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Transforming Growth Factor-β Stimulates Articular Chondrocyte Cell Growth through p44/42 MAP Kinase (ERK) Activation

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Abstract. Transforming growth factor-β1 (TGF-β1) stimulates articular chondrocyte cell proliferation and extracellular matrix formation. We reported previously that immediate and transient expression of c-fos mRNA through protein kinase C activation is required for the mitogenic effect of TGF-β1 on cultured rat articular chondrocytes (CRAC). In gel kinase assays using myelin basic protein (MBP) showed that total cell lysates from cells treated with TGF-β1 caused rapid phosphorylation of MBP, which suggests the involvement of mitogen-activated protein kinase (MAPK) activation. To identify specific MAPK pathways activated by TGF-β1, we performed in vitro kinase assays using specific substrates. TGF-β1 induced a rapid activation of extracellular signal regulated kinase (ERK) with a peak at 5 min, which decreased to basal levels within 240 min after TGF-β1 stimulation. In contrast, the c-jun N-terminal kinase activity increased only about 2.5-fold after 240 min of stimulation and p38 MAPK activity did not change significantly. ERK activation by TGF-β1 was also confirmed by in vivo phosphorylation assays of Elk1. However, a specific MEK1 inhibitor, PD98059, significantly decreased TGF-β1 induced Elk1 phosphorylation in a dose-dependent manner. Furthermore, PD98059 reduced the TGF-β1-induced cell growth by 40%. These results indicate that TGF-β1 specifically activates MEK1 and subsequent ERK pathways in CRAC, and that the activation of this MAPK pathway plays a role in the mitogenic response to TGF-β1.

Key words: TGF-β1, Articular chondrocyte, Cell growth, MAPK, ERK

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TRANSFORMING growth factor-β1 (TGF-β1) is known to regulate a wide variety of cellular processes including cell proliferation, differentiation, motility, organization, and extracellular matrix production [1, 2]. TGF-β1 mediates signaling through two transmembrane serine/threonine kinase receptors, the type I and type II TGF-β receptors. The constitutively active type II receptor recruits the type I receptor upon ligand binding and then phosphorylates serine and threonine residues in the GS domain of the type I receptor [3, 4]. Once phosphorylated, the type I receptor is activated and propagates the signal to downstream targets. Recent evidence indicates that TGF-β1 transduces signals through two different pathways, Smads and the mitogen-activated protein kinase (MAPK) family. Smad proteins were first isolated from genetic screens in *C. elegans* and in *Drosophila*, and to date ten different Smads have been identified in vertebrates. In the case of TGF-β1 signaling, Smad2 or Smad3 is phosphorylated by the type I receptor and forms a complex with Smad4.

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This complex translocates to the nucleus whereby it activates transcription of target genes [5].

MAPK, members of the cytoplasmic serine/threonine kinase family, are also involved in TGF-β receptor signaling [6]. MAPK cascades are comprised of many kinases and are intricately regulated. However to simplify the picture, downstream pathways can be classified into three major groups mediated by the following kinases: p44/42 MAPK (extracellular signal regulated kinase, ERK) [7–11], c-jun N-terminal kinase (JNK) [11] and p38 MAPK. The propagation of TGF-β signals by these molecules has been shown in many cell types. Recently the TGF-β-specific MAPK kinase kinase, TGF-β-activated kinase 1 (TAK1), was also identified as a modulator of TGF-β signaling [12].

TGF-β1 inhibits cell growth of epithelial and endothelial cells, but this ligand can also work as a potent stimulator of cell growth for certain cell types. We have previously reported that TGF-β1 stimulates cell proliferation and extracellular matrix formation in cultured rat articular chondrocytes (CRAC) [13]. TGF-β1 is stored in large amounts in cartilage matrix in an inactive or latent form [14, 15]. When cartilage destruction takes place, TGF-β1, generated as a result of proteinase activation by matrix proteolysis, may subsequently act to stimulate cell proliferation and synthesis of collagen and proteoglycan to restore a homeostatic balance. Recently we have demonstrated that the mitogenic effects of TGF-β1 depend on the direct induction of the c-fos gene [16]. We also identified the TGF-β-responsive element on the promoter of the c-fos gene. However, it is still unclear whether intervening molecules lead to the transactivation of the c-fos gene. Since the identified promoter contains several cis-elements that could be potential targets for different MAPK cascades, we focused on identifying which MAPK cascades are specifically activated by TGF-β1.

Materials and Methods

Materials

Five-week-old male Sprague-Dawley (SD) rats were purchased from Charles River Laboratories (Shizuoka, Japan). Fetal bovine serum (FBS) and Dulbecco's Modified Essential Medium (DMEM) were purchased from Gibco Oriental Co. (Tokyo, Japan). Recombinant human TGF-β1 was purchased from AUSTRAL Biologicals (San Ramon, CA). Cell culture plates were purchased from Becton Dickinson Co. (Rutherford, NJ), Falcon (Lincoln Park, NY), Corning (Corning, NY), and Costar (Cambridge, MA). p44/42 MAPK Assay Kit, SAPK/JNK Assay Kit, p38 MAPK Assay Kit, and specific MEK1 Inhibitor (PD98059) were all purchased from New England Biolabs, Inc. (Beverly, MA). Polyvinylidene difluoride (PVDF) membrane, Clear Blot Membrane-P®, was purchased from Atto, Co. (Tokyo, Japan). X-ray films were ordered from Eastman Kodak (Rochester, NY). PathDetect™ Elk1 trans-Reporting System was purchased from STRATAGENE, Inc. (La Jolla, CA). LIPOFECTIN® Reagent was from Life Technologies, Inc. (Palo Alto, CA). Dual-Luciferase™ Reporter Assay System was from Promega Co. (Madison, WI). A Cell Counting kit was purchased from DOJINDO (Kumamoto, Japan). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell cultures

Articular chondrocytes were prepared as described previously [17]. Briefly, articular cartilage slices were dissected from the shoulder, the hip, and the knee joints of 5-week-old male SD rats. Isolated chondrocytes were obtained by enzymatic digestion in a spinner bottle with 0.25% trypsin and 10 mM EDTA in phosphate-buffered saline (PBS) for 3 h at 37°C, followed by an overnight 0.2% collagenase treatment in DMEM with 10% FBS. Digested cells were seeded at 3×10^4 cells/cm^2 in 9.6 cm diameter culture dishes with DMEM containing 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) in a 5% CO environment, and were fed every 3 days. Five days after seeding, upon reaching confluence, cells were passaged with trypsin/EDTA and were reseeded in a 9.6-cm diameter culture dish, or in plastic culture plates with either 24 or 96 wells. Only the first subcultures were used in the present study.

In gel assays of MBP kinase activity

Total cell lysates prepared from CRAC were subjected to electrophoresis in a SDS-4% polyacrylamide stacking gel and a SDS-10% polyacrylamide

separating gel. Myelin basic protein (MBP) (0.2 mg/ml gel) was added to the separation gel just prior to polymerization. Following electrophoresis, SDS was removed from the gels by washing with 20% 2-propanol in 50 mM Tris-HCl (pH 7.5) for 1 h and then 50 mM Tris-HCl (pH 7.5) containing 5 mM β-mercaptoethanol for 1 h. Proteins were denatured twice with 6 M guanidine HCl for 1 h and renatured with 0.04% Tween 20 (250 ml × 5) at 4°C for 16 h. The gel was subsequently incubated in a buffer of 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.1 mM EDTA, 10 mM MgCl and 0.4 mM DTT for 30 min at 30°C, followed by the same buffer containing 50 μM ATP and 30 μCi [γ-^{32}P]-ATP for 1 h. Then, the gels were washed with 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate to remove unlabeled ATP. The gels were dried and analyzed by autoradiography.

In vitro kinase assay

ERK activity was determined using a p44/42 MAPK Assay Kit according to manufacturer's instructions. Briefly, CRAC were lysed with the kit lysis buffer supplemented with 1 mM phenyl methyl sulfonyl fluoride, and the concentration of the proteins were measured by a Bio-Rad Bradford [16] protein assay. Equal amounts of protein were then subjected to immunoprecipitation with anti-phospho-specific p44/42 MAPK antibody. In vitro kinase reactions were performed with bacterially expressed Elk1 protein (307–428) as substrate, in the presence of cold ATP. Samples were separated by electrophoresis in 12% SDS-PAGE, transferred onto PVDF membranes and immunoblotted with phospho-specific Elk1 (Ser383) antibody. A similar method was employed for quantification of p38 MAPK activity by using p38 MAPK antibody and GST-ATF2 (19–96). For JNK in vitro kinase assays, endogenous JNK was pulled-down with c-jun (1–84) conjugated beads and an anti-phospho-c-jun (Ser63) antibody detected phosphorylated c-jun.

Elk1 phosphorylation assay

Quantification of Elk1 phosphorylation was determined using PathDetect™ Elk1 trans-Reporting System according to the manufacturer's protocol. This system is designed to detect endogenous ERK activity in vivo. The pFA-Elk1 vector expresses a fusion protein of the functional domain of Elk1 and the GAL4 DNA binding domain. The reporter vector, pFR-Luc contains a GAL4-response element upstream of the luciferase gene. Phosphorylation of Elk1 by ERK causes a homodimerization that induces DNA binding through GAL4 responsive element, and results in the activation of the reporter gene. For this experiment, CRAC in 24-well plates were transiently cotransfected with 200 ng of pFA-Elk1 and 1 μg of pFR-Luc by the LIPOFECTIN® method. PRL-TK with 200 ng was also cotransfected to monitor the transfection efficiency. After 24 h, the cells were incubated with or without TGF-β1 (10 ng/ml) together with varying concentrations of PD98059 for 24 h. The cell lysates were prepared and assayed for luciferase activity using the Dual-Luciferase™ Reporter Assay System per the manufacturer's instructions. Light emission was measured for 12 sec to 24 sec with a luminometer.

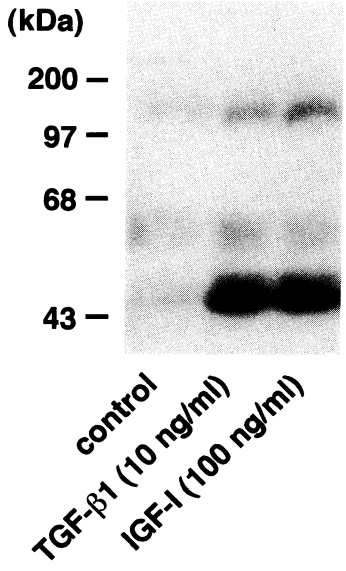

Fig. 1. In gel MBP kinase assay. CRAC were serum-starved for 24 h and treated with TGF-β1 (10 ng/ml) or IGF-I (100 ng/ml) for 5 min. Total cell lysates were electrophoresed with 10% SDS-PAGE containing myelin basic protein (MBP) (0.2 mg/ml gel), followed by an in gel kinase assay with [γ-^{32}P]-ATP. After removing unlabelled ATP, gels were analyzed by autoradiography. Note that both TGF-β1 and IGF-I caused significant increase of MBP phosphorylation near the 43 kDa position.

Quantification of cell viability

The Cell Counting kit, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to a formazan dye by the mitochondrial dehydrogenase of viable cells, was used to quantify cell viability. Briefly, subconfluent articular chondrocytes in 96-well microtiter plates were serum-starved with DMEM for 24 h, and were incubated with or without TGF-β1 (5 ng/ml) together with varying concentrations of PD98059 for 48 h. The ready-to-use WST-1 reagent was added to the cells and incubated for 1 h at 37°C. Absorbance at 405 nm was measured using a multi-well spectrophotometer (IMMUNO-MINI NJ-2300, Inter Med, Tokyo, Japan).

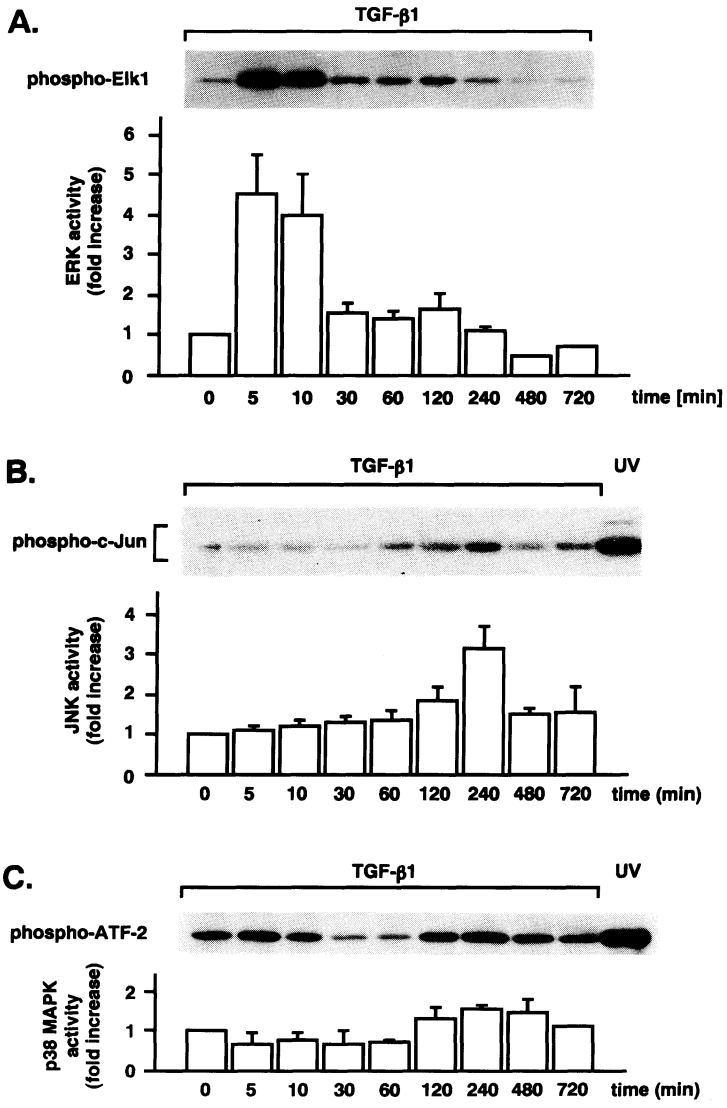

Fig. 2. In vitro kinase assays of ERK, JNK and p38 MAPK. CRAC were serum-deprived for 24 h and stimulated with TGF-β1 (5 ng/ml) for the indicated times. Equal amounts of total cell lysates were immunoprecipitated with anti-phospho-ERK (A) or anti-p38 MAPK (C) antibody. For JNK (B), endogenous JNK was pulled-down with c-jun conjugated sepharose beads. Immunoprecipitated complexes were subjected to in vitro kinase assays using the appropriate substrates as indicated, electrophoresed in 10% gel and immunoblotted with specific antibodies against phosphorylated forms of each substrate. In JNK and p38 MAPK assays, ultraviolet light irradiation (50 J/m^2) for 10 min was used as a positive control. The upper blots in each panel show one of the three representative experiments. In contrast, lower graphs indicate fold increases over control values with mean±SE from three independent experiments.

Results

In gel kinase assay with MBP

To address whether the activation of a MAPK cascade is involved in TGF-β1 signaling in CRAC, total cell lysates treated with or without TGF-β1 for 5 min were loaded in SDS-PAGE containing MBP. Endogenous proteins were renatured in the gel and then incubated with [γ-^{32}P]-ATP (Fig. 1). We used IGF-I, another growth factor, as a positive control for MAPK activation. As expected, treatment of CRAC with IGF-I caused phosphorylation of MBP observed at approximately the 43 kDa position. Similar to IGF-I, TGF-β1 induced the phosphorylation of MBP, which implicates the involvement of some MAPK cascade in TGF-β1 signaling.

In vitro kinase assays for ERK, JNK and p38 MAPK

Since MBP could be a substrate of variety serine/threonine kinase families, we next attempted to identify which signaling MAPK cascades were activated by TGF-β1. For this, we performed in vitro kinase assays using specific antibodies and their target substrates. We analyzed three different pathways: ERK, JNK and p38 MAPK. Serum-starved chondrocytes were treated with TGF-β1 for the

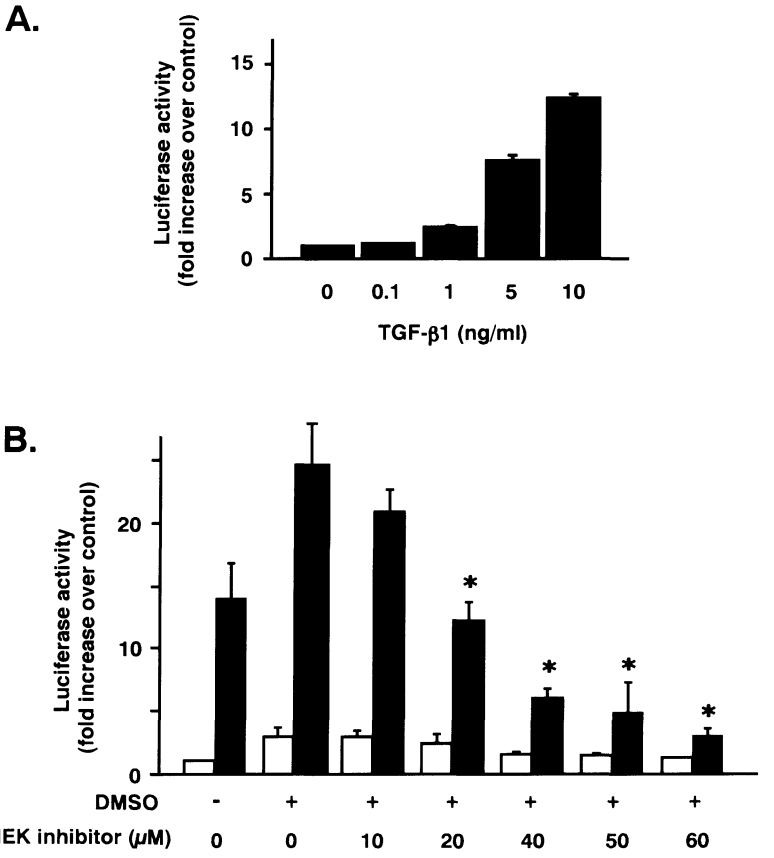

Fig. 3. Elk1 phosphorylation induced by TGF-β1 through endogenous ERK. (A) CRAC were co-transfected with the fusion trans-activator plasmid (pFA-Elk1) and the reporter plasmid (pFR-Luc) as described in Materials and Methods. Twenty-four hours after transfection, the cells were incubated with indicated concentrations of TGF-β1 for 24 h. Extracts prepared thereafter were assayed for luciferase activity using the Dual-Luciferase™ Reporter Assay System. Data are expressed as fold increases over control values (mean±SE, n=6). (B) CRAC transfected with the same constructs as indicated above were incubated with (closed bars) or without (open bars) TGF-β1 (10 ng/ml) together with the indicated concentrations of PD98059 for 24 h. Data are expressed as fold increases above untreated control values (mean±SE, n=4). An asterisk indicates statistically significant difference from TGF-β1-treated controls, $P<0.05$ (Mann-Whitney test).

indicated time periods and cell lysates were immunoprecipited using the phospho-specific antibodies against ERK or p38 MAPK or by c-jun conjugated beads. The precipitated enzymes were then subjected to in vitro kinase reactions with specific substrates followed by immunoblotting with phospho-specific antibodies against those substrates. In ERK kinase assays, the phosphorylation of Elk1 was observed at 5 min and this activation was steady until 10 min of TGF-β1 stimulation. Subsequently, however, the kinase activity of ERK decreased and gradually returned to basal levels at 240 min of stimulation (Fig. 2A). The phosphorylation of c-jun by JNK also increased significantly due to TGF-β1, but the peak was observed after 240 min of stimulation (Fig. 2B). In contrast, no obvious phosphorylation of ATF-2 by p38 MAPK was observed throughout these time course studies (Fig. 2C).

Elk1 phosphorylation by TGF-β1

To confirm that TGF-β1 activates ERK, we studied in vivo phosphorylation of Elk1 by ERK using a trans-reporter assay. CRAC were transiently cotransfected with an Elk1 expression vector together with the pFR-Luc plasmid that contains a luciferase reporter gene. Transfected cells were subsequently treated with varying concentrations of TGF-β1. The luciferase activity in this experiment reflects endogenous ERK activity. Incubation with TGF-β1 increased luciferase activity in a dose dependent manner (Fig. 3A). The highest dose of TGF-β1 (10 ng/ml) caused a 15-fold increase in luciferase activity. To test whether the phosphorylation of Elk1 by ERK was due to the activation of MEK1 (which lies upstream of ERK), we used PD98059, a MEK1 specific inhibitor in the same assay. Prior to use of this inhibitor, we tested the effect of DMSO, a resolvent of PD98059 on Elk phosphorylation and obtained a slight increase of luciferase activity in both basal and ligand dependent induction (Fig. 3B). In contrast, treatment with PD98059 that was resolved in the same volume of DMSO decreased the luciferase activity in a dose dependent manner, wherein the inhibition was strong in TGF-β1 induced activation compared with basal levels.

Cell growth by TGF-β1 through ERK activation

To confirm the requirement of ERK activation for the mitogenic response to TGF-β1 in rat articular chondrocytes, CRAC were incubated with or without TGF-β1 together with two different concentrations of PD98059. Incubation with TGF-β1 alone increased cell proliferation of chondrocytes to about 2.5-fold of control. In contrast, treatment with PD98059 resulted in a partial but significant decrease of TGF-β1-induced cell proliferation, while basal levels of cell proliferation were not affected (Fig. 4).

Discussion

Our major findings in this study are that, within MAPK cascades, TGF-β1 transduces signaling through ERK but not through JNK or p38 MAPK in

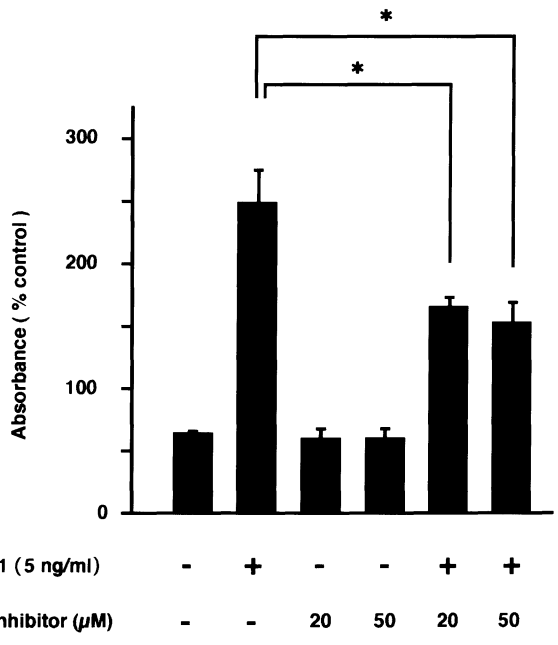

Fig. 4. Effects of MEK1 inhibitor on TGF-β1-induced cell growth. CRAC were incubated with or without TGF-β1 (5 ng/ml) together with the two different concentrations of PD98059, a MEK1 inhibitor, for 48 h. The WST-1 reagent was added to the cells and was incubated for 1 h at 37°C and the absorbance at 405 nm was measured. Data are expressed as the percentage absorbance of the control (mean±SE, n=5). An asterisk indicates statistically significant differences, $P<0.05$ (Mann-Whitney test).

cultured rat articular chondrocytes (CRAC), and that ERK activation is required in part for the mitogenic activity of TGF-β1. We have demonstrated previously that TGF-β1 is a potent stimulator of rat articular chondrocyte cell growth and that this mitogenic activity of TGF-β1 does not depend on secondary factors, such as platelet-derived growth factor, but requires c-fos gene induction [16]. Stimulation of CRAC with TGF-β1 causes rapid and transient expression of c-fos mRNA through protein kinase C. We also identified the TGF-β1 responsive element on the c-fos promoter. Since these findings seemed to be a specific phenomenon for chondrocytes, we attempted to further investigate the signaling mechanism of TGF-β1 receptor and its role in the proliferation of chondrocytes. Our previous result concerning c-fos gene activation implicated a role for MAPK cascades in TGF-β1 signaling [16]. Thus, we performed in gel MBP kinase assays and sought to obtain evidence of the activation of some MAPK cascade in response to TGF-β1. MBP, however, is known to be a substrate for a wide range of MAPK pathways. The MAPKs of 38, 42, 44, 54, 57, 63, 85, 97, 105, and 130 kDa are all capable of phosphorylating MBP [19]. Thus, we tried to identify precisely which particular MAPK cascades were being activated by TGF-β1.

MAPK cascades consist of numerous enzymes, but the majority of downstream pathways can be classified broadly into three pathways: classical Ras-Raf-MEK1-ERK-Elk1, JNK and p38 MAPK. The MKK family regulates the latter two of these cascades. In this study, we analyzed the activation of these three pathways by in vitro kinase assays with candidate substrates as well as by an in vivo phosphorylation study of Elk1. Our results demonstrate that the activation of ERK is rapid and transient, while JNK activation is significantly delayed compared to that of ERK, indicating that TGF-β1 receptors transduce signaling directly through ERK activation. The activation of JNK is likely due to a secondary event. By using a specific inhibitor against MEK1, we also confirmed that ERK activation by TGF-β1 is mediated through MEK1. We observed slight but significant activation of Elk1 by DMSO alone, which remained to be explained, however, DMSO itself can also activate 3TP-lux in CRAC (data not shown).

TGF-β1 has been demonstrated to activate MAPK cascades directly. Activation of ERK is known to stimulate cell proliferation and extracellular matrix formation [20, 21]. However, TGF-β1-induced ERK activation does not consistently lead to cell proliferation. TGF-β1 activates ERK in NIH 3T3 [7] and RFL-6 rat lung fibroblast cells [22], but the proliferation of these cell types is inhibited or unaffected by TGF-β1 treatment. In contrast, the activation of JNK and p38 MAPK is linked to other cellular responses, such as apoptosis [23, 24]. Indeed, UV irradiation activates these MAPK cascades in CRAC as shown in Fig. 2.

Compared to JNK and p38 MAPK, the physiological role of ERK activation by TGF-β1 is still obscure, although the partial but significant inhibition by MEK1 inhibitor strongly suggests the requirement for MEK1 activation in TGF-β1-induced cell proliferation of articular chondrocytes. Our previous results which demonstrate a complete inhibition of TGF-β1-induced cell growth by an inhibitor of protein kinase C also support the likelihood of an essential role for MAPK cascades in TGF-β1-induced CRAC cell growth. It is well known that ERK phosphorylates Elk1 and phosphorylated Elk1 form a heteromeric complex with serum response factor, which can bind to the c-fos promoter sequence through serum response element [25]. The interaction between ERK activation and c-fos gene induction has not been directly proven yet, though it is possible that the sequential activation of ERK cascades by TGF-β1 leads to c-fos gene induction, resulting in cell proliferation of chondrocytes. However, the MEK1 inhibitor we employed could not completely inhibit cell proliferation, which suggests the probable involvement of signaling pathways other than MAPK cascades. It is conjectured that a balance of MAPK activation and Smad-mediated signaling may influence the fate of cell growth.

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