Endoglin Modulation of TGF- β 1-Induced Collagen Synthesis is Dependent on ERK1/2 MAPK Activation

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Key Words

L6E9 myoblasts • Endoglin • Transforming growth factor beta (TGF- β) • TGF- β receptor system • Extracellular matrix (ECM) • Collagen • p38 • ERK1/2

Abstract

Background/Aims: Transforming growth factor- β 1 (TGF- β 1) plays a pivotal role in the extracellular matrix accumulation observed in fibrotic diseases. Endoglin is an important component of the TGF- β receptor complex highly expressed in tissues undergoing fibrotic processes. Endoglin expression regulates the effect of TGF- β on extracellular matrix synthesis. The purpose of our study has been to understand the molecular mechanism by which endoglin exerts its effects on fibrosis and the possible role of MAP kinases in these effects. **Methods:** We have assessed in mock and in endoglin-transfected L6E9 myoblasts the effect of TGF- β 1 on collagen mRNA by Northern blot and effect of TGF- β 1 on collagen

content in the cultured medium by [³H]-Proline incorporation into collagen proteins. Total and activated MAPK and their role on collagen synthesis were assessed by Western blot. **Results:** TGF- β 1 induced an increase on $\alpha_2(I)$ collagen mRNA expression and collagen accumulation in mock-transfected myoblasts, whereas the response was much lower in endoglin-transfected cells. TGF- β 1 activated the ERK1/2 and p38 MAPK pathways but not the JNK pathway in L6E9 myoblasts. TGF- β 1-induced $\alpha_2(I)$ collagen mRNA expression and collagen accumulation were completely inhibited by SB203580, in either mock or endoglin-transfected myoblasts. PD98059 increased TGF- β 1 induced-collagen synthesis and accumulation in endoglin-transfected myoblasts but not in mock cells. **Conclusion:** Our studies demonstrate that TGF- β 1-induced collagen synthesis is mediated by p38 MAPK activation in L6E9 myoblasts. Furthermore, endoglin expression reduces basal and TGF- β 1 induced collagen synthesis when ERK1/2 pathway is operating.

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Introduction

Transforming growth factor beta 1 (TGF- β 1) plays a pivotal role in the extracellular matrix accumulation observed in chronic progressive tissue fibrosis [1, 2] and in the pathogenesis of several chronic fibrotic diseases characterized by increased expression of TGF- β 1 and excessive accumulation of extracellular matrix (ECM) [3, 4]. For this reason, numerous investigators have focused on dissecting the signalling pathways induced by TGF- β in order to identify targets to design effective therapies that could revert or prevent fibrotic diseases. TGF- β 1 is a member of a large family of polypeptide factors that exert their function through binding to specific receptors, including receptors type I (R-I), type II (R-II), betaglycan and endoglin [5].

Endoglin/CD105 is a 180-kDa homodimeric type-I integral membrane protein that functions as an auxiliary receptor, binds TGF- β 1, TGF- β 3, activin-A, BMP2 and BMP-7 in the presence of the signaling receptor type I and II [6-8], and modulates TGF- β 1-induced synthesis of ECM-related molecules including collagen, fibronectin, plasminogen activator inhibitor-1 (PAI-1), and lumican [6, 9]. We have previously demonstrated that endoglin is up-regulated in several experimental models of renal fibrosis [10, 11]. Interestingly, endoglin was found to be expressed in mesangial cells, which are a major source of extracellular matrix in fibrotic kidneys, and this expression was further up-regulated by TGF- β 1 [12, 13]. In addition, it has been shown that endoglin expression reduces both basal and TGF- β 1-stimulated collagen and PAI-1 accumulation in L₆E₉ myoblasts [7, 14]. L6E9 rat myoblasts are a useful model to study the effect of endoglin on cell biology because these cells do not express endoglin neither in basal conditions nor after TGF- β stimulation and it is possible to obtain stable endoglin transfectants [7, 8, 14].

The TGF- β signaling downstream the cell surface receptor complex triggers Smad-dependent and Smad-independent pathways [15]. The mitogen-activated protein kinase (MAPK) pathway is activated by TGF- β in a Smad-independent fashion [16, 17]. There are three well characterized cascades of MAPKs: i) the extracellular signal-regulated kinase (ERK); ii) the p38 MAPK; and iii) the c-Jun N-terminal kinases (JNK) [18, 19]. These MAPK components operate in a pleiotropic way regulating a wide variety of physiological functions, including cell growth, differentiation, apoptosis, immune responses, and fibrogenesis. TGF- β regulates several MAPK pathways, but the mechanisms of activation appear to be cell-

type specific and remain poorly understood [20-24]. While TGF- β triggers a rapid induction of ERK1/2 and JNK activity in mesangial cells, specific blockade of ERK reduces TGF- β 1-induced collagen expression. On the other hand, TGF- β 1 induces collagen synthesis and accumulation via p38 MAPK pathway in cultured L6E9 myoblasts and in myofibroblasts [25, 26]. Given the modulator role of endoglin in the synthesis of collagen and other ECM components induced by TGF- β [12-14, 19], and the fact that TGF- β -induced collagen synthesis is mediated by MAPK [25], here we have investigated the effect of endoglin expression on MAPK activation and its potential role as mediator of TGF- β 1-induced ECM accumulation.

Materials and Methods

Stable transfectants and growth factor stimulation

Generation of stable transfectants of rat myoblast cell line L₆E₉ expressing human L-endoglin and the corresponding control (mock transfectants) have been already described [7]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FBS (Biowhittaker) and 100 U/ml of penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Cells were plated in 6 x 4 well plates (2 x 10⁴ cells/well), for collagen determination, and in 100-mm culture dishes (4 x 10⁵ cells/dish) for Northern and Western blot analyses. Twenty-four hours later, cells were serum starved for 24 hours, and after adding fresh serum-free medium, cells were treated with various concentrations of active human recombinant TGF- β 1 (R & D Systems; dilutions made from a 1 μ g/ml stock solution in 4 mM HCl containing 1 mg/ml BSA) or control vehicle during 24 hours.

Inhibitors used in these experiments included the MEK-1 inhibitor PD98059 (50 μ M) (New England Biolabs), and the p38 MAPK inhibitor, SB203580 (10 μ M) (Calbiochem). Pre-treatments with these inhibitors were for 30 min.

Collagen determination

The collagen content in the cultured medium was quantified by measuring the incorporation of [³H]-Proline into collagen proteins as previously described [14, 25]. L-[³H]-proline (2 μ Ci/well) was added at the same time as TGF- β 1 to the cells.

Northern blot analysis

Total RNA was isolated by cell lysis using the guanidinium thiocyanate-phenol-chloroform method. A 20 μ g aliquot of total RNA was electrophoresed in 1.2% denaturing formaldehyde agarose gel and blotted onto Hybond-N (Amersham). The radiolabeled probes were hybridized at 60°C in a hybridization solution (1% w/v SDS, 10% w/v dextran sulphate, and 1 M NaCl). Blots were washed twice in 3 M NaCl, 30 mM sodium citrate (pH 7.0), 1% (w/v) SDS for 30 min at 60°C, followed by a last wash with 0.3 M NaCl, 3 mM sodium citrate (pH 7.0), 0.1%

Fig. 1. Effects of endoglin expression on collagen synthesis and accumulation induced by TGF- β 1 in L₆E₉ myoblasts. Quiescent mock and endoglin-transfected myoblast were incubated with or without TGF- β 1 (10 ng/ml) for 24 hours. (A) The levels of specific transcripts of α_2 (I) collagen mRNA were detected by Northern blot analysis. The levels of collagen mRNA in mock and endoglin transfected myoblasts were analyzed by densitometric analysis and corrected by their respective 18S. (B) Total collagen accumulation in culture media was measured by metabolic radiolabeling with [³H]-proline. Results are expressed as percentage over mock control. Each value represents the mean $\pm$ SEM of 3 experiments. * p <0.05 versus control. + p <0.05 versus mock+ TGF- β 1.

(w/v) SDS for 1 hour at room temperature. The rat pro- α_2 (I) collagen cDNA inserted in pUC18 plasmid (kindly provided by Dr. Diego Rodríguez-Puyol, University of Alcalá de Henares, Spain), was digested with PstI and the resulting 600-pb fragment was used as a probe. To control for relative equivalence of RNA loading, blots were hybridized with a [³²P]-labeled 18S rRNA probe.

Western blot analysis

Myoblasts were treated with TGF- β 1 (10 ng/ml) for different periods of time leading up to simultaneous harvest. Total cell extracts were obtained by lysis in RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS] containing protease and phosphatase inhibitors (1 mM PMSF, 1 mM EDTA, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, and 1 mM Na₃VO₄). Equal amounts of proteins were electrophoresed by SDS-PAGE and then electroblotted onto Immobilon-P PVDF membranes. The membranes were incubated with anti-ERK2 (C-14), anti-phospho-ERK (E-4), anti-phospho p38, anti-p38, or anti-JNK (C-17) antibodies (Santa Cruz) for two hours at room temperature, followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase (HRP) for 30 minutes at room temperature. Signal development was assessed by chemiluminescence.

Immunoprecipitation of JNK was performed by binding 2 μ g of JNK polyclonal antibody (C-17, Santa Cruz) with 60 μ l Protein A Sepharose beads and one milligram of protein from whole cell extracts. Precipitated proteins were separated by 10% SDS-PAGE. The membranes were incubated with the anti-

p-JNK (G-7, Santa Cruz) antibody. Western analysis and autoradiographic detection were performed as above.

Statistical analysis

Statistical differences between experimental groups were determined by analysis of variance using NCSS 2000 software for PC. Values of p <0.05 were considered significant.

Results

Endoglin expression decreases basal and TGF- β 1-induced collagen synthesis and accumulation through the MAPK pathway in L₆E₉ myoblasts

To study the role of endoglin in ECM accumulation, we used the rat myoblasts cell line stably transfected with human endoglin (endoglin) and the corresponding mock-transfected L₆E₉ cells (mock). We have previously demonstrated that the corresponding parental myoblasts cell

Fig. 2. Effect of p38 MAPK inhibition on TGF-β1 induced α₂(I) collagen mRNA expression and collagen accumulation in mock and endoglin transfected L₆E₉ myoblasts. Mock and endoglin transfected L₆E₉ rat myoblasts were pretreated with (+) or without (-) SB203580 (10 μM) and incubated in the presence (+) or absence (-) of exogenous TGF-β1 (10 ng/ml) for 24 h. (A) Northern blot analyses of pro-α₂(I) collagen mRNA as in Figure 1A (B) Collagen accumulation was measured as in Figure 1B. Each value represents the mean ± SEM of two experiments each one in triplicate. *p<0.05 versus mock control; #p<0.05 versus endoglin control. SB203580 was dissolved in DMSO (0.2%).

line do not express endogenous endoglin [14]. Endoglin-transfected myoblasts showed a lower α₂(I) collagen mRNA expression than mock-transfectants (Figure 1A). Incubation with exogenous TGF-β1 induced a three-fold increase on α₂(I) collagen mRNA expression in mock-transfected myoblasts, whereas the response was much lower in endoglin-transfected cells (Figure 1A). Accordingly, collagen accumulation increased after treatment with TGF-β1 in both endoglin and mock-transfected myoblasts (Figure 1B), although collagen accumulation was significantly higher in mock- than in endoglin-transfected myoblasts. In addition, basal collagen levels were significantly lower in endoglin than in mock-transfectants (Figure 1B).

Since TGF-β1 activates the ERK1/2 and p38 MAPK pathways in this cell line [25], we next investigated whether the effect of endoglin expression on TGF-β1-induced collagen accumulation was mediated by the modulation of these pathways. For this purpose we used inhibitors of ERK1/2 and p38 MAPK pathways. Mock

and endoglin transfected myoblasts were pretreated for 30 min with PD98059, a specific inhibitor of the ERK1/2 kinase MEK 1/2, or with SB203580, a selective inhibitor of the p38 MAPK, and then treated with TGF-β1 for 24h. TGF-β1-induced α₂(I) collagen mRNA expression and total collagen accumulation was assessed either in the presence or in the absence of the inhibitors. TGF-β1-induced α₂(I) collagen mRNA expression and collagen accumulation were completely inhibited by SB203580, in either mock or endoglin-transfected myoblasts (Figures 2A and 2B). In addition, PD98059 increased TGF-β1 induced-collagen synthesis and accumulation in cells transfected with endoglin but not in mock cells (Figure 3A and 3B). As a control, PD98059 efficiently prevented the activation of ERK1/2 MAPK pathway induced by TGF-β1 (Figure 3C). No alterations in the expression and accumulation of collagen were observed when myoblasts were treated with the MAPK inhibitors solvent (DMSO) (data not shown).

Fig. 3. Effect of ERK1/2 MAPK inhibition on TGF-β1 induced α₂(I) collagen mRNA expression and collagen accumulation in mock and endoglin transfected L₆E₉ myoblasts. Mock and endoglin transfected L₆E₉ rat myoblasts were pretreated with (+) or without (-) PD98059 (50 μM) and incubated in the presence (+) or absence (-) of exogenous TGF-β1 (10 ng/ml) for 24 h. (A) Northern blot analyses of pro-α₂(I) collagen mRNA as in Figure 1A. (B) Collagen accumulation was measured as in Figure 1B. Each value represents the mean ± SEM of two experiments, each one in triplicate. *p<0.05 versus mock control; +p<0.05 versus endoglin control; #p<0.05 versus endoglin+TGF-β1. (C) Representative Western blot of ERK1/2 phosphorylation using anti-phospho-ERK antibody (phospho ERK1/2). TGF-β1 (10 ng/ml) and PD98059 (10 μM) treatments are indicated. Cell extracts from mock and endoglin myoblasts stimulated by osmotic shock (0.7 M sorbitol, 60 min) served as a positive control (C+).

Effect of endoglin expression on the MAPK pathways activation induced by TGF-β

Since inhibition of the ERK1/2 MAPK pathway seems to counteract the negative effect of endoglin on the TGF-β-induced collagen expression, we analyzed whether endoglin expression would affect the TGF-β-induced ERK 1/2 activation. As shown in Figure 4A, expression of total ERK1 and ERK2 do not change upon treatment with TGF-β1 in both types of myoblasts. However, there is a reduction in total ERK1 in the endoglin cells compared with mock transfectants, and an increase in total ERK2 in the endoglin cells compared with mock transfectants (Figure 4Aa). The representative Western blot of ERK1 and ERK2 phosphorylation in the figure 4Ab shows that TGF-β1 induced ERK1 and ERK2 phosphorylation in L6E9 myoblasts that is maximal at 30 minutes of treatment. This phosphorylation was always higher

in mock than in endoglin transfected myoblasts. We analyzed the kinetics of TGF-β-induced ERK1 and ERK2 phosphorylation related to total ERK1 and ERK2 respectively in mock *versus* endoglin transfected myoblasts (Figure 4A). Peak levels for TGF-β-induced ERK1 phosphorylation were reached after 5 min of stimulation in mock and 15 min of stimulation in endoglin transfected myoblasts (Figure 4Ac). Peak levels for TGF-β-induced ERK2 phosphorylation were reached after 30 min of stimulation in mock and 5 min of stimulation in endoglin transfected myoblasts (Figure 4Ac). Thus, TGF-β-induced ERK1 phosphorylation is higher and faster in mock than in endoglin transfected myoblasts. TGF-β-induced ERK2 phosphorylation is higher, slower and much more stable in mock than in endoglin transfected myoblasts (Figure 4Ac).

The expression of total p38 was found to be similar

Fig. 4. Effect of endoglin expression on TGF- β 1-induced MAPK activation. Mock and endoglin L₆E₉ rat myoblasts were incubated with exogenous TGF- β 1 (10 ng/ml) for the indicated times in absence of serum. (Aa) Densitometric analysis of Western blot for total ERK1 and ERK2 protein expression in mock and endoglin transfected myoblasts. (Ab) Representative Western blot of ERK1 and ERK2 phosphorylation from cellular extracts using anti-ERK- antibody (phospho-ERK1/2) in mock and endoglin-transfected myoblasts. Cell extracts from L₆E₉ myoblasts stimulated by osmotic shock (0.7 M sorbitol, 60 min) served as a positive control (C+). (Ac) Densitometric analysis of Western blot for phosphorylated

in mock- and -endoglin transfected myoblasts both untreated and TGF- β 1-treated samples (Figure 4B). p38 MAPK phosphorylation was increased at 30 min of TGF- β 1 treatment in both mock- and endoglin-transfected myoblasts. As opposed to the ERK1/2 and p38 pathways, there was no phosphorylation of JNK upon TGF- β 1 treatment in either mock- or endoglin-transfected L₆E₉ myoblasts (Figure 4C). Total JNK protein in cellular extracts did not change along the experiment (data not shown).

Discussion

L₆E₉ rat myoblasts are a useful model to study the effect of endoglin on cell biology because these cells do not express endoglin and it is possible to obtain stable endoglin transfectants [7, 8, 14]. In fact these cells have been previously used for this purpose [7, 28-31].

In addition, these cells synthesize and release collagen, and this synthesis is enhanced by TGF- β 1 [14, 25, 27, 28].

Our results on the inhibitory activity of endoglin on TGF- β -induced extracellular matrix synthesis are supported by previous reports. Letamendia *et al.* used rat myoblasts to demonstrate the inhibitory role of endoglin on TGF- β 1-induced PAI-1 synthesis and promoter activity [7]. Furthermore, inhibition of TGF- β 1-mediated synthesis and secretion of $\alpha_2(I)$ collagen by endoglin in a mouse fibroblast cell line has been reported [13], and endoglin expression regulates basal and TGF- β 1-induced extracellular matrix synthesis in cultured L6E9 myoblasts [14]. The observation that overexpression of endoglin in NIH3T3 cells led to a reduction in the TGF- β 1-induced promoter activity of connective tissue growth factor (CTGF) also supports our results [23]. Previous studies have reported that endoglin overexpression reduces other biological effects of TGF- β 1 [7, 9, 29]. The classical mechanism of cell signaling by TGF- β is the activation of the Smad cascade [32]. The mechanism that we show here is an additional one, involving regulation of MAP kinase activation by endoglin. It has been already demonstrated that TGF- β 1 induces extracellular matrix synthesis in mesangial cells by mechanisms that involve MAP-kinase activation in addition of SMAD activation [33].

In the present study, TGF- β 1 did not induce JNK phosphorylation either in mock or endoglin-transfected myoblasts, thus suggesting that in this cell line JNK does not participate in the regulation of collagen synthesis by TGF- β 1. It has been already demonstrated that p38 mediates TGF- β 1-induced collagen synthesis in L6E9 myoblasts [25]. However, the present study demonstrates that when cells are transfected with endoglin, p38 phosphorylation is not modified, and that p38 inhibition has the same effect in mock and endoglin-transfected myoblasts. These data demonstrate that the effect of

endoglin expression on TGF- β 1-induced collagen synthesis is not mediated by p38 activation.

Our results clearly demonstrates that, compared with mock-transfected cells, myoblasts expressing endoglin has lower levels of ERK1 and ERK2 phosphorylation either in basal conditions or after incubation with TGF- β 1. In mock-transfected cells, that do not express endoglin, blocking ERK1/2 phosphorylation with PD98059 has no effect on TGF- β 1-induced collagen synthesis. However, in endoglin-transfected cells, incubation with PD98059 induced a marked increase in TGF- β 1-induced collagen synthesis. Thus this study demonstrates that in the presence of endoglin, ERK1/2 activation blocks TGF- β 1-induced collagen synthesis. Similar to our results, and in the same cells, Tiffin *et al.* [34] have reported that only when ERK1/2 phosphorylation is inhibited, IGF-I can stimulate myogenin mRNA expression. The mechanism by which ERK1/2 inhibition stimulates TGF- β -induced collagen synthesis in endoglin-transfected cells can not be deduced from the present studies. However, it has been reported in human mesangial cells that cross-talk between ERK1/2 MAPK and Smad signaling pathways enhances TGF- β -dependent collagen synthesis [33].

In summary, our studies demonstrate that although p38 phosphorylation is necessary for TGF- β 1-induced collagen synthesis, in the presence of endoglin it must be accompanied by a decrease in ERK1/2 phosphorylation to obtain the maximal effect.

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