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Transforming growth factor-β1 induces collagen synthesis and accumulation via p38 mitogen-activated protein kinase (MAPK) pathway in cultured L_6E_9 myoblasts

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Abstract Transforming growth factor-β (TGF-β) plays a pivotal role in the extracellular matrix accumulation observed in chronic progressive tissue fibrosis, but the intracellular signaling mechanism by which TGF-β stimulates this process remains poorly understood. We examined whether mitogen-activated protein kinase (MAPK) routes were involved in TGF-β1-induced collagen expression in L_6E_9 myoblasts. TGF-β1 induced p38 and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation whereas no effect on Jun N-terminal kinase phosphorylation was observed. Biochemical blockade of p38 but not of the ERK MAPK pathway abolished TGF-β1-induced α_2(I) collagen mRNA expression and accumulation. These data indicate that TGF-β1-induced p38 activation is involved in TGF-β1-stimulated collagen synthesis.

Key words: Extracellular matrix; Fibrosis; Signaling; Transforming growth factor-β1

1. Introduction

Transforming growth factor-β1 (TGF-β1) is a member of a large family of polypeptide factors that participates in a wide array of biological activities such as development and wound repair, as well as pathological processes [1–3]. TGF-β1 plays a pivotal role in the extracellular matrix accumulation observed in chronic progressive tissue fibrosis [2,4] and in the pathogenesis of several chronic inflammatory diseases including pulmonary fibrosis [5], glomerulonephritis [6] and proliferative vitreoretinopathy [7]. Each of these diseases is characterized by increased expression of TGF-β1 and excessive accumulation of extracellular matrix (ECM). TGF-β1 induces the synthesis of a number of ECM proteins in vivo and in vitro, including types I and III collagen, fibronectin, laminin, and proteoglycans [8–12]. Type I collagen is a major structural component of the ECM, which is synthesized by fibroblasts and vascular smooth muscle cells.

Biological actions of TGF-β are mediated by two transmembrane Ser/Thr kinases, types I and II TGF-β receptors [3,13], which are co-expressed by most cells, including L_6E_9 myoblasts [14,15]. Several studies suggest that TGF-β1 may act as a physiological regulator of myogenic differentiation in L_6E_9 myoblasts. Thus, it can act as an inducer of myogenic differentiation in mitogen-rich environments [16], although in the absence of mitogens, TGF-β1 acts as an inhibitor of myoblast differentiation [17]. This inhibition seems to be mediated by TGF-β1-induced upregulation of type I collagen expression and deposition in L_6E_9 myoblasts, because a fibrillar type I collagen layer inhibits L_6E_9 myoblast differentiation [17]. Ignotz et al. [9] have demonstrated in rat L_6E_9 myoblasts that TGF-β1 induces collagen and fibronectin synthesis.

The routes that participate in this myogenic response to TGF-β1 are still unknown. TGF-β1 has been shown to activate the Smad cascade, leading to the regulation of specific genes. In addition, the mitogen-activated protein kinase (MAPK) routes have also been implicated in signal transduction by TGF-β receptors. However, to which extent the later routes may participate in collagen deposition triggered by TGF-β1 in L_6E_9 cells is unresolved. This investigation was thus undertaken to determine whether TGF-β1 activates specific MAPK signaling pathway(s) in cultured rat L_6E_9 myoblasts and whether this MAPK activation mediates the biological effect of TGF-β1 on type I collagen synthesis and accumulation. Our findings clearly show that TGF-β1 activates extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK pathways, and that p38 MAPK activation is involved in TGF-β1-induced collagen synthesis.

2. Materials and methods

2.1. Cell culture and growth factor stimulation

L_6E_9 rat myoblasts were seeded at a density of 4×10^5 cells/plate on 100 mm petri dishes for Northern and Western blot analysis and at 2×10^4 cells/well on 24 well plates for ECM measurements. Cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Biowhittaker) and 100 U/ml of penicillin/streptomycin at 37°C in the presence of 5% CO_2. When cultures achieved 80–90% confluence, cells were serum-starved for 24 h. Cells were treated with active human recombinant TGF-β1 (R&D Systems) or control vehicle during 24 h in the absence of serum.

Inhibitors used in these experiments included the MEK-1 inhibitor PD98059 (50 μM) (New England Biolabs), and the p38 MAPK in-

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hibitor, SB203580 (10 μM) (Calbiochem). Pretreatments with these inhibitors were for 30 min.

2.2. Collagen determination

The collagen content in the medium was quantified by measuring the incorporation of [^{3}H]proline into collagen proteins, as previously described [18]. Protein content of cell extracts was determined by the Bradford assay (Bio-Rad).

2.3. Northern blot analysis

Total RNA was isolated by cell lysis with the guanidinium thiocyanate–phenol–chloroform method [19]. A 20 μg 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 sulfate, 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 each at 60°C followed by a last wash with 0.3 M NaCl, 3 mM sodium citrate (pH 7.0), 0.1% (w/v) SDS for 1 h at room temperature. The rat pro-α_2(I) collagen (600 bp *Pst*I fragment) cDNA probe was obtained by digestion of the corresponding cDNA inserted into plasmid pUC18, and was kindly provided by Dr. Diego Rodríguez-Puyol (University of Alcalá de Henares, Madrid, Spain). To control for relative equivalence of RNA loading, blots were hybridized with ^{32}P-labeled 18S rRNA probe.

2.4. Cell lysates and Western blot analysis

Myoblasts were treated with 50 pM TGF-β1 for different periods of time leading up to simultaneous harvest. Total cell extracts were obtained by cell 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 phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM Na_3VO_4). Cell extracts were then centrifuged at 15 000×*g* for 10 min at 4°C, supernatants collected and protein content determined as above. Fresh cell extracts were prepared in Laemmli buffer (final concentration: 125 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol). Samples were resolved by SDS–PAGE and then electroblotted onto Immobilon-P polyvinylidene difluoride membranes. The membranes were incubated with anti-ERK2 antibody C-14, 0.04 μg/ml; anti-phospho-ERK antibody, E-4, 0.02 μg/ml; anti-phospho p38, 0.2 μg/ml; anti-p38 1 μg/ml, or anti-Jun N-terminal kinase (JNK) antibody, C-17 (Santa Cruz) for 2 h at room temperature followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature. Signal development was by chemiluminescence. The levels of activated MAPK were quantitated by densitometric analysis using the National Institutes of Health (NIH) image program. Results shown were corrected for total MAPK.

Immunoprecipitation of JNK was performed by binding 2 μg of JNK polyclonal antibody C-17 (Santa Cruz) with 60 μl protein A Sepharose beads and 1 mg of protein from whole cell extracts. Immunoprecipitates were eluted by boiling in 2×SDS Laemmli buffer prior to 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.

3. Results

3.1. Stimulation of pro-α_2(I) collagen mRNA expression and collagen accumulation by TGF-β1

Treatment with exogenous TGF-β1 increased pro-α_2(I) collagen mRNA expression in a dose-dependent fashion (Fig. 1A). To assess that increased collagen mRNA expression was followed by collagen deposition, we measured [^{3}H]proline incorporation into collagen proteins. Similarly to the mRNA data, exogenous TGF-β1 increased collagen accumulation in the culture media of L_6E_9 myoblasts in a dose-dependent fashion (Fig. 1B).

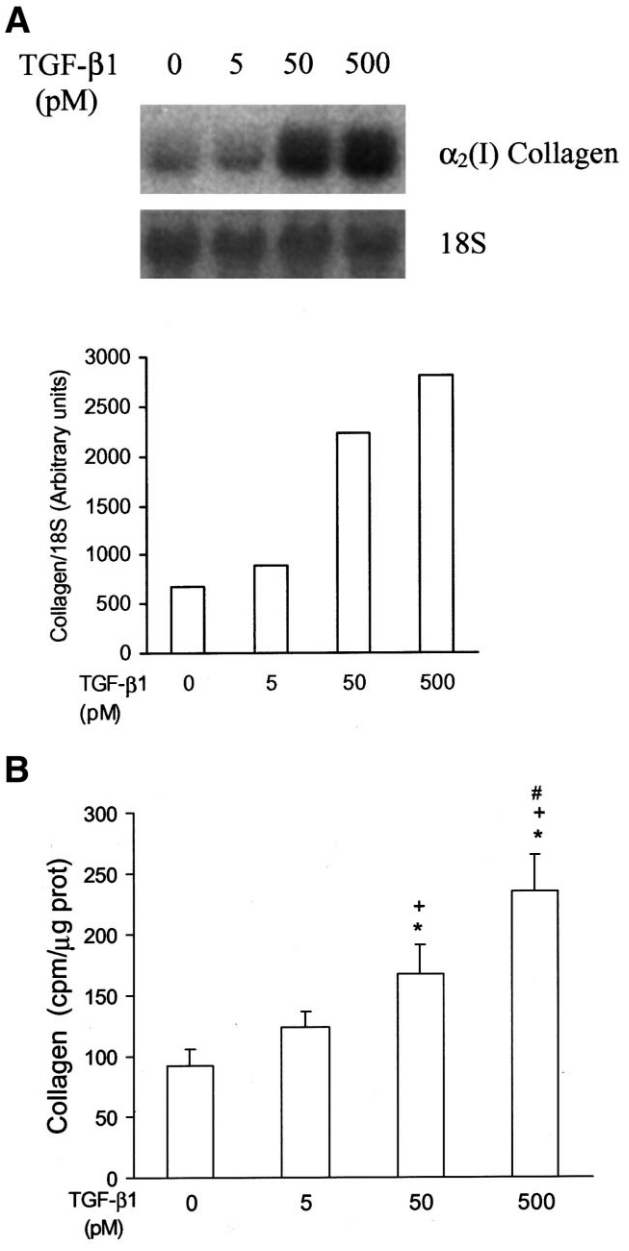

Fig. 1. Effect of TGF-β1 on induction of pro-α_2(I) collagen mRNA expression and accumulation in L_6E_9 rat myoblasts. Cultured L_6E_9 myoblasts were incubated with increasing concentrations of TGF-β1 for 24h. A: Total RNA was extracted and isolated for Northern analyses where pro-α_2(I) collagen mRNA appeared at 5.2 kb. The autoradiograms were analyzed by optical scanning densitometry and values are expressed as arbitrary units with normalization of the results according to the corresponding transcripts levels for the ribosomal 18S gene. Figure shows a representative experiment of three performed in the same conditions. B: Metabolic radiolabeling with [^{3}H]proline was carried out for 24 h. Collagen accumulation was measured according to the amount of radioactivity incorporated into the protein fraction of the medium. Each value represents the mean ± S.E.M. of three experiments, each in triplicate. $^*P < 0.001$ versus basal; $^+P < 0.001$ versus TGF-β1 (5 pM); $^\#P < 0.001$ versus TGF-β1 (50 pM).

3.2. Activation of p38 and ERK1/2 in L_6E_9 myoblasts by TGF-β1

Previous reports have suggested that some of the biological actions of TGF-β1 are mediated by activation of MAPK sig-

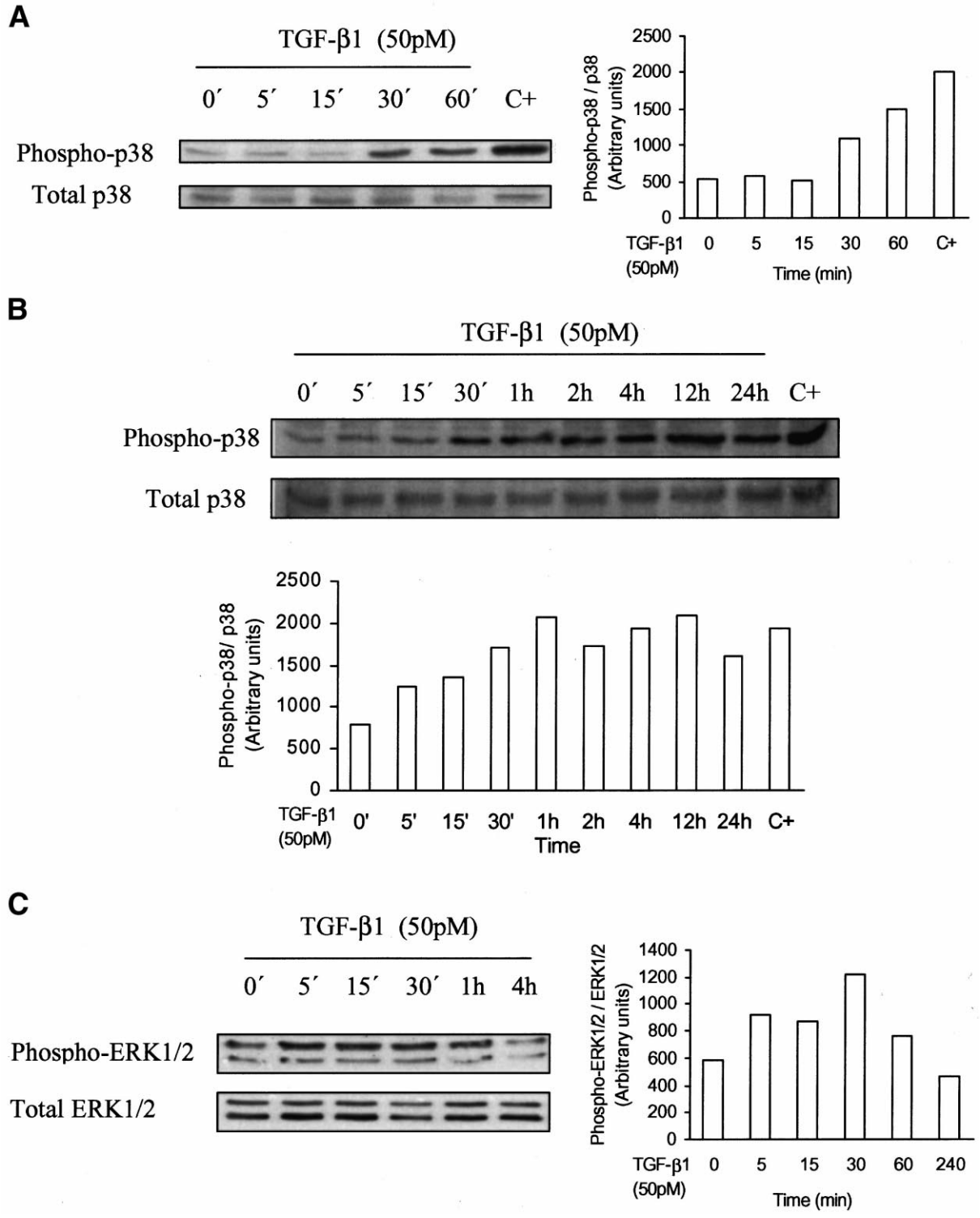

Fig. 2. MAPK pathway activation by TGF-β1. L_6E_9 rat myoblasts were incubated with exogenous TGF-β1 (50 pM) for the times indicated. The figure shows a representative experiment of three performed in the same conditions. A: Phosphorylation of p38 MAPK from cellular extracts was determined using Western blot with anti-phospho-p38 antibody (phospho-p38). Western blot of total p38 protein (total p38) in cellular extracts is also shown. Cell extracts from L_6E_9 myoblasts stimulated by osmotic shock (0.7 M sorbitol, 60 min) served as a positive control (C+). Bar graphs shown the levels of activated p38 corrected for total p38. B: Phosphorylation of p38 MAPK from cells treated with TGF-β1 for 24 h. Western blot of total p38 protein (total p38) in cellular extracts is also shown. Cell extracts from L_6E_9 myoblasts stimulated by osmotic shock (0.7 M sorbitol, 60 min) were used as a positive control (C+). C: Phosphorylation of ERK1/2 MAPK from cellular extracts was determined using Western blot with phospho-p44/42 MAPK antibody (phospho-ERK1/2). Western blot of total ERK1/2 protein (total ERK1/2) in cellular extracts is also shown. Bar graphs shown the levels of activated ERK1/2 corrected for total ERK1/2.

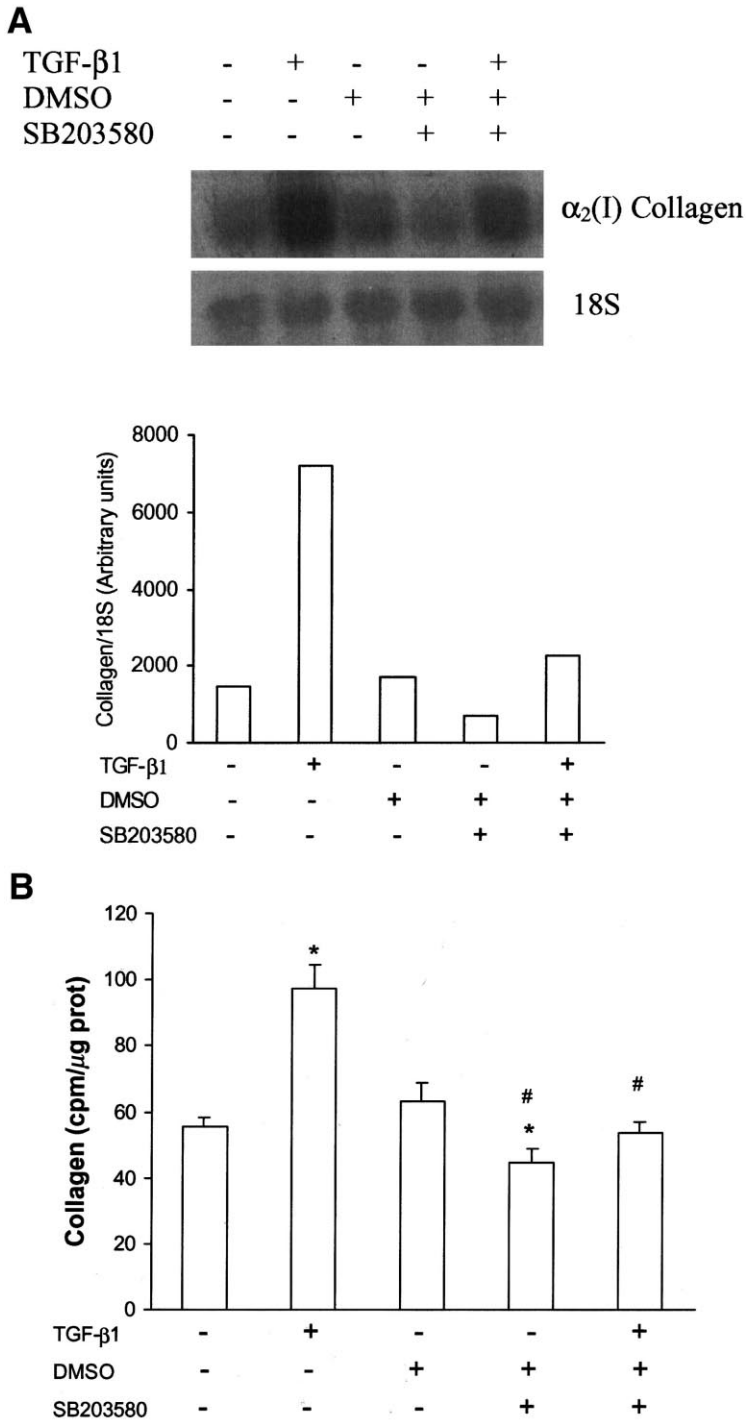

Fig. 3. Inhibition of the p38 MAPK pathway with SB203580 blocks TGF-β1-induced pro-α_2(I) collagen mRNA expression and collagen accumulation. L_6E_9 rat myoblasts were pretreated with (+) or without (−) SB203580 (10 μM) and incubated in the presence (+) or absence (−) of exogenous TGF-β1 (50 pM) for 24 h. A: Northern analyses of pro-α_2(I) collagen mRNA as in Fig. 1A. B: Collagen accumulation was measured as in Fig. 1B. Each value represents the mean ± S.E.M. of two experiments, each in triplicate. $^{*}P < 0.001$ versus basal; $^{\#}P < 0.001$ versus TGF-β1 (50 pM). SB203580 was dissolved in DMSO (0.2%). Cells treated with the SB203580 solvent (DMSO) are also shown.

naling pathways [20,21]. To investigate the potential intermediate role of MAPKs on TGF-β1-induced accumulation of α_2(I) collagen, we analyzed the effect of this factor on collagen deposition, as well as on the phosphorylation status of MAPK. This latter study was carried out using antibodies that specifically recognize the phosphorylated form of these MAPKs. In addition, we assessed the effect of inhibiting the different MAPK routes on TGF-β1-induced collagen deposition.

As shown in Fig. 2A, exogenous TGF-β1 (50 pM) increased the phosphorylation of p38 MAPK at 30 min of stimulation and p38 remains phosphorylated at least 24 h (Fig. 2B). The amount of the total p38 MAPK remained unchanged during the experiment, as indicated by Western blotting of the lysates

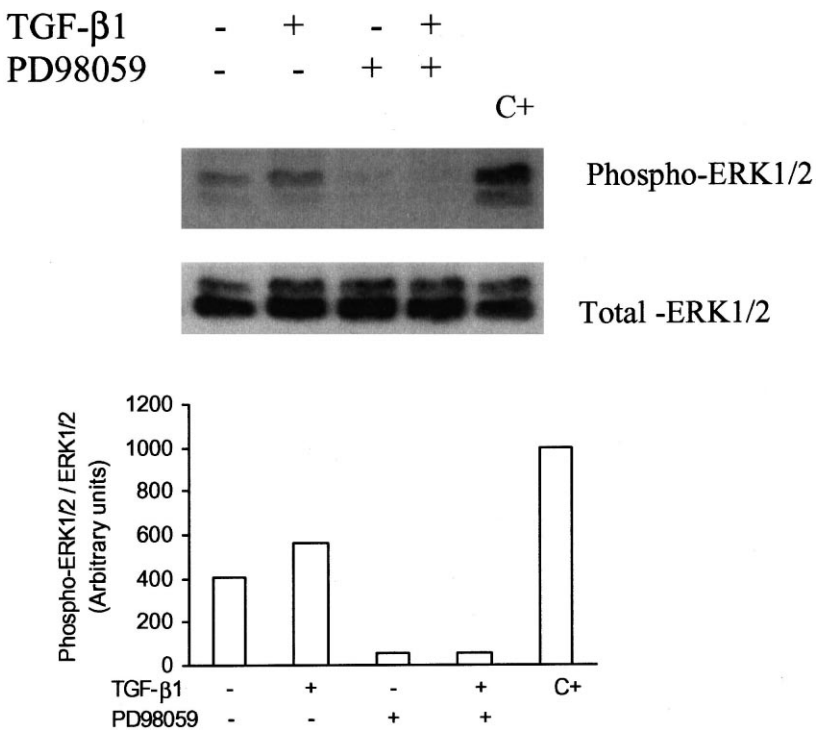

Fig. 4. Inhibition of the ERK1/2 pathway with PD98059 blocks TGF-β1-induced ERK activation. Representative blot of phosphorylation of ERK from cellular extracts determined using Western blot with anti-phospho-ERK antibody (phospho ERK1/2). Western blot of total ERK1/2 protein (total ERK1/2) in cellular extracts is also shown. Where indicated, cells were incubated with TGF-β1 (50 pM) and PD98059 (10 μM) for 24 h. Cell extracts from L_6E_9 myoblasts stimulated by osmotic shock (0.7 M sorbitol, 60 min) served as a positive control (C+). Bar graphs shown the levels of activated ERK1/2 corrected for total ERK1/2.

using antibodies that detect total (phosphorylation state-independent) p38 protein. As a positive control, L_6E_9 cell lysates stimulated by osmotic shock (0.7 M sorbitol, 1 h) were analyzed in the same gels. Osmotic shock strongly activated p38 phosphorylation to levels higher than those induced by 50 pM TGF-β1.

To determine whether TGF-β1 activated the ERK1/ERK2 MAPK pathway in cultured L_6E_9 myoblasts, we performed Western blot analyses using an anti-phospho-p44/42 MAPK monoclonal antibody. ERK1 and ERK2 phosphorylation increased slightly as early as 5 min after stimulation with exogenous TGF-β1 and persisted for as long as 60 min, declining thereafter. No changes in total ERK1/2 proteins were observed when blots were probed with antibodies that detect the total ERK1/2 proteins (Fig. 2C).

We also analyzed whether TGF-β1 activated the SAPK/JNK pathway in cultured rat L_6E_9 myoblasts. In contrast to the p38 and ERK1/ERK2 MAPKs, there were no significant increases in the phosphorylation of SAPK/JNK MAPK upon TGF-β1 treatment (data not shown). As a positive control, UV irradiation strongly stimulated JNK phosphorylation.

3.3. Inhibition of TGF-β1 α_2(I) collagen mRNA expression by p38 MAPK but not by ERK1/2 inhibition

Preincubation of L_6E_9 myoblasts with SB203580, a specific inhibitor of p38 MAPK, efficiently prevented TGF-β1-induced α_2(I) collagen mRNA expression (Fig. 3A). In addition, SB203580 also inhibited TGF-β1-induced collagen accumulation (Fig. 3B). In addition, PD98059, an ERK1/2 MAPK route inhibitor, prevented the activation of the ERK1/ERK2 MAPK pathway (Fig. 4), but failed to inhibit TGF-β1-induced α_2(I) collagen mRNA expression (Fig. 5A) and total collagen accumulation (Fig. 5B).

4. Discussion

This study examined the role of the MAPK signaling cascades in mediating the effect of TGF-β1 in collagen synthesis in rat L_6E_9 myoblasts. Treatment of L_6E_9 myoblasts with exogenous TGF-β1 resulted in p38 and ERK1/ERK2 phosphorylation. Thus, our findings demonstrate for the first time a rapid activation of p38 and ERK1/ERK2 MAPK in rat L_6E_9 myoblasts, occurring within 5–30 min of TGF-β1 addition. It is interesting to note that p38 MAPK remains activated for at least 24 h. However, no apparent activation of JNK was observed within the same time periods of TGF-β1 treatment. JNK and p38 MAPKs are strongly activated by environmental stresses and inflammatory cytokines, and are inconsistently activated by insulin and growth factors [22]. In almost all instances, the same stimuli that recruit JNKs also recruit p38 MAPK [23]. However, in the present study we found a dissociation between JNK and p38 activities as TGF-β1 treatment activated p38 but not JNK. This is compatible with the failure of a rapid JNK activation by TGF-β1 also observed in studies by Chin et al. [21] in mesangial cells.

With respect to the roles of MAPKs in myoblasts, Jones et al. [24] demonstrated that fibroblast growth factor-stimulated ERK1/2 signaling is required during the G1 phase of the cell cycle for commitment of DNA synthesis, but is not required for mitosis once cells have entered the S-phase. Moreover, ERK1/2 signaling is not required either to repress differentiation, to promote skeletal muscle gene expression, or to pro-

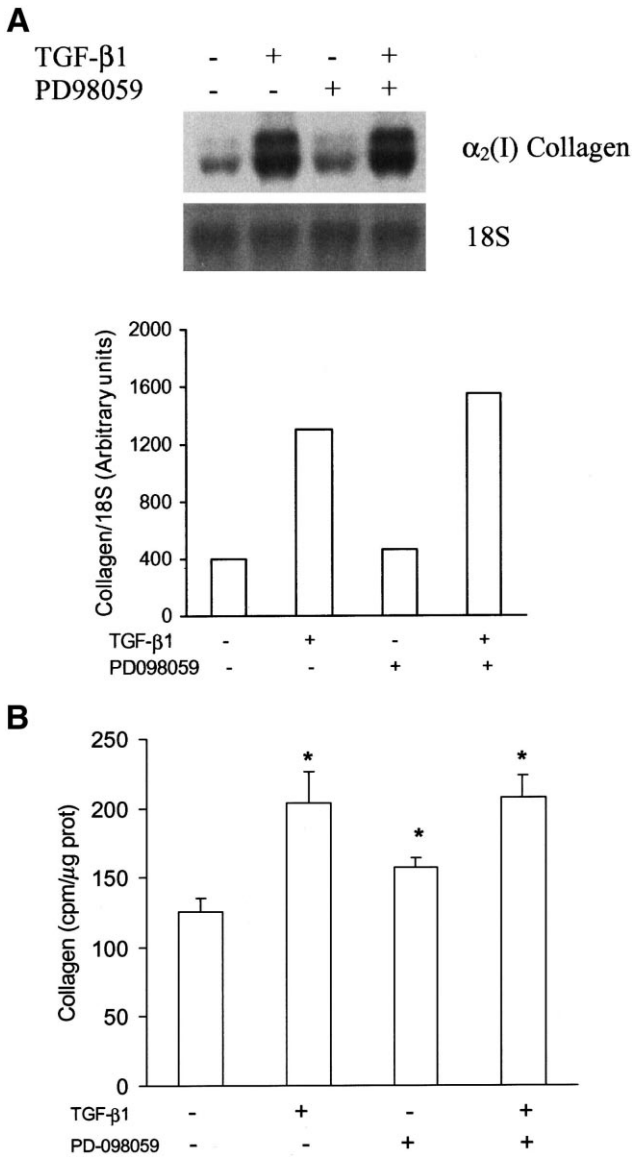

Fig. 5. Effect of the ERK pathway inhibitor PD98059 on TGF-β1-induced pro-α_2(I) collagen mRNA expression and total collagen accumulation in L_6E_9 rat myoblasts. L_6E_9 rat myoblasts were pretreated with (+) or without (−) PD98059 (50 μM) and incubated in the presence (+) or absence (−) of exogenous TGF-β1 (50 pM) for 24 h. A: Northern blot analysis of pro-α_2(I) collagen mRNA was performed as in Fig. 1A. B: Collagen accumulation was measured as in Fig. 1B. Each value represents the mean ± S.E.M. of two experiments, each in triplicate. $^*P < 0.001$ versus basal.

mote myoblast fusion. In addition, activation of the insulin-like growth factor (IGF) autocrine loop is required for myogenic differentiation leading to a sustained activation of phosphoinositide 3′-kinase (PI3-kinase) [25,26], p38 [26] and ERK1/ERK2 [27]. Furthermore, these authors demonstrated that blocking the Raf/MEK/ERK cascade with PD98059 does not block myogenic differentiation; however, myotubes do not form. Thus, PI3-kinase, in association with IRS-1, is involved in an ERK-independent signaling pathway in myoblasts required for IGF-dependent myogenic differentiation and in inducing sustained activation of ERKs necessary for later stages of differentiation. In addition, recent studies demonstrated that phosphorylation by p38 induced transcriptional activity of myocyte enhancer factor 2 [28], which plays a pivotal role in muscle gene transcription and differentiation of skeletal myoblasts [29]. Thus, MAPKs seem to play a major role in regulating myoblast proliferation and differentiation.

In the present study, we also evaluated the effect of inhibiting the ERK1/ERK2 or the p38 MAPK pathways on collagen synthesis. Blockade of the ERK cascade by PD98059, a specific inhibitor of MEK1 that prevents downstream activation of ERK1/ERK2, did not prevent induction by TGF-β1 of α_2(I) collagen mRNA or collagen I accumulation in L_6E_9 myoblasts. By contrast, in the presence of SB203580, a specific inhibitor of the p38 MAPK cascade, exogenous TGF-β1 was unable to induce both α_2(I) collagen mRNA and collagen I accumulation in L_6E_9 myoblasts. Our present data demonstrate that p38 MAPK activation mediates TGF-β1-induced collagen I synthesis in L_6E_9 myoblasts. These data are in agreement with those of Hanafusa et al. [30] demonstrating the involvement of the p38 MAPK pathway in TGF-β1-induced gene expression. In addition, Chin et al. [21] have recently reported that in rat mesangial cells, TGF-β1 rapidly induced ERK1/ERK2 activation, p38 phosphorylation and pro-α1(I) collagen mRNA expression. Their findings suggest that the p38 MAPK pathway functions as a component in the signaling of pro-α_1(I) collagen induction by TGF-β1 in mesangial cells, which is in agreement with the results of the present study. Also very recently, it has been demonstrated that p38 MAPK mediates the TGF-β1-induced expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts [31].

In summary, our results demonstrate that TGF-β1 activates different MAPK routes in L_6E_9 myoblasts and support the involvement of the p38 MAPK pathway in TGF-β1-induced collagen synthesis and accumulation in L_6E_9 myoblasts. The activation of multiple pathways may account for the pleiotropic actions of TGF-β1 in these cells.

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