Signaling by ALK5 mediates TGF-β-induced ET-1 expression in endothelial cells: a role for migration and proliferation

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Accepted 29 January 2007
Journal of Cell Science 120, 1256-1266 Published by The Company of Biologists 2007
doi:10.1242/jcs.03419

Summary

Endothelin-1 (ET-1) is a potent endothelial-derived 21-amino-acid vasoconstrictor peptide and its expression is potently regulated by the cytokine transforming growth factor-β (TGF-β). Most cell types contain a TGF-β type I receptor form known as activin receptor-like kinase 5 (ALK5). However, endothelial cells coexpress an additional type I receptor named ALK1. These forms do not constitute redundant receptors with the same function, but they activate different Smad-mediated expression programmes leading to specific endothelial phenotypes. The aim of our study was to characterize the TGF-β-induced pathway leading to ET-1 expression in endothelial cells and the contribution of the TGF-β-mediated enhancement of ET-1 to the regulation of the endothelial cell migration and proliferation capacity. Our experiments indicate that TGF-β induces ET-1 expression preferentially through the ALK5/Smad3 pathway. Specific ALK5 inhibition totally blocked the anti-angiogenic effect of TGF-β. Antagonism of ET receptors partially reverted the effect of TGF-β, indicating that a significant portion of the anti-migratory and anti-proliferative actions of this cytokine is mediated by ET-1 acting in an autocrine manner on endothelial cells.

Key words: Vascular endothelial cells, Cell migration and proliferation, Angiogenesis, Endothelin-1, Transforming growth factor-β, ALK5

Introduction

It is widely accepted that the vascular endothelium is not a simple barrier between the bloodstream and the vascular bed. Endothelial cells play a key role in the regulation of vascular tone through the synthesis and release of various vasoactive substances that act on these cells or other vascular cells in an autocrine or paracrine manner. This concept emerged after the discovery of the endothelium-derived relaxing factor in 1980, later characterized as nitric oxide, and was further extended in 1988 with the discovery and characterization of the vasoconstrictor peptide endothelin-1 (ET-1) (Furchgott and Zawadzki, 1980; Palmer et al., 1987; Yanagisawa et al., 1988).

ET-1 is synthesized as a precursor protein of 212 amino acids, the preproET-1. This precursor undergoes several proteolytic cleavage steps to yield the bioactive form of 21 residues (Inoue et al., 1989a; Miyauchi and Masaki, 1999). Although there is some evidence suggesting that ET-1 might be stored in endothelial-specific subcellular granules, the Weibel-Palade bodies, it is well accepted that the peptide is released from cells as it is synthesized (Inoue et al., 1989b; Kawana et al., 1995; Lee et al., 1991; Rondaij et al., 2006). Therefore, modulation of its expression occurs mainly by increasing or decreasing levels of ET-1 mRNA. One of the most potent regulators of ET-1 levels is the cytokine transforming growth factor-β (TGF-β) (Kurihara et al., 1989). Recently, we have described the molecular mechanism by which TGF-β induces expression of the endothelin-1 gene (*EDN1*) in vascular endothelial cells (Rodriguez-Pascual et al., 2003; Rodriguez-Pascual et al., 2004). TGF-β activates the Smad signaling pathway and the cooperation between Smad and activator protein-1 (AP-1) transcription factors at specific sites within the ET-1 promoter.

Cellular responses to TGF-β are elicited through specific transmembrane type I and type II Ser/Thr kinase receptors (Massague and Wotton, 2000; ten Dijke and Hill, 2004). The signaling pathway is initiated by TGF-β binding to the TGF-β type II receptor. After ligand binding, TGF-β type II receptor recruits and phosphorylates TGF-β type I receptor, also known as activin receptor-like kinase (ALK), which propagates the signal to the nucleus through members of the Smad family (Itoh et al., 2000; Moustakas et al., 2001). Most cell types contain a form of TGF-β type I receptor known as ALK5. However, endothelial cells are unique because they coexpress an additional type I receptor named ALK1. Interestingly, whereas activated ALK5 induces the phosphorylation of Smad2 and Smad3, activated ALK1 has been shown to induce the phosphorylation of Smad1 and Smad5 (Goumans et al., 2003a; Oh et al., 2000; Ota et al., 2002). The pattern of physiological effects resulting from the activation of these Smad-mediated signaling pathways is different. The ALK5/Smad3 pathway leads to inhibition of cell migration and proliferation and is associated with a mature endothelium with

increased expression of genes such as collagen type I or plasminogen activator inhibitor-1 (PAI-1). ALK1/Smad1/5 activates cell proliferation and migration and is more related to the angiogenic state with the expression of inhibitor of DNA binding 1 (Id-1) and endoglin, among others (Byfield and Roberts, 2004; Goumans et al., 2003b; Goumans et al., 2002; Wu et al., 2006). TGF-β has been described to either activate or repress the process of angiogenesis (Madri et al., 1992; Roberts et al., 1986; Vinals and Pouyssegur, 2001; Yang and Moses, 1990). The existence of these receptors and their downstream responses may then reconcile contradictory TGF-β-mediated effects observed in endothelial cells. Therefore, cellular responses to TGF-β are highly dependent on the presence, ratio and functionality of both receptors, and ultimately on the specific target genes that the corresponding signaling pathway is able to transactivate.

Recent reports from the literature have extended the role of ET-1 as a vasoconstrictor molecule to an essential modulator of several physiological processes such as the deposition of extracellular matrix components or lung metastasis (Titus et al., 2005; Xu et al., 2004). Nevertheless, its role in the endothelial cell migration and proliferation has not been explored in depth, especially considering that ET-1 is a downstream target of TGF-β action and therefore may mediate effects of this cytokine on the angiogenic process. The aim of our study was to characterize the TGF-β-induced pathway leading to ET-1 expression in endothelial cells and the contribution of the TGF-β-mediated enhancement of ET-1 expression to the regulation of the activation state of the endothelium. Here we describe that TGF-β induces ET-1 expression preferentially through the ALK5/Smad3 pathway. Specific ALK5 inhibition totally blocked the anti-angiogenic effect of TGF-β observed in our culture model of bovine aortic endothelial cells (BAEC). Interestingly, blocking of ET receptors partially reverted the effect of TGF-β, indicating that a significant portion of the anti-migratory and anti-proliferative actions of this cytokine is mediated by ET-1 acting in an autocrine manner on endothelial cells.

Results

TGF-β induces ET-1 expression through the activation of the ALK5/Smad3 pathway

Incubation of vascular endothelial cells in culture with TGF-β induced a time-dependent increase in the steady-state ET-1 mRNA levels compared with cells incubated under basal conditions (Fig. 1A). This increase in mRNA levels was accompanied with an enhancement in the accumulation of ET-1 peptide as determined by specific ELISA (Fig. 1B). Cells were also transfected with a luciferase reporter driven by a –650/+172-bp fragment of the human ET-1 promoter or with the empty vector (pGL3-Basic) as control. Transfected cells were incubated with TGF-β for different time periods and promoter activity estimated as a measure of luciferase associated to the cells. Luciferase activity driven by pGL3-Basic empty vector was almost negligible and not affected by TGF-β incubation. The fragment of the human ET-1 promoter yielded basal activity, which was significantly increased by TGF-β (Fig. 1C).

We have previously described that the human ET-1 promoter is induced by TGF-β through activation of the Smad signaling pathway (Rodriguez-Pascual et al., 2003). The activation involves the functional cooperation between Smad and AP-1 transcription factors at their corresponding binding sites within the ET-1 promoter: a proximal AP-1 site (–108/–102) and a Smad-binding element (–193/–171). As shown in Fig. 2A, specific mutation of the Smad-binding element or the AP-1 site

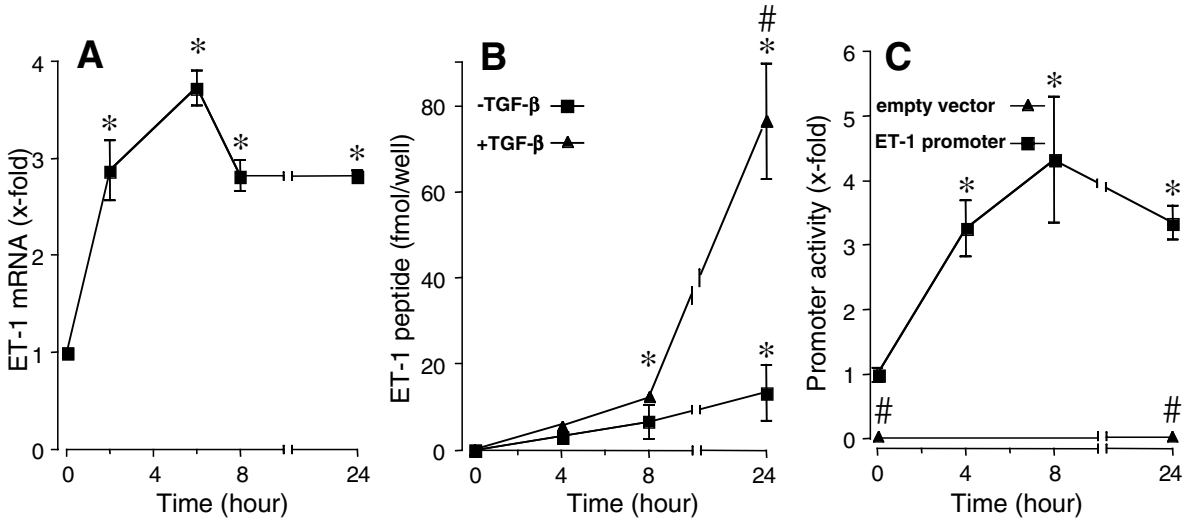

Fig. 1. TGF-β-mediated induction of the ET-1 gene. BAEC were incubated with or without 5 ng/ml TGF-β for different time periods as indicated, and changes in the expression of ET-1 at the level of mRNA, peptide and promoter activity were determined as described in the Materials and Methods. (A) ET-1 mRNA levels were detected and analyzed by RT-qPCR. Values are represented as fold induction (mean ± s.d., n=4, *P<0.05 versus zero time). (B) ET-1 peptide levels were detected by specific ELISA and expressed as fmol/well (mean ± s.d., n=3, *P<0.05 versus zero time, #P<0.05 versus treated cells). (C) Transcriptional activation of the human ET-1 promoter was estimated from cells transfected with a –650/+172-bp fragment of the human ET-1 promoter, compared with empty vector pGL3-Basic. Basal and TGF-β-induced luciferase activity was measured by luminometry and expressed as fold induction with respect to the activity of human ET-1 promoter under basal conditions (mean ± s.d., n=4, *P<0.05 versus zero time, $^{\#}P$<0.001 versus human ET-1 promoter for the indicated times).

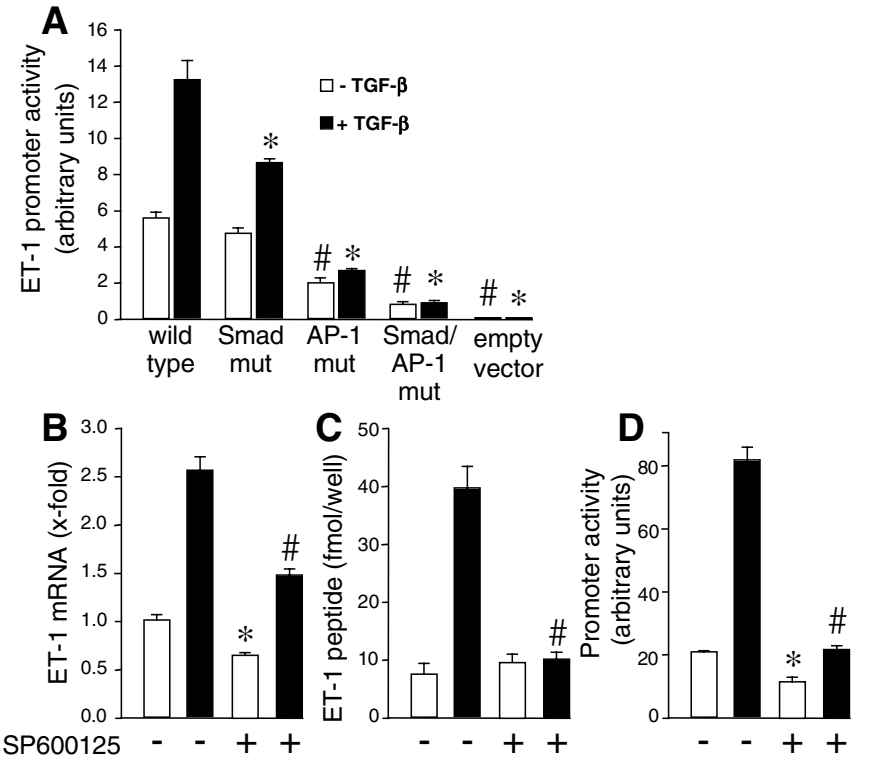

Fig. 2. Smads and AP-1 cooperate to mediate TGF-β induction of the ET-1 gene through activation of the Smad signaling pathway. (A) Specific point-mutations were introduced in the human ET-1 promoter-luciferase construct (wild type) that alter the Smad-binding element and/or the AP-1 site. BAEC transfected with this set of constructs were incubated with TGF-β for 18 hours or left under basal conditions. Luciferase activity was measured and expressed as normalized arbitrary units (mean ± s.d., n=4, *P<0.05 versus wild type in the presence of TGF-β, #P<0.05 versus wild type in the absence of TGF-β). The involvement of JNK/AP-1 in the induction of ET-1 gene expression by TGF-β was analyzed in endothelial cells incubated with the JNK inhibitor SP600125 (25 μM). (B) ET-1 mRNA levels were detected and analyzed by RT-qPCR. Values are represented as fold induction. (C) ET-1 peptide levels were detected by specific ELISA and expressed as fmol/well. (D) Human ET-1 promoter was estimated from cells transfected with the ET-1 promoter-luciferase construct and expressed as arbitrary units normalized to total protein content (mean ± s.d., n=4, *P<0.05 versus basal without the inhibitor, #P<0.05 versus TGF-β stimulation without the inhibitor).

significantly reduced the TGF-β induction of the ET-1 promoter. The requirement of an intact AP-1 site for basal and TGF-β-stimulated responsiveness suggests that c-Jun N-terminal kinases [(JNKs) or stress-activated protein kinases (SAPKs)] may also regulate ET-1 gene transcription. The effect of the pharmacological blockade of JNK activity on ET-1 expression was analyzed in endothelial cells. JNK inhibitor SP600125 significantly reduced TGF-β-induced ET-1 expression at mRNA and peptide levels (Fig. 2B,C), as well as it was able to block the TGF-β induction of the ET-1 promoter (Fig. 2D).

Our results suggest that Smads and AP-1 through activation of the Smad signaling pathway cooperate to mediate TGF-β induction of the ET-1 gene. Since TGF-β transduces via two distinct Smad signaling pathways through activation of ALK1 and ALK5 in endothelial cells, we aimed to analyze the form of TGF-β type I receptor leading to ET-1 expression. For that purpose, we overexpressed in BAEC wild type (wt) and mutant [kinase-deficient (kd) inactive and constitutively active (ca)] forms of type I receptors ALK1 and ALK5, as well as Smad isoforms. Overexpression was followed by western blot and immunofluorescence microscopy. ALK1 and ALK5 overexpression was detected either by using the corresponding anti-ALK1 or anti-ALK5 antibodies, respectively, or using anti-haemagglutinin (HA), as these plasmids also possess a HA-tag (Fig. 3A). Anti-ALK1 or anti-ALK5 antibodies were also able to detect faint bands corresponding to endogenous ALK1 and ALK5 forms that were seen in control plasmid pCMV5-transfected cells. Fig. 3A also shows significant overexpression of Smad3, Smad1 and Smad5 forms in BAEC after transfection with the corresponding expression vectors for these forms. The overexpression of the Smad isoforms was also confirmed by immunofluorescence microscopy, as shown in Fig. 3B.

Once we detected overexpression of ALK1, ALK5 and Smad forms in BAEC, functional validation of the overexpression was performed by cotransfection of constructs together with ALK5- and ALK1-specific luciferase reporters, as shown for constitutively active forms of ALK5 and ALK1 in Fig. 4A,B. As an ALK5-specific reporter, we have used a luciferase construct driven by a synthetic tandem repeat based on the upstream Smad3-binding element from the human PAI-1 promoter, whereas a luciferase reporter under the control of the Smad1/5 binding sites present in the mouse Id-1 promoter was employed as a specific ALK1 reporter.

Cotransfection of ALK1, ALK5 and Smad isoforms together with the ET-1 promoter luciferase construct suggests that TGF-β induces upregulation of ET-1 promoter preferentially through the ALK5/Smad3 pathway (Fig. 4C,D). As shown in Fig. 4C, in the absence of TGF-β, ALK5 ca was more effective than ALK1 ca in potentiating ET-1 promoter activity. Under conditions of stimulation with TGF-β, only ALK5 wt was able to potentiate activity, whereas ALK1 was without any effect. In the same way, whereas ALK5 kd produced a significant inhibition of TGF-β-induced promoter activity in a dominant-negative fashion, ALK1 kd was devoid of effect. When looking at the effect of the overexpression of Smad isoforms, Smad3 was the most potent compared with Smad1 and Smad5 (Fig. 4D).

Selective downregulation of ALK5 and Smad3 expression with siRNA ALK5/Smad3 or pharmacological inhibition of ALK5 impaired TGF-β induction of the ET-1 gene

Overexpression experiments constitute very useful approaches to investigate the role of specific proteins in physiological processes. However, apart from dominant-negative forms,

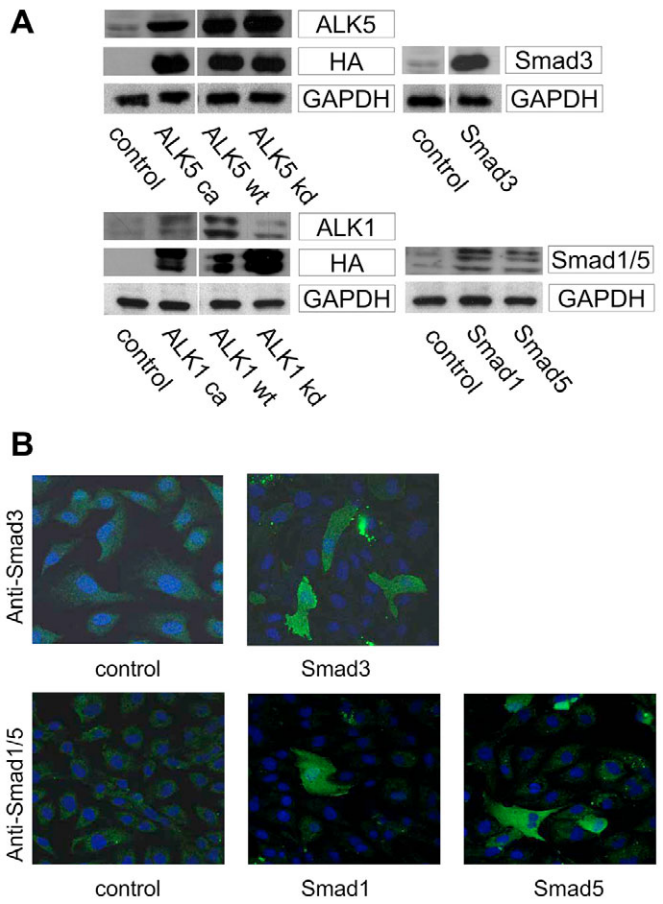

Fig. 3. Overexpression of TGF-β receptors and Smad isoforms in endothelial cells. Cells were transiently transfected with expression constructs for wild type (wt), constitutively active (ca) and kinase-deficient (kd) forms of type I receptors ALK1 and ALK5, and Smad3, Smad1 and Smad5 isoforms. (A) For western blot analyses, whole-cell extracts were fractionated by 10% SDS-PAGE and transferred to blots. The membranes were then incubated with specific antibodies against ALK5, ALK1 and HA (epitope tag for receptor expression plasmids) and Smad3, Smad1 and Smad5. Bands show the presence of endogenous (control) and corresponding overexpressed levels of these proteins. On some occasions, for example for ALK1 and Smad1/5, multiple specific bands were detected. The housekeeping gene GAPDH was detected with an anti-GAPDH antibody for loading control purposes.
(B) Immunofluorescence microscopy was used to detect endogenous (control) and overexpressed levels of Smad3, Smad1 and Smad5 in BAEC. Primary anti-Smad3 or anti-Smad1/5 were coupled to FITC-labeled secondary antibodies (green signal). Nuclei were stained with Hoechst 33342 (blue signal).

these experiments provide limited information on the participation of the endogenous forms and hence loss-of-function approaches are generally needed to address this issue. To confirm the preferential role of endogenous ALK5 and Smad3 for the TGF-β action on ET-1 induction, we knocked down the expression of TGF-β type I receptors and Smad isoforms Smad1 and Smad3 by small interfering RNA (siRNA) technology. We transfected BAEC with specific ALK1, ALK5, Smad1 and Smad3 siRNA duplexes and compared their effects with an siRNA control (Fig. 5). The efficiency of endogenous ALK1, ALK5, Smad1 and Smad3 knockdowns by siRNA was checked by western blotting as shown in Fig. 5A. We also validated siRNA by cotransfection with ALK5 ca/ALK1 ca and ALK1-/ALK5-specific reporters. As shown in Fig. 5B,C, activation of the ALK1-specific reporter was inhibited by cotransfection with siRNA ALK1 and Smad1, but not significantly affected by siRNA ALK5 and Smad3, whereas ALK5-specific reporter activity was abolished by siRNA ALK5 and Smad3, but not modified by siRNA ALK1 and Smad1. We checked then whether these siRNA were able to modify TGF-β-dependent responses of the ET-1 gene. Fig. 5D,E shows that siRNA ALK5 and Smad3 impaired both the activation of the ET-1 promoter and the accumulation of ET-1 peptide in the extracellular medium. The siRNAs for ALK1 and Smad1 had no effect.

In addition, we have taken advantage of the use of a commercial and ALK5-specific inhibitor known as SB-431542. We previously tested the capacity of this compound to inhibit ALK5 signaling in our system by cotransfection of ALK1/ALK5 ca and ALK1-/ALK5-specific reporters. As shown in Fig. 6A,B, increasing concentrations of SB-431542 completely abolished ALK5 ca-dependent activation of ALK5-reporter (IC_{50} value of 0.589±0.115 μM), but was without any inhibitory effect on ALK1 ca-dependent ALK1-reporter activity. Interestingly, SB-431542 concentrations able to block ALK5-dependent activation produced an enhancement of ALK1-dependent activity. Fig. 6C-E shows that SB-431542 dose dependently inhibited TGF-β-induced ET-1 mRNA, peptide and promoter activity. Basal levels of ET-1 mRNA, peptide accumulation and promoter activity were also reduced by this inhibitor.

In conclusion, these results obtained using specific downregulation of expression of endogenous ALK5 and Smad3 and selective pharmacological inhibition of ALK5 with SB-431542 are consistent with the concept that TGF-β responses of the ET-1 gene in endothelial cells are mediated by the ALK5 receptor and the Smad3 isoform.

Role of ET-1 in TGF-β-mediated endothelial cell migration and proliferation

Our results so far indicate that TGF-β preferentially induces ET-1 expression by activation of the ALK5 receptor in endothelial cells. TGF-β/ALK5 signaling is also related to the activation of genes such as collagen type I or fibronectin and, more generally, with a cellular context of inhibition of proliferation and migration that evolves into a mature and quiescent endothelium. We therefore asked whether ET-1 might also be modulating this endothelial phenotype. For that purpose, we studied the migration capacity of the endothelial cells and its modulation by TGF-β. We first used Transwell assays in which cells are seeded in an upper chamber on top of a porous filter, migrating from this surface through the pores of the filter under the appropriate stimulus. With this experimental set-up, TGF-β treatment was associated with a decreased number of migrating cells compared with untreated control cells (Fig. 7A,B). This action of TGF-β was completely abolished by co-incubation with SB-431542, the specific inhibitor of the ALK5 receptor, indicating that under our culture conditions, TGF-β inhibits cell migration by activation of ALK5 signaling. Interestingly, incubation with the dual

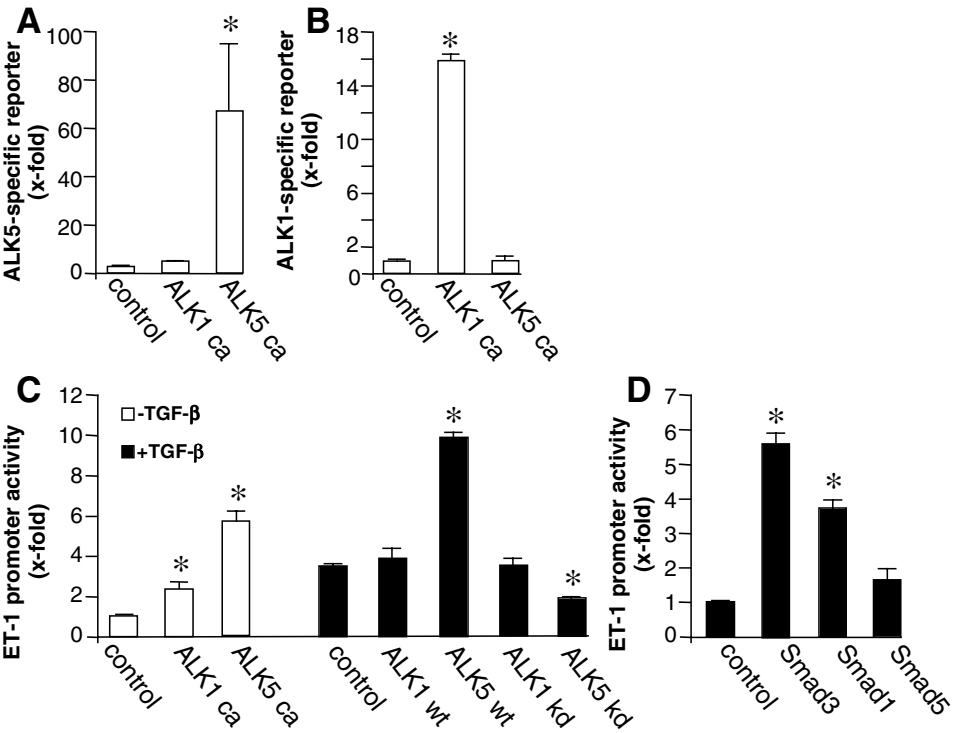

Fig. 4. Effect of the overexpression of ALK5, ALK1 and Smad isoforms on ALK5- and ALK1-specific reporters and on human ET-1 promoter. The effect of the overexpression of constitutively active (ca) forms of ALK5 and ALK1 on (A) ALK5- and (B) ALK1-specific luciferase reporters was analyzed by cotransfection experiments in BAEC. Overexpression plasmids were cotransfected together with a –650/+172-bp fragment of the human ET-1 promoter linked to a luciferase gene. (C) Effect of ALK5 and ALK1 ca in the absence of TGF-β (open bars), and of ALK5 and ALK1 wild type (wt) and kinase-deficient (kd) forms in the presence of 5 ng/ml TGF-β (filled bars). (D) Effect of Smad3, Smad1 and Smad5 overexpression in the presence of TGF-β. Luciferase activity was measured by luminometry and expressed as fold induction with respect to control pCMV5 empty vector (mean ± s.d., n=4, $^{*}P$<0.05 versus control).

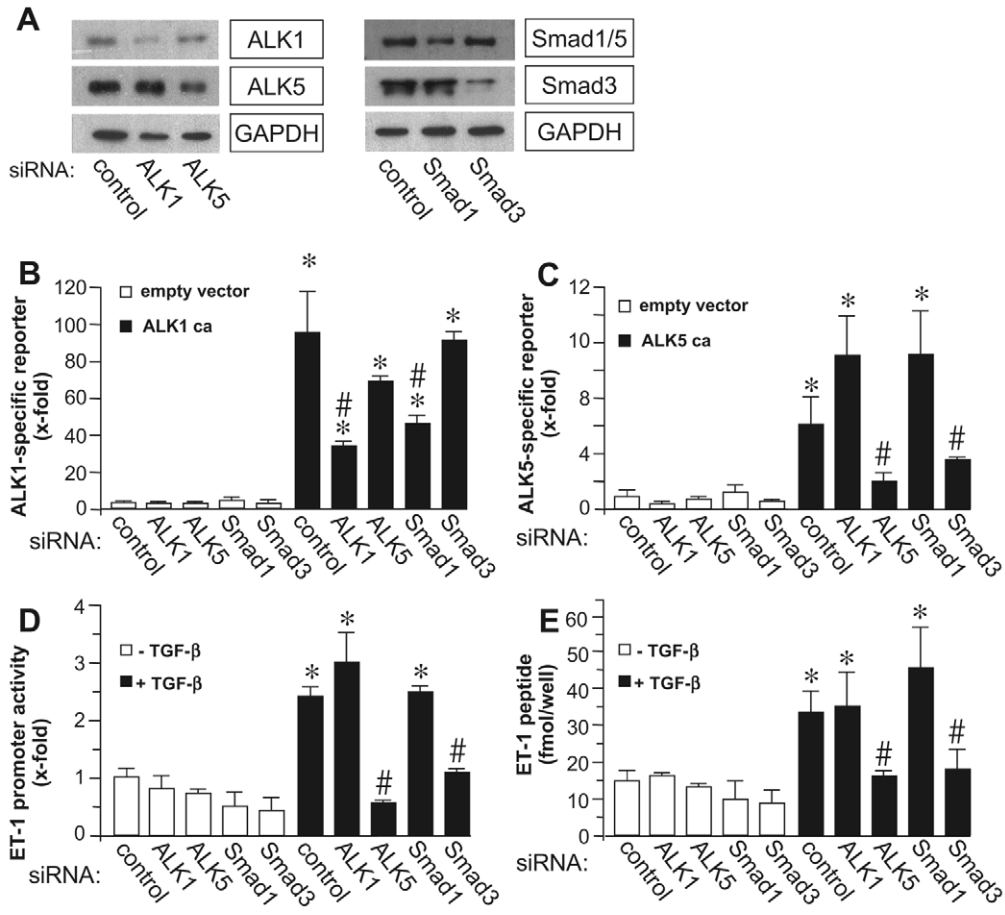

Fig. 5. Effect of selective downregulation of ALK1 and ALK5 receptors and Smad1 and Smad3 isoforms by siRNA on TGF-β-induced ET-1 expression. (A) Efficiency of endogenous protein knockdowns by siRNA was confirmed by western blot assays. Functional validation of ALK1, ALK5, Smad1 and Smad3 siRNA was done by cotransfection with ALK1- and ALK5-specific reporters under overexpression of ALK1 constitutively active (ca) (B) and ALK5 ca (C) forms. Results are compared with the effect of an siRNA control. TGF-β-induced ET-1 expression under specific downregulation of ALK1 and ALK5 receptors and Smad1 and Smad3 isoforms was analyzed at the level of ET-1 promoter activity (D) and peptide secretion (E), as previously described. Values are expressed as fold induction with respect to siRNA control with pCMV5 empty vector (B,C) or without TGF-β (D,E) (mean ± s.d., n=3, $^{*}P$<0.05 versus control without activation, $^{\#}P$<0.05 versus control with activation).

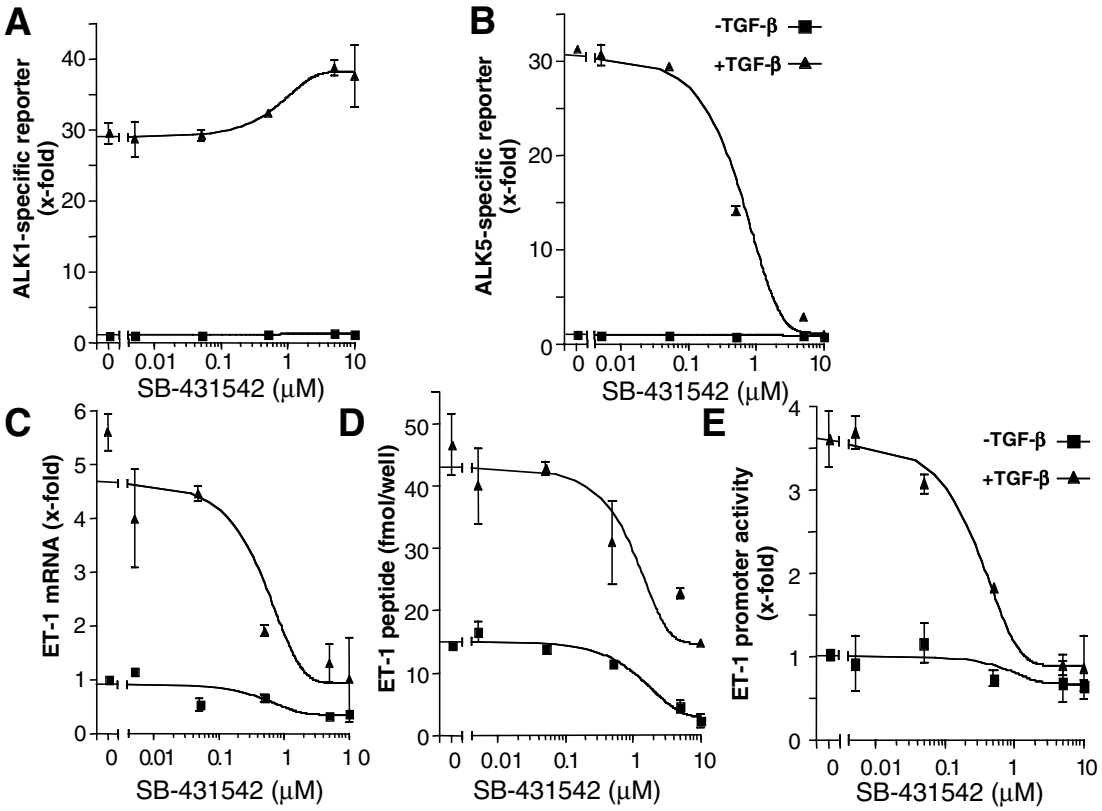

Fig. 6. Pharmacological inhibition of ALK5 with SB-431542 impaired TGF-β-induction of the ET-1 gene. Validation of compound SB-431542 (5 nM-10 μM) was done in BAEC transfected with (A) ALK1- and (B) ALK5-specific luciferase reporters together with ALK1 constitutively active (ca) and ALK5 ca forms, respectively. ET-1 mRNA levels, peptide accumulation and promoter activity were also analyzed in cells treated with SB-431542. (C) ET-1 mRNA expression was investigated using RT-qPCR to detect and quantify the level of transcripts. Values are represented as fold induction with respect to untreated cells. (D) Accumulation of ET-1 peptide in the extracellular medium was analyzed by specific ELISA and expressed as fmol/well. (E) ET-1 promoter activity was estimated by luminometry and values expressed as fold induction with respect to the corresponding control in the absence of TGF-β (mean ± s.d., n=3).

ETA/ETB receptor antagonist, bosentan, also reverted the effect of TGF-β. SB-431542 and bosentan alone did not modify the rate of migration (data not shown). Control experiments of ALK1 and ALK5 signaling using the specific promoter-reporters and the overexpression of ALK1 ca/ALK5 ca in the presence of bosentan were performed in order to rule out any significant effect of this drug on primary TGF-β signaling (Fig. 7C,D). In addition, we confirmed the results of the migration experiments using a wound healing model of endothelial cell migration. As shown in Fig. 8, TGF-β significantly slowed down the closure of a damaged endothelial monolayer compared with untreated control cells, an effect that was observed 24 hours after injury. Incubation with 10 μM SB-431542 completely abolished this effect. Bosentan also partially but significantly reverted the effect of TGF-β (69.60±5.20 for TGF-β plus bosentan versus 55.67±4.71 for TGF-β alone, P<0.05). SB-431542 and bosentan alone did not alter the rate of wound closure (data not shown). We then looked at whether ET-1 is sufficient to promote inhibition of cell migration by analyzing the effect of exogenous ET-1 on the reversion by ALK5 inhibition of TGF-β-induced suppression of migration. As shown in Fig. 9A, the addition of exogenous ET-1 did not restore the anti-migratory response of TGF-β under blockade of ALK5 signaling. In addition, ET-1 alone did not show any significant effect.

Previous work has also shown that TGF-β regulates the proliferation of endothelial cells (Madri et al., 1992; Yang and Moses, 1990). In order to check in our model whether TGF-β also exerts an anti-proliferative effect, we performed experiments of bromodeoxyuridine (BrdU) incorporation as a means to evaluate cell proliferation in cells incubated under basal conditions and with TGF-β in the absence or presence of SB-431542 or bosentan. As shown in Fig. 9B, TGF-β significantly reduced BrdU incorporation compared with cells under basal conditions. This effect is mediated by ALK5 receptor activation as it is abolished by incubation with SB-431542. Bosentan also reverted the effect of TGF-β (61.77±12.08 for TGF-β versus 85.58±6.54 for TGF-β plus bosentan or 83.90±8.75 for TGF-β plus SB-431542, P<0.05). SB-431542 and bosentan alone did not modify cell growth rate (data not shown).

Taken together, these results indicate that in our model of aortic endothelial cell in culture, TGF-β exerts anti-migratory and anti-proliferative effects, which rely completely on ALK5 signaling. TGF-β-induced ET-1 expression also plays a role in this effect as the antagonism of ET receptors partially reverts the effect of TGF-β, indicating that a significant

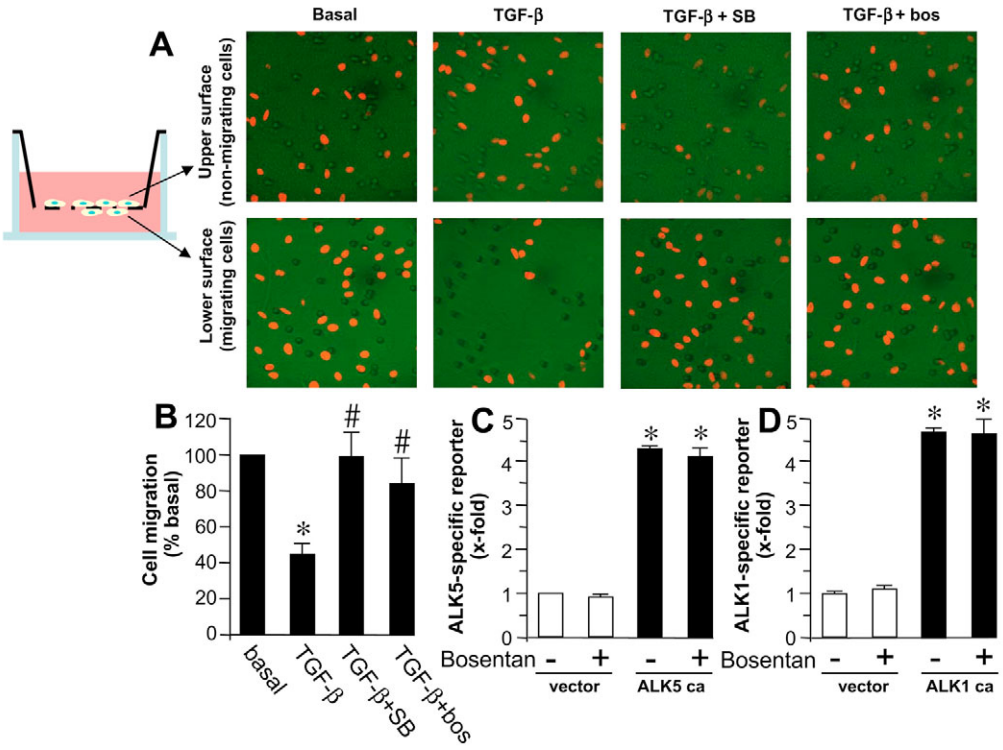

Fig. 7. Effect of TGF-β-induced ET-1 on endothelial cell migration. Transwell assays: BAEC were seeded on Transwell inserts and cell-migration capacity analyzed as described in the Materials and Methods. (A) Schematic representation of filter device and representative pictures taken at corresponding sides of the filter membrane showing non-migrating cells (upper surface) and migrating cells (lower surface) for the indicated experimental conditions (basal, TGF-β alone, TGF-β plus SB-431542 and TGF-β plus bosentan). (B) Cell-migration capacity is shown in the bar graph as the ratio of migrating versus non-migrating cells expressed as a percentage of control (untreated cells) (mean ± s.d., n=3, *P<0.05 versus basal, $^{\#}P$<0.05 versus TGF-β alone). The potential effect of bosentan on TGF-β signaling was analyzed by transfection of ALK5- and ALK1-specific reporters under overexpression of ALK5 constitutively active (ca) (C) and ALK1 ca (D) forms in cells treated with medium alone or with 10 μM bosentan. Promoter activity was estimated by luminometry and values expressed as fold induction with respect to the corresponding control without activation (mean ± s.d., n=3, *P<0.05 versus control without activation).

portion of the anti-migratory and anti-proliferative effects of TGF-β are mediated by ET-1. Our results also suggest that an active TGF-β response mediated by the ALK5 receptor is required for ET-1 to exert its effect, suggesting that ET-1 is acting in conjunction with other TGF-β-activated factors.

Discussion

TGF-β is a multifunctional growth factor that regulates many biological processes controlling the development and physiology of organs and tissues (Blobe et al., 2000). TGF-β signaling is a complex phenomenon, involving several cellular polypeptides, from receptors to transcription factors or accessory proteins (Shi and Massague, 2003). At the plasma membrane, signaling is initiated by specific binding of the cytokine to the TGF-β type II receptor and then transmitted by phosphorylation to the type I form, also named ALK, for which there are different isoforms. Whereas ALK5 is expressed in most tissues and cell types, the expression of ALK1 is almost restricted to the endothelium. The coexistence in endothelial cells of two distinct ALK/Smad-mediated signaling pathways, the ALK5/Smad3 and the ALK1/Smad1/5 pathways, therefore results in an additional level of complexity and converts responses to TGF-β highly dependent on the pattern of genes activated in a given circumstance.

Previous work in the literature reflects a detailed characterization of genes either activated by ALK5 or by ALK1 (or both) (Goumans et al., 2002; Ota et al., 2002; Wu et al., 2006). This allowed us to establish the existence of clusters of genes associated with ALK5 or ALK1, and therefore the assignment of defined biological functions for both receptors in endothelial cell physiology. For example, genes related to enhanced deposition of extracellular matrix, such as collagen type I, fibronectin or PAI-1, are targets of ALK5, suggesting a role for ALK5 in the process of endothelial maturation. However, genes involved in proliferation or inhibition of differentiation, such as Id-1, are activated by ALK1, which indicates an essential role of ALK1 in the activation state of angiogenesis (Goumans et al., 2003a). ET-1 is also a target gene for the action of TGF-β, an effect with important implications for the pathophysiology of the vascular system. In contrast to that observed in lung fibroblasts, where the induction of the ET-1 gene by TGF-β relies only on the JNK/AP-1 signaling pathway, in vascular endothelial cells activation of the Smad signaling pathway and cooperation between Smads and AP-1 transcription factors mediates the induction of the ET-1 gene (Rodriguez-Pascual et al., 2003; Shi-Wen et al., 2006). To date, no study has addressed the precise ALK/Smad pathway leading to enhanced ET-1

expression in the endothelium. The results presented here clearly demonstrate that ET-1 induction by TGF-β is mediated by ALK5/Smad3: (1) results obtained by overexpression of wild-type and mutant ALK1 and ALK5 forms are compatible with a major role of ALK5/Smad3 compared with ALK1/Smad1/5; (2) siRNA-mediated suppression of endogenous ALK5 and Smad3 abolishes TGF-β-induced ET-1 expression; and (3) inhibition of ALK5 by the compound SB-431542 also impairs ET-1 induction by TGF-β.

According to the literature, ALK5 and ALK1 signaling are intricately regulated (Byfield and Roberts, 2004; Goumans et al., 2003b). ALK1 signaling requires intact ALK5 for activation by TGF-β, and ALK5/Smad3 is directly antagonized by ALK1, probably by interference of Smad3 complexes with Smad1/5 members. We did not investigate this in detail in our model of aortic endothelial cells in culture. However, by using specific suppression of ALK1 by siRNA, we did not observe any potentiation of ALK5/Smad3-mediated ET-1 induction by TGF-β. Therefore, it is unlikely that ALK1 is antagonizing the action of TGF-β on ET-1 expression, although this cannot be completely ruled out.

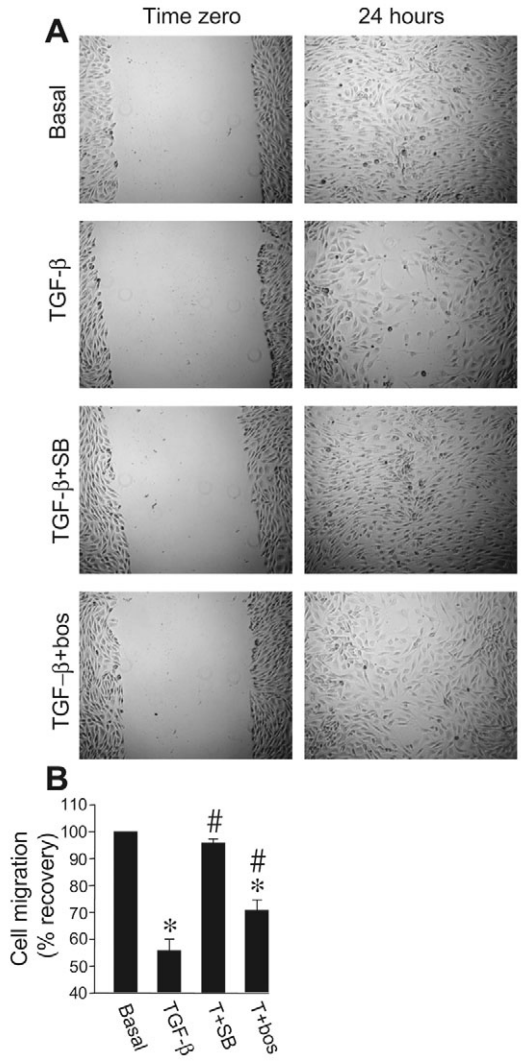

Fig. 8. Effect of TGF-β-induced ET-1 on endothelial cell migration. Wound healing assays: BAEC seeded on 24-well plates after confluency were wounded with a yellow pipette tip and incubated during reendothelization with medium alone (basal) or medium containing 5 ng/ml TGF-β, TGF-β plus 10 μM SB-431542 or TGF-β plus 10 μM bosentan. (A) Representative images of the monolayers at 10× magnification showing initial lesions (zero time) and after 24 hours for the selected experimental conditions. (B) Estimation of cell-migration capacity was obtained by measurement of the wounded area expressed as the percentage of recovery (see Materials and Methods for details) (mean ± s.d., n=3, $^{*}P$<0.05 versus basal, $^{\#}P$<0.05 versus TGF-β alone).

TGF-β plays a pivotal role during vascular remodeling and reports describing this cytokine as either an inhibitor or promoter of blood vessel formation have been published (Madri et al., 1992; Roberts et al., 1986; Vinals and Pouyssegur, 2001; Yang and Moses, 1990). In general terms, TGF-β has been shown to participate in the resolution phase of angiogenesis. Under most culture conditions, TGF-β inhibits the proliferation and migration of endothelial cells and this is in total agreement with our observations. TGF-β significantly reduced the migration capacity of endothelial cells in Transwell assays and also in the classical wound healing experiment. In addition, we observed that TGF-β exerts a significant inhibition of the proliferation. According to the proposed role for ALK5, TGF-β-induced inhibition of migration and proliferation was completely abolished by incubation with the ALK5-specific inhibitor SB-431542. ALK5-activated gene targets involved in these effects comprise PAI-1, a known inhibitor of angiogenesis in vivo, and several extracellular matrix components, i.e. collagen type I alpha 2 or fibronectin 1, which together support vascular stabilization. We hereby present results showing that the ALK5 target ET-1 also participates in the anti-angiogenic properties of TGF-β in this cell culture system. Blockade of ET receptors by incubation with the mixed ETA/ETB antagonist bosentan unmasked these anti-migratory and anti-proliferative actions. Conflicting reports exist in the literature about the potential pro- or anti-migratory or proliferative properties of ET-1. For example, ET-1 has been described to reduce cell migration and proliferation in human bronchial epithelial cells or in the epithelial-like 293 cell line (Dosanjh and Zuraw, 2003; Yamauchi et al., 2002). However, human umbilical vein-derived endothelial cells proliferate and migrate when exposed to ET-1 (Morbidelli et al., 1995; Salani et al., 2000). Apart from being a different cell system, this may be a different scenario compared with ours in which cells are challenged by TGF-β to produce enhanced ET-1. In this respect, cultured endothelial cells accumulate ET-1 in the extracellular medium by basal, constitutive release of the peptide. However, bosentan did not modify the rate of cell migration and proliferation under these conditions (data not shown). Therefore, it is plausible that the coexistence of signals activated under TGF-β incubation confers endothelial cells with autocrine ET-1-dependent anti-angiogenic properties. The fact that exogenously added ET-1 did not rescue the effect of TGF-β under blockade of the ALK5 receptor with SB-431542 indicates that active TGF-β signaling by ALK5 is required for ET-1 to exert its effect. Therefore, our results suggest that ET-1 is acting in conjunction with other TGF-β-activated factors. Indeed, we have recently observed that TGF-β also increases the expression of ETB receptors in BAEC, which might on confer the cells more responsiveness to the secreted ET-1 (F.R.-P., unpublished). Supporting this

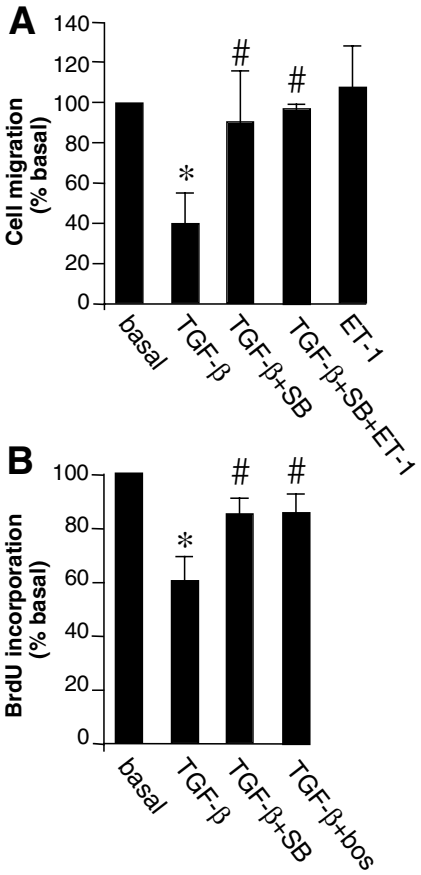

Fig. 9. Role of ET-1 in TGF-β-mediated endothelial cell migration and proliferation. (A) Effect of exogenous ET-1 on endothelial cell migration. BAEC were seeded on Transwell inserts and cell-migration capacity analyzed for the indicated experimental conditions (basal, TGF-β alone, TGF-β plus SB-431542 and TGF-β plus SB-431542 in the presence of 100 nM ET-1). A control experiment with ET-1 alone is also shown. Cell-migration capacity is shown as the ratio of migrating versus non-migrating cells expressed as a percentage of basal (mean ± s.d., n=3, *P<0.05 versus basal, $^{\#}P$<0.05 versus TGF-β alone). (B) Effect of TGF-β-induced ET-1 on endothelial cell proliferation. BrdU incorporation: BAEC seeded on six-well plates were treated with TGF-β, TGF-β plus SB431542 or TGF-β plus bosentan in the presence of 10 μM BrdU for 24 hours. They were then fixed, treated with pepsin/HCl, washed with PBS and incubated with anti-BrdU FITC-conjugated antibody. Estimation of cell proliferation was obtained by measurement of the mean fluorescence intensity from the FITC signal expressed as a percentage of basal (mean ± s.d., n=3, *P<0.05 versus basal, $^{\#}P$<0.05 versus TGF-β alone).

notion of coincidence of signals, it has been described that TGF-β and ET-1 synergize to induce the expression of the potent fibrotic mediator connective tissue growth factor (CTGF) in vascular smooth muscle cells, in the same way that functional interactions between ET-1 and angiotensin II have been reported to promote collagen type I transcription in vivo (Fakhouri et al., 2001; Rodriguez-Vita et al., 2005). ET-1, like TGF-β, is a potent profibrotic agent for fibroblasts and other cell systems (Horstmeyer et al., 2005; Xu et al., 2004). In a similar fashion to that described here, deregulated TGF-β-induced autocrine ET-1 expression contributes to the pathological pulmonary fibrosis observed in scleroderma patients (Shi-Wen et al., 2006). The question regarding how ET-1 exerts the anti-angiogenic actions observed here remains open. Candidates for such analysis are extracellular matrix protein genes as well as matrix-degrading enzymes. We are currently investigating this particular mechanism.

In conclusion, our results demonstrate that TGF-β induces ET-1 expression preferentially through the activation of the ALK5/Smad3 pathway. TGF-β shows anti-angiogenic properties in our culture model of BAEC, an effect completely abolished by ALK5 inhibition. TGF-β-induced enhancement of ET-1 expression contributes significantly to the anti-migratory and anti-proliferative properties of TGF-β in endothelial cells. These observations may be relevant for clinical settings where the TGF-β-ET-1 axis is an important player of cell proliferation and migration, such as atherosclerosis.

Materials and Methods

Cell culture

BAEC were isolated from thoracic aortas using previously described methods (Rodriguez-Pascual et al., 2003). Cells were seeded on gelatin-coated cultured plates and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% calf serum (BioWhittaker), 100 U/mL penicillin and 100 μg/mL streptomycin.

ET-1 peptide determination by ELISA

Cells were seeded on six-well plates and cultured until they reached confluence. They were then incubated in 1 ml of complete medium (basal) or with TGF-β [TGF-β1, Chinese hamster ovary (CHO) cell-derived; R&D Systems Europe] and/or pretreated with SB-431542 (potent and selective ALK5 inhibitor, IC_{50}=94 nM; Tocris Bioscience) or with siRNA as described above (Inman et al., 2002). At the end of the experiment, cell supernatants were taken for solid-phase extraction and further ELISA determination. Briefly, 0.5 volumes of 12% acetic acid were added to the supernatants and these were then loaded onto preactivated (methanol, water, 4% acetic acid) Sep-Pak C18 cartridges (Waters Corporation). Columns were washed with water and then with 25% ethanol and were eluted with 4 ml of a solution of 4% acetic acid and 86% ethanol. An amount of this eluate (200 μl) was evaporated in a SpeedVac (Savant) and resuspended in 50-200 μl ET-1 ELISA assay buffer. ELISA determinations were performed following the instructions of the manufacturer (Endothelin-1 Biotrak ELISA System, Amersham).

Detection and quantification of specific mRNA by reverse transcription-quantitative PCR (RT-qPCR)

For RNA experiments, BAEC were seeded on six-well plates and cultured until they reached confluence. After the incubation time with reagents, monolayers were washed with phosphate-buffered saline (PBS) and processed for RNA isolation by guanidium thiocyanate/phenol/chloroform extraction. Relative expression levels were determined by RT-qPCR.

Briefly, 1 μg of total RNA was reverse transcribed into single-strand cDNA using M-MLV reverse transcriptase (Invitrogen). Reverse transcription reaction was performed for 2 minutes at 70°C, 1 hour at 37°C and 10 minutes at 70°C. Real-time qPCR was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The following primers were used for the amplification of a bovine ET-1 cDNA 145-bp fragment (Accession number NM_181010): forward, 5′-AGCAGGAAAAGAACTCAGGG-3′ and reverse, 5′-TGATGGCCTCCAAC-CTTCTT-3′. ET-1 expression was normalized to the housekeeping gene 18S (VIC/MGB TaqMan probe, Applied Biosystems). Thermal conditions were: 2 minutes at 50°C, 10 minutes at 95°C, and 45 cycles of 15 seconds at 95°C, 30 seconds at 56°C and 30 seconds at 72°C. Values are expressed as the amount of target ET-1 mRNA normalized to the endogenous reference 18S mRNA considering the following equation: $2^{-\Delta\Delta\ Ct}$.

Reporter plasmids and cell transfection

Luciferase reporters driven by –650-bp and –193-bp (wild-type and mutated Smad-binding element and AP-1 site) fragments of the human ET-1 promoter (–650 ppET1-prom-luc) were generated as described (Rodriguez-Pascual et al., 2003). The ALK5/Smad3-reporter (CAGA-luc) consisted of 12 tandem repeats of the upstream Smad3-binding element from human PAI-1 promoter linked to a viral minimal promoter and to a luciferase gene, and was a generous gift from Aris Moustakas (Biomedical Center, Uppsala, Sweden) (Dennler et al., 1998). ALK1/Smad1/5-reporter (BRE-luc) construct consisted of two tandem repeats of the Smad elements from mouse Id-1 linked to a cassette minimal promoter-luciferase, and was

generated as described previously (Korchynskyi and ten Dijke, 2002). Wild-type and mutant overexpression plasmids (pCMV5-derived) for ALK5 and ALK1 were generated as described (Goumans et al., 2002). Overexpression plasmids for Smad1, Smad3 and Smad5 (pCMV5-derived) were obtained from Liliana Attisano (University of Toronto, Ontario, Canada). Transient transfection experiments were performed with BAEC seeded on gelatin-coated 24-well plates (60-70% confluency) and promoter activity was estimated by luminometry as described previously using pRL-CMV plasmid (a Renilla luciferase under the control of the CMV promoter) for normalization purposes (Rodriguez-Pascual et al., 2003). Experiments involving the use of the JNK inhibitor SP600125 (Sigma) were normalized to total protein content (Bradford) as this inhibitor altered CMV promoter-driven Renilla luciferase activity from cotransfected pRL-CMV. Overexpression of TGF-β type I receptor and Smad forms was followed by western blot analyses and immunofluorescence microscopy using specific antibodies.

Western blot analyses

BAEC were seeded on six-well plates and protein isolation and immunoblotting was performed as described previously (Rodriguez-Pascual et al., 2003). Blots were probed with anti-Smad2/3 (1:500 dilution, mouse IgG1 monoclonal antibody; BD Biosciences Clontech, France), anti-Smad1/5/8 (1:1000 dilution, goat polyclonal antibody; sc-6031, Santa Cruz), anti-ALK5 (TGF-β RI, V22, 1:1000 dilution, rabbit polyclonal antibody; sc-398, Santa Cruz), anti-ALK1 (D-20, 1:1000 dilution, goat polyclonal antibody; sc-19546, Santa Cruz) and anti-HA (clone 3F10, 1:1000 dilution, rat monoclonal antibody; Roche Applied Science), followed by horseradish peroxidase (HRP)-coupled secondary antibodies at 1:2000 dilution, and immunocomplexes were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham). As a control for protein loading and transfer, membranes were assayed with anti-glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) antibody (6C5, 1:2000, monoclonal).

Immunofluorescence microscopy

Cells were seeded on 10 mm-diameter glass coverslips in 24-well plates. They were then transfected as previously described. Cells were fixed with 3.5% paraformaldehyde and permeabilized with 70% methanol. Specific antibodies were used to detect Smad3 (1:50 dilution), Smad1 (1:100 dilution) and Smad5 (1:100 dilution). Secondary goat anti-mouse FITC (1:100 dilution, Sigma F-1010) and swine anti-goat FITC (1:100 dilution, Innogenetics G50001) were used. Nuclear staining was performed with Hoechst 33342 (Sigma-Aldrich B2261). Cell fluorescence was visualized by confocal microscopy (Radiance 2100, Bio-Rad).

Depletion of endogenous ALK1, ALK5, Smad1 and Smad3 by siRNA

siRNA technology was employed to reduce the content of endogenous ALK1, ALK5, Smad1 and Smad3 in cultured endothelial cells. siRNA duplex oligonucleotides, designed to target both bovine and human ALK1 (sense 5′-UUCGUCAACCACUACUGCUUU-3′ and antisense 5′-AGCAGUAGUGGUUGACGAACU-3′), ALK5 (sense 5′-CAUAUUGCUGCAACCAGGAtt-3′ and antisense 5′-UCCUGGUUGCAGCAAUAUGtt-3′), Smad1 (sense 5′-CACUGGUGCUCUAUUGUCUtt-3′ and antisense 5′-AGACAAUAGAGCACCAGUGUU-3′), Smad3 (sense 5′-GAUCUUCAACAACCAGGAGUU-3′ and antisense 5′-CUCCUGGUUGUUGAAGAUCUU-3′) and siRNA control (Ambion reference 4611) were purchased from Ambion. Cells were seeded on six-well plates and transfected with siRNA (1.25 μg) using Lipofectamine 2000 (Invitrogen) as a transfection reagent following the instructions of the manufacturer. Simultaneous transfection of siRNA and luciferase reporter DNA plasmids was performed using cells seeded on 24-well plates with 0.3 μg siRNA and 0.25 μg DNA. After transfection, cells were either stimulated with TGF-β or left under basal conditions for 24 hours. Afterwards, they were processed for ELISA determinations (six-well) or luminometry (24-well) as explained above.

Transwell assays

Cell migratory assays were performed using Transwell chambers with filter membranes of 8 μm pore size (Corning, Life Sciences). Filter membranes were coated with 0.5% gelatine and chambers were inserted in 24-well cultured plates. BAEC were seeded into the upper chamber (3×10^4 cells per well in 1% calf serum in RPMI) and allowed to attach for 12 hours at 37°C. Subsequently, cells were treated with 5 ng/ml TGF-β, TGF-β plus 10 μM SB-431542 or TGF-β plus 10 μM bosentan (antagonist for ETA/ETB receptors, kindly donated by Actelion) for 24 hours (medium alone, with SB-431542 or with bosentan were used as controls) (Clozel et al., 1994). The ligand ET-1 (100 nM) (Bachem AG) was also included in some experiments. Filter membranes were removed, fixed with 70% ethanol and stained with 0.003% propidium iodide including RNase A (0.1 mg/ml) for 30 minutes at 37°C. Migration activity was quantified by counting both the migrating (cells that reached the lower surface of the filter) and non-migrating cells (still attached on the upper surface) using a confocal microscope (Radiance 2100, Bio-Rad). Data are reported as the ratio of migrating versus non-migrating cells expressed as a percentage of control (untreated cells).

Wound healing assays

BAEC seeded on 24-well plates were cultured until confluency. They were then scratched with a yellow pipette tip, washed with PBS and further incubated with 5 ng/ml TGF-β, TGF-β plus 10 μM SB-431542 or TGF-β plus 10 μM bosentan in RPMI medium supplemented with 2% calf serum (medium alone, with SB-431542 or with bosentan were used as controls). Images were taken at fixed time intervals of 6, 9 and 24 hours with an Axiovert 200 M SP LSM5 Zeiss microscope. The reendothelization rate was quantified by selecting a field of view, which covered approximately 8% of the total well area. The image analysis system used (Axio Vision LE 4.3.0.101, Carl Zeiss Vision) allowed the measurement of the lesion area in each field of view. For normalization purposes, the wounded area was expressed as the percentage of recovery (%R) using the equation: %R=[1–(wounded area at T_t/wounded area at T_0)]×100%, where T_t is the number of hours post-injury and T_0 is immediately post-injury (Lauder et al., 1998).

Proliferation assays

Cells seeded on six-well plates were cultured until 50% confluency. They were then treated with TGF-β, TGF-β plus SB-431542 or TGF-β plus bosentan in the presence of 10 μM BrdU for 24 hours. Afterwards, they were fixed in 70% ethanol and treated with pepsin in acidic medium to achieve the DNA denaturation required for detection of the BrdU epitope. They were then washed with PBS and stained with anti-BrdU FITC-conjugated antibody (1:20 dilution; 347583, Becton Dickinson) for 1 hour at room temperature. Fluorescence intensity was analyzed in a CyAn MLE-R (Dako-Cytomation) cytometer. Data are reported as the mean of fluorescence intensity expressed as a percentage of control (untreated cells).

Statistical analysis

Experimental data were analyzed by unpaired Student's *t*-test in the case of normal distribution of data, or using nonparametric tests as appropriate. The *P* values obtained are indicated in the figure legends when statistically significant. Dose-response analyses were performed using the GraphPad Prism 3 statistical software (GraphPad Software).

This work was supported by grants SAF2003-01039 and SAF2006-02410 from Plan Nacional de I+D+I to S.L., GR/SAL/0484/2004 from the Consejeria de Educación de la Comunidad de Madrid to F.R.-P., and EC Angiotargeting Project 504743 to P.t.-D. C.C. holds a fellowship funded by the Plan Nacional de I+D+I (SAF2003-01039). F.R.-P. is the holder of a Ramon y Cajal contract from the Ministerio de Educación y Ciencia. We thank Alberto Alvárez-Barrientos, Elvira Arza, Pilar Torralbo and Raquel Nieto from the Citometry Unit (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) for their technical assistance, and Lia Pardali (Leiden University Medical Center, The Netherlands) for helpful discussion.

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