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Propranolol as antiangiogenic candidate for the therapy of hereditary haemorrhagic telangiectasia

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Summary

The β -blocker propranolol, originally designed for cardiological indications (angina, cardiac arrhythmias and high blood pressure), is nowadays, considered the most efficient drug for the treatment in infantile haemangiomas (IH), a vascular tumour that affects 5–10% of all infants. However, its potential therapeutic benefits in other vascular anomalies remain to be explored. In the present work we have assessed the impact of propranolol in endothelial cell cultures to test if this drug could be used in the vascular disease hereditary haemorrhagic telangiectasia (HHT). This rare disease is the result of abnormal angiogenesis with epistaxis, mucocutaneous and gastrointestinal telangiectases, as well as arteriovenous malformations in several organs, as clinical manifestations. Mutations in *Endoglin* (*ENG*) and *ACVLR1* (*ALK1*) genes, lead to HHT1 and HHT2, respectively. Endoglin and ALK1 are involved in the TGF- β 1 signalling pathway and play a critical role for the

proper development of the blood vessels. As HHT is due to a deregulation of key angiogenic factors, inhibitors of angiogenesis have been used to normalise the nasal vasculature eliminating epistaxis derived from telangiectases. Thus, the antiangiogenic properties of propranolol were tested in endothelial cells. The drug was able to decrease cellular migration and tube formation, concomitantly with reduced RNA and protein levels of *ENG* and *ALK1*. Moreover, the drug showed apoptotic effects which could explain cell death in IH. Interestingly, propranolol showed some profibrinolytic activity, decreasing PAI-1 levels. These results suggest that local administration of propranolol in the nose mucosa to control epistaxis might be a potential therapeutic approach in HHT.

Keywords

Propranolol, HHT, endoglin, ALK1, epistaxis

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Introduction

Propranolol is a non-cardio-selective β -blocker capable of antagonising peripheral β 1- and β 2-adrenergic receptors with the same affinity. It was originally designed for cardiological indications and is actually used for angina, cardiac arrhythmias and high blood pressure. Propranolol is an antagonist of serotonin, a neurotransmitter heavily implicated in the physiopathology of migraine attacks (1). The most recent application of this drug is the treatment of infantile haemangioma (IH) (2–4). IH is a vascular tumour made up of endothelial cells that proliferate under the action of VEGF (vascular endothelial growth factor) and bFGF (fibroblastic growth factor). These haemangiomas are the most common benign tumours of infancy, affecting 5–10% of all infants, and up to 30% of premature babies. In 2008, the antiproliferative effect of propranolol in IH was described (5) and since then, this drug has become the first choice of therapy in these patients. Several mech-

anisms (6) of propranolol action in IH have been postulated: i) vasoconstriction, inducing a reduction of blood flow within the haemangioma with a visible change in colour (5); ii) inhibition of angiogenesis reducing VEGF, FGF or MMP9 expression on endothelial cells (7, 8); and iii) induction of apoptosis (9). These functional effects on the vasculature suggest the therapeutic use of propranolol in other vascular anomalies such as hereditary haemorrhagic telangiectasia (HHT) or Rendu Osler Weber syndrome. This autosomal dominant vascular disease whose clinical manifestations are epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations in the pulmonary, cerebral or hepatic circulation (10, 11), has an average prevalence between 1:5,000 and 1:8,000. Mutations in *Endoglin* (*ENG*) and *ACVLR1* (*ALK1*) genes, cause HHT type 1 or HHT type 2, respectively, in a 90% of the patients (12, 13). In approximately 2% of all HHT patients, the origin of the disease is a mutation in *MADH4* gene, which codes for Smad4 co-activator, leading to the combined

syndrome of juvenile polyposis and HHT (JPHT) (14, 15). A common property of all these genes is that they encode proteins involved in the transforming growth factor (TGF)- β 1 signalling pathway, critical for the proper development of the blood vessels. TGF- β 1 binds to receptor II and the resulting complex recruits and phosphorylates receptor I (RI). In endothelial cells ALK1 is the specific RI, whereas ALK5 is the ubiquitous RI in most of the cell types. The receptor complex also contains the auxiliary receptor Endoglin. RI phosphorylates R-Smads which then associate with Co-Smads, namely, Smad4. The R-Smad/Co-Smad complex translocates to the nucleus to regulate the target genes by binding TGF- β 1 responsive elements in their promoter regions.

The most frequent clinical manifestation of HHT is epistaxis which significantly interferes with the quality of life (16–18). The origin of these epistaxes is the existence of telangiectases on the nasal mucosa, which are very sensitive to slight traumata and even to the air when breathing, giving rise to nose bleeds. Treatments to control epistaxis include minor and major surgeries and pharmacological therapies (19). The drugs to reduce nosebleeds act through different mechanisms of action: antifibrinolytics (tranexamic acid, aminocaproic acid) (20, 21), antioxidants (N-acetylcysteine) (22), or oestrogens, among them raloxifene, a selective oestrogen receptor modulator (SERM) which is, up to now, the only orphan drug designed for the treatment of HHT (23).

Because vascular lesions in HHT are thought to originate from a deregulation of the angiogenic process, inhibitors of angiogenesis could be an option to decrease abnormal vasculature. Indeed, in the last years, studies with two antiangiogenic drugs, bevacizumab and thalidomide, have been conducted, observing a decrease in the epistaxis and gastrointestinal bleeding (24) and vessel normalisation, respectively (25). However, these drugs have poor specificity, affecting a range of physiological processes with severe side effects. Because of the necessity to look for appropriate antiangiogenic drugs, propranolol has been tested in this report. This β -blocker has been used in IH in a large range of doses, with no important side effects so could be an appropriate inhibitor of angiogenesis to normalise the nasal vasculature eliminating epistaxis.

Materials and methods

Cell culture

The *human microvascular endothelial cell* line HMEC-1 (26) was cultured in MCDB131 (GIBCO, Grand Island, NY, USA); Primary *human umbilical vein endothelial cells* HUVEC (LONZA, Walkersville, MD, USA) and from the umbilical cord of an HHT2 newborn, were cultured in EBM (Endothelial Basal Medium, LONZA); and the EOMA (*Mouse Hemangioendothelioma Endothelial Cells*) cell line (27) was cultured in DMEM (Dubecco's Modified Eagle Medium). All culture media were supplemented with 10% fetal bovine serum (FBS, GIBCO) and 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. EGM-2 SingleQuots (LONZA) were added to EBM medium. Plates were previously coated with 0.2%

gelatin in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA). Endothelial cells were treated with propranolol (0–100 μ M) (Sigma). Scratch Wound healing and tube formation assays were carried out in propranolol-treated or untreated HUVEC or EOMA cells as described (24).

Flow cytometry

Propranolol-treated and untreated cells were incubated with anti-Endoglin (P4A4, DSHB, Iowa University) or anti-ALK1 (MAB370; R&D Systems, Minneapolis, MN, USA) mouse monoclonal antibodies and analysed by immunofluorescence flow cytometry as described (28).

Real-time RT-PCR

Total cellular RNA was extracted from HMEC-1 using the RNAeasy kit (Qiagen, Germantown, MD, USA). One microgram of total RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), using random primers. SYBR Green PCR system (BioRad, Hercules, CA, USA) was used to carry out the real-time PCR. Oligonucleotides used were: *ENG* Forward: 5'-AGCCTCAGCCCCACAAGT-3'; *ENG* Reverse: 5'-GTCACCTCGTCCCTCTCG-3'; *ALK1* Forward: 5'-ATCTGAGCAGGGCGACAC-3'; *ALK1* Reverse: 5'-ACTCCCTGTGGTGCAGTCA-3'; *uPA* Forward: 5'-GGCAGG-CAGATGGTCTGTAT-3'; *uPA* Reverse: 5'-GGACTACAG-CGCTGACACG-3'; *PAI-1* Forward: 5'-CACCTCAG-CATGTTTCATTG-3'; *PAI-1* Reverse: 5'-GGTCATGTT-GCCTTTCCAGT-3'; and *18S* as endogenous control, *18S* Forward: 5'-CTCAACACGGGAAACCTCAC-3'; *18S* Reverse: 5'-CGCTCCACCAACTAAGAACG-3'. Samples were used in triplicates and each experiment was repeated twice.

Cell transfections and reporter assays

Transient transfections of HMEC-1 cells were carried out in P-24 plates using 1 μ g of reporters for the *ENG* promoter, pCD105 (-350/+350) in pXP2 (pENG/pXP2) (29), the *ALK1* promoter, pALK1 (-1035/+209) in pGL2 (pALK1/pGL2) (30), the *uPA* promoter, puPA (-2345/+32) in pGL3 (pUPA/pGL3) (31), and the *PAI-1* promoter, p800 (-800/+71) in pUC19luc (pPAI-1/pUC19luc) (32). The constructs of BRE-luc and CAGA-luc, kindly provided by Dr. P. ten Dijke (Leiden University Medical Center, The Netherlands) contain artificial promoters consisting of repeated Smad binding consensus sequences. After transfection, cells were incubated in the absence or presence of propranolol for 24 h. Relative luciferase units were measured in a TD20/20 luminometer (Promega, Madison, WI, USA). Samples were cotransfected

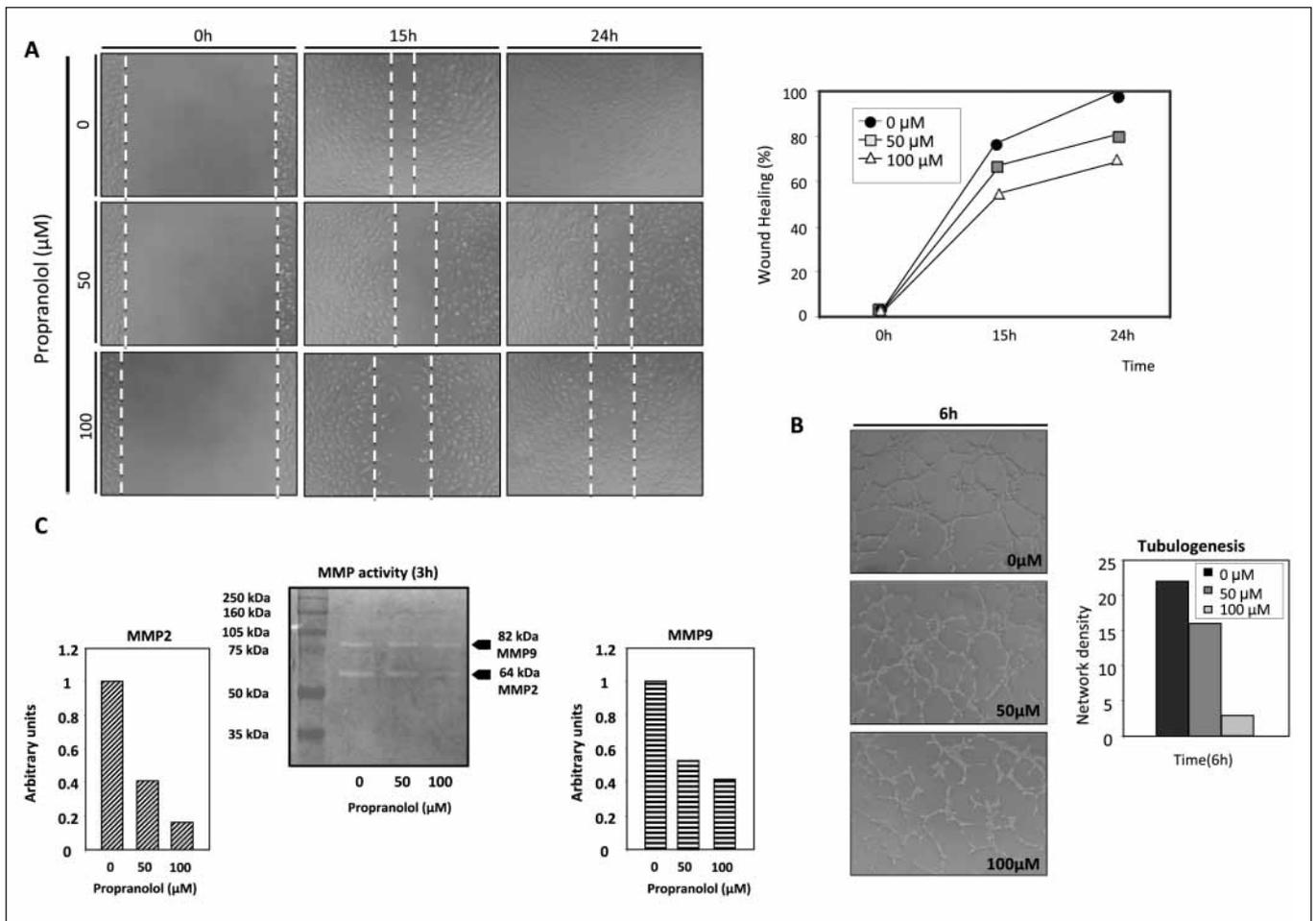


Figure 1: Effect of propranolol as anti-angiogenic drug. A) Scratch-Wound healing. Confluent HUVEC monolayers with or without 50 μM or 100 μM of propranolol, were disrupted with a pipette tip to test the speed of migration with which cells were able to close the wound. Pictures were taken at different times and the speed of migration was quantified by densitometry of the filled space in the wound at each timepoint. The complete closure in untreated cells was between 20–24 h. White discontinuous lines mark the migration edges. Experiments were repeated twice and a representative picture is shown. B) Tubulogenesis assay. Cells were incubated on Matrigel (P-24) coated-plates at 37°C in the absence or presence of propranolol. The cord network formation was measured taking pictures at different times up

to 6 h after cell plating. The appearance of a complete network is observed in untreated and 50 μM treated cells while, at 100 μM there is an incomplete network due to an inhibitory effect of propranolol on endothelial cell tubulogenesis. The picture shown is representative of the whole plate. C) Metalloproteases activity in HMEC-1. Zymography of propranolol-treated HMEC-1 cells shows the presence MMP2 and MMP9 active forms (64 and 82 kDa) expressed in HMEC-1 cells. The activity of both MMPs, decreased in a dose-dependent manner to 0.2- and 0.4-fold, respectively, to untreated cells. This decrease in the endothelial metalloproteases expression promotes the inhibition of migration by propranolol.

with 20 ng/ml of SV40- β -galactosidase vector to correct for transfection efficiency. β -galactosidase activity was measured by using Galacto-light (Tropix, Bedford, MA, USA). Transfections were made in triplicates and repeated in three independent assays. Representative experiments are shown in the figures.

Western blot analysis

Cell lysates were centrifuged at 14,000 g for 5 min. Similar amounts of proteins from aliquots of cleared cell lysates were boiled in SDS

sample buffer and analysed by 10% SDS-PAGE under non-reducing conditions. Proteins from gels were electrotransferred to nitrocellulose membranes, followed by immunodetection with anti-procaspase3 (RB-1197-P1; Thermo Scientific, UK), anti-caspase3 (9662, Cell Signaling, Danvers, MA, USA) or anti- β -actin (A-2103, Sigma). Secondary antibodies were horseradish peroxidase conjugates from Dako (Glostrup, Denmark). Membranes were developed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

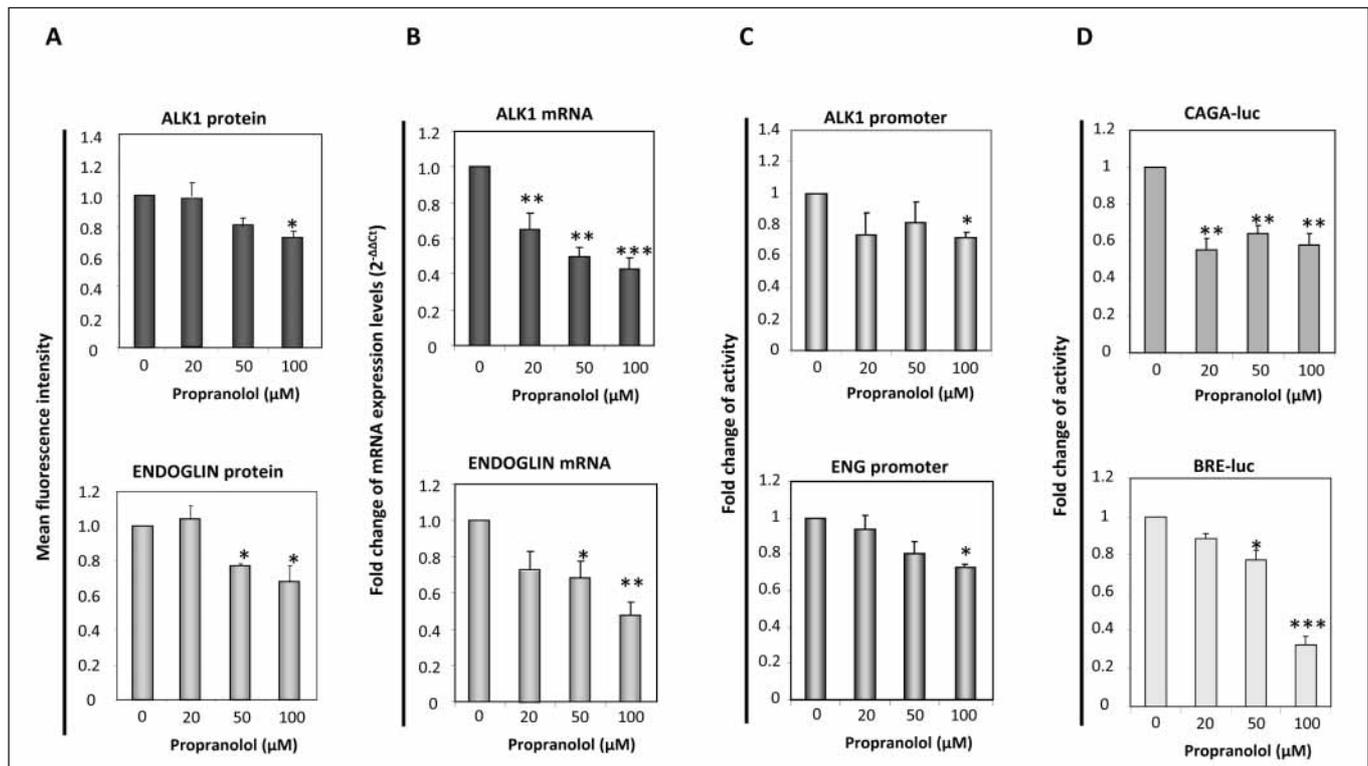


Figure 2: Effect of propranolol in Endoglin and ALK1 expression in HMEC-1. A) Effect on protein expression. HMEC-1 were cultured in the absence or presence of propranolol for 24 h. Levels of Endoglin and ALK1 were measured by flow cytometry. A decreased expression of these proteins (20%) after propranolol treatment was observed. Experiments were performed in duplicate, and each experiment was repeated at least three times. The figure shows a representative experiment. B) Effect on mRNA levels. Real-time PCR experiments were carried out, extracting total RNA from untreated and propranolol-treated HMEC-1. RNA was retro-transcribed and amplified. Endoglin and ALK1 transcription levels were compared with the endogenous control of 18S ribosomal RNA. Propranolol treatment leads to a gradual decrease in both mRNAs (Endoglin and ALK1). Samples were in triplicate in each assay, and the experiment was repeated three times. C) Effect on the

promoter activity. HMEC-1 cells were transiently transfected with the pCD105 (-350/+350) ENG promoter reporter vector or the pGL2 (-1035/+209) ALK-1 promoter construct. Luciferase activity was measured in cells treated with three different propranolol concentrations or in untreated cells. The activity of ENG and ALK1 promoters decreased 0.20-fold and 0.30-fold, respectively. Samples were in triplicate in each assay, and the experiment was repeated three times. D) Effect on specific reporters of TGF- β 1 pathway, BRE-luc and CAGA-luc. The BRE-luc vector contains binding sites for p-Smad proteins that signal via ALK1 pathway, whereas the CAGA-luc construct contains binding sites for p-Smad that signal via the ALK5 pathway. The activity of both reporters was decreased when endothelial cells were treated with increasing doses of propranolol.

Immunofluorescent microscopy

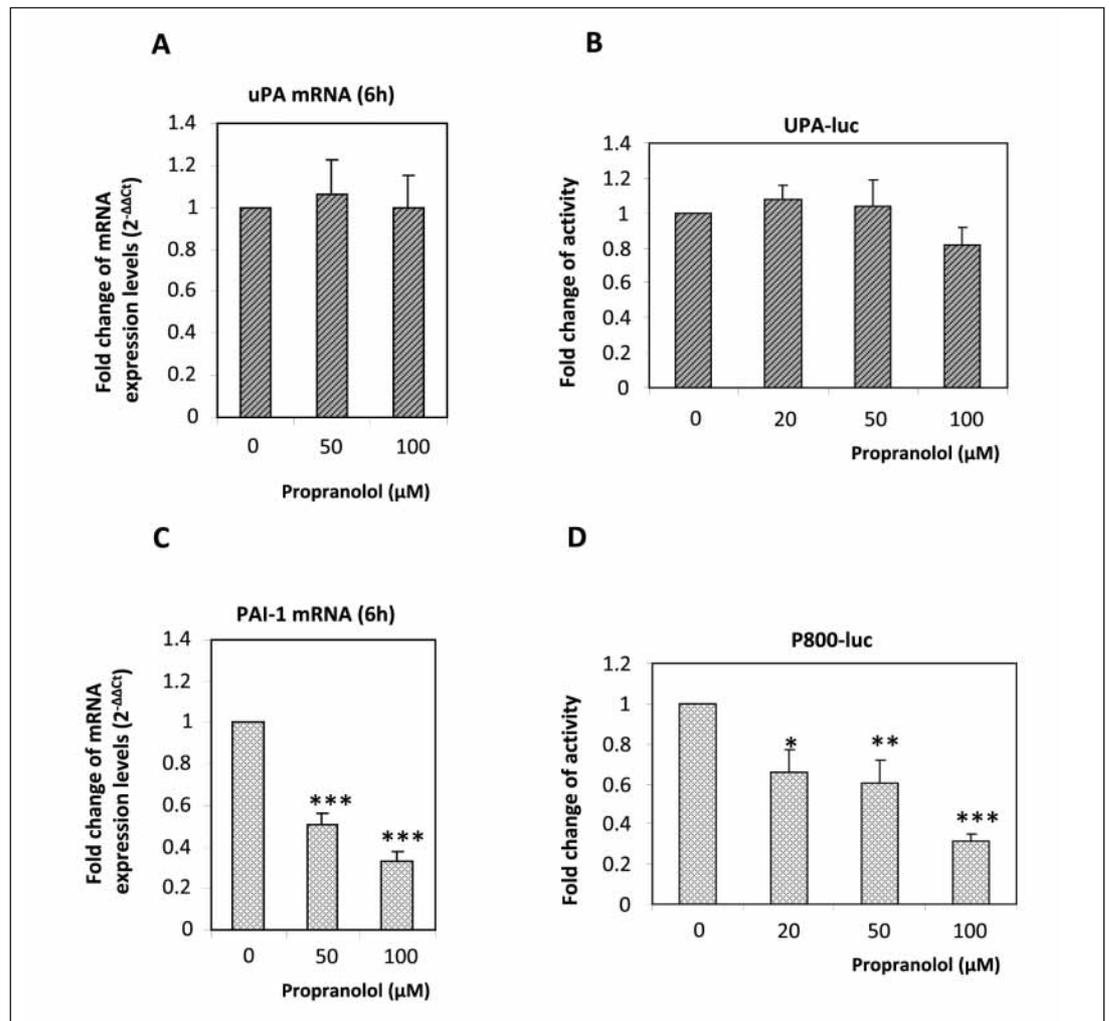
Propranolol-treated cells were grown onto glass coverslips, previously coated with 0.2% gelatin. Cells were incubated with 100 μ g/ml L- α -lysophosphatidylcholine (Sigma), 5 U/ml Phalloidine-Alexa 546 (Molecular Probes, Eugene, OR, USA) and 3.5% formaldehyde (Merck, Whitehouse Station, NJ, USA) in PBS for 30 min at 4°C. Coverslips were mounted with Prolong Gold with DAPI (Molecular Probes) and observed with a spectral confocal microscope Leica TCS SP2 (Leico Microsystems, Nussloch, Germany). Nuclear staining of detached cells was made after collection by centrifugation at 1,500 rpm for 5 min and resuspension in 75 μ l of cold ethanol 70%. Then, 5 μ l of DAPI was added and 25 μ l of the total sample was placed in a slide with a coverslip to be observed in the fluorescence microscope. To study apoptosis, phosphatidylserine (PS) were stained with the Annexin V-FITC Fluorescence Micro-

scopy Kit (BD Pharmingen™). Treated and untreated cells grown onto glass coverslips were incubated during 15 min at room temperature with Annexin V-FITC diluted in Binding Buffer. Coverslips were mounted with Prolong Gold with DAPI and cell images were acquired with an Axioplan Universal microscope (Carl Zeiss, Jena, Germany) and a Leica DFC 350 FX CCD camera.

Proliferation assay with MTT

HMEC-1 and EOMA cells were treated with propranolol (20 μ M, 50 μ M and 100 μ M) during 24 h and 48 h. Then, cells were incubated with MTT (methyl thiazolyl tetrazolium) (Sigma). This tetrazolium salt is reduced by the mitochondrial dehydrogenases to purple formazan in viable cells. The absorbance was measured at

Figure 3: Effect of propranolol in fibrinolysis of HMEC-1. A, B) Levels of uPA RNAm of HMEC-1 were measured by real time PCR. Propranolol treatment did not affect uPA expression or the promoter activity when cells were transfected with the uPA promoter reporter. C, D) mRNA levels of the plasminogen activator inhibitor-1 (PAI-1) were measured. The drug caused a significant decrease in mRNA expression to 0.5- and 0.3-fold with 50 μ M and 100 μ M propranolol, respectively. Transfected cells with the reporter of PAI-1 promoter (p800luc) showed a decrease from the lowest assayed concentration to 0.65-, 0.60- and 0.30-fold with doses of 20 μ M, 50 μ M and 100 μ M, respectively. Propranolol acts as profibrinolytic in HMEC-1 by decreasing the expression of PAI-1, and therefore favouring plasmin activity.



560nm in a Novaspec Plus Visible Spectrophotometer (Amersham Biosciences).

Gelatin zymography

An aliquot of 30 μ l of the serum starved culture media of propranolol-treated and untreated HMEC-1 cells was mixed with sample Laemmli buffer and subjected to SDS-PAGE in a 10% polyacrylamide gel containing 1 mg/ml gelatin. The gel was incubated in 2.5% Triton X-100 three times and washed in distilled water. The gel was incubated o/n at 37°C in an enzymatic reaction buffer stained with 0.5% Coomassie and then destained in 10% acetic acid, 40% methanol in H₂O. Matrix metalloproteinases (MMP) gelatinolytic activity was detected as unstained bands on a blue background.

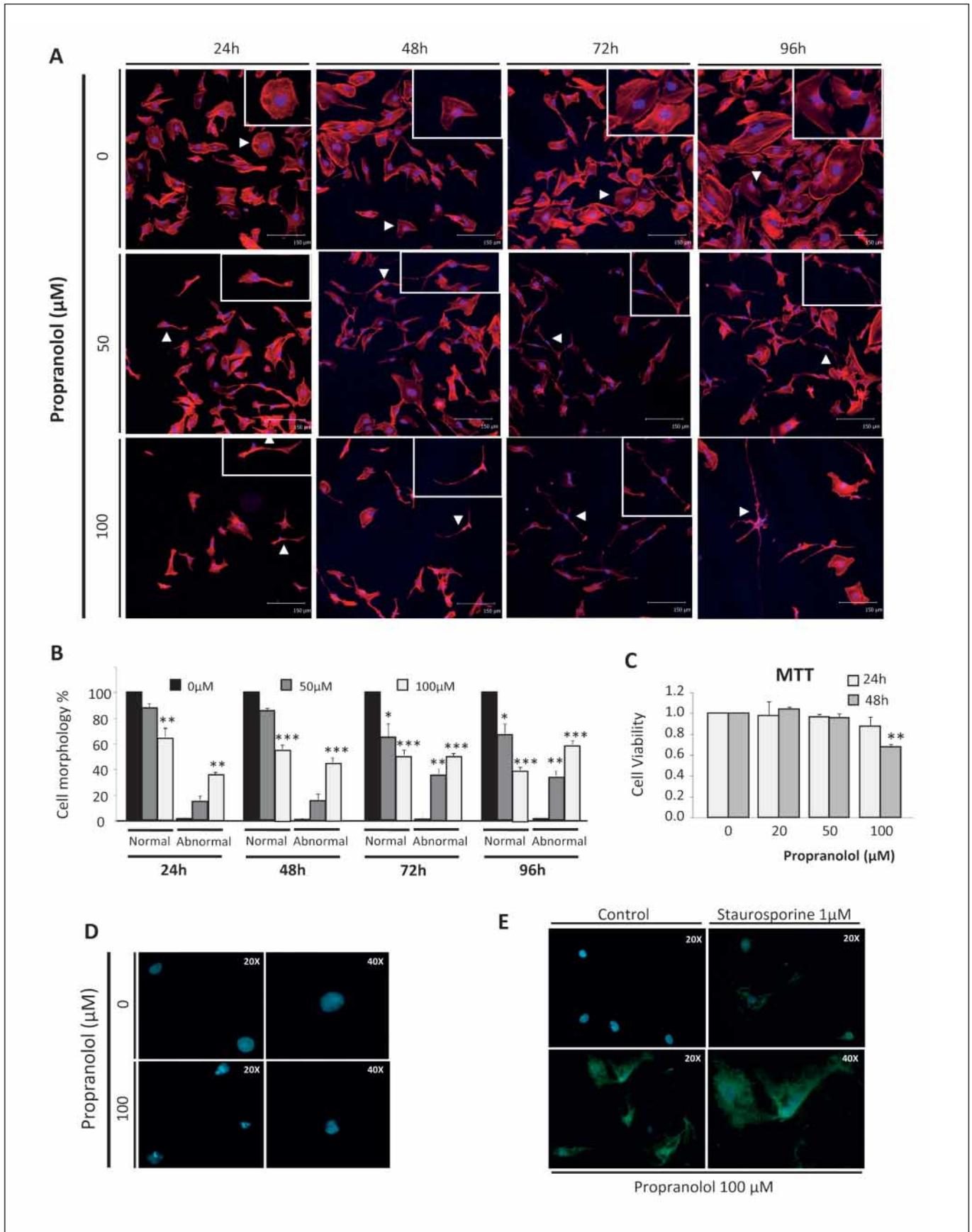
Statistics

Data were subjected to statistical analysis and results are shown as mean \pm SD. Differences in mean values were analysed using Student's t-test. In the figures, the statistically significant values are marked with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$).

Results

Propranolol acts as antiangiogenic drug decreasing HUVECs migration and tube formation

The effect of different doses of propranolol on angiogenesis and scratch wound healing was checked in HUVECs (► Fig. 1A). After scratching the endothelial monolayer (wound), untreated HUVECs, migrated faster than propranolol-treated ones. Indeed, there was a clear delay in the migration of the latter, mainly at 100 μ M propranolol. At 24 h after wounding, untreated cells com-



pletely closed the wound, while propranolol-treated cells still showed the discontinuity in the monolayer. In the tube formation assay in matrigel, HUVEC network developed slower in propranolol-treated cells, being decreased with 50 μM treatment and was completely inhibited at 100 μM (► Fig. 1B). The results of these two functional experiments clearly show the antiangiogenic effect of propranolol on normal endothelial cells.

To assess the MMP activity, HMEC-1 cells were treated with propranolol at doses of 50 μM and 100 μM in serum-starved EBM medium for 3 h. The zymography in ► Figure 1C shows the presence in the culture supernatant of two bands with MMP activity of an approximate size of 64 and 82 kDa. These bands fit with the molecular weights of the MMP2 and MMP9 active forms, respectively. The activity of both bands decreased in a propranolol dose-dependent manner to 0.2- and 0.4-fold, with respect to untreated cells (► Fig. 1C). These results are suggestive of an inhibition of migration by propranolol, due to a decrease in the endothelial metalloproteinases expression, a finding that is in agreement with the scratch-wound healing experiments (► Fig. 1A).

Propranolol treatment decreases ALK1 and Endoglin expression in HMEC-1 cells

In addition to being the target genes mutated in HHT, Endoglin and ALK1 act functionally in the angiogenic process, promoting endothelial cell migration, proliferation and tube formation. At the same time, they inhibit differentiation and activate metalloproteinases' action to degrade the cellular matrix, giving rise to the

new vessel ramification (33). So next, we wanted to check if the anti-migratory and anti-angiogenic effects of propranolol were derived from altered Endoglin and ALK1 expression levels. We assessed the effect of propranolol on Endoglin and ALK1, by measuring the levels of both proteins in *in vitro* cultures of endothelial cells, after 24 h of propranolol treatment, and at different doses, ranging from 0 to 100 μM . In ► Figure 2A, the impact in protein expression of Endoglin and ALK1 after propranolol treatment, relative to untreated cells, is shown. The amount of these proteins decreased at least to 0.8- and 0.7-fold at 50 μM and 100 μM of propranolol treatment (► Fig. 2A).

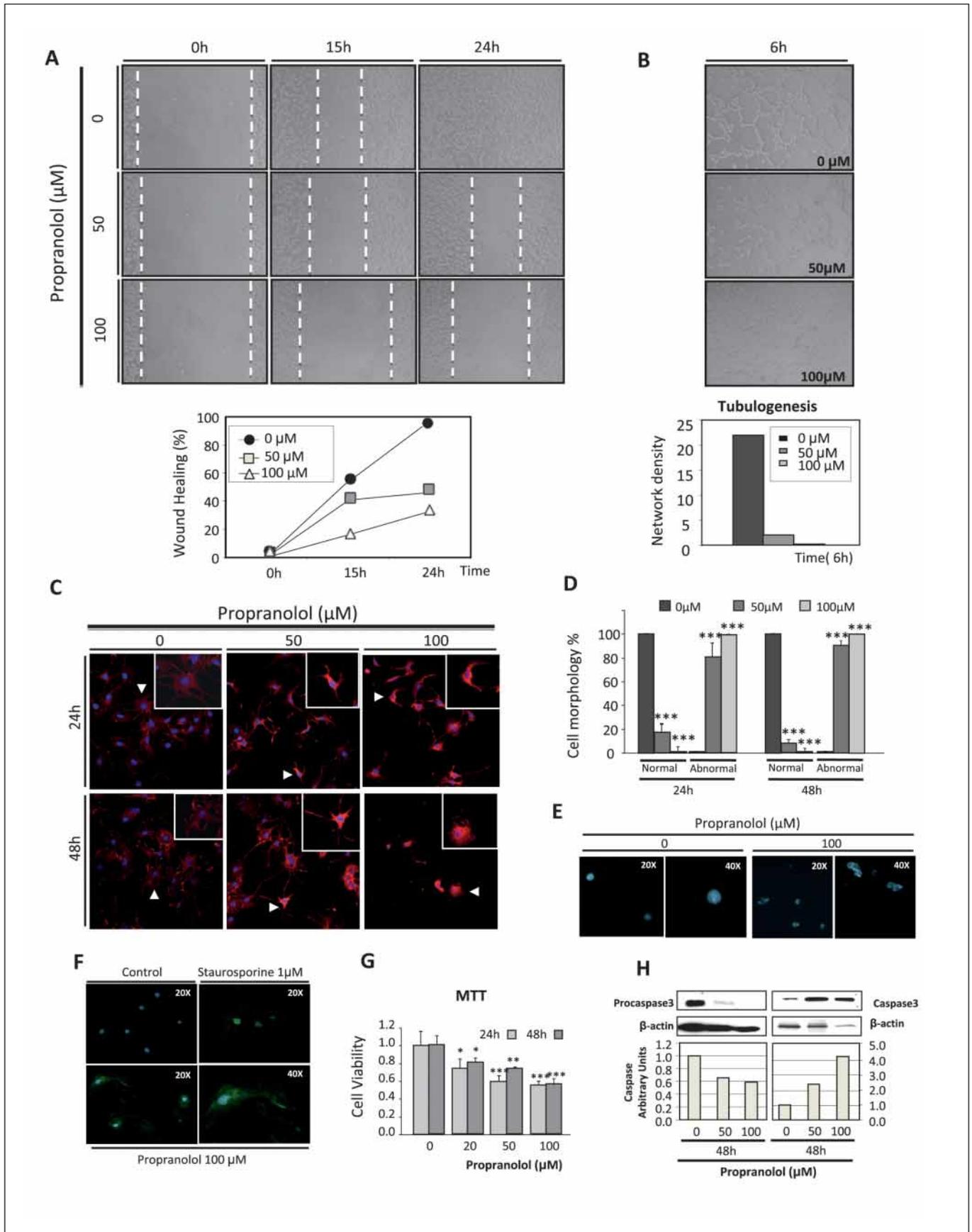
Given that propranolol decreased Endoglin and ALK1 protein levels at the cell surface, we carried out experiments to ascertain if this effect was due to a parallel decrease at the messenger RNA level. Real time-PCR analysis revealed that *ENG* mRNA levels were significantly decreased by 0.73-, 0.67- and 0.46-fold with treatments of 20 μM , 50 μM and 100 μM , respectively. In the case of ALK1, the decrease was of 0.65-, 0.5- and 0.42-fold with treatments of 20 μM , 50 μM and 100 μM , when levels of ALK1 RNA were measured (► Fig. 2B).

As propranolol significantly decreased the levels of *ENG* and ALK1 mRNA, we proceeded to investigate if these effects were due to a decrease in the promoter activity of *ENG* and *ALK1*. HMEC-1 cells were transfected with reporters driven by the respective promoters, and treated in the absence or presence of propranolol. As shown in ► Figure 2C, cells treated with this drug showed a 20% decrease of the luciferase activity driven by *ENG* promoter in treatments with 50 μM and 100 μM of propranolol. Moreover, the *ALK1* promoter activity was even more affected than that of Endoglin from the lowest concentration (20 μM), reaching a 30% decrease.

Altogether, these results suggest that Propranolol inhibits the transcriptional activity of *ENG* and *ALK1* leading to decreased levels of mRNA and proteins from *ENG* and *ALK1*. Since it is known that *ENG* and *ALK1* promote migration, proliferation and angiogenesis, acting through the TGF- β /ALK1/Smad1/5, signalling pathway, the anti-angiogenic properties of propranolol shown in ► Figure 1, could be mediated, at least partially, through the *ENG* and *ALK1* decrease. Because Endoglin and ALK1 are receptors of the TGF- β superfamily members, we next assessed the impact of propranolol treatment on the TGF- β pathway by transfection with specific reporters. BRE-luc and CAGA-luc are reporter vectors that contain artificial promoters consisting of repeated Smad binding consensus sequences; in particular, these are reporters for TGF- β 1/ALK1 and TGF- β 1/ALK5 pathways, respectively. BRE-luc consists of two BRE sites (BMP-responsive elements; GTCT) upstream of the pGL3 luciferase reporter which can be bound by Smad1/5/8. CAGA-luc contains 12 CAGA Smad binding motifs, upstream the pGL3 luciferase reporter.

After propranolol treatment, BRE-luc activity decreased in a dose dependent manner to 0.9-, 0.8- and 0.35-fold with treatments of 20 μM , 50 μM and 100 μM , respectively, while the CAGA-luc activity decreased to 0.6-fold in the 20–100 μM range (► Fig. 2D).

Figure 4: Propranolol promotes a dose-dependent apoptosis effect in endothelial cells. A, B) HMEC-1 cells were treated at different doses and for different periods of time (from 24 h to 96 h) with propranolol. To study cell morphology, actin cytoskeleton and nucleus, cells were stained with DAPI and Phalloidin-Alexa 546 and examined by fluorescence microscopy. Significant changes in morphology in HMEC-1 propranolol-treated cells were observed. Some cells disappeared from the coverslips, by detachment, as a consequence of death. After 96 h of treatment, only very few cells with death morphology still remained on the coverslip. White arrows mark cells magnified in the upper right insets. The graph shows a decrease in the number HMEC-1 cells (panel A) with normal morphology and an increase in the number of cells with abnormal morphology after propranolol treatment. The average of three fields was recorded. C) Propranolol decreases cell viability in HMEC-1. The MTT assay shows the decrease in cell viability after propranolol treatment; it was only statistically significant at 100 μM and 48 h treatment. D) Propranolol causes chromatin condensation in endothelial cells supporting the induction of cellular apoptosis. Nuclei of detached control cells appeared in a round shape, in HMEC-1 cells, while in 100 μM propranolol-treated cells; the nucleus morphology is dramatically affected. Left: magnification 20X; right: magnification 40X. E) HMEC-1 cells were left untreated (top left panel), treated with staurosporine, as positive control (1 μM , 5 h) (top right panel), or with propranolol (100 μM , 9 h) and stained with Annexin V-FITC. Untreated cells were negative for the staining, whereas propranolol-treated cells show staining.



Propranolol affects fibrinolysis in HMEC-1 cells

Propranolol was long ago reported as a profibrinolytic drug (34), due to an increase in the amount of uPA mRNA in human brain endothelial cells (HBMEC). Since HHT telangiectases show high fibrinolytic activity (35, 36), we assessed the properties of propranolol in relation to fibrinolysis in endothelial cells. Treatment of HMEC-1 cells with propranolol did not cause any significant effect in the uPA mRNA expression measured by real time PCR (► Fig. 3A). Similarly, there were no significant differences on the activity of uPA promoter when HMEC-1 cells were treated with 20–100 μ M propranolol (► Fig. 3B).

On the other hand, levels of the plasminogen activator inhibitor-1 (PAI-1) mRNA were measured, and as shown in ► Figure 3C, a significant decrease in mRNA expression to 0.5- and 0.3-fold was observed with 50 μ M and 100 μ M propranolol, respectively. In parallel, cells were transfected with the reporter of PAI-1 promoter (p800-luc). The PAI-1 promoter activity showed a decrease to 0.65-, 0.60- and 0.30-fold with 20 μ M, 50 μ M and 100 μ M, respectively (► Fig. 3D). These results suggest that propranolol acts as profibrinolytic drug in HMEC-1 by decreasing the expression of PAI-1, and therefore favouring the plasmin activity.

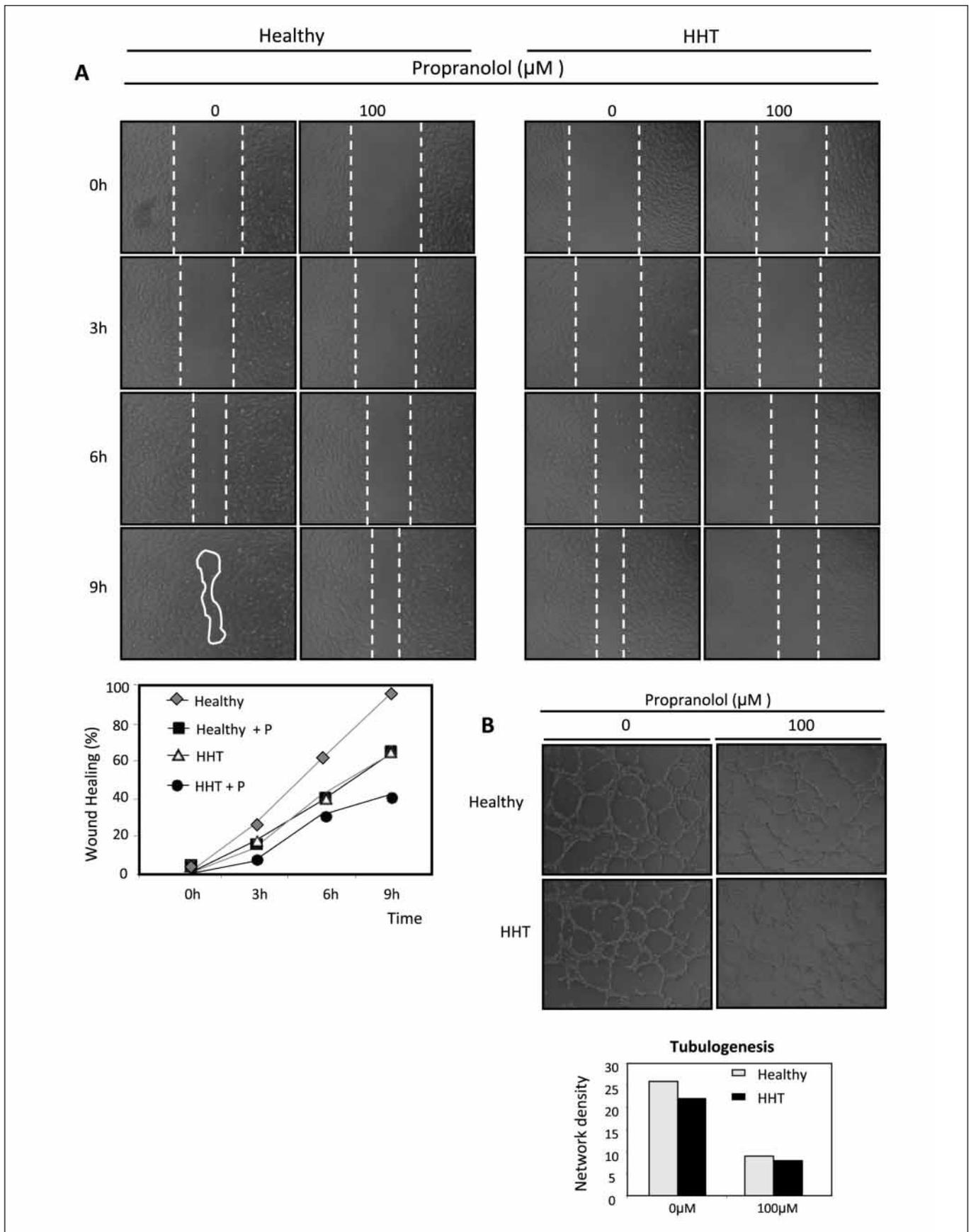
Figure 5: Effect of propranolol on scratch-wound healing and tube formation in EOMA cells. A) A dramatically delayed migration was observed in endothelioma cells with different treatments between 15–24 h. White discontinuous lines mark the migration edges. B) After 6 h of different propranolol treatments, a clear inhibition in tube formation that was complete at 100 μ M was observed. C, D) Propranolol promotes dose-dependent apoptosis in EOMA cells. EOMA cells were treated at different doses and different periods of time with propranolol. Cells were stained with DAPI and Phalloidin-Alexa 546 and examined by fluorescence microscopy. After propranolol treatment cells disappeared from the coverslips, by detachment, as a consequence of death (EOMA being more sensitive than HMEC-1). Only very few cells with death morphology remained on the coverslips. White arrows mark the cells magnified in the upper right corner insets. The graph (panel D) shows the decrease in the number EOMA cells (from panel C) with normal morphology and an increase in the number of cells with abnormal morphology after propranolol treatment at 24 h and 48 h. The average of three fields was recorded. E) Propranolol causes changes in chromatin condensation in apoptotic EOMA cells. Nuclei of control detached cells appear in a round shape at 100 μ M propranolol. The nucleus morphology is dramatically affected, with stronger impact than in HMEC-1 cells, with micronuclei resulting from the cleavage of the original nuclei. Left: magnification 20X; right: magnification 40X. F) EOMA cells were left untreated (top left panel), treated with staurosporine as positive control (1 μ M, 5 h) (top right panel) or with propranolol (100 μ M, 9 h) and stained with Annexin V-FITC. Untreated cells were negative for the staining, whereas propranolol treated cells show apoptotic effects. G) Propranolol decreases cell viability in EOMA. The MTT assay shows the decrease of cell viability from the lowest dose of 20 μ M propranolol, until the highest one of 100 μ M, where the survival decreases to 50%. H) Cell lysates of EOMA treated cells were separated by SDS-PAGE electrophoresis and subjected to Western blot assay. Propranolol decreases the expression of procaspase3 and increases the expression of caspase 3 promoting apoptosis in EOMA cells. As loading control β -actin was used.

Propranolol promotes a dose-dependent apoptosis effect in endothelial cells

Since propranolol has been recently used as a major therapeutic tool in the treatment of haemangiomas, we investigated the mechanism by which propranolol suppresses these benign tumours. Because programmed cell death not only occurs in normal physiology but also in disease states or upon medical treatments, we assessed the possible involvement of apoptosis in propranolol treatments. Apoptosis is characterised by fragmentation of nuclear chromatin, shrinkage of cytoplasm, loss of membrane and activation of different signalling pathways. To study this process, HMEC-1 cells were treated at different doses (20–100 μ M) and for different periods of time (24 h–96 h) with propranolol. Cells were examined by fluorescence microscopy with DAPI and Phalloidin-Alexa 546 staining to study cell morphology, actin cytoskeleton and nucleus. ► Figure 4A, shows that HMEC-1 cells treated with 50 μ M of propranolol undergo significant changes in morphology from a cobblestone-rounded shape in untreated conditions to an elongated, shrank and thin shape, more pronounced with time. During the course of the treatment, some cells detached from the coverslips, as a consequence of death. At the highest dose (100 μ M), the process of cell death is relatively fast. After 24 h, many cells have already lost adherence. The morphology of detached HMEC-1 cells treated with different doses of propranolol was observed by fluorescence microscopy. White arrows mark the cells shown at higher magnification in the insets. ► Figure 4B shows the proportion of each morphology (cobblestone/normal or elongated/abnormal) in the images. Normal morphology decreased with propranolol treatments. Accordingly, in the MTT assays, propranolol decreased significantly proliferation and viability of HMEC-1 at 100 μ M after 48 h treatment (► Fig. 4C). As shown in ► Figure 4D, nuclei of control detached cells appear with a round shape, while in 100 μ M propranolol-treated cells, the nucleus morphology is dramatically affected. Moreover, at 100 μ M, HMEC-1 cells have an irregular shape with centers of condensate chromatin, a property characteristic of apoptotic cells. In ► Figure 4E we can observe how propranolol induced translocation of phosphatidylserine (PS), from the inner (where is normally present) to the outer leaflet of the plasma membrane, a key feature of apoptotic cells. In fact, AnnexinV-FITC stained staurosporine (positive control) and propranolol-treated cells, but not untreated ones. Altogether, we can conclude that propranolol promotes apoptosis in endothelial cells.

Murine model of haemangioendothelioma and propranolol treatment

As propranolol is used to treat IH, we next assessed the effect of this treatment on an endothelioma model of murine origin (EOMA). The results of functional assays of angiogenesis were similar to those shown in ► Figure 1, but with stronger differences between untreated and propranolol-treated cells. The speed of migration to



close the wound in propranolol-treated cells was much slower in EOMA than in HUVECs. Indeed, at 100 μ M propranolol treatment, EOMA cells did not migrate at all (► Fig. 5A). Furthermore, the inhibition of tube formation in EOMA was even stronger than in HUVECs, almost without cellular connections at 100 μ M (► Fig. 5B). Thus, the anti-angiogenic and anti-migratory effect of propranolol was very pronounced in EOMA cells which are otherwise particularly proliferative. We also examined the effect of this drug in the apoptotic process. EOMA cells were treated at different doses (20–100 μ M) and for different periods of time (24 h – 96 h) with propranolol. Cells were examined by fluorescence microscopy with DAPI and Phalloidin-Alexa 546 staining to study cell morphology, actin cytoskeleton and nucleus. As shown in ► Figure 5C, EOMA cells are much more sensitive than HMEC-1 cells (► Fig. 4A). Treatments for 24 h and 48 h were sufficient to impact the cell viability. In the absence of treatment the morphology of these cells is round with many “dendritic-like” extensions, whereas at 50 μ M propranolol, cells are detached from the substrate, and the remaining ones show extremely thin cell bodies with longer and thinner connections. At 100 μ M propranolol, very few cells are viable, with strange morphology, cropped on the coverslips and showing clear signs of apoptosis. White arrows mark the cells at higher magnification in the insets. ► Figure 5D shows that the percentage of normal morphology decreased with propranolol treatment. The detached EOMA cells were observed by fluorescence microscopy after treatment with 100 μ M propranolol. As depicted in ► Figure 5E, nuclei of control cells appear with a round shape, while in propranolol-treated cells, the nuclear morphology is dramatically affected. Also, treated cells have an irregular shape with centres of condensate chromatin. Overall EOMA showed similar results as HMEC-1 cells in response to propranolol, but with stronger impact, appearing micronuclei as a result of the original nuclei cleavage. Moreover, as in HMEC-1 cells, Propranolol induced translocation of phosphatidylserine (PS), from the inner to the outer leaflet of the plasma membrane (► Fig. 5F). The staining with AnnexinV-FITC also revealed apoptotic effects in staurosporine (positive control) and propranolol treatments, but not in untreated cells. In addition, the MTT assay showed that propranolol decreases proliferation and viability of EOMA cells from the lowest dose used. The decrease is higher at 100 μ M, with only 50% of survival (► Fig. 5G). To further support the apoptotic origin of cell death after propranolol treatment, caspase 3 expression was studied. Caspase 3 is one of the key executioners of apoptosis, as it is responsible for the proteolytic cleavage of many key proteins. It

is synthesized as inactive procaspase 3 (32 kDa) and upon activation is cleaved generating two subunits of 17 and 12 kDa. The expression of procaspase3 decreased in propranolol-treated cells, disappearing at the highest dose, while the expression of the cleaved active caspase 3 increased with propranolol, promoting apoptosis (► Fig. 5H). This is one of the therapeutic mechanisms by which propranolol inhibits the growth of IH.

Propranolol treatment reduces migration and angiogenesis of HHT cells

As propranolol effects were tested in HMEC-1 and in EOMA cells, next we wanted to assess the response of endothelial HHT cells. To this end, we performed the scratch-wound healing and tubulogenesis assay with normal and HHT2 HUVECs. The results show the same type of behaviour as shown in ► Figures 1A-B and 5A-B. Basically, propranolol acts as an antiangiogenic drug decreasing both migration and angiogenesis in both types of HUVECs (► Fig. 6A, B). Moreover, the delayed migration and tubulogenesis inhibition were more pronounced in HHT than in control cells.

Discussion

One of the current main applications of propranolol is the treatment of IH, endothelial vascular tumours that proliferate under the action of VEGF and bFGF (3, 4, 6, 7). Nothing is known about its applications in other vascular diseases, so in the present work we have tested the molecular and functional properties of this drug in endothelial cells to explore from an experimental *in vitro* scenario, the possible efficacy of propranolol in the treatment of HHT or Rendu Osler Weber syndrome. The results obtained suggest the possible application of the drug in a controlled cohort of HHT patients.

It is generally accepted that HHT1 and HHT2 pathogenicity is triggered by Endoglin or ALK1 haploinsufficiency. Currently, several drugs have been used, with a certain degree of success, to control epistaxis and gastrointestinal bleeding. These drugs act by at least one of the following strategies: i) Increasing the expression of *ALK1* or *ENG* to compensate the haploinsufficiency, as the SERM raloxifene, or the immunosuppressor FK506 (23, 28); ii) Stimulation of the procoagulant cascade, triggering fibrinolysis inhibition, as ϵ -aminocaproic or tranexamic acid (20, 21); and iii) Normalisation of the abnormal vasculature of the HHT mucosa by antiangiogenesis, thalidomide or bevacizumab (24, 25, 38).

As HHT is due to an imbalance of the angiogenic process, leading to vascular malformations, inhibitors of angiogenesis could be an option to decrease the abnormal vasculature (antiangiogenesis). Recently, the first results with two antiangiogenic drugs, bevacizumab and thalidomide were reported, with a certain success in the bleeding control but with severe side effects, as drawbacks. Thus, we looked for alternative antiangiogenic drugs with fewer

Figure 6: Effect of propranolol on scratch-wound healing (A) and tubulogenesis (B) in HHT HUVECs cells. A) Confluent HUVEC monolayers, treated with or without 100 μ M propranolol, were disrupted with a pipette tip to test the speed of migration. In healthy as well as in HHT HUVECs there was a delayed migration when closing the wound when these cells were treated with propranolol (P). White discontinuous lines mark the migration edges. B) After 6 h of growth on Matrigel (P-24) coated-plates at 37°C, the tube formation was measured and a delay in migration was observed in propranolol-treated cells.

side effects than propranolol, to normalise the anomalous vasculature in HHT patients. The *in vitro* concentrations of propranolol assayed in this report (20–100 μ M) are within the range of the ones used in previous reports (8, 34). These concentrations are also comparable to the administration of 0.5–15 mg/kg/day used in IH patients (3, 4, 6).

We have observed, in addition to apoptotic effects in endothelial cells, that propranolol decreases significantly ENG and ALK1 expression in HMEC-1. This decrease is due to a downregulation of both genes at the transcription level. Thus, propranolol seems to be antiangiogenic, not only because it inhibits VEGF synthesis, but also because of the decrease of both proangiogenic proteins, ENG and ALK1 (► Fig. 2A-C) (37). In addition to MMP decrease, observed as a consequence of propranolol treatment (8, 39), we postulate that the decrease of Endoglin and ALK1 protein levels is also contributing to the inhibition of angiogenesis. In HHT patients the levels of Endoglin and ALK1 are already lower than in normal population, so propranolol would act synergistically lowering Endoglin and ALK1 levels even more, reducing furthermore migration and tubulogenesis in HHT cells. Non-HHT patients taking propranolol systemically might have a slightly decreased wound healing, probably negligible since there are many people taking propranolol for arrhythmias or hypertension, and this effect on wound healing has not been observed by clinicians.

Moreover, propranolol is cheaper than other antiangiogenic drugs, and with fewer deleterious side effects. While writing the

manuscript, a case report of an HHT patient treated with topical timolol was published. Timolol is a β -blocker similar to propranolol, used in glaucoma and superficial haemangiomas. This 48-year-old man experienced 3–4 nosebleeds/day lasting more than 30 min, but after one month of timolol treatment (1 drop in each nostril 3 times/day), his epistaxes decreased to an average of 1–2/week lasting less than 5 min (40). This case supports our *in vitro* results at the clinical level and has encouraged the use of propranolol by local administration to a cohort of controlled HHT patients.

HHT patients have frequent epistaxes due to an abnormal vasculature and the highly fibrinolytic activity of this vasculature (35, 36). Interestingly, Peracchia et al. published that propranolol increased the urokinase plasminogen activator (uPA) expression, involved in fibrinolysis (34). This is in agreement with our results, suggesting that propranolol has fibrinolytic properties. Indeed, although uPA levels were not affected, PAI levels were significantly decreased after propranolol treatment in HMEC-1 cells. The decrease in PAI-1 protein, the natural uPA inhibitor, allows an increase in the active uPA protein, giving rise to fibrinolysis stimulation. At this point, a warning should be made in relation to systemic propranolol administration. The compassionate use of propranolol in a five-year-old girl, affected by HHT, with multiple pulmonary microfistulae led to epistaxis and vaginal bleeding without improvement of the lung condition. In addition, another HHT affected 42-year-old woman was prescribed propranolol for murmur and arrhythmia, leading to a sudden increase of her epistaxes. The withdrawal of systemic propranolol was recommended and epistaxes decreased (unpublished cases from the Spanish HHT Association). The increased fibrinolysis in HHT patients may be only physiologically relevant at the telangiectases sites, where the fibrinolytic activity is very high (35, 36, 40). However, outside the HHT fistulae, and in non-HHT patients, there is no reason to believe that systemic propranolol treatment might lead to bleeding. In this sense, we are not aware of references in the literature describing increased bleeding in patients upon treatment with β -blockers. Of note, propranolol has only been studied in coagulopathies, not affecting the factor VIII levels (41, 42).

Altogether, our results suggest that, given the anti-angiogenic properties of propranolol, this drug could be topically used on nasal mucosa of HHT patients to reduce epistaxis, where there is an excess of abnormal vascularisation that needs to be corrected. Ideally, the treatment should be combined with an antifibrinolytic drug to counteract potential bleeding, especially in the first days after propranolol administration. By contrast, our results do not recommend the systemic administration of propranolol in HHT due to the profibrinolytic effect of this drug in HHT patients.

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Conflicts of interest

None declared.

What is known about this topic?

- Propranolol has been successfully used in therapy of infantile haemangioma (IH) since 2008.
- The effect of propranolol in other vascular diseases, different from IH, has not been assessed so far.
- Antiangiogenesis, a good therapeutic option for bleeding in hereditary haemorrhagic telangiectasia (HHT) is hampered by the side effects derived from drugs under trial: Bevacizumab and Thalidomide.

What does this paper add?

- Propranolol acts as an *in vitro* antiangiogenic drug in endothelial cell lines, in endothelioma-derived cells, and in HHT HUVECs, decreasing the migration and the tubulogenesis, and increasing apoptosis.
- Propranolol decreases the promoter activity of *Endoglin* and *ALK1*, the genes mutated in 90% of the HHT patients, decreasing the surface levels of both proteins, and contributing in this way, to the reduced angiogenesis, a function partially dependent on these proteins.
- The therapeutic use of propranolol in HHT to decrease the anomalous irrigated nose mucosa is promising with no expected side effects. The profibrinolytic effect shown in this work, would advice the topical use of propranolol, or in combination with antifibrinolytics.

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