelial cells (Fig. 1A). The effect on the endothelial marker PECAM was used as a control, and an irrelevant antibody was taken as negative control. The amount of cell-surface endoglin was increased 3–4 times, even at the lowest concentration (0.5 mM). In another set of experiments, at 2 mM TA (since it yields good response), the amount of endoglin and ALK-1 increased by 1.5– and 1.75-fold, respectively (Fig. 1B). In Figure 1C, the endoglin and ALK-1 induction after 2 mM TA in BOECs (other type of primary endothelial cells) (28) is shown. The upregulation of endoglin and ALK-1 by TA treatment was also explored after AC treatment and the plasmin protease inhibitor AP, in a dose-response experiment, to investigate if other antifibrinolytic agents were behaving similarly. In this case the endothelial established cell line HMEC was used for purposes of homogeneity of results, and they were grown on Dubbecco's Modified Eagle Medium (DMEM). This cell line has lower basal levels of endoglin which allowed us to observe stronger induction changes after treatments than in primary cultures. As can be seen in Figure 1D and E all of them seem to increase the protein levels of endoglin and ALK-1, with TA being the most efficient in increasing endoglin levels at the concentrations used. TA and AP were equally efficient stimulating protein levels of ALK-1 on the cell surface. These experiments show that antifibrinolytic drugs, TA, AC and AP increase the levels of endoglin and ALK-1 on the endothelial cell surface, but the mechanism involved may be either post-transcriptional stabilization of these surface proteins, induction at transcriptional level or both mechanisms at the same time. To discriminate among these possibilities, the effects of TA, AC, and AP on endoglin and ALK-1 RNA levels were investigated. To this end, HMECs were treated with TA, AC (2mM) and AP (5 μg/ml). These single doses were selected as the most convenient leading to significant protein increase, and good cell viability after the results shown in Figure 1D and E. As shown in Figure 2A, the levels of endoglin and ALK-1 RNA are significantly induced after treatments. However, the most efficient induction is achieved in the case of TA, with the effect of AC and AP induction on endoglin and ALK-1 RNA levels being very weak and not significant. Since TA doubles *endoglin/ALK-1* mRNA levels while the corresponding protein levels are induced by 10- to 14-fold, at this point we must argue that TA is affecting both, transcriptional and post-transcriptional processes, while the effects of AC and AP at the RNA level may be too weak to be observed after 24 h. Since TA has significant affects at *endoglin* and *ALK-1* RNA levels, the transcriptional effects will be only explored after TA treatment.

Thus, the effect of TA was assessed at the transcriptional level on *endoglin* and promoters. Figure 3A shows the results of transfecting endothelial cells (BOECs) with constructs representing *endoglin* and *ALK-1* promoters. In both cases, the TA treatment doubles the transcription rate of *endoglin* and *ALK-1* promoter constructs.

As published previously (28), ALK-1 affects *endoglin* promoter activity. Accordingly, using a dominant negative ALK-1 form (ALK-1-KR), *endoglin* promoter activity is blocked, while a constitutively active ALK-1 mutant (ALK-1-QD) increases *endoglin* promoter activity (Fig. 3A). In both cases TA treatment does not affect this response which argues in favour of TA acting, either directly or indirectly, through the TGF-β pathway.

Effect of TA on the TGF-β, ALK-5 and ALK-1/endoglin pathways

Since TA enhances endoglin and ALK-1 expression, at protein, RNA and promoter levels, we decided to ascertain if TA also has an effect on the TGF-β pathways active in endothelial cells, ALK-5 and ALK-1/endoglin (32, 34). A general TGF-β reporter

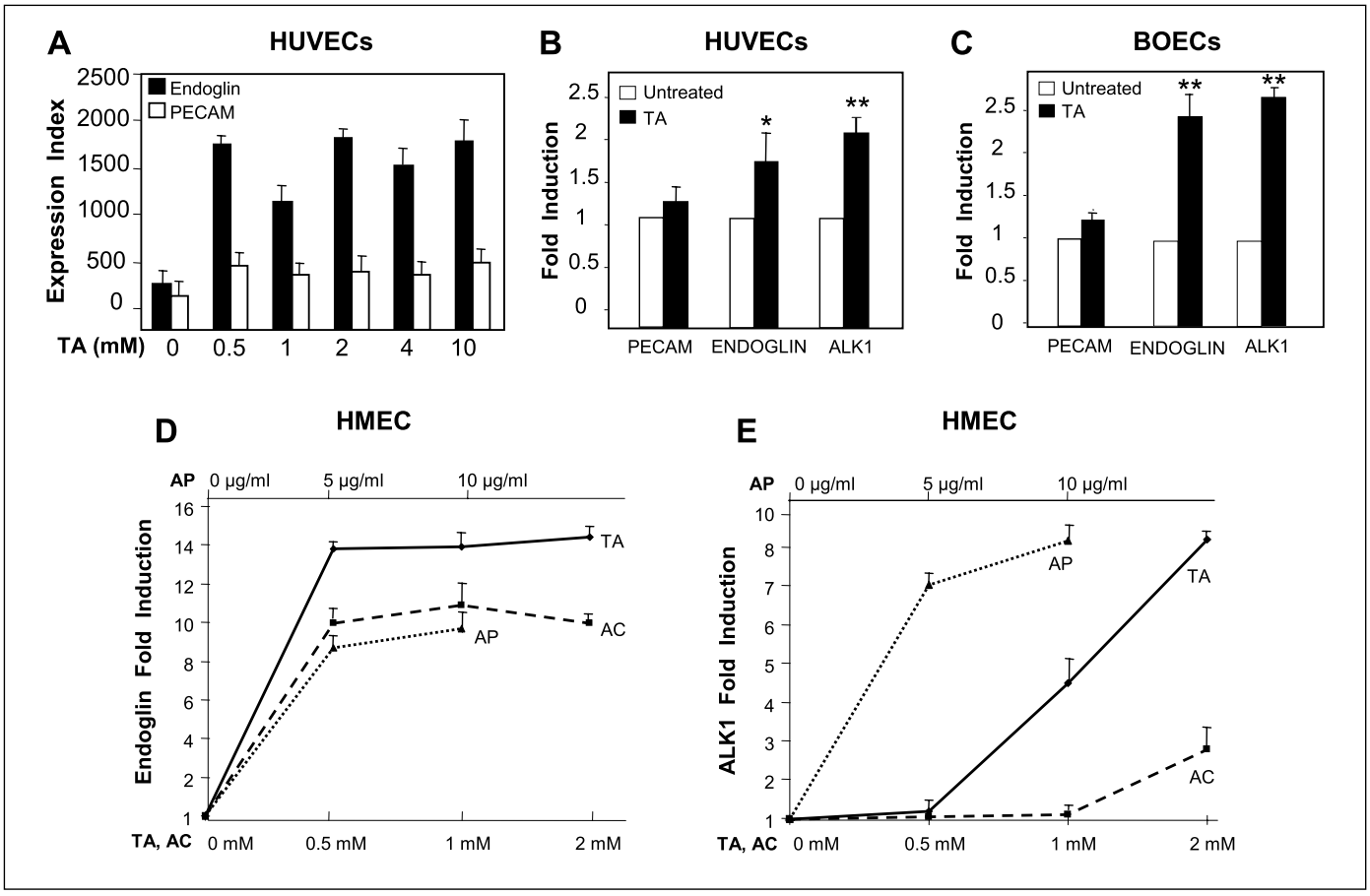

Figure 1: Effect of TA on the surface expression of endoglin and ALK-1 in endothelial cells. A) HUVECs were cultured in the absence or presence of increasing concentrations of TA (0.5 mM to 10 mM) for 24 h. Endoglin and PECAM levels were measured by flow c ytometry. B) HUVECs were incubated in the absence or in the presence of 2 mM TA and the levels of endoglin, ALK-1 and PECAM were measured after 24 h and compared with untreated cells. The results of the flow cytometry are expressed in fold induction levels. *P< 0.05; **P<0.01. C) BOECs were treated with 2 mM TA, and the levels of endoglin, ALK-1, and PECAM were measured after 24 h. The results of flow cytometry are expressed in fold induction with respect the untreated control. D and E) Endoglin (D) and ALK-1 (E) content was measured by flow cytometry in endothelial cells untreated or treated with different amounts of TA, AC, and AP, as indicated. The dose of 20 µg/ml AP was also tested, but the survival of cells was significantly reduced, and therefore the corresponding data is not included in the figure. The results of the flow cytometry are normalized with PECAM expression and expressed in fold induction with respect the untreated control. Experiments were made in triplicates, repeated at least twice, and a representative experiment in each panel is shown.

derived from the PAI-1 promoter, 3TP-lux, an ALK5-pathway specific reporter, $(CAGA)_{12}$-luc, and the ALK-1/endoglin-pathway specific reporter, BRE-luc, were used. The 3TP-lux and the $(BRE)_2$-luc reporters showed a significant increase of their activity following TA treatment, but, while 3TP-lux activity was slightly increased (~1.5-fold), $(BRE)_2$-luc reporter was strongly stimulated (~4-fold). The $(CAGA)_{12}$-luc reporter did not show any change after TA treatment. Since 3TP-lux contains PAI-1 proximal promoter encompassing AP1 and other transcription factor sites in addition to the ALK-5/Smad3 responsive element, the 1.5-fold increase of the 3TP-lux reporter by TA may be the consequence of other trancription factor(s) induced by TA, and it is interesting in itself due to the PAI-1antifibrinolytic function.

In summary, TA seems to specifically stimulate the TGFβ/ALK-1/endoglin pathway, though we do not know the mechanism yet.

Plasminogen inhibitors favour endothelial cell functions depending on the ALK-1/endoglin pathway: cord formation and migration

We have just shown that TA is specifically stimulating the TGFβ/ALK-1/endoglin pathway, without affecting TGFβ/ALK5 signalling. Therefore we performed two types of functional experiments under control of this pathway according to literature (32, 34): cord formation (angiogenesis) and cell migration after wound healing.

Cord formation, is an in-vitro experiment mimicking the angiogenic function of endothelial cells. Figure 4 shows cord formation in a time-course experiment. As can be seen, tubes develop faster in TA-treated endothelial cells. After six hours we can observe that the cord network is completely developed in TA treated cells; this happens around eight hours in AC and AP treatment and only after 12 h in untreated controls.

TA, AC and AP promote a faster migration of cells, so that an artificial monolayer disruption is closed between 15 and 24 h. In

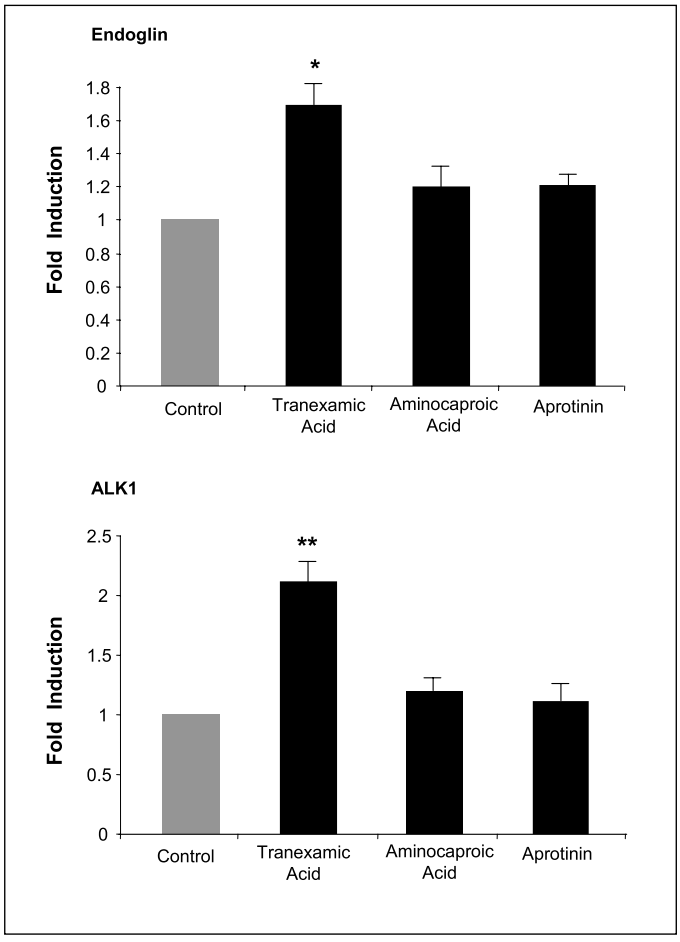

Figure 2: Effect of TA on endoglin and ALK-1 transcription. Endothelial cells were treated for 24 h with TA (2 mM), AC (2 mM) and AP (5 μg/ml). For real-time PCR, total RNA was extracted, retro-transcribed and amplified. Quantification of the number of ALK-1 and endoglin RNA molecules was carried out and compared to endogenous controls of 18S ribosomal RNA and *GAPDH*. The experiment was made in triplicates, and repeated for the three types of endothelial cells. A representative experiment of HMECs is shown,
although similar results were obtained with HUVECs and BOECs.

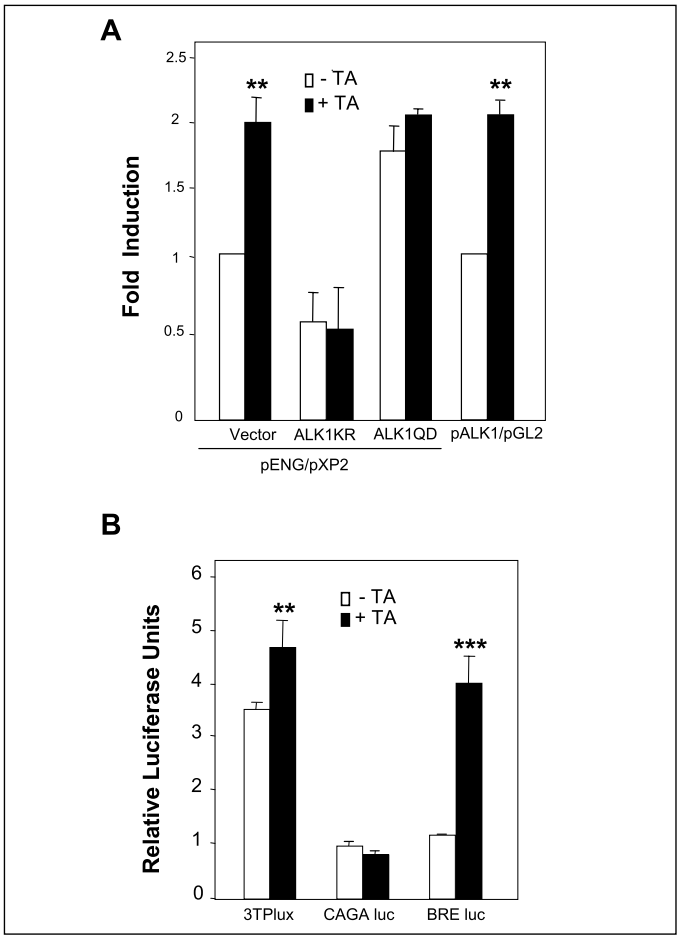

Figure 3: Effect of TA on the TGFβ pathways in endothelial cells. A) Effect of TA on endoglin and ALK-1 promoter activity. BOECs were transiently transfected with a construction of endoglin promoter, pENG/pXP2, and a construct of ALK-1 promoter in pGL2. Kinase dead ALK-1 (ALK-1-KR), constitutively active ALK-1 (ALK-1-QD) or empty expression vector (pcDNA3) were cotransfected, as indicated. Luciferase activity was measured in 2 mM TA-treated cells and expressed as fold induction with respect the untreated cells. B) BOECs were transiently transfected with different reporters for the TGFβ pathway, the general TGFβ responsive 3TP-lux, the ALK-5 specific CAGA-luc, and the ALK-1/endoglin-specific BRE-luc. Transfected cells were incubated in the presence or in the absence of 2 mM TA. Reporter activity is expressed in relative luciferase units. All the experiments were made by triplicate and repeated at least three times. The results shown are a representative experiment.

untreated cells (controls), some remaining layer discontinuity is still observed after 24 h. TA treatment is the strongest in stimulating cell migration with a closure around 18 h followed by AC and AP closing the wound at 24 h (Fig. 5).

These two functional experiments support the involvement of TA action in triggering the TGFβ/ALK-1/endoglin pathway in endothelial cells. On the other hand, the experiments suggest that TA way of action is indirect through plaminogen activation inhibition, since treatment with AC and AP, both inhibitors of this activation, leads to similar results.

Discussion

Epistaxis is the most common symptom in HHT patients, appearing in 90% of them. Epistaxis shows up before the third decade of age, but patients normally worsen with age, and epistaxis becomes very severe in 18% of the cases leading to anemia as well as to a decrease in the patient's life quality (12). Different treatments have been assayed in the past including cauterization, estrogen/progesterone treatment, the injection of sclerosing agents (i.e. ethyblock) in the telangiectases, septodermoplastia, embolization or radiotherapy, all of them with a limited and temporal efficiency (33, 35, 36). After all these treatments, an increase in the fibrinolytic activity of the telangiectasic tissue is later observed (37). In all cases, this activity was mediated by a plasminogen activator increase.

From these observations, several authors began the use of antifibrinolytic drugs such as ε-aminocaproic acid initially, and TA later for the treatment of HHT patients (18–21). TA is an

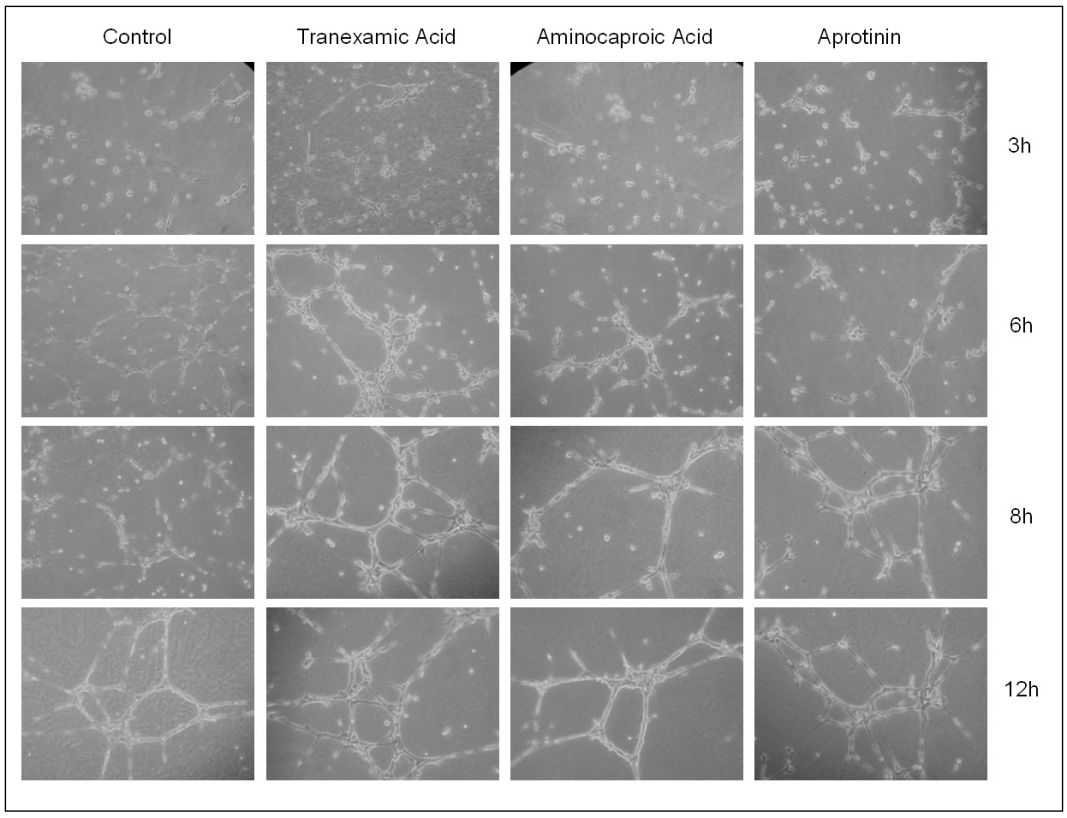

Figure 4: Effect of TA, AC and AP on cord formation. Around 300,000 HMECs were seeded in P-6 matrigel-containing plates and either untreated (Control) or treated with TA (2 mM), AC (2 mM), or AP (5 µg/ml). Cells were photographed at different time points from 3 h to 24 h post-seeding.

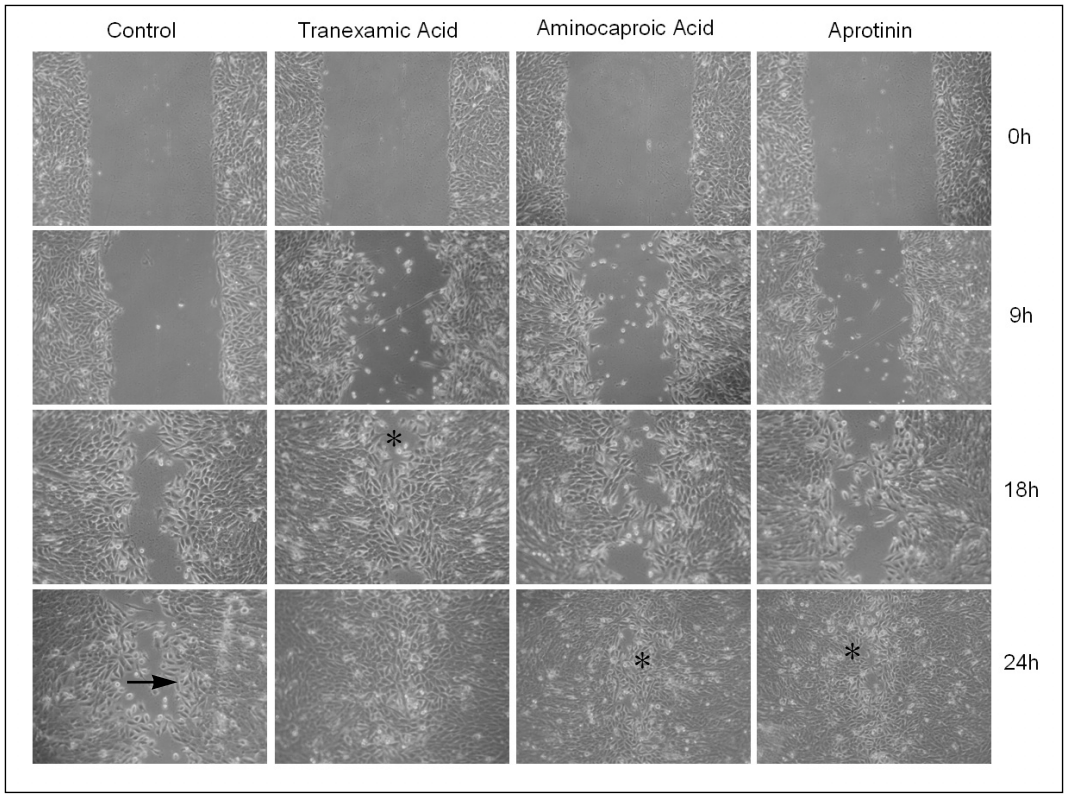

Figure 5: Effect of TA, AC and AP on cell migration after wound healing. HMEC confluent monolayers were disrupted with a pipette tip, and culture medium was removed after the injury. Cells were washed in PBS, reincubated in fresh endothelial medium and either untreated (Control) or treated with TA (2 mM), AC (2 mM), or AP (5 µg/ml). At different time points (from 0 to 24 h) cells were photographed. Asterisks mark the time points when the cells close the discontinuity. The arrow in Control after 24 h, points to the slight discontinuity still present.

antifibrinolytic agent 10-fold more potent than aminocaproic acid and with longer half-life. Thus, it has been postulated that TA would act on HHT patients by inhibiting the fibrinolysis on the wall of telangiectases, where there is an increase of fibrinolytic activity (36, 37). The only contraindication would be in those patients prone to suffer thrombosis (22).

The reports of TA performance in HHT patients are not very abundant in literature and the few known are dealing with a li-

mited number of patients. In these sparse and limited studies, the patients showed a significant decrease in the nose bleeds with a subsequent increase in the hemoglobin levels. None of them showed side effects, nor did they need any blood transfusions. Perez-Molino (33) reported the control of a massive, life threatening haemorrhage in a HHT patient, by oral administration of TA.

The 14 patients included in the present study showed epistaxis interfering with their quality of life. All of them improved significantly, decreasing the nose bleeds and improving the levels of hemoglobin. The need for blood transfusions almost disappeared in four patients who had been depending on them before the treatment. None of the patients showed side effects derived from the TA treatment. Therefore, we can conclude that at the doses applied, up to 1 g/8 h, TA is a safe treatment for HHT patients.

Given the good results obtained with the TA therapy, we have explored its mechanism of action by in-vitro cellular studies, since little or nothing had been reported so far in relation to HHT. Since endoglin/ALK-1 haploinsufficiency is the general admitted origin for the HHT symptoms (38–41), TA effects on endoglin and ALK-1 were assessed. We compared TA effects with AC, an anti-fibrinolytic less potent than TA, and with a plasmin inhibitor, AP, acting at the same level as both anti-fibrinolytic drugs. We have shown that TA improves the protein levels of endoglin and ALK-1 at the surface of the endothelial cell. AC and AP act in the same direction as TA, although AP's effect is only comparable to TA at ALK-1 protein levels, and AC is less potent increasing both endoglin and ALK-1 protein levels.

Moreover, TA also acts at the RNA level for *endoglin* and *ALK-1*, as proven by real-time PCR in endothelial cells. The use of AC and AP also shows a tendency to increase endoglin and ALK-1 RNA level, but the effect is not significant, at least after 24 h.

Finally, the reporter experiments with constructs representing *ALK-1* and *endoglin* promoters showed that TA acts at the transcriptional level, observing about a two-fold increase in both promoters after TA treatment.

In endothelial cells, TGFβ may signal through ALK-1 or ALK-5, and ALK-1 cooperates with endoglin in the transmission of the TGFβ signal (42). The use of a specific TGF-β/ALK-1/endoglin reporter, $(BRE)_2$-luc, and the corresponding ALK-5 reporter, $(CAGA)_{12}$-luc, showed that TA was stimulating preferentially the ALK-1/endoglin alternative, and had no effect on the ALK-5 pathway. A weak, but interesting, effect is the TA stimulation of the PAI-1 promoter in the 3TP-lux reporter. Thus, the main conclusion is that TA, in addition to the described anti-fibrinolytic effects, may trigger an increase in ALK-1 and endoglin protein levels, with the consequent stimulation of the ALK-1/endoglin pathway, affected in HHT patients. This may be an additional reason why TA is more effective in HHT than in other non-HHT haemorrhages.

The way by which TA is able to stimulate this TGFβ pathway remains unclarified. Nevertheless, functional experiments like cord formation and wound healing seem to indicate that TA promotes functions dependent on the TGFβ/ALK-1/endoglin pathway (32, 34), since it increases the angiogenic ability and the migration rate of endothelial cells. It is worth mentioning that angiogenesis involves also migration and disruption of extracellular matrix; however, while angiogenesis and wound-healing migration is dependent on metalloproteinases (not inhibited by antifibrinolytic drugs as TA, AC or AP), fibrinolysis is caused by the serine protease plasmin (43). At this point, the fact that aprotinin mimicks the increase in migration and cord formation would mean that TA stimulates this pathway indirectly through an inhibition of plasminogen activation. This fact opens new perspectives in the search for a link between plasmin activation and the TGFβ pathway in endothelial cells.

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