L- and S-endoglin differentially modulate TGFβ1 signaling mediated by ALK1 and ALK5 in L_6E_9 myoblasts

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Summary

TGFβ regulates cellular processes by binding to type I and type II TGFβ receptors (TβRI and TβRII, respectively). In addition to these signaling receptors, endoglin is an accessory TGFβ receptor that regulates TGFβ signaling. Although there are two different alternatively spliced isoforms of endoglin, L-endoglin (L, long) and S-endoglin (S, short), little is known about the effects of S-endoglin isoform on TGFβ signaling. Here, we have analyzed the TGFβ1 signaling pathways and the effects of L- and S-endoglin in endoglin-deficient L_6E_9 cells. We found that TGFβ activates two distinct TβRI-Smad signaling pathways: ALK1-Smad1-Id1 and ALK5-Smad2-PAI1, in these cells. Interestingly, L-endoglin enhanced the ALK1-Id1 pathway, while S-endoglin promoted the ALK5-PAI1 route. These effects on signaling are supported by biological effects on TGFβ1-induced collagen I expression and inhibition of cell proliferation. Thus, while L-endoglin decreased TGFβ1-induced collagen I and CTGF expression and increased TGFβ1-induced proliferation, S-endoglin strongly increased TGFβ1-induced collagen I and CTGF expression, and reduced TGFβ1-induced cell proliferation.

Key words: TGFβ, L-endoglin, S-endoglin ALK1, ALK5, Id1, PAI1, Smads, Collagen I, Proliferation

Introduction

Transforming growth factor β (TGFβ) is a family of multifunctional growth factor that regulates biological processes controlling the physiology of organs and tissues. TGFβ proteins regulate cell proliferation, migration, extracellular matrix (ECM) production and the differentiation of a wide variety of cell types (Piek et al., 1999), and play a pivotal role during embryonic development and adult homeostasis (Massague, 2000). TGFβ family members elicit cellular responses by binding to a heteromeric complex of specific type I and II serine/threonine kinase receptors and their downstream nuclear effectors, termed Smads (Shi and Massague, 2003). TGFβ type I receptor (TβRI), also known as activin receptor-like kinase (CAVRL1, hereafter referred to as ALK), acts downstream of TGFβ type II (TβRII) receptor and propagates the signal to the nucleus by phosphorylating specific members of the Smad family, receptor-regulated (R)-Smads, at their C-terminal serine residues. Phosphorylated R-Smads form complexes with the common partner (Co)-Smad, i.e. Smad4, which accumulate in the nucleus where they participate in transcriptional regulation of target genes (Massague and Gomis, 2006). In most cells cell types, TGFβ1 binds to the ubiquitously expressed ALK5 receptor that activates Smad2 and Smad3 (Massague and Gomis, 2006). In a few other cell types, TGFβ activates ALK1 in the presence of functional ALK5, resulting in phosphorylation of Smad1 and Smad5 (Goumans et al., 2002; Lebrin et al., 2005; Scherner et al., 2007). It has been proposed that ALK1 activation triggers proliferation and migration, whereas ALK5 activation has the opposite effects in endothelial cells (Goumans et al., 2002; Lebrin et al., 2005). However, other authors have reported rather different results (David et al., 2007). Besides these classical signaling receptors, two accessory receptors, i.e. betaglycan (TβRIII) and endoglin (CD105), have been described (Piek et al., 1999; Shi and Massague, 2003; Gougos and Letarte, 1990; Duff et al., 2003). Endoglin binds different members of the TGFβ superfamily in the presence of the signaling receptors types I and II (Cheifetz et al., 1992; Yamashita et al., 1994; Letamendia et al., 1998). The functional interaction between endoglin and TGFβ receptors has been thoroughly analyzed in endothelial cells (Lebrin et al., 2004; Lebrin et al., 2005; Blanco et al., 2005) and only recently in L_6E_9 myoblasts (Scherner et al., 2007). Molecular cloning of the human endoglin cDNA has demonstrated the existence of two protein variants, arising by alternative splicing. L-endoglin, the predominant isoform, has a cytoplasmic domain of 47 residues, whereas the minor isoform, S-endoglin, contains a cytoplasmic tail of only 14 amino acids (Bellon et al., 1993; Perez-Gomez et al., 2005). Both endoglin forms are able to bind ligand (Bellon et al., 1993), but differ in their level of phosphorylation (Lastres et al., 1994), and in their capacity to regulate certain TGFβ-dependent responses (Lastres et al., 1996). As L-endoglin is the predominant isoform, its role in the TGFβ system has been analyzed by several laboratories. However, little is known about the function of S-endoglin (Perez-Gomez et al., 2005; Lastres et al., 1994; Lastres et al., 1996). Here, we analyze the effect of L- and S-endoglin on TGFβ1 signaling and on collagen I synthesis and cell proliferation in L_6E_9 myoblasts.

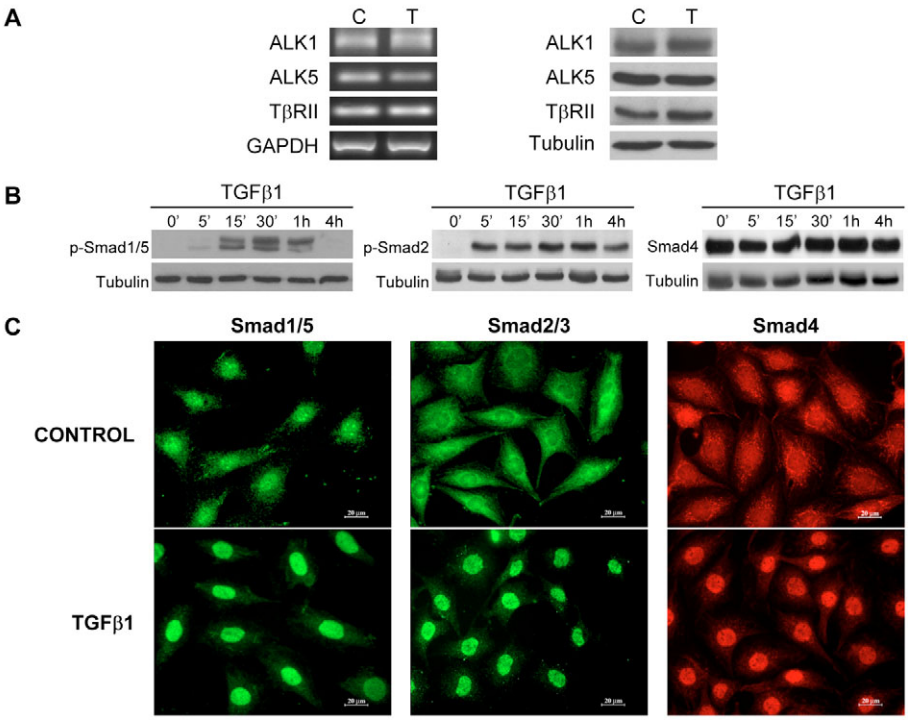

Fig. 1. Expression of TGFβ receptors and Smads in L_6E_9 cells. L_6E_9 cells were serum starved for 24 hours before TGFβ1 treatment. (A) The expression of ALK1, ALK5 and TβRII from control (C) or 500 pM TGFβ1-treated (T) cells (24 hours) was analyzed by RT-PCR and western blot. (B) Myoblasts were stimulated with TGFβ1 for the indicated time periods. Total proteins extracts were analyzed by western blot with anti-phospho-Smad1, anti-phospho-Smad2, anti-Smad2/3 and anti-Smad4 antibodies. Loading controls included GAPDH, β-actin and tubulin. A representative blot from three independent experiments is shown. (C) Immunofluorescence of Smad1/5, Smad2/3 and Smad4 in L_6E_9 cells untreated or treated with TGFβ1 for 24 hours.

Results

TGFβ1 activates ALK1 and ALK5 signaling pathways

The presence of ALK1, ALK5 and TβRII mRNA and protein expression in L_6E_9 cells was clearly detected by RT-PCR and western blot (Fig. 1A). Furthermore, they were unchanged after 24 hours of TGFβ1 treatment. As evidenced by western blot analysis of total cellular extracts (Fig. 1B) or isolated nuclei (data not shown), stimulation of L_6E_9 with TGFβ1 resulted in phosphorylation and nuclear accumulation of Smad1/5 (Fig. 1B) and Smad2/3 (Fig. 1B). Smad4 expression in total cellular extracts was unchanged after TGFβ1 treatment (Fig. 1B), but was increased in nuclear extracts (data not shown). Immunofluorescence studies revealed that under basal conditions Smad1/5, as well as Smad2/3 and Smad4 are homogenously distributed in the cytoplasm with perinuclear and nuclear accumulation. However, after 30 minutes of stimulation with TGFβ1, Smad1/5, Smad2/3 and Smad4 accumulated mainly in the nucleus (Fig. 1C). TGFβ-induced Smad phosphorylation was abrogated in the presence of the synthetic ALK5 inhibitor SB431542 (Fig. 2A). We found an increased expression of the specific downstream target of TGFβ-ALK1 pathway Id1 (Goumans et al., 2002) in L_6E_9 cells (Fig. 2B). Id1 expression induced by 500 pM TGFβ1 was maximal at 1 hour and decreased after 4 hours (data not shown). We also found a small activation of the $(BRE)_2$-Luc reporter upon 24 hours of TGFβ1 treatment (Fig. 2C). Expression of the specific downstream target of TGFβ-ALK5 pathway, plasminogen activator inhibitor 1 (Serpine1, hereafter referred to as PAI1) (Goumans et al., 2002), was strongly induced by 500 pM TGFβ1 (Fig. 2D). As shown in Fig. 2E, the reporter activity $(CAGA)_{12}$-Luc was powerfully stimulated by TGFβ1 treatment for

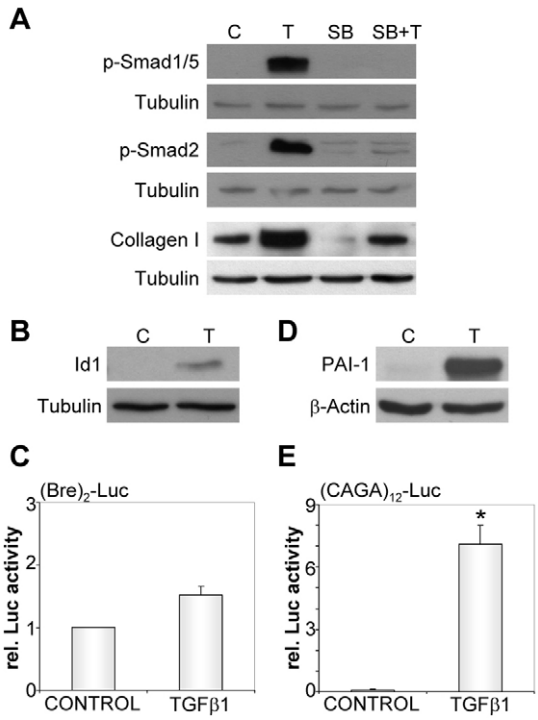

Fig. 2. TGFβ1-ALK1 and TGFβ1-ALK5 signaling pathways in L_6E_9 cells. Cells were serum starved for 24 hours before TGFβ1 treatments (30 minutes for p-Smads, 1 hour for Id1 and 24 hours for PAI1). (A) Cells were treated with the ALK5 inhibitor SB431542 (SB, 5 μM) 1 hour before treatment with TGFβ1. Whole-cell extracts were analyzed by western blot with anti-pSmad1, anti-pSmad2 and collagen I. Total protein extracts from control (C) or TGFβ1-treated (T) myoblasts (1 hour) were analyzed by western blot with anti-Id1 (B) and anti-PAI1 (D). L_6E_9 were transiently transfected with $(Bre)_2$-Luc (C), and $(CAGA)_{12}$-Luc (E) reporters; cells were incubated or not with TGFβ1 for 24 hours, before measuring the luciferase activity. Results are represented as fold induction of the TGFβ1 treated over the untreated counterparts. The histogram represents the mean of three independent experiments. *P<0.05, Student's t-test.

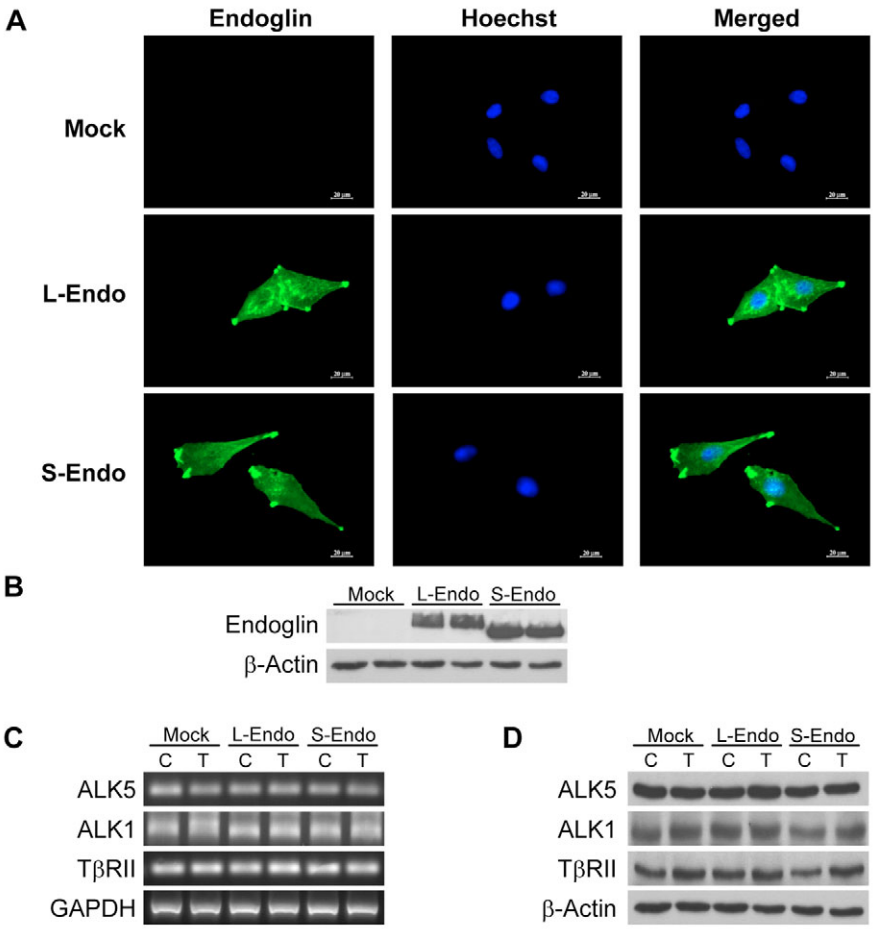

Fig. 3. L- and S-endoglin expression and their effects on TGFβ receptors.
(A) Immunofluorescence and (B) western blot of endoglin in L_6E_9 mock, L-endoglin (L-Endo) and S-endoglin (S-Endo). (C,D) Mock-, L-Endo- and S-Endo-transfected cells were serum starved for 24 hours before TGFβ1 treatment. The expression of ALK1, ALK5 and TβRII in control (C), or TGFβ1-treated (T) cells was analyzed by RT-PCR (C) and western blot (D). A representative blot from three independent experiments is shown.

24 hours. These experiments revealed that TGFβ1 was sufficient to activate Smad2 and Smad1/5. Interestingly, both phosphorylation of Smad2 and Smad1/5 were blocked in the presence of SB431542 (Fig. 2A). Because ALK1 is insensitive to this inhibitor (Laping et al., 2002), this finding indicated that ALK1 signaling itself is ALK5 dependent in L_6E_9 cells. In addition, TGFβ-induced collagen I expression was reduced in the presence of SB431542 (Fig. 2A).

L- and S-endoglin expression in L_6E_9 cells, and their effect on TGFβ receptors and Smad activation

Ectopic expression of L- and S-endoglin in L_6E_9 transfectants was assessed by immunofluorescence and western blot analysis. Both L- and S-endoglin were found to be homogeneously distributed on the cell membrane and accumulated at the adhesion points to the substratum (Fig. 3A). L- and S-endoglin were detected also by western blot (Fig. 3B). As we already reported (Obreo et al., 2004), no endoglin expression was found in mock L_6E_9 cells. RT-PCR and western blot analysis revealed that L- or S-endoglin expression in L_6E_9 cells did not modify ALK1, ALK5 or TβRII mRNA (Fig. 3C) and protein expression (Fig. 3D). Stimulation of mock, L- or S-endoglin L_6E_9 myoblasts with TGFβ1 resulted in Smad1, Smad2 and Smad3 phosphorylation (Fig. 4A). No significant differences were found in phosphorylated Smad1, Smad2 and Smad3 between mock, L- or S-endoglin in untreated cells (data no shown). TGFβ1-induced Smad3 phosphorylation was higher in S-endoglin cells than in L-endoglin and mock, whereas no significant differences were found in phospho-Smad2 (Fig. 4A). TGFβ1-induced Smad1, Smad2 and Smad3 phosphorylation was abrogated in the presence of the synthetic ALK5 inhibitor SB431542 in mock, L- and S-endoglin-transfected cells (Fig. 4B).

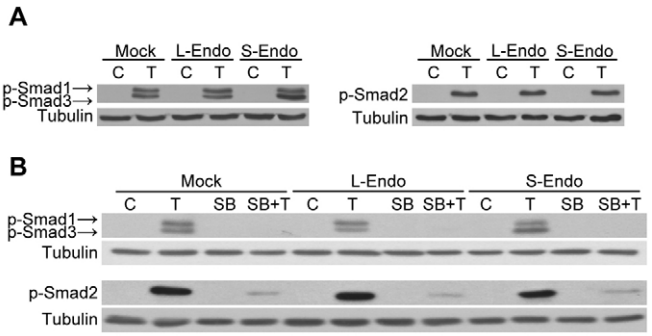

Fig. 4. Effects of L- and S-endoglin on Smad phosphorylation. Mock-, L-Endo- and S-Endo-transfected cells were serum starved for 24 hours before 30 minutes of TGFβ1 treatment. (A) Total protein extracts from control (C) or TGFβ1-treated (T) myoblasts were analyzed by western blot with anti-phospho-Smad1/3 or anti-phospho-Smad2 antibodies; anti-tubulin was used as a loading control. (B) Mock-, L-Endo- and S-Endo-transfected cells were treated with the ALK5 inhibitor SB431542 (SB, 5 μM) 1 hour before treatment with TGFβ1. Total protein extracts from control (C) or TGFβ1-treated (T) myoblasts were analyzed by western blot with anti-phospho-Smad1/3, anti-phospho-Smad2 and anti-tubulin antibodies. A representative blot from three independent experiments is shown.

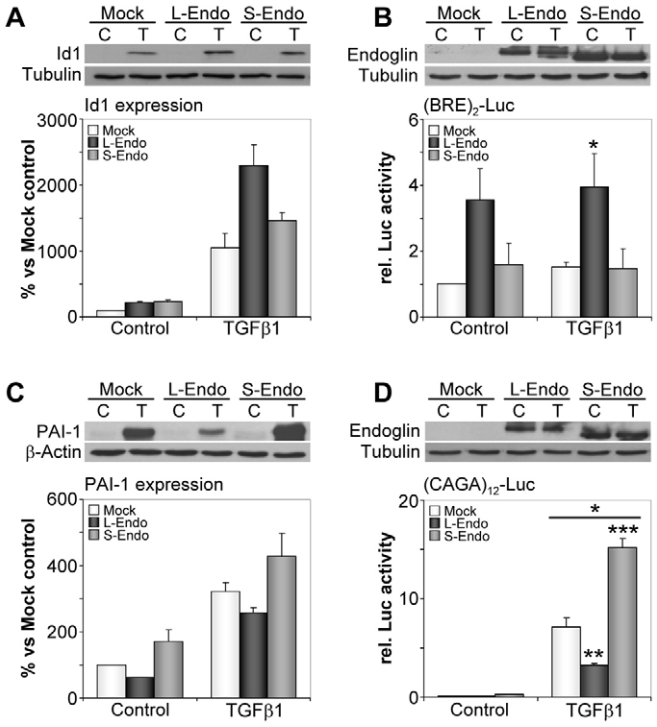

Fig. 5. Effects of L- and S-endoglin on TGFβ1-ALK1 and TGFβ1-ALK5 signaling pathways. Cells were serum starved for 24 hours before TGFβ1 treatment. Total protein extracts from control (C) or TGFβ1-treated (T) myoblasts analyzed by western blot with anti-Id1 (A) and anti-PAI1 (C). Measures of densitometry of each band were performed and relative values are represented. Id1 and PAI1 histogram represents the mean of three different extracts. (B,D) Western blots of endoglin in L_6E_9 mock, L-endoglin (L-Endo) and S-endoglin (S-Endo). L_6E_9 cells were transiently transfected with $(Bre)_2$-Luc reporter (*P<0.05 compared with mock and S-Endo) (B), and $(CAGA)_{12}$-Luc reporter (*P<0.05 TGFβ1 compared with control; **P<0.05 compared with mock and S-Endo; ***P< 0.05 compared with mock and L-Endo). (D) Cells were incubated or not with TGFβ1 for 24 hours before measuring the luciferase activity.

Differential effects of L- and S-endoglin on ALK1 and ALK5 signaling pathways

TGFβ1-induced Id1 expression in L-endoglin-transfected L_6E_9 was significantly higher than in mock cells (Fig. 5A). In addition, Id1 expression in L-endoglin was higher than in S-endoglin cells (Fig. 5A). Accordingly, the trans-activation activity of the $(BRE)_2$-Luc reporter was 3.5 times higher in L-endoglin than in mock cells, whereas S-endoglin $(BRE)_2$-Luc reporter activity was 1.5 times higher than in mock cells (Fig. 5B). However, the $(BRE)_2$-Luc reporter was barely increased by TGFβ1 (Fig. 5B). Fig. 5B also shows a representative western blot of both L- and S-endoglin expression in L_6E_9 myoblasts. Altogether, these results show that L-endoglin expression promotes signaling through the ALK1 receptor.

TGFβ-induced PAI1 expression was also modified by endoglin expression. Whereas L-endoglin significantly decreased PAI1 expression, S-endoglin strongly augmented this response (Fig. 5C). As shown in Fig. 5D, the reporter activity of $(CAGA)_{12}$-Luc was stimulated upon TGFβ treatment in mock cells, whereas the TGFβ-mediated response of this ALK5-dependent reporter was diminished by L-endoglin and strongly enhanced by S-endoglin. Fig. 5D also shows a representative western blot of both L- and S-endoglin expression in L_6E_9 myoblasts. Thus, S-endoglin expression in L_6E_9 promotes signaling through the ALK5 receptor.

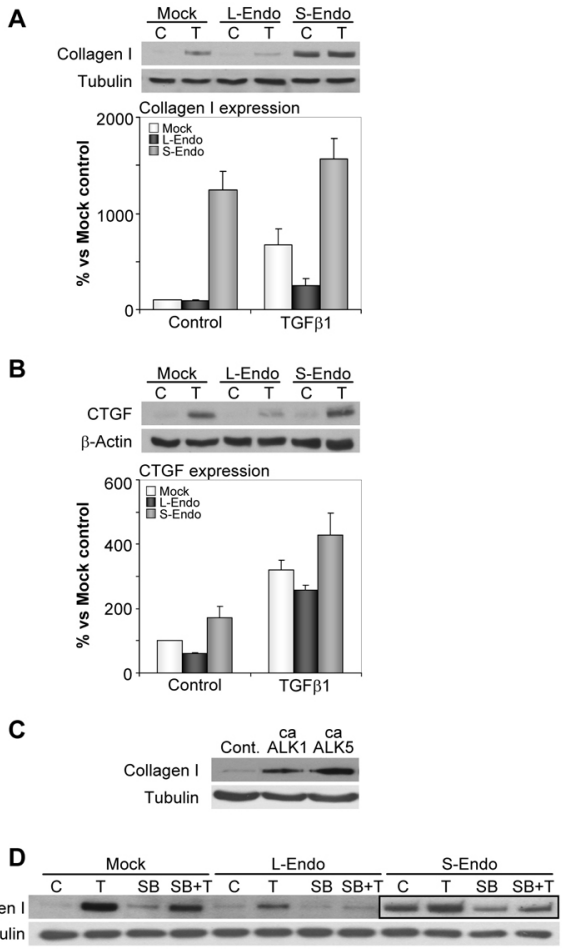

Fig. 6. Effect of L- and S-endoglin on collagen I and CTGF expression. (A,B) Mock, L- and S-endoglin cells were treated (T) or not (C) with TGFβ1 for 24 hours in serum-free medium. Total protein extracts were analyzed by western blot using a specific antibody for collagen I (A) or CTGF (B). Measures of densitometry of each band were performed and relative values are represented. Collagen I and CTGF histogram represents the mean of three different extracts. (C) L_6E_9 cells were transfected with a vector expressing either caALK1 or caALK5, and collagen expression analyzed by western blot. (D) Mock, L-Endo and S-Endo-transfected cells were treated with the ALK5 inhibitor SB431542 (SB, 5 μM) 1 hour before treatment with TGFβ1. Total protein extracts from control (C) or TGFβ1-treated (T) myoblasts were analyzed by western blot with anti-collagen I and anti-tubulin antibodies. A representative blot from three independent experiments is shown. The blot of the S-endoglin samples is under-exposed in order to visualize the differences caused by the SB431542 treatment.

Differential effects of L- and S-endoglin on collagen I accumulation

The effect of L- and S-endoglin on collagen I expression was analyzed in L_6E_9 cells. In basal conditions, collagen I was markedly augmented in S-endoglin but not in L-endoglin cells (Fig. 6A). Collagen I levels increased after TGFβ1 treatment in both mock, L- and S-endoglin L_6E_9 cells (Fig. 6A), although the increase was much higher in S-endoglin and lower in L-endoglin cells than in mock L_6E_9 cells.

To investigate the role of ALK1 and ALK5 signaling pathways on collagen I accumulation, we transfected L_6E_9 cells with a vector expressing either constitutively active (ca) ALK1 or ALK5, and analyzed the effect on collagen I expression. caALK1 and caALK5

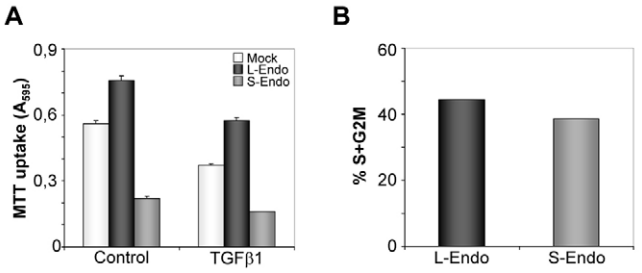

Fig. 7. L- and S-endoglin modify cell proliferation. (A) Proliferation was assessed by the number of cells determined by MTT assay. Cell proliferation was analyzed at day 0 (day of treatment, data not shown) and 3 days after treatment. A representative experiment of three independent experiments using quadruplicate samples is shown. (B) Representative flow cytometry graph showing a higher proportion of L-endoglin cells in S and G2-M phases of the cell cycle.

induced an increase in the collagen I expression that was higher in cells expressing ALK5 than in cells expressing ALK1 (Fig. 6C). These results indicate that ALK1 and ALK5 mediate collagen expression in L_6E_9 cells. In addition, TGFβ1-induced collagen I expression was reduced in the presence of SB431542 in mock, L- and S-endoglin-transfected myoblasts (Fig. 6D); however, the differences in collagen expression between the three groups are preserved.

As connective tissue growth factor (CTGF) seems to be a key mediator of the profibrotic effects of TGFβ1 (Chen et al., 2002; Gupta et al., 2000; Riser et al., 2000), we assessed whether L- or S-endoglin could modify CTGF expression. We observed that under basal conditions, L-endoglin had lower and S-endoglin higher CTGF expression than mock cells (Fig. 6B). TGFβ1 treatment induced a similar percentage of increase in CTGF expression in both types of cells (Fig. 6B). However, the amount of CTGF detected in L-endoglin after TGFβ1 treatment was lesser than in mock, while S-endoglin showed a higher TGFβ1-induced increase in CTGF than did mock L_6E_9 cells (Fig. 6B).

Differential effects of L- and S-endoglin on cell proliferation

We and others have reported the effects of endoglin expression on cell proliferation in the presence or absence of TGFβ1 (Letamendia et al., 1998; Obreo et al., 2004). Here, cells were subcultured in 24-well plates, allowed to attach for 12 hours and then treated or not with 500 pM TGFβ1. Under these conditions, the number of cells in both mock, L- or S-endoglin after 1 day of culture was the same (data not shown). In the absence of TGFβ1 treatment, the number of cells after 4 days of plating was higher in L-endoglin and lower in S-endoglin than in mock cells (Fig. 7A). Incubation with TGFβ1 decreased in a similar way the number of cells in both mock, L- and S-endoglin cells (Fig. 7A). Flow cytometry analysis revealed a greater percentage of L-endoglin cells in S and G2-M phase (44.5%) than in S-endoglin (38.6%) cells (Fig. 7B). These results suggest that L-endoglin promotes, whereas S-endoglin reduces, L_6E_9 proliferation.

Discussion

In the present studies, we investigated the function of L- and S-forms of endoglin on TGFβ1 signaling pathways using the rat myoblast cell line L_6E_9. This is a well-established cellular model reported to be highly responsive to TGFβ1 that, interestingly, lacks endoglin expression (Letamendia et al., 1998; Guo et al., 2004; Obreo et al., 2004; Scherner et al., 2007). As the actual tools used to detect endoglin on tissues and primary cultured cells barely distinguish between L- and S-endoglin, this is an excellent model system with which to analyze the role of endoglin isoforms in modulating TGFβ signaling pathways by transfecting cDNA encoding L- and S-endoglin into the L_6E_9 cells.

The effects of TGFβ1 on target gene expression are well described and attributed to the ubiquitous signaling pathway encompassing the type I receptor ALK5, Smad2 and Smad3. The ALK1-Smad1/5 pathway has been analyzed almost exclusively in endothelial cells (Goumans et al., 2002; Lebrin et al., 2004; Lebrin et al., 2005; Blanco et al., 2005) and only recently its involvement in myoblasts has been suggested (Scherner et al., 2007). In our study, it was noted that L_6E_9 cells express both TGFβ type I and II receptors, and show responsiveness towards TGFβ1, as Smad1/5 and Smad2/3 (respectively) were activated. Moreover, the reporters $(CAGA)_{12}$-Luc and $(BRE)_2$-Luc were activated by TGFβ1. In addition, we showed that TGFβ1 induces Id1 and PAI1 expression in these cells. Our data are consistent with those from Scherner et al. (Scherner et al., 2007) showing that both ALK1- and ALK5-TGFβ1-dependent signaling pathways are present and functional in L_6E_9 myoblasts. One feature in ALK1 signaling is the dependence on ALK5 activity that was previously observed in ALK5-deficient endothelial cells (Goumans et al., 2003) and in L_6E_9 cells (Scherner et al., 2007). We confirmed this characteristic in L_6E_9 cells because TGFβ-dependent Smad2/3 and Smad1/5 phosphorylation was completely blocked in the presence of the ALK5 inhibitor SB431542, which does not interfere with ALK1 group receptors (Laping et al., 2002).

Generally, the studies regarding endoglin are referred to its predominantly expressed long isoform L-endoglin. Although L-endoglin isoform appears to be the predominant endoglin transcript expressed in mouse tissues and cell lines, significant levels of S-endoglin mRNA are co-expressed with L-endoglin in several tissues, such as liver and lung, as well as in endothelial cultured cells (Bellon et al., 1993; Perez-Gomez et al., 2005). S-endoglin rises from an alternative splicing mechanism by which a 136 bp intron between exon 12 and 13 is not eliminated. Consequently, a premature stop codon appears in the reading frame; thus, both endoglin isoforms differ by their cytoplasmic tails. Whereas the cytoplasmic region of L-endoglin contains 47 amino acids, that of S-endoglin has only 14 residues, with the seven juxtamembrane amino acids in common. Recently, it has been reported the existence of the mouse endoglin short isoform (Perez-Gomez et al., 2005), but the role of S-endoglin on TGFβ signaling pathway has not been analyzed in detail.

Our data support the view that endoglin is a modulator of the balance between TGFβ1-ALK1 and TGFβ1-ALK5 signaling pathways. We show that L- and S-endoglin expression increased the ALK1 signaling pathway by increasing Id1 expression in response to TGFβ1, but the effect of L-endoglin was much higher than that of S-endoglin. This view is in agreement with reports showing that L-endoglin promotes endothelial cell proliferation via TGFβ1-ALK1 signaling, while it interferes with the TGFβ1/ALK5 pathway (Lebrin et al., 2004; Blanco et al., 2005). Id1 has been reported to enhance proliferation (Lin et al., 2000) and to serve as an effector for the TGFβ1-ALK1 pathway in mediating the stimulatory effect on proliferation. Consistent with this notion, we found that TGFβ-induced decrease in proliferation is abolished in cells expressing L-endoglin and increased in cells expressing S-endoglin. Lastres et al. (Lastres et al., 1996) found similar response

on TGFβ1-induced [^{3}H]-thymidine uptake in U-937 L- and S-endoglin-transfected monocytes. Besides, S-endoglin highly increased ALK5 signaling pathway that, by contrast, was reduced in L-endoglin cells. This last observation is in agreement with a large number of reports describing the role of L-endoglin as an antagonist of specific ALK5-mediated TGFβ1 responses, including inhibition of cellular proliferation, apoptosis triggering or induction of extracellular matrix synthesis (Letamendia et al., 1998; Obreo et al., 2004; Diez-Marques et al., 2002; Li et al., 2003). It is important to note that after 3 days of serum depletion, L-endoglin increased, whereas S-endoglin decreased cell proliferation, suggesting that a TGFβ-independent pathway is implicated in this biological effect of endoglin. Because the extracellular domain of endoglin is common in both L- and S-isoforms, the cytoplasmic tails would be responsible for the antagonist effects observed.

However, in the presence of S-endoglin, L_6E_9 cells accumulate much more collagen I and express more CTGF, while L-endoglin cells accumulate less collagen I and express less CTGF than mock-transfected cells. Furthermore, caALK5-transfected cells accumulate more collagen than do caALK1-transfected cells. These data suggest that S-endoglin expression could favor ALK5-mediated TGFβ1 responses such as induction of extracellular matrix synthesis. Ectopic expression of L-endoglin in cell lines has been reported to modulate their responses to TGFβ1, possibly by favoring signaling via the ALK1 pathway (Letamendia et al., 1998). Our results show that S-endoglin plays an important role in TGFβ1 signaling in L_6E_9 cells by increasing ALK5-PAI1 pathway while L-endoglin enhances ALK1-Id1 pathway. Studies in the human promonocytic line U-937 showed that S-endoglin transfectants produced a higher amount of extracellular matrix components such as fibronectin in response to TGFβ1 than L-endoglin, but less than mock cells (Lastres et al., 1996). These data indicate a different pattern of cellular response modulation by TGFβ, depending on the endoglin isoform. We show that the TGFβ1-induced collagen I synthesis occurs, in part, via upregulation of ALK1, as caALK1 increases collagen expression in L_6E_9 cells. The short endoglin isoform is able to exert opposite effects to that of the largely expressed L-endoglin on the TGFβ signaling (Bellon et al., 1993; Perez-Gomez et al., 2005), including the positive and negative cooperation with ALK5 and ALK1, respectively. Interestingly, we have found that ALK5 and ALK1, as well as TβRII, levels remain unchanged in L- or S-endoglin-transfected L_6E_9 cells. Therefore, balancing the TGFβ signal through ALK5 or ALK1 would depend on the S-endoglin : L-endoglin ratio present on the cells. However, S-endoglin could not cooperate with the ALK1 signaling pathway owing to the low affinity of the interaction, thus leading the signal through ALK5. Supporting this hypothesis, an anti-angiogenic effect of S-endoglin has been recently suggested, in contrast to the pro-angiogenic role attributed to L-endoglin (Perez-Gomez et al., 2005). In S-endoglin-transfected cells without treatment, collagen I expression is higher than in mock-transfected myoblasts, thus suggesting that a TGFβ-independent pathway is involved in this biological effect of endoglin. In this regard, we show here that ALK1 inhibition reduces only the collagen synthesis induced by TGFβ1, and we have already demonstrated the dependence of collagen I synthesis on p38 MAPK in L_6E_9 myoblasts (Rodríguez-Barbero et al., 2002).

Taken together, our data demonstrate a different and sometimes opposed effect of L and S isoforms of endoglin on the regulation of TGFβ-induced responses and signaling in L_6E_9 cells. Furthermore, some of the effects of the endoglin isoforms could be independent of TGFβ.

Materials and Methods

Cell culture

The rat myoblast cell line L_6E_9 and their stable transfectants expressing human L-endoglin and S-endoglin were obtained and maintained as described (Letamendia et al., 1998). In brief, clone 3.3 in pUC13 was digested with *Bbr*PI and *Bam*HI. The endoglin fragment was made blunt and inserted into the mammalian expression vector pcEXV, yielding pcEXV-EndoS. The lack of leader sequence in the L-endoglin cDNA was overcome by the construction of pcEXV-EndoL. pcEXV-EndoS was digested with *Mlu*I and *Bam*HI and ligated to the 563 bp *Mlu*I-*Bam*HI fragment specific of L-endoglin cDNA, resulting in pcEXV-EndoL as previously described (Bellon et al., 1993). Myoblast transfectants were generated by co-transfecting pcEXVEndoL or pcEXVEndoS vectors and pSV2neo plasmid (Clontech) that contains a neomycin resistance gene at a 10:1 ratio. 10 mg of plasmid DNA were mixed with 20 mg of Lipofectin (Life Technologies) in serum-free medium according to the protocol provided by the manufacturer. Positive clones were selected in the presence of 400 mg/ml of the antibiotic G418. Parallel transfections with psV2neo alone yielded endoglin-negative mock transfectants. Parental and transfectant cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science) containing 10% fetal bovine serum (FBS, Gibco) and 100 U/ml of penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere. 24 hours after plating, cells were serum starved for 24 hours and treated with the active human recombinant TGFβ1 at 500 pM.

Antibodies and reagents

Purified TGFβ1 was purchased from R&D Systems. ALK5 inhibitor SB431542 was from Tocris. Antibodies against ALK5 (sc-399), ALK1 (sc-19546), β-actin (sc-1616), Smad1/5 (sc-7965), Smad2/3 (sc-6032), Smad4 (sc-7966), Id1 (sc-488) and CTGF (sc-14939) were from Santa Cruz Biotechnology. Antibodies against phosphorylated Smad1/5, and phosphorylated Smad2 were generated as described (Persson et al., 1998). Antibody against phosphorylated Smad1/3 (CS-9514) was from Cell Signaling. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-mouse IgG and anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology. Anti-α-tubulin antibody was from Calbiochem, anti-TβRII was from Upstate Biotechnology. Anti-collagen I antibody was from Chemicon. Anti-endoglin antibody was P3D1, which has been previously described (Pichuantes et al., 1997).

RT-PCR analysis

Total RNA was isolated using NucleoSpin RNAII (Macherey-Nagel) according to the manufacturer's instructions. First-strand cDNA was generated from 2 μg of total RNA using poly-dT as primers with the M-MLV reverse transcriptase (Promega), 0.5 mg of cDNA were used in a standard 50 ml PCR mixture with 2 ng/μl of each primer and 2 U of FastStart Taq DNA polymerase (Roche). The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The primers were designed for specific sequences and checked by BLAST algorithm (Altschul et al., 1997).

Western blot analysis

Western blot analysis was performed basically as described (Rodriguez-Barbero et al., 2001). Cells were lysed on ice-cold lysis buffer and protein concentrations were determined (Bradford, BioRad). Protein samples were separated by SDS-PAGE, blotted onto PVDF membranes, and incubated with the primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-tubulin and β-actin antibodies were used to confirm equal loading of protein in each lane. Protein expression was quantified by densitometry using Scion Image software (Scion). Some membranes were re-probed with several antibodies using a stripping solution (Chemicon) following the manufacturer's instructions.

Immunofluorescence staining

Immunofluorescence staining was performed as described (Rodriguez-Barbero et al., 2001). Cells were plated onto glass coverslips, fixed, permeabilized and incubated with primary antibodies for 1 hour. After washing, cells were incubated with the appropriate Cy3 or Alexa Fluor 488-conjugated secondary antibodies (Jackson Immunoresearch) for 30 minutes. Slides incubated only with the secondary antibody were used to control for non-specific binding. Cells were washed in 0.2% BSA-PBS, rinsed briefly in 2 mM Hoechst (Sigma) to stain the nuclei, and mounted with mowiol (Hoechst). Stained cells were photographed using a Zeiss fluorescence microscope (Carl Zeiss) equipped with a digital camera.

Plasmids, transfection and luciferase reporter assay

The TGFβ-responsive vectors used as reporters were the ALK5 specific $(CAGA)_{12}$-Luc (Dennler et al., 1998) and $p(BRE)_2$-Luc that contains the crucial ALK1-specific response elements of the Id1 promoter (Korchynskyi and ten Dijke, 2002). Expression plasmids for mutant ALK5 and ALK1 have been described (Goumans et al., 2002). In luciferase assays, the expression plasmid pRL-TK vector containing the Renilla luciferase gene (Promega) and the pGLE2 and pGLE3-basic vectors served as internal controls to correct for transfection efficiency. Cells were transfected for 5 hours using jetPEI transfection reagent (Polyplus transfection) according to the manufacturer's instructions. Cells were grown in FBS-free medium for 18 hours and treated with

TGFβ1 (500 pM) for 24 hours. Then cells were lysed for western blot or reporter assays. Luciferase and renilla activities were measured using a dual-reporter assay kit (Promega).

Cell proliferation assay

Subconfluent monolayer cultures were plated in 24-well plates to a density of 12,000 cells per well. 12 hours after plating, cells were serum starved and treated with TGFβ1. Cell proliferation was analyzed after TGFβ1 treatment by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay (Roche). Cell proliferation was measured based on absorbance at 595 nm using a Sunrise plate reader (Bio-Tek Instruments). Each experiment was performed in quadruplicate and repeated three times. The amount of color produced is directly proportional to the number of viable cells and is represented as the MTT uptake. For cell cycle studies, cells were plated in 100 mm plates, cultured for 24 hours and serum starved for an additional period of 24 hours. Then, cell cycle analysis was evaluated by flow cytometry.

Data analyses

All numerical data are presented as mean±s.e.m. and were analyzed by one way ANOVA and the Student's *t*-test. The entire statistical tests were performed using SPSS 14.0 software.

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