Estrogen therapy for hereditary haemorrhagic telangiectasia (HHT): Effects of raloxifene, on Endoglin and ALK1 expression in endothelial cells

Virginia Albíñana1,2; Maria E. Bernabeu-Herrero1,4; Roberto Zarrabeitia1,3; Carmelo Bernabeu1,2; Luisa M. Botella1,2

1Centro de Investigaciones Biológicas, Spanish Research Council (CSIC), Madrid, Spain; 2Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Instituto de Salud Carlos III (ISCIII), Madrid, Spain; 3Spanish HHT Unit, Hospital de Sierallana, Torrelavega, Santander, Spain; 4Present address: Siemens S.A., Sector Healthcare, Getafe, Spain

Summary

Hereditary haemorrhagic telangiectasia (HHT), or Rendu-Osler-Weber syndrome, is an autosomal dominant vascular disease. The clinical manifestations are epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations. There are two predominant types of HHT caused by mutations in Endoglin (ENG) and activin receptor-like kinase 1 (ALK1) (ACVRL1) genes, HHT1 and HHT2, respectively. No cure for HHT has been found and there is a current need to find new effective drug treatments for the disease. Some patients show severe epistaxis which interferes with their quality of life. We report preliminary results obtained with Raloxifene to treat epistaxis in postmenopausal HHT women diagnosed with osteoporosis. We tried to unravel the molecular mechanisms involved in the therapeutic effects of raloxifene. ENG and ACVRL1 genes code for proteins involved in the transforming growth factor β pathway and it is widely accepted that haploinsufficiency is the origin for the pathogenicity of HHT. Therefore, identification of drugs able to increase the expression of those genes is essential to propose new therapies for HHT. In vitro results show that raloxifene increases the protein and mRNA expression of ENG and ALK1 in cultured endothelial cells. Raloxifene also stimulates the promoter activity of these genes, suggesting a transcriptional regulation of ENG and ALK1. Furthermore, Raloxifene improved endothelial cell functions like tubulogenesis and migration in agreement with the reported functional roles of Endoglin and ALK1. Our pilot study provides a further hint that oral administration of raloxifene may be beneficial for epistaxis treatment in HHT menopausal women. The molecular mechanisms of raloxifene involve counteracting the haploinsufficiency of ENG and ALK1.

Keywords

HHT, Endoglin, ALK1, epistaxis, raloxifene

Introduction

Hereditary haemorrhagic telangiectasia (HHT), or Rendu-Osler-Weber syndrome, is an autosomal dominant vascular disease with incomplete penetrance characterised by localised angiodysplasia. This is manifested as epistaxis, mucocutaneous and gastrointestinal telangiectases, and arteriovenous malformations (AVM) in the pulmonary, cerebral or hepatic circulation (1). The prevalence is on average between 1:5,000/8,000, 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 Spain has been calculated to be around one in 8,000, according to HHT studies in progress since 2003 (4, 5). There are two main HHT types, type 1 and type 2, which are caused by mutations in Endoglin (ENG) and ALK1 (ACVRL1) genes, respectively (6, 7). In around 2% of all HHT patients the origin of the disease is a mutation in the MADH4 gene, leading to the combined syndrome of juvenile polypsisis and HHT (JPHT) (8). A common property for all these genes is the peculiarity of coding for proteins involved in the transforming growth factor β (TGF-β)-signalling pathway, critical for the proper development of the blood vessels. Recently, the third and fourth HHT loci for unknown genes have been described in chromosomes 5 (HHT3) and 7 (HHT4), respectively (9, 10). It is generally accepted that Endoglin or ALK1 haploinsufficiency is the origin for the pathogenicity of the disease (11). Therefore, the identification of drugs able to increase the expression of those genes is essential to propose new therapies for HHT.

The most frequent clinical manifestation of HHT is epistaxis (nose bleeds), normally from light to moderate (12–14). However, some patients show severe epistaxis which notably interferes with their quality of life. The origin of this epistaxis is the existence of telangiectases on the nasal mucosa. 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 proposed but none of them with conclusive results. The use of antifibrinolytic agents, such as e-aminocaproic or tranexamic acids, systemically administered using oral administration show satisfactory results with an improvement in epistaxis and the associated anemia (15, 16).
However, this is contraindicated in those patients prone to suffer thrombosis. It is necessary to screen for levels of coagulant factors VIII and V and von Willebrand factor, elevated among the HHT population before starting an antifibrinolytic treatment (17). In patients with high levels of coagulation factors, the therapies to avoid bleeding may lead to the risk of suffering deep venous thromboembolism (18). Therefore we need to look for alternative therapeutic sources to counteract HHT epistaxis. Hormonal therapy, using estradiol/norethindrone for epistaxis and gastrointestinal management of HHT, has shown a variable degree of efficacy depending on the patient. Zacharski and co-workers described a case based report with long-term cessation of epistaxis using tamoxifen in a postmenopausal woman (19). The conclusion was that systemic estrogen at doses used for oral contraception may eliminate bleeding in symptomatic HHT and is a reasonable initial option in fertile women. On the other hand, tamoxifen, well tolerated in postmenopausal women, should be considered for randomised clinical trials.

In our Spanish HHT unit, the efficacy of raloxifene is currently assessed in postmenopausal women diagnosed of osteoporosis. Raloxifene is a SERM (selective estradiol receptor modulator) drug of the same group as tamoxifen, and with advantages on bone mineralisation, cardiovascular and gynaecological cancer prevention. Taking into account the initial promising clinical results, an in vitro study on raloxifene effects in cultured cells was carried out to unravel the molecular mechanisms involved in the therapeutic effects. This drug actually increases the expression of Endoglin and ALK1 at the endothelial cell surface, thus counteracting the haemposaliciency of these HHT genes. In relation to the thrombotic risks due to raloxifene treatment, a recent paper (20) shows that in postmenopausal women at increased risk for coronary events, the incidences of venous thromboembolism were higher in those assigned raloxifene (rate of 0.39) versus placebo (rate of 0.27, p=0.02), therefore treatment decisions about raloxifene should be based on a balance of projected absolute risks and benefits.

### Cell culture

HMEC-1 (Human Microvascular Endothelial Cells) is an immortalised cell line that expresses endothelial specific markers and shows an endothelial phenotype (22). This cell line has been kept at least for 30 passages in our lab maintaining the endothelial properties, i.e. expression of endothelial markers. HMEC-1 cells were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium, Gibco, New York, NY, USA) with 10% bovine fetal serum (FBS, Gibco), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Before treatment with raloxifene (Sigma, St. Louis, MO, USA), cells were cultured for 24 hours (h) in DMEM completed with charcoal dextran-treated free hormones FBS (Cambrex, Walkersville, MD, USA). Estrogen treatment was carried out by the incubation of the cells with raloxifene in the latter medium.

### Flow cytometry

Endoglin and ALK1 levels on the cellular surface were analysed by flow cytometry (FACS; Fluorescence-Activated Cell Sorting), using mouse monoclonal antibodies PAA4 (anti-endoglin; DSHB, Iowa University, USA), MAB370 (anti-ALK1; R&D, Minneapolis, MN, USA) and W6/32 (anti-HLA-I, Sigma). Cells were incubated with anti-Endoglin or anti-ALK1 antibodies for 60 minutes (min) at 4°C. After two washes with phosphate-buffered saline (PBS), FITC-F(ab)2 anti-mouse IgG was added and incubated for 30 min at 4°C. Finally cells were washed twice and the fluorescence was measured in a Coulter Epics XL flow cytometer (High Wycombe, UK). The results obtained were expressed as expression index, resulting of the product of positive cell percentage multiplied by the medium fluorescence intensity of the total population.

### Real-time RT-PCR

Total cellular RNA was extracted from HMEC-1 cells using a commercial kit (RNAeasy, Qiagen, Germantown, MD, USA). One microgram of total RNA was reverse transcribed in a final volume of 20 μl with the Kit First Strand cDNA Synthesis (Roche, Mannheim, Ger-
many), using random primers. SYBR Green PCR system (Applied Biosystems, Foster City, CA, USA) was used to carry out the real-time PCR. The sequence of the oligonucleotides was used for ENG, ALK1 and 18S, as endogenous control, were: ENG Forward: 5’- AGCCTCAGGCCCCACAAGT 3’; Reverse: 5’- GTCACCTGTCCCTCTCG 3’; ALK1 Forward: 5’- ATCTGAGCAGGCCGACAC 3’; Reverse: 5’- ACTCCCTGTGTGACCTA 3’; 18S RNA Forward: 5’- CTCAAACGCGGAAACCTCAC 3’; Reverse: 5’- CGCCTCCACCAACTAGAACG 3’.

The samples were used in triplicates and the experiment was repeated twice.

Cell transfections and reporter assays

Transient transfections of HMEC-1 cells were carried out using reporters for the ENG promoter, pCD105 (+350/+350) in pXP2 (pENG/pXP2), and the ALK1 promoter, pALK1 (-1035/+209) in pGL2 (pALK-1/pGL2) in the presence of a commercial transfection reagent (Superfect, Qiagen). After transfection, cells were incubated in the absence or presence of raloxifene 0.2 nM for 24 h. Relative luciferase units 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, Bedford, MA, USA). Transfections were made in duplicates and repeated at least in three independent experiments. Representative experiments are shown in the figures.

Chromatin immunoprecipitation

To obtain nuclear extracts, HUVECs (Human Umbilical Vein Endothelial Cells) subconfluent cell cultures were either treated with 0.2 nM raloxifene for 16h in 10% FBS (charcoal absorbed serum) or left untreated and then collected. Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT Express kit (Active motif, Rixensart, Belgium) following the manufacturer’s instructions. Briefly, HUVECs were grown in three 15 cm² plates until confluence. Then, they were fixed with formaldehyde 1% in Optimum medium (Gibco). Fixation solution was poured off and cells were washed by adding ice-cold PBS. The fixation reaction was stopped by adding glycine stop fix solution and then washed. Cells were scraped in presence of PMSF and lysed with lysis buffer provided with the kit. Nuclei were separated by using a dounce homogeniser and digested with enzymatic shearing cocktail for 15 min. One aliquot of this sheared chromatin was used as “input chromatin” and the rest was incubated with protein G magnetic beads, ChIP buffer 1, Protease inhibitor cocktail and 2 μg rabbit antibody against human Sp1 (specificity protein 1 transcription factor) as positive control (SC-59) and ERβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control anti-human IgG was used. Immunocomplexes were incubated on a rolling shaker for 4 h at 4°C. Protein G magnetic beads with the immunocomplexes were pelleted, washed and eluted with elution buffer provided with the kit. Then the crosslinking was reversed and the samples were incubated with ChIP buffer 2, NaCl and proteinase K during 1h at 37°C. The proteinase K was stopped and centrifuged. The supernatants were used for PCR. Primers used for PCR were selected by mapping the proximal region of promoter sequence; –147 to +7. Sequences of the two primers were as follows:

Fwd ENG 5’- GTGCTTGGAGACACAGCCA 3’
Rev ENG 5’- GGAGGCAGAAATGCCTC 3’

Wound healing and tube formation assay

In vitro-scratched wounds were created by scraping confluent HMEC-1 monolayers in P-24 plate wells with sterile disposable pipet tips. The remaining cells were washed with PBS and incubated with DMEM in the absence or presence of raloxifene for up to 24 h. Endothelial cell migration into the denuded area was monitored by photography of the plates at different times. For tube formation assays, HMEC-1 were plated in DMEM culture medium in the absence or presence of raloxifene on P-6 well matrigel plates (BD Biosciences, Bedford, MA, USA) and incubated at 37°C, as indicated by the manufacturer. Tube formation was monitored for 24 h.

Statistics

Data were subjected to statistical analysis and results are shown as mean ± SD. Differences in mean values were analysed 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 (* p<0.05).

Results

Raloxifene therapy for epistaxis in HHT patients

Based on previous reports describing a good response of epistaxis to tamoxifen, an estrogen receptor modulator, the efficacy of raloxifene in 19 postmenopausal HHT women was assessed. These women, diagnosed of osteoporosis with ages ranging from 47 to 74 years, had no contraindication for the hormonal therapy, and were good candidates for a hormonal substitutive therapy. At the beginning of the treatment, patients had coagulation values, especially of factors VIII and V, on the average. Of the nineteen patients following the treatment, 11 were HHT2, six HHT1, and two with Curaçao criteria for HHT, but without molecular assessment. All patients met, at least three out of the four Curaçao criteria to be considered HHT patients. All of them had epistaxis, telangiectases and
familiar inheritance. Not all of them had the complete screening made; therefore, we cannot include data on the existence of AVMs. All of them showed an improvement of the HHT symptoms concerning epistaxis. The effects on epistaxis were evaluated after 6 months based on the scale by Sadick et al (21). This scale evaluates the amount and frequency of nose bleeds. A decrease of at least one grade in the scale in frequency and quantity of epistaxis was observed in all patients according to Figure 1. Also, as shown in Figure 1 (right hand histograms), the average of Sadick frequency in the nineteen patients before the treatment was 2.36, and after treatment 1.31. On the other hand, in the same figure, to the right bottom the average of Sadick quantity is shown as 2.26 before the treatment and 1.42 after raloxifene. As shown, the difference in both cases was statistically significant. In addition, an average raise in haemoglobin levels of 9.25 % (before treatment 11.18 ± 0.10 and after treatment increased to 12.08 ± 0.15) was observed. There were no relevant side effects, although liver transaminase levels were about double of the usual values, but there were no alterations of hepatic function. In spite of the reported increases in coagulation factors due to raloxifene treatment (23), the coagulation values were normal. There were no thromboembolic side effects. Tolerance was good in all cases, without digestive or vegetative side effects detected. These results suggest that raloxifene may be useful in the management of epistaxis offering a wide spectrum of indications and benefits especially in women. Given these positive results we next assessed the in vitro effects of raloxifene on endothelial cells.

**Effect of raloxifene on ALK1 and Endoglin protein expression at the surface of endothelial cells**

As HHT1 and HHT2 are caused by haploinsufficiency in Endoglin and ALK1, respectively, we explored the putative increase in both proteins after raloxifene treatment in in vitro cultures of endothelial cells. The effect of raloxifene on ALK1 and Endoglin levels was measured by flow cytometry after drug treatment, in a range from 0 to 10 nM concentration. A total of four independent experiments were carried out and the expression index of Endoglin and ALK1 and quantity was calculated for the nineteen patients before and after raloxifene treatment. Means with the standard deviations are shown in the histograms. Differences were statistically significant according to t-test.

*p value of 0.05.

---

Figure 1: Raloxifene as a therapy for epistaxis in HHT patients. The frequency and quantity of epistaxis, measured by the Sadick scale, is shown in postmenopausic women before and after six months of treatment with 60 mg of raloxifene every day. To the right side an average of the Sadick frequency and quantity was calculated for the nineteen patients before and after raloxifene treatment. Means with the standard deviations are shown in the histograms. Differences were statistically significant according to t-test.

*p value of 0.05.
was recorded. The levels of HLA-I membrane protein were taken as reference. Expression index of Endoglin was increased in cells treated with 0.1 nM raloxifene, but decreased at 1 nM (Fig. 2A). In a range from 0.1 to 0.5 nM the highest Endoglin expression was reached at 0.2 nM raloxifene (Fig. 2B). The behaviour of ALK1 was similar to Endoglin (Fig. 2C and D). In summary, doses of raloxifene between 0.05–0.2 nM gave rise to an increase in the expression of Endoglin and ALK1 at the cell surface, 0.2 nM being the optimal concentration.

The concentrations used in the in vitro experiments cannot be extrapolated directly to the therapeutic doses applied with raloxifene (60 mg is equivalent to 22 μM). In vitro experiments normally deal with higher doses in order to be able to detect significant effects within a short time of observation. In fact, the concentration used in cell culture to study drug effects is normally in the range of micromolar, 100 μM being in the upper limit. In the present study, the range of treatment falls within the lower range (0.05–10 nM), with an optimal concentration of ~0.2 nM. In postmenopausal women the physiological level of estrogens circulating in blood are around 9.8 pM. This means 500 times less than the doses we have applied to the cells, but the parameters are not directly comparable.

Effect of raloxifene on the expression of ENG and ALK1 mRNA

Given that raloxifene increased the levels of Endoglin and ALK1 proteins at the surface of endothelial cells, we investigated if the effect was due to an increase in the levels of the messenger RNA from these genes. Experiments of real-time RT-PCR were performed and we observed that levels of mRNA of ENG and ALK1 were stimulated by 1.5 and 2-fold, respectively, when cells were treated with 0.2 nM raloxifene (Fig. 3A and B).

Effect of raloxifene on the promoter activity of ENG and ALK1

As raloxifene not only increased the levels of protein, but also those of mRNA we analysed if these effects were due to an increase in promoter activity of ENG and ALK1. To this end, cells were transfected with two reporters driven by ENG and ALK1 promoters and treated in the absence or presence of raloxifene. As shown in Figure 4, cells treated with raloxifene showed promoter stimulation...
in both genes of 3.5- and 2.2-fold for ENG (Fig. 4A) and ALK1 (Fig. 4B), respectively.

Although, the proximal promoter of ENG contains functional Sp1 binding sites essential for its basal transcriptional activity (24), a theoretical in silico-analysis of ENG promoter does not reveal any consensus dimer for the estrogen receptor (ERα) in the region stimulated by the raloxifene in the luciferase assay (-350 to transcription start). Only half a site for estrogen consensus is found at position (-308) (25). However, estrogen receptor could still be bound to ENG promoter through its interaction with other transcription factors. To analyse whether ERα is bound to ENG promoter, HUVECs were treated with raloxifene and the crosslinked chromatin was used for a Chromatin immunoprecipitation assay using a couple of primers encompassing the region stimulated by raloxifene in the luciferase assay (-350 to transcription start). As shown in Figure 5, both Sp1 and ERα factors are bound in vivo to ENG proximal promoter, region located between -147 to +7 positions.

Wound healing and tube formation

We performed two types of functional experiments to measure in vitro effects of raloxifene: tube formation as an angiogenesis test, and wound healing as a cell migration assay. Figure 6 shows cord formation in a time-course experiment after raloxifene treatment compared to untreated HMEC-1. Tubes develop faster in raloxifene-treated endothelial cells and the cord network is completely developed in raloxifene-treated cells after 4 h, whereas it requires around 8 h in untreated cells. On the other hand, raloxifene promotes a faster migration of cells after disruption of endothelial monolayers, so while the wound is closed between 20 and 24 h in treated cells, in control cells the closure occurs later, around 48–52 h (Fig. 7). The results of these two functional experiments are in agreement with the observed increase in ALK1 and Endoglin protein levels, and therefore in the proangiogenic effect derived of the functionality of these proteins.
**Discussion**

The pathogenesis in HHT is commonly considered a result of cell haploinsufficiency for Endoglin and ALK1, the two proteins whose genes are affected in 90% of the cases. Therefore, one of the therapeutic approaches is to identify drugs increasing the transcription level of the genes involved in the disease, namely, *Endoglin/ENG* and *ACVRL1/ALK1*. The transcriptional regulation of *ENG* has been elucidated for certain stimuli increasing the transcription such as TGF-β, hypoxia, LXRα agonists or vascular injury through Smads, HIF-1α, Sp1, or KLF6 transcription factors (26, 27, 28). However, many of the transcription factors involved in the regulation of *ENG* promoter remain unknown. On the other hand, little is known about the regulation of *ACVRL1*, whose promoter characterisation is currently being conducted in our group. In this scenario, it was interesting to try the effect of estrogens, which are prescribed for postmenopausal women with osteoporosis, and with probable beneficial effects in the case of HHT epistaxis and gastrointestinal bleeding, according to previous literature reports (29). Since there are no previous references in the literature about the use of raloxifene in men, we cannot discard the putative use of this drug also in HHT male patients.

**Figure 5**: ERα and Sp1 bind to ENG proximal promoter. Chromatin immunoprecipitation was carried out with nuclear extracts of HUVECs. Cells were treated or not with raloxifene 0.2 nM. A pair of oligonucleotides encompassing 130 bp to the transcription start region of ENG promoter were used. As positive control Sp1 antibody was used. IgG was the negative control. The input lane represents the proximal 130 bp fragment of ENG promoter. The arrow points to the 130 bp fragment amplified by PCR after immunoprecipitation.

**Figure 6**: Tube formation. Cells were incubated on Matrigel plates at 37°C in absence or presence of raloxifene 0.2 nM. The cord network formation was measured taking photos at different times up to 20 h after cell plating. A complete network appearance is achieved by 20 h in the case of raloxifene-treated cells, while in untreated controls cells remain partially in tubes with patches of disorganised, sparse cells. The picture shown is representative of the whole plate. In the case of the control plate, approximately half of the cells were not arranged in tubes as shown in the figure. The amount of closed cells in the network of tubes was counted and represented by histograms as a quantitative measure of the network density. Experiments were repeated three times and a representative picture is shown.
Raloxifene, a second generation SERM, exhibits an improved clinical profile versus that of tamoxifen and is approved for the treatment and prevention of postmenopausal osteoporosis. Like tamoxifen, raloxifene reduces the risk of estradiol receptor-positive invasive breast cancer (29). In addition, a procoagulant state characterised by increased factor VIII, XI, and XII plasma levels and by reduced activated protein C (APC) sensitivity has been reported after raloxifene therapy in postmenopausal women (23), although this fact was not observed in the series of patients (data not shown). Whether this procoagulant effect of raloxifene contributes to the therapeutic action on HHT is unknown, but it should not be discarded.

The present study was conducted in postmenopausal women whose indication for treatment was moderate to severe epistaxis and deficient bone mineralisation. As a result of this survey, women reported a decrease in the frequency and amount of nose bleedings. In parallel, in vitro assays were devised to look for the effects of raloxifene at the protein level. Only half a site for any consensus dimer for the estrogen receptor (ERα) in the region Endoglin and ACVRL1, which are haploinsufficient in HHT. Since the levels of these proteins are increased as a consequence of raloxifene treatment, we assessed that both the RNA amount and the transcriptional rate was upregulated by this SERM. Transcriptional activation by the estrogen receptors is achieved through autonomous activation functions (AFs): a constitutive N-terminal AF-1 domain and a C-terminal, ligand-dependent AF-2 domain that comprises a motif conserved between estrogen (ERα) and other nuclear receptors (30). SERMs mimic the agonistic activities of estradiol, the main ERα ligand in inhibiting bone resorption or lowering serum cholesterol levels while preventing the effects of estradiol in uterus and breast tissues. This indicates that SERMs are ERα ligands that have the properties of both, receptor antagonist and agonist. There have been several demonstrations of intrinsic SERM agonistic activity in transcription. McDonnell showed that hydroxytamoxifen can transactivate the estradiol-responsive Complement component 3 (C3) promoter in hepatocarcinoma cells (31). Others have demonstrated that both estradiol and raloxifene can stimulate the activity of the TGF-β3 promoter in vitro (32). Moreover, treatment of ERα-transfected cells with raloxifene, hydroxytamoxifen or ICI-164384 has been found to stimulate an activator protein 1-controlled promoter (33, 34).

The tissue-specific effects of partial estrogen agonists have been attributed to several non-mutually exclusive mechanisms in the literature. The leading view is that there are differences in the interaction of the ligand-occupied receptor with other co-activators and co-repressors bound to the promoters. This has been demonstrated for tamoxifen and raloxifene in breast and endometrial cell lines, and it may explain some of their biological activities on these tissues in vivo. Shang and Brown demonstrated that, at least in breast cells, hydroxytamoxifen and raloxifene act as antagonists by recruiting a co-repressor to a subset of genes (35). In contrast, in uterine cells, hydroxytamoxifen, but not raloxifene, recruited the steroid receptor co-activator SRC-1. Scafones et al. have recently shown that estradiol and SERMs act as agonists in repressing the ERα-controlled promoter from the matrix metalloproteinase 1 (MMP1) promoter (36). This repression is likely a complex process that is controlled by more than one molecular event or interaction.

If transcription is involved in the effect of raloxifene on ENG and ACVRL1 in endothelial cells, it would be interesting to elucidate the mechanism of this particular SERM on the promoters of these two genes. The sequence of ENG promoter does not contain any consensus dimer for the estrogen receptor (ERα) in the region stimulated by raloxifene in the luciferase assay. Only half a site for estrogen consensus is found at position (-308) (25). On the other
hand, the polypurine tract identified as raloxifene responsive element (RRE) is not present in ENG promoter (25). Another possibility is to consider that the estrogen raloxifene, may be bound to the promoter through the interaction with an essential factor for ENG transcription. In this sense, in the promoter of the Fas ligand, the stimulation by estrogens occurs indirectly, by facilitating Sp1-binding to the promoter (37).

As it has been reported that Sp1 is essential for endoglin basal transcription and also for the TGF-β stimulatory effect, we examined whether ERα and Sp1 are binding in vivo to the proximal promoter of ENG. As shown in results, both factors, ERα and Sp1 are present in ENG promoter, and it seems that raloxifene acts by slightly increasing the binding of ERα to the endoglin proximal promoter. We could speculate that the same may occur in ALK1 promoter (manuscript in preparation).

Hardman et al. have shown clear beneficial effects of the SERMs tamoxifen and raloxifene on cutaneous wound healing (38). The in vitro functional effects of raloxifene, on the migration promotion, measured by wound healing assay, and the stimulation of the angiogenic capacity by the tube formation assay, agree with the stimulation of Endoglin and ALK1 triggered by this drug. In fact, both proteins are related to migration and angiogenesis in endothelial cells (39, 40, 41). Consequently, the treatment of HHT patients with raloxifene, decreased the epistaxis significantly, according to the expected increase of Endoglin and ALK1.

Altogether, we can conclude that raloxifene treatment induces in vitro cultured endothelial cells an increase in the amount of Endoglin and ALK1 proteins present in the cell surface, and this is probably due to an upregulation at the promoter level, as shown in results. We also find that raloxifene results in an enhanced endothelial cell number of the HMEC-1 cell line as measured by migration and tubulogenesis. Whether the behaviour of raloxifene-treated HMEC-1 cells in vitro can be extrapolated to that of endothelial cells in vivo where other cell types like connective tissue cells may be involved, remains to be determined. In the HHT patient series studied here, raloxifene decreases the epistaxis, suggesting its potential interest for bleeding management. One factor to be aware of, is that raloxifene treatment increases the odds of suffering deep venous embolism (62% increase), or pulmonary thromboembolism (54% increase), as has been reported (42). However, it is not associated with an acquired resistance to APC (43). Therefore, treatment decisions about raloxifene should be based on a balance of projected absolute risks and benefits.

The search for stimulatory factors in the transcription of ENG and ACVR1L genes is a useful tool for the search of therapeutic drugs overcoming the haploinsufficiency threshold present in the disease. If estrogens really are able to partially counteract ALK1 or Endoglin haploinsufficiency due to HHT, they might not only have a therapeutic, but also a prophylactic effect.

Acknowledgements

This paper is dedicated to the HHT Association of Spanish patients, and in general to all families affected by HHT. We thank Dr. Calvin Vary (Maine Medical center Research Institute, USA) for the ALK1 promoter construct. This work was supported by grants from Ministerio de Ciencia e Innovacion (SAF05–01090 and SAF08–01218 to LMB, and SAF2007–61827 to CB), Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Instituto de Salud Carlos III (ISCIII-CIBER CB/06/0038), Fundación Ramón Areces of Spain, and Instituto Fundación Marqués de Valdecilla (IFIMAV).

References


