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Expression of Endoglin mRNA and Protein in Human Vascular Smooth Muscle Cells

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Endoglin, the gene linked to the autosomal dominant vascular disorder hereditary hemorrhagic telangiectasia type 1 (HHT1), encodes a 95-kDa membrane-bound proteoglycan which binds TGFβ1 and regulates signaling via the type I and II TGFβ receptors on the surface of vascular endothelial cells. Using reverse-transcription polymerase chain reaction (RT-PCR) and Northern blot analysis we have shown that endoglin mRNA is expressed in both cultured human VSMCs and VSMCs freshly isolated from human aortas. Northern blot analysis was also used to demonstrate that endoglin expression decreased in serum-stimulated cultured human VSMCs but could be maintained by exogenous TGFβ1. Endoglin protein expression in human VSMCs was shown by immunocytochemistry. These data, the first describing the existence of endoglin in VSMCs, suggest that through regulating TGFβ1 signaling endoglin may mediate the effects of TGFβ1 on VSMC behavior *in vitro* and *in vivo*.

The ability of vascular smooth muscle cells (VSMCs) to modulate their phenotype, proliferate and secrete extracellular matrix underlies their crucial role in the development and progression of a number of vascular disorders [1]. Each of these aspects of VSMC behavior can be modified by transforming growth factor beta 1 (TGFβ1), an autocrine growth factor thought to play a significant role in the pathogenesis of vascular diseases [2-7].

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Abbreviations used: VSMCs, vascular smooth muscle cells; TGFβ, transforming growth factor beta; RT-PCR, reverse transcription polymerase chain reaction; HHT, hereditary hemorrhagic telangiectasia.

Endoglin is a membrane-bound proteoglycan, similar to betaglycan, which has been shown to bind TGFβ as well as the type I and II TGFβ receptors (TβRI/II), thus, it is often referred to as the type III TGFβ receptor. Human endoglin consists of two 95 kDa disulphide-linked subunits containing N- and O-linked oligosaccharides. Its primary sequence contains a 561 amino acid extracellular domain, a single transmembrane region, and a short cytoplasmic tail of 43 amino acid residues. Endoglin was found to bind TGFβ1 and β3 with high affinity, furthermore, immunoprecipitation experiments using antibodies specific for endoglin or TβRII implied a role in TGFβ signaling as endoglin co-immunoprecipitated with both the type I and II receptors in the presence of TGFβ ligand [8]. Endoglin was originally identified in human vascular endothelial cells and was shown to be highly expressed on human umbilical vein endothelial cells in culture [9]. It was subsequently found to be expressed in murine ovary, uterus, heart, and skeletal muscle, and at low levels in placenta and spleen. Endoglin tissue distribution is remarkably similar to that of TGFβ1, particularly in the heart and uterus, suggesting that it may be involved in TGFβ1 signaling in stromal fibroblast-like cells [10]. However, it is unclear what role endoglin has in TGFβ1 signaling since no signal transduction domains are present in its cytoplasmic domain, thus it cannot mediate TGFβ signaling on its own. Therefore, it has been suggested that endoglin acts as a reservoir and/or presenter of TGFβ1 ligand to the heteromeric TβRI/II signaling receptor complex.

A dramatic example of the role of endoglin in the regulation of arteriovenous development was identified with the discovery that the endoglin gene, which maps to 9q34 [11], is linked to hereditary hemorrhagic telangiectasia (HHT) [12]. HHT, the first human disease defined by a mutation in a member of the TGFβ receptor complex, is an autosomal dominant disorder characterised by multi-systemic vascular dysplasia and recurrent hemorrhage from vascular lesions [13]. The most

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severe cases of HHT involving pulmonary arteriovenous malformations are strongly linked to the endoglin locus [14]. Indeed, a recent study by Pece *et. al.* [15] demonstrated that mutant endoglin in HHT type 1 is only transiently expressed intracellularly and does not form heterodimers at the cell surface. If the initiating event in the formation of a HHT vascular lesion is the inactivation of the normal endoglin allele, then this suggests that endoglin is required for maintaining vascular structure and regulating vasculogenesis.

Thus, given its role in regulating TGFβ1 signaling and its involvement in a vascular disorder, it was important to determine whether endoglin mRNA and protein were present in human VSMCs. In the present study, the expression of endoglin mRNA and protein in human aortic VSMCs were investigated. We provide the first evidence for the existence of both endoglin mRNA and protein in VSMCs and suggest that endoglin may have a role in regulating the effects of TGFβ1 on human VSMCs.

MATERIALS AND METHODS

Human VSMC culture. A primary culture of human VSMCs was established by explanting 2 mm^2 pieces of aortic tunica media freshly isolated from the thoracic aorta of an organ transplant donor. Cells grown from the explants were identified as VSMCs by their typical appearance and growth characteristics, as well as by positive immunofluorescence with anti-α-SM-actin and anti-calponin antibodies. Cultured VSMCs were grown in M199 supplemented with 20% fetal bovine serum (FBS), incubated at 37°C in 5% CO_2, and split 1:2 (vol/vol) on reaching confluence. VSMCs were made quiescent by incubating 50% confluent cultures in FBS-free M199 for 72 hours. Since the population doubling time of human VSMCs derived by the explant technique is approximately 35-40 hours [16], this was long enough to induce growth arrest (Go). Periodic microscopic analysis was used to measure approximate cell numbers before and after the re-application of serum. Fifth to eighth passage human VSMCs were used for these studies.

Isolation of total RNA from cultured and enzyme dispersed human aortic VSMCs. Isolation of RNA from human medial VSMCs first required enzymatic dispersion of the human aortic tissue. Segments of thoracic aorta from two female donors where obtained at the time of transplantation and kept moist in ice cold M199. After cutting open the vessel, the endothelium was completely removed by scraping the lumenal surface with a scalpel. Strips of tunica media containing the VSMCs were removed with forceps, taking care not to remove any of the adventitia, and chopped into 1-2 mm^2 pieces in a petri dish containing 3 ml M199. These were enzyme digested using 10 ml collagenase (3 mg/ml) (Sigma) and 5 ml elastase (1 mg/ml) (Sigma) at 37°C in a shaking water-bath until a single cell suspension was obtained. Cultured VSMCs were isolated from the surface of plastic culture flasks by washing twice in EBSS followed by incubation in 1 ml 1× trypsin/EDTA solution for 3 minutes.

Freshly dispersed cells and trypsinised cultured cells where washed twice in ice-cold 1× PBS and collected by centrifugation at 900 rpm. Total cytoplasmic RNA was isolated from these cells by lysis in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM $MgCl_2$ and 0.5% (vol/vol) Nonidet P-40 for 4 minutes on ice. Nuclei and cell debris were peletted at 3000 rpm for 5 minutes and the supernatant was made 1.5% with sodium dodecyl sulphate. This was extracted twice with citrate buffered phenol (pH 4.5) and centrifuged at 3000 rpm. RNA was precipitated and resuspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water. RNA stocks were aliquoted to prevent repetitive freeze thawing and stored at−-70°C.

RT-PCR and DNA sequence analysis. cDNA was synthesised from 1 μg of total human VSMC RNA and made up to 12.5 μl with DEPC-treated H_2O. The RNA was denatured at 75°C for 3 minutes, spun briefly and placed on ice. Moloney Murine Leukemia Virus reverse transcriptase (M-MLVRT) was used to synthesise first-strand cDNA in a 20 μl reaction containing; 12.5 μl denatured RNA, 1× RT buffer, 0.5 mM each dNTP, 1 μM oligo(dT)-18 primer, and 200 units M-MLVRT. This was incubated at 42°C for 1 hour and the reaction terminated by further incubation at 94°C for 5 minutes. The cDNA products were diluted 5-fold by adding 80 μl DEPC-treated H_2O. Amplification of an 805 bp coding region of human endoglin (position 231-1037, EMBL sequence X72012) was achieved using 20mer sense (5′-TGCCACTGGACACAGGATAA-3′; 231-250) and anti-sense (5′-GATGAGGACGGCATCGAGAT-3′; 1018-1037) oligonucleotide primers in a total reaction volume of 50 μl. The composition of the PCR mixture was as follows; 10 μl human VSMC cDNA, 1× PCR buffer, 1 mM $MgCl_2$, 100 μM each dNTP, 20 pmol of each primer, and 2.5 units of Taq. polymerase. Reactions were overlaid with one drop of mineral oil and subjected to 30 PCR cycles of 94°C denaturation (2 minutes), 55°C annealing (1.5 minutes), 72°C extension (2 minutes), followed by a final extension at 72°C for 6 minutes. Amplified cDNA products were resolved on 1% agarose gels, purified, and cloned into the pT7Blue dT:dA cloning vector (Novagen). The cloned insert was sequenced using the M13 (−40) forward and T7 reverse sequencing primers within the pT7Blue vector. Sequence information was compared with the human endoglin sequence (EMBL accession number X72012).

Northern blot analysis. Between 10-15 μg of human VSMC total RNA (in 10 μl) was denatured in 30 μl denaturation buffer (19 μl formamide, 7 μl formaldehyde, 4 μl 10× MOPS), heated at 60°C for 5 minutes, then placed on ice. 5 μl loading buffer was added and the samples electrophoresed at 160 mA in 1.5% agarose gels containing 2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA, and 0.5 μg/ml ethidium bromide. RNA was transferred from the gel by capillary blotting to nylon membrane and cross-linked using 254 nm UV radiation at 1.5 J/cm^2 before being probed and exposed to X-ray film for 48-72 hours at −70°C with intensifying screens. Radiolabelled endoglin cDNA probes were generated by random hexamer priming and Klenow extension with [α-^{32}P]dCTP.

Immunocytochemistry. Human VSMCs were seeded onto glass cover-slips in six-well plates and left for 24 hours in 10% FBS to adhere and flatten. Cells were washed twice in ice-cold 1× PBS then fixed in 1 ml 3% formaldehyde/PBS at 4°C for 45 minutes. Following two more washes in ice-cold PBS the cells were permeabilised in 0.5 ml 0.5% Nonidet-P40/PBS at room temperature for 3.5 minutes, then washed three times in PBS before being 'blocked' overnight in 1 ml 3% BSA/PBS (blocking buffer) at 4°C. The monoclonal anti-endoglin antibody was prepared by the Developmental Studies Hybridoma Bank, Johns Hopkins University School of Medicine, University of Iowa, USA, and was raised against endoglin purified from human umbilical vein endothelial cells in RBF/DnJ mice. Primary anti-endoglin and anti-α-SM-actin (Sigma) antibodies were made up in 200 μl blocking buffer and incubated on the cells for 1 hour at room temperature. In addition, mouse IgG was used to control for non-specific binding of the anti-endoglin antibody. After removal of the primary antibodies or control IgG a secondary FITC conjugated antibody (Sigma), diluted to 200 μl in blocking buffer, was added to the cells in the absence of light for 30 minutes at room temperature. Cells were washed twice in blocking buffer, rinsed briefly in 150 μl of 20 μg/ml bisbenzimide (Sigma) to stain the nuclei, and washed twice in PBS. Immunofluorescence was studied using a Zeiss Axioskop fluorescence microscope and photographs were taken using Fujicolour 100 film at exposures of 30 or 60 seconds.

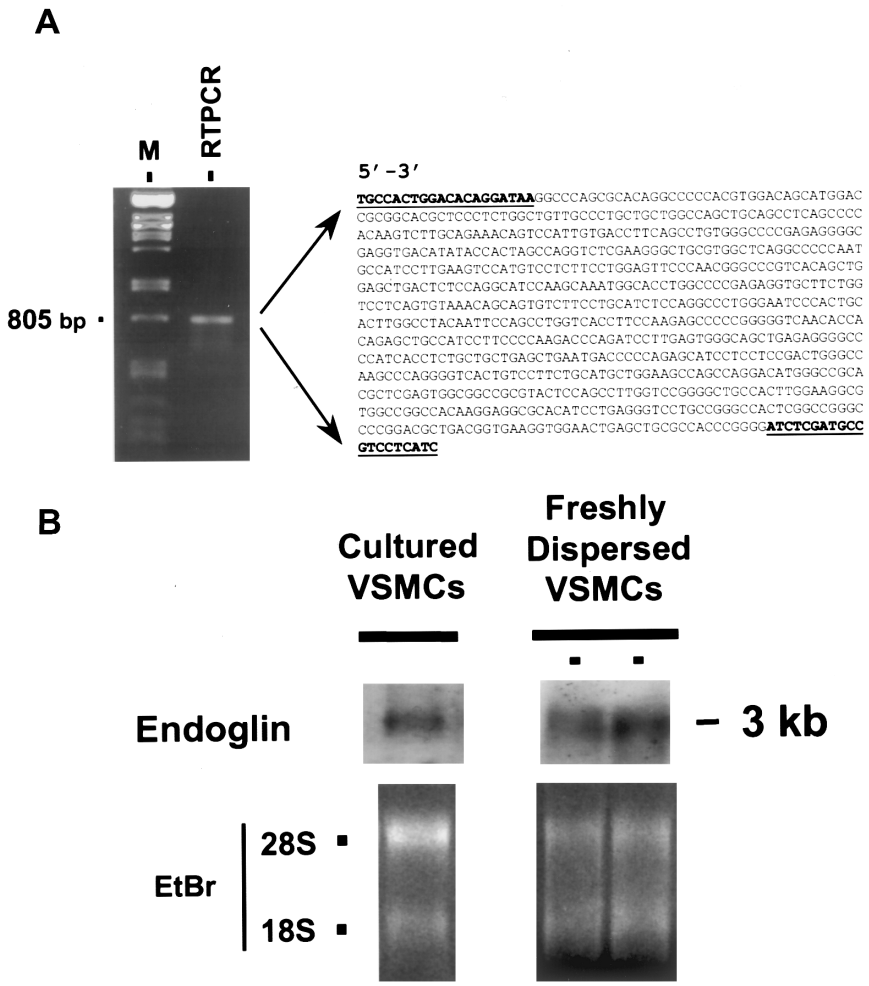

FIG. 1. Endoglin mRNA expression in human VSMCs. (A) RNA isolated from cultured human VSMCs stimulated with TGFβ1 for 8 hours was used to detect an 805 bp fragment of human endoglin by RT-PCR (region 321-1037, EMBL sequence X72012) The 20mer endoglin PCR primers used are shown bold and underlined. The 805 bp PCR product was purified, cloned, and the DNA sequenced to confirm its identity. M; Pst 1 cut lambda phage DNA marker. (B) The endoglin cDNA was radiolabelled and used to probe Northern blots containing RNA from cultured and freshly dispersed human VSMCs. Medial VSMCs were isolated from two separate female donor aortas (expression of the 3 kb endoglin transcript in each is shown). Ethidium bromide staining of the 28S and 18S ribosomal RNAs is shown to indicate RNA loading.

RESULTS

Endoglin is expressed in human VSMCs. Quiescent human VSMCs were re-stimulated with serum + 10 ng/ml TGFβ1 for 8 hours before the VSMCs were harvested and the RNA extracted. Using primers specific to human endoglin, RT-PCR was used to amplify a single 805 bp PCR product of the predicted size from the VSMC RNA (Figure 1A). To confirm that this band represented endoglin, it was purified, cloned into a T:A vector, and sequenced to reveal a 100% match to human endoglin (EMBL accession number 72012). This endoglin cDNA was radiolabelled and used to probe Northern blots containing RNA from both cultured human VSMCs and aortic VSMCs freshly dispersed from two healthy donor vessels. Northern blot analysis identified the expression of a 3 kb endoglin transcript in the cultured VSMCs as well as in both aortic VSMC samples (Figure 1B).

Effect of exogenous TGFβ1 on endoglin expression in cultured human VSMCs. To examine the effect of exogenous TGFβ1 on VSMC endoglin expression, quiescent cultured human VSMCs were stimulated with serum $\pm$ 10 ng/ml TGFβ1 for 8 and 24 hours respectively. After isolation of RNA from each set of VSMCs Northern blot analysis was used to demonstrate that Endoglin was expressed at similar levels in control and TGFβ1 treated VSMCs at 8 hours. However, at 24 hours expression of the 3 kb endoglin transcript was downregulated in the control VSMCs but significantly higher in the presence of TGFβ1 (Figure 2A). Indeed, scanning densitometry revealed that TGFβ1 main-

tained endoglin expression 2.5-fold higher than that in the control VSMCs (Figure 2B).

Identification of endoglin protein in human VSMCs. To demonstrate that endoglin protein was present in human VSMCs *in vitro,* an anti-endoglin antibody was used to stain four separate 4.5 cm^2 wells containing freshly-plated cultured human VSMCs. As shown in Figure 3A, endoglin protein was easily detectable in the human VSMCs, although it was found that the level of staining was not uniform in each VSMC (compare Figure 3A with Figure 3B indicating the location of each VSMC in the field by bisbenzimide nuclear staining). VSMCs which were treated with a mouse IgG control antibody did not show any background staining, indicating that the endoglin staining was specific (data not shown). To demonstrate that the cells studied were indeed VSMCs they were also stained with an antibody to α-SM actin, this showed high reactivity to actin-containing stress fibers indicating that the cells were VSMC in origin (Figure 3C).

DISCUSSION

The present study is the first to demonstrate that endoglin, a membrane proteoglycan with a role in TGFβ1 signaling and vasculogenesis, is expressed at the mRNA and protein level in human VSMCs.

When endoglin riboprobes were hybridised *in situ* to several murine tissues it was found that uterine

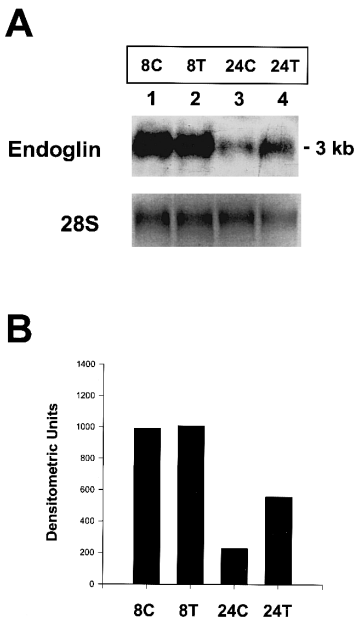

FIG. 2. Effect of exogenous TGFβ1 on endoglin expression in serum-stimulated cultured human VSMCs. (A) Quiescent cultured human VSMCs were re-stimulated with serum ± 10 ng/ml TGFβ1 for 8 and 24 hours. At each time point VSMCs were harvested, the RNA extracted, and endoglin expression measured by Northern blot analysis; VSMCs which had been re-stimulated with serum − (C) or + (T) 10 ng/ml TGFβ1 for 8 hours (lanes 1 and 2) or 24 hours (lanes 3 and 4). Ethidium bromide staining of the 28S ribosomal RNA is shown to indicate RNA loading. (B) Scanning densitometric analysis indicating the levels of endoglin expression shown in A.

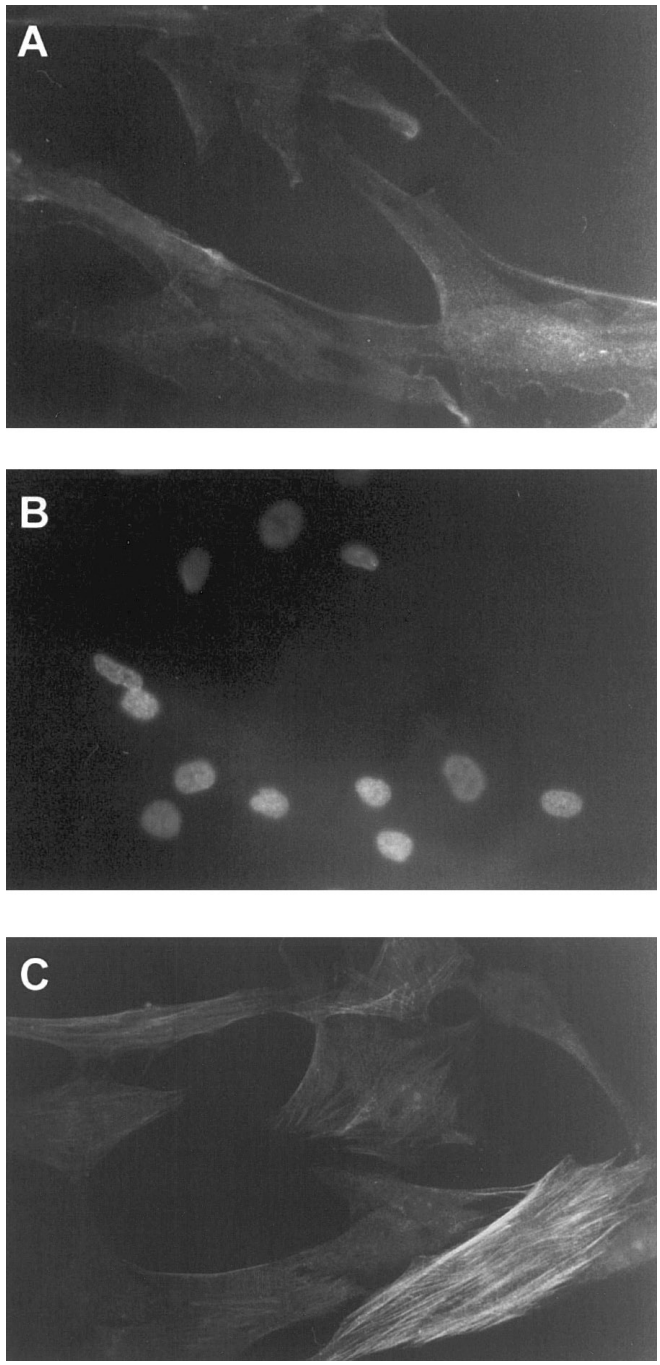

FIG. 3. Endoglin protein expression in cultured human VSMCs. Immunocytochemistry was performed on freshly plated human VSMCs. The reactivity of an antibody specific for endoglin is shown in A. The location of each VSMC is indicated by bisbenzimide staining of the nuclei (B) VSMCs were identified by positive immunoreactivity to an anti-smooth muscle α-actin antibody (C).

smooth muscle did not express endoglin [10]. This suggests that endoglin expression is not common to all smooth muscle containing tissues and may have a specific function in the regulation of TGFβ1 signaling in VSM. The finding that exogenous TGFβ1 maintained endoglin expression in proliferating VSMCs in culture is consistent with the findings of Lastres, *et al.*. [17] who showed that TGFβ1 stimulated the expression of endoglin in cultured human monocytes. The decrease in endoglin expression 24 hours after serum stimulation may be a consequence of proliferation; proliferating VSMCs may down-regulate the expression of endoglin as a feedback mechanism to inhibit TGFβ1 signaling and its anti-proliferative property. Indeed, there is evidence for such a role for endoglin in endothelial cells where it has been shown that, unlike TGFβ2, TGFβ1 + β3, both of which bind strongly to endoglin, inhibit endothelial cell proliferation [18]. Therefore, endoglin may regulate the proliferation of VSMCs as well as endothelial cells and may explain why quiescent, freshly dispersed aortic VSMCs express endoglin.

Could VSMC endoglin play a role in the pathogenesis of hereditary hemorrhagic telangiectasia (HHT)? The recognised malformations of HHT are all due to abnormalities in vascular structure [reviewed in 12]. In fully developed telangiectasia, the vessels are markedly dilated and convoluted with excessive layers of smooth muscle without elastic fibers. Indeed, there may be as many as eleven layers of smooth-muscle cells. These findings are consistent with a role for endoglin in regulating VSMC proliferation. Non-functional endoglin protein in VSMCs in HHT may reduce TGFβ1-induced VSMC growth control and allow the proliferation of SMCs in the vasculature. Similarly, since TGFβ1 is known to increase elastin expression in VSMCs *in vivo*, this may also explain why in HHT the layers of VSMCs lack elastic fibers.

In summary, although further studies are required to determine the precise role of endoglin in TGFβ signaling, the finding that endoglin is expressed in human VSMCs suggests that it may play an important role in regulating the effects of TGFβ1 on VSMCs in a number of vascular disorders.

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