Smad3 Mediates Transforming Growth Factor-β-induced Collagenase-3 (Matrix Metalloproteinase-13) Expression in Human Gingival Fibroblasts

EVIDENCE FOR CROSS-TALK BETWEEN Smad3 AND p38 SIGNALING PATHWAYS*

Received for publication, July 1, 2002, and in revised form, September 16, 2002
Published, JBC Papers in Press, September 20, 2002, DOI 10.1074/jbc.M206535200

Suvi-Katri Leivonen‡§, Andrew Chantry‡, Lari Hääkinen‡, Jiahuai Han**,* and Veli-Matti Kähäri†††††

From the ‡Centre for Biotechnology, University of Turku and Åbo Akademi University, FIN-20520 Turku, Finland, the §Department of Medical Biochemistry and Department of Dermatology, University of Turku, FIN-20520, Turku, Finland, the ¶School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom, the ‡Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, the **Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Transforming growth factor-β (TGF-β) is a potent inducer of collagenase-3 (MMP-13) gene expression in human gingival fibroblasts, and this requires activation of the p38 mitogen-activated protein kinase pathway. Here, we have constructed recombinant adenoviruses harboring genes for hemagglutinin-tagged Smad2, Smad3, and Smad4 and used these in dissecting the role of Smads, the signaling mediators of TGF-β, in regulation of endogenous MMP-13 gene expression in gingival fibroblasts. Adenoviral expression of Smad3, but not Smad2, augmented the TGF-β-elicited induction of MMP-13 expression. In addition, adenoviral gene delivery of dominant negative Smad3 blocked the TGF-β-induced MMP-13 expression in gingival fibroblasts. Co-expression of Smad3 with constitutively active MKK3b and MKK6b, the upstream activators of p38, resulted in nuclear translocation of Smad3 in the absence of TGF-β and in induction of MMP-13 expression. The induction of MMP-13 expression by Smad3 and constitutively active mutants of MKK3b or MKK6b was blocked by specific p38 inhibitor SB203580 and by the dominant negative form of p38α. These results show that TGF-β-induced expression of human MMP-13 gene in gingival fibroblasts is dependent on the activation of two distinct signaling pathways (i.e. Smad3 and p38α). In addition, these findings provide evidence for a novel type of cross-talk between Smad and p38 mitogen-activated protein kinase signaling cascades, which involves activation of Smad3 by p38α.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent metalloendopeptidases collectively capable of degrading several distinct classes of pericellular substrates, including growth factors, cytokines, chemokines, their receptors, and components of the extracellular matrix (ECM) (1, 2). MMPs function in development, tissue repair, inflammation, and tumor invasion. The MMP gene family consists of at least 21 human members, which can be classified into subgroups of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs according to their substrate specificity and structure (1, 2). Collagenases (MMP-1, -8, and -13) are the principal proteinases capable of cleaving native fibrillar collagens. Human collagenase-3 (MMP-13) has a wide substrate specificity, and its expression appears to be limited to physiologic situations, in which rapid and effective remodeling of collagenous ECM is required (e.g. fetal development of bone, postnatal bone remodeling, and gingival and fetal skin wound repair) (3–6). Transforming growth factor-β (TGF-β) is a potent stimulator of MMP-13 expression in human gingival and fetal skin fibroblasts, and this requires activation of p38 mitogen-activated protein kinase (MAPK) pathway (5, 6). However, the activation of p38α alone or in combination with extracellular signal-regulated kinase 1/2 (ERK1/2) is not sufficient to induce the expression of MMP-13, suggesting that additional signaling pathways are involved in regulating TGF-β-elicited induction of MMP-13 expression (5, 6).

Smad transcription factors are intracellular mediators of TGF-β signaling (7–9). Receptor-activated Smad2 and Smad3 are phosphorylated by activated TGF-β receptor complex, and after phosphorylation these Smads associate with a common mediator Smad, Smad4. Subsequently, this hetero-oligomeric complex translocates into the nucleus, where Smads bind to DNA or associate with other transcription factors (e.g. FAST-1/2, TFE-3, activating transcription factor-2, AP-1, CREB-binding protein/p300, and Sp1) and control the transcription of various TGF-β-responsive genes (7–9). Smad7 is an inhibitory Smad, the expression of which is induced by TGF-β and which is capable of inhibiting phosphorylation of Smad2 and Smad3 by competitively interacting with TGF-β receptor complex (10).

Smads have been shown to mediate the effects of TGF-β on deposition of ECM (9). It has been shown that the transcriptional activation of plasminogen activator inhibitor-1 (PAI-1) protein kinase; MKK, MAPK kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MOI, multiplicity of infection; PAI, plasminogen activator inhibitor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HA, hemagglutinin; TIMP-1, tissue inhibitor of metalloproteinases-1.
and type I and type VII collagen gene expression by TGF-β involves direct binding of Smad3 and Smad4 to specific Smad-binding elements in the respective promoters (11–14). In addition, suppression of collagenase-1 (MMP-1) gene expression in dermal fibroblasts by TGF-β involves Smad3 and Smad4 (15). Recent studies have shown that Smad signaling is not only activated by TGF-β receptors but is also regulated through crosstalk with other kinase signaling cascades (e.g., the MAPK signaling pathways and calcium-calmodulin-dependent protein kinase II) (16–19).

In this study, we have constructed recombinant adenoviruses coding for hemagglutinin (HA)-tagged wild-type Smad2, Smad3, and Smad4 and used these to examine the role of Smads in the regulation of MMP-13 expression in human gingival fibroblasts. In addition, we have examined the cross-talk between the Smad and p38 MAPK pathways in the regulation of MMP-13 expression. Our results show that Smad3 mediates the induction of MMP-13 expression by TGF-β, whereas Smad2 is not involved in this context. Activation of p38α with its upstream activators MKK3b and MKK6b and simultaneous co-expression of Smad3 results in the induction of MMP-13 expression. In addition, activation of p38α induces the nuclear translocation of Smad3, indicating that Smad3 is activated by p38α either directly or indirectly via endogenous mediator. Together these results indicate that Smad3 is involved in TGF-β-elicited induction of the expression of MMP-13 in human gingival fibroblasts and that this involves cross-talk between Smad3 and the p38 MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents**—Normal human gingival fibroblasts were established from a healthy 25-year-old female and a 32-year-old male (5). Fibroblasts and HaCaT cells, a spontaneously immortalized nontumorigenic human adult epidermal keratinocyte cell line (20), were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 µg/ml streptomycin. Human recombinant TGF-β was obtained from Sigma, and specific p38 inhibitor SB203580 was from Calbiochem.

**Construction of Recombinant Smads 2, 3, and 4 Adenoviruses**—Replication-deficient (E1- and E3-) adenoviruses RAdSmad2, RAdSmad3, and RAdSmad4 harboring human Smad2, Smad3, and Smad4 cDNAs, respectively, with hemagglutinin (HA) tag in the N terminus of Smad2 and Smad3 and in the C terminus of Smad4 were constructed as described previously (21, 22). Briefly, cDNAs coding for HA-tagged Smad2, Smad3, and Smad4 (19) were subcloned into a pCA3 shuttle vector under the control of the cytomegalovirus immediate early promoter (Microbix Biosystems, Toronto, Canada). Human embryonic kidney-293 cells were cotransfected with the resultant plasmids and adenoviral backbone plasmid pBHG10 using the CalPhosMaximizer kit (Clontech, Palo Alto, CA). For constructing an empty control virus, RAdpCA3, human embryonic kidney-293 cells were cotransfected with the empty pCA3 shuttle vector and pBHG10. After 3 weeks, plates were visible, cell layers were harvested, viruses were subjected to plaque purification, and positive recombinants were identified by PCR using primers specific for pCA3 (sense, 5′-GA ATT TGT GAT CTT ATG GGG AGA G-3′; antisense, 3′-ATT GC-3′), and RAdpCA3 (sense, 5′-GGG AGA G-3′; antisense, 3′-ATT GC-3′). One positive clone of each recombinant Smad adenovirus was chosen for preparation of CEI-purified high titer virus stock.

For determining the expression of corresponding HA-Smads, HaCaT cells in DMEM containing 1% FCS were infected with RAdSmad2 and RAdSmad3, and RAdSmad4, or with empty control virus RAd66 or RAdLacZ at MOI 500, plated, and incubated for 18 h in DMEM containing 1% FCS. Thereafter, the medium was changed into DMEM without FCS. After a 24-h incubation, the cells were treated with TGF-β1 (5 ng/ml) for 24 h, and the conditioned medium was analyzed with Western blotting for the phosphorylation of Smad3 and Smad4. Thereafter, the filters were stripped and reprobed with monoclonal anti-HA antibody to confirm equal loading and with total Smad3 and Smad4 antibodies to detect the total amounts of Smad3 and Smad4.

In experiments with adenoviruses together with adenoviruses RAdp38α, RAdMKK3bE, RAdMKK6bE, RAdMEK1CA, or RAdp38AF, human gingival fibroblasts were infected as described above, either treated with SB203580 (10 µM) or left untreated, and incubated for 24 h. The conditioned media were analyzed for the levels of pro-MMP-13, pro-MMP-14, and TIMP-1, and the cell layers were harvested for RNA extraction or for determination of the activation of p38α and ERK1/2.

**Immunoblotting and Antibodies**—Aliquots of conditioned media or cell lysates were fractionated on SDS-polyacrylamide gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membranes were blocked against nonspecific binding using 5% skim milk. Proteins were detected using specific primary antibodies and peroxidase-conjugated secondary antibodies. Antibodies against phospho-Smad2 (PS2) and phospho-Smad3 (PS3), which shows cross-reactivity with phosphorylated Smad3, were kind gifts from Dr. Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) (28, 29). Polyclonal Smad2 and Smad3 antibodies were from Zymed Laboratories Inc. (San Francisco, CA), mouse monoclonal Smad4 antibody was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA), rat monoclonal anti-HA 5F10 antibody was from Roche Applied Science (Indianapolis, IN), mouse monoclonal anti-human MMP-15 antibody was from Calbiochem, and polyclonal anti-TIMP-1 was from Chemicon International Inc. (Temecula, CA). Polyclonal rabbit anti-serum against human MMP-1 was a kind gift from Dr. Henning Birkedal-Hansen (NIDCR, National Institutes of Health, Bethesda, MD). Polyclonal antibodies for phospho-p38, p38, phospho-ERK1/2, and phospho-Smad2 were from Cell Signaling Technology (Beverly, MA). TIMP-1 antibodies were visualized by the ECL detection system (Amersham Biosciences).

**Northern Blot Hybridizations**—Total cellular RNA was extracted with the Qiagen rapid RNA purification kit. Equal aliquots of total RNA were fractionated on 3% agarose gel containing 2.2% formaldehyde, transferred to Zeta Probe nylon membrane (Bio-Rad) by vacuum transfer, baked at 80°C for 20 min, and immobilized by UV cross-linking and heating at 80°C for 30 min. The filters were probed with several plasmids containing cDNAs labeled with [α-32P]dCTP using random priming. For hybridizations, fragments covering the coding region and part of the 3′-untranslated...
region of human MMP-13 cDNA (altogether 1.9 kb) were used (30). In addition, a 2.0-kb human MMP-1 cDNA (31), a 0.7-kb human pro-α(I) collagen cDNA (32), human plasminogen activator inhibitor (PAI-1) cDNA (33), and a 1.3-kb rat GAPDH cDNA (34) were used. Specific hybridization was visualized with autoradiography.

**Immunofluorescence Analysis**—To examine the nuclear translocation of HA-Smads produced by adenoviruses, human gingival fibroblasts were infected in suspension with RADSmad2, RADSmad3, and RADSmad4 and with control virus RAD66 at MOI 500 as described above, plated on sterile coverslips, and incubated for 18 h. Thereafter, the medium was changed to DMEM without FCS, and the incubations continued for 24 h. The cells were treated with TGF-β1 (5 ng/ml) for 2 h, fixed with methanol at −20°C for 6 min, and stained with rat monoclonal anti-HA antibody using rhodamine-conjugated anti-rat secondary antibody (Calbiochem).

In experiments investigating the effect of MKK3bE and p38α on the activation and nuclear translocation of Smad3, gingival fibroblasts were infected in suspension with adenoviruses for HA-tagged Smad3, FLAG-tagged p38α, constitutively active MKK3b, or dominant negative p38α and with empty control virus RAD66 at MOI 500. The cells were plated on sterile coverslips in DMEM without FCS and incubated for 12 h in the absence or presence of SB203580 (5 μM). Thereafter, the cells were fixed with MeOH and stained with mouse monoclonal anti-FLAG M2 antibody (Sigma) and rat monoclonal anti-HA antibody using fluorescein isothiocyanate-conjugated anti-mouse and rhodamine-conjugated anti-rat secondary antibodies (Calbiochem).

**RESULTS**

**Construction and Characterization of Recombinant Smad2, Smad3, and Smad4 Adenoviruses**—To examine the role of Smads in TGF-β-elicited induction of human MMP-13 expression, we constructed replication-deficient adenoviruses RADSmad2, RADSmad3, and RADSmad4, as described under “**Experimental Procedures**.” Smad2 and Smad3 cored by these viruses contain N-terminal HA-tags, and Smad4 has an HA tag in the C terminus. The cDNAs coding for HA-tagged Smads were subcloned to pCA3 shuttle vector under the control of cytomegalovirus immediate early promoter and cotransfected with adenoviral backbone plasmid pBHG10 into 293 cells, in which the adenoviruses were generated by recombination and packaged. To characterize the recombinant adenoviruses for HA-tagged Smad2, Smad3, and Smad4, HaCaT keratinocytes were infected with adenoviruses for Smad2 and Smad3 at MOI 500, and the expression of the corresponding Smads was analyzed with Western blotting using anti-HA-antibody. As shown in Fig. 1A, the expression of HA-tagged Smad2, Smad3, and Smad4 was detected in HaCaT cells infected with the corresponding Smad adenoviruses 24 h after infection.

Receptor-activated Smad2 and Smad3 have a C-terminal consensus motif, SSXS, in which the two last serine residues are phosphorylated by activated TGF-β receptor complex (35, 36). After the phosphorylation, Smad2 and Smad3 oligomerize with common mediator Smad4, and this complex translocates to the nucleus (37). To confirm the proper activation of adenovirally expressed HA-tagged Smads, we first analyzed the phosphorylation of Smad2 and Smad3 after TGF-β treatment by Western blot analysis using phospho-specific antibodies against phospho-Smad2 and phospho-Smad1, which cross-reacts with phosphorylated Smad3 (28, 29). Human gingival fibroblasts were infected with RADSmad2 and RADSmad3 and treated with TGF-β (5 ng/ml) for different periods of time, as indicated (Fig. 1B). Adenovirally produced HA-Smad2 and endogenous Smad2 could not be distinguished on SDS-PAGE (not shown). Therefore, to confirm the activation of adenovirally expressed Smad2, HA-tagged Smad2 was immunoprecipitated from lysates of RADSmad2-infected cells followed by Western blot analysis. Phosphorylation of HA-Smad2 was detected after 30- and 60-min TGF-β treatment (Fig. 1B). Adenovirally expressed HA-Smad3 was phosphorylated 30 min after TGF-β treatment, and the phosphorylation was maximal after 60 min of TGF-β treatment (Fig. 1B). Adenovirally expressed HA-Smad3 and endogenous Smad3 were separated on SDS-PAGE, the upper bands representing adenovirally produced HA-Smad3 and the lower bands representing endogenous Smad3 (Fig. 1B). The activation of the endogenous and adenovirally expressed Smad3 appeared with similar kinetics (Fig. 1B).

For examination of the nuclear translocation of HA-Smad2,
Smad3 Regulates Collagenase-3 Expression

HA-Smad3, and HA-Smad4, human gingival fibroblasts were infected with the corresponding RAdSmads, treated with TGF-β for 2 h, and stained with anti-HA antibody. As shown in Fig. 1C, in untreated cells, adenovirally expressed HA-tagged Smad2, Smad3, and Smad4 were located predominantly in the cytoplasm, but after a 2-h TGF-β treatment, HA staining was detected in the nucleus. Together, these results indicate that infection of cells with RAdSmad2, RAdSmad3, and RAdSmad4 results in production of the corresponding functional Smad proteins, which are phosphorylated and subsequently translocated to the nucleus upon TGF-β treatment.

Adenoviral Expression of Smad3 Enhances TGF-β-elicted Induction of MMP-13 Expression—We have previously noted that TGF-β induces the expression of MMP-13 in human gingival and fetal skin fibroblasts and that this requires the activity of p38 MAPK (5, 6). However, the activation of p38 alone or in combination with ERK1/2 is not sufficient to induce the expression of MMP-13 in these cells, suggesting that additional signaling pathways are involved (5, 6). To elucidate the roles of Smad2 and Smad3 in this context, we infected human gingival fibroblasts with RAdSmad2 and RAdSmad3 and treated the cells with TGF-β. In accordance with our previous findings (5), a 24-h treatment of gingival fibroblasts with TGF-β resulted in induction of the expression of MMP-13 mRNAs, as compared with untreated cells (Fig. 2A). Infection of cells with empty control virus RAd66 slightly reduced the TGF-β-elicted induction of MMP-13 mRNA levels (Fig. 2A). Interestingly, the expression of MMP-13 mRNAs was markedly (by 22-fold) enhanced by Smad3 overexpression after 24-h TGF-β treatment, as compared with RAd66-infected cells (Fig. 2A). In contrast, overexpression of Smad2 had no effect on induction of MMP-13 expression by TGF-β (Fig. 2A).

Previous studies have identified human PAI-1 as a Smad-responsive gene (11, 38, 39). As shown in Fig. 2A, TGF-β induced the expression of PAI-1 mRNAs, and the overexpression of Smad3 augmented the TGF-β-elicted induction of PAI-1 mRNA levels by 2-fold, as compared with RAd66-infected cells, indicating that PAI-1 is a Smad3-responsive gene in human gingival fibroblasts. Adenovirally expressed Smad2 and Smad3 had no effect on MMP-13 or PAI-1 expression in the absence of TGF-β (Fig. 2A). In comparison, the up-regulatory effect of TGF-β on pro-α1(I) collagen mRNA levels was not markedly affected in response to Smad3 overexpression (Fig. 2A).

To further elucidate the role of Smad3 in the regulation of MMP-13 expression, we examined the effect of Smad3 together with Smad4 on TGF-β-elicted induction of pro-MMP-13 production. Human gingival fibroblasts were infected with adenoviruses for Smad3 and Smad4 and treated with TGF-β for 24 h. Western blot analysis of conditioned media showed that adenoviral expression of Smad3 alone and in combination with Smad4 augmented the TGF-β induction of pro-MMP-13 production, as compared with control virus RAd66-infected cells (Fig. 2B). However, the production of pro-MMP-13 was not further augmented when Smad4 was co-expressed with Smad3, suggesting that the endogenous levels of Smad4 are sufficient to match the levels of adenovirally expressed Smad3 (Fig. 2B).

Co-expression of Smad2 and Smad4 had no effect on TGF-β-induced production of MMP-13 (data not shown). Adenoviral expression of Smad3 alone or in combination with Smad4 had
induced by treatment of infected fibroblasts with p38 inhibitor (Fig. 3A, lower panels).

Next, we studied whether the MKK3b-induced nuclear translocation of Smad3 is dependent on activation of p38α MAPK. In accordance with the observations above, activation of endogenous p38 by constitutively active MKK3b (MKK3bE) induced nuclear translocation of Smad3, and this could be inhibited by treatment of infected fibroblasts with p38 inhibitor SB203580 (5 μM) and by adenosinergic co-expression of dominant negative p38α (p38αF) (Fig. 3B).

Expression of Smad3 and Activation of p38α Induces MMP-13 Expression in the Absence of TGF-β—To examine whether p38-dependent activation of Smad3 also affects the expression of Smad3-dependent genes, gingival fibroblasts were infected with recombinant adenoviruses for Smad3 and Smad4 together with adenoviruses for wild-type p38α and constitutively active MKK3b and incubated for 24 h. As shown in Fig. 4A, the activation of endogenous p38α by MKK3bE and simultaneous co-expression of Smad3 resulted in the induction of expression of MMP-13 mRNAs in the absence of TGF-β. Co-expression of Smad4 did not markedly enhance the effect of constitutively active MKK3b and Smad3 on MMP-13 mRNA levels (Fig. 4A). The abundance of MMP-1 mRNA was reduced in response to overexpression of Smad3, whereas the activation of p38α by constitutively active MKK3b enhanced the expression of MMP-1 (Fig. 4A), as noted recently (40). In contrast, the levels of pro-α1(I) collagen mRNA were not significantly altered in this experiment (Fig. 4A). To exclude the possibility that the induction of MMP-13 expression could be due to endogenous expression of TGF-β, we analyzed the expression of Pai-1, which was shown to be highly TGF-β-responsive in these cells (Fig. 2A). Activation of p38α by MKK3bE resulted in induction of Pai-1 mRNAs, whereas expression of Smad3 or Smad4 had no effect on MKK3bE-elicited induction of Pai-1 mRNAs in the absence of TGF-β (Fig. 4A). This provides evidence that activation of Smad3 and Smad4 in this context

To confirm that the levels of Smad3 and Smad4 are elevated by infection of fibroblasts with corresponding adenoviruses, we examined the levels of Smad3 and Smad4 in the cells by Western blot analysis. As shown in Fig. 2C, elevated levels of Smad3 and Smad4 were detected in cells infected with RAdSmad3 or RAdSmad4 alone or in combination.

To further examine the role of Smad3 in mediating the effect of TGF-β on MMP-13 expression, gingival fibroblasts were infected with an adenovirus coding for dominant negative Smad3 (RAdSmad3DN) in parallel with RAdSmad3 and the control virus RAd66 and treated with TGF-β for 24 h. In accordance with the observations above, adenosinergic expression of Smad3 augmented (by 7-fold) the up-regulatory effect of TGF-β on the expression of pro-MMP-13, as compared with RAd66-infected cells (Fig. 2D). Interestingly, adenosinergic expression of dominant negative Smad3 resulted in potent (by 90%) inhibition of pro-MMP-13 production in the presence of TGF-β (Fig. 2D). In comparison, the production of TGF-1 was not markedly altered by dominant negative Smad3 or TGF-β (Fig. 2D). Together, these observations show that Smad3 mediates the TGF-β-elicited induction of MMP-13 expression in human gingival fibroblasts, whereas Smad2 appears not to have a role in this context.

Activation of p38α Induces Nuclear Translocation of Smad3—Recent studies have revealed cross-talk between the Smad pathway and other cellular signaling pathways (e.g. p38, ERK1/2, c-Jun N-terminal kinase, and Ca2+-calmodulin-dependent protein kinase II signaling pathways) (16–19). In addition, we have previously noted that the induction of MMP-13 expression by TGF-β requires p38 MAPK activity (5, 6). In order to study the cross-talk between Smad3 and the p38 MAPK pathway, human gingival fibroblasts were first infected with adenoviruses for p38α containing FLAG tag (RAdp38αF), constitutively active MKK3b (RAdMKK3bE), and Smad3 with HA tag (RAdSmad3). After 12 h of incubation, the cells were fixed and stained with anti-FLAG and HA antibodies to analyze the activation and nuclear translocation of p38α and Smad3, respectively. As shown in Fig. 3A, when expressed alone, p38α and Smad3 were detected predominantly in the cytoplasm of infected fibroblasts. However, co-expression of p38α with the constitutively active mutant of its upstream activator, MKK3b, resulted in translocation of FLAG-tagged p38α into the nucleus, and simultaneous expression of Smad3 had no effect on the activation and nuclear localization of p38α (Fig. 3A). Interestingly, activation of endogenous or adenovirally expressed p38α by constitutively active MKK3b induced the nuclear translocation of adenosinergic delivered Smad3 (Fig. 3A). Furthermore, activated p38α and Smad3 showed nuclear co-localization (Fig. 3A, lower panels).

no effect on pro-MMP-13 production in the absence of TGF-β (Fig. 2B). The production of TIMP-1 was not affected in response to Smad3 or Smad4 expression in the absence or presence of TGF-β (Fig. 2B).
does not result in induction in the production of TGF-β and that the expression of MMP-13 is not due to autocrine stimulation by TGF-β. This notion is also supported by our observation that the levels of TGF-β mRNA were not induced under these conditions (data not shown).

We have previously observed that TGF-β also activates ERK1/2 in human gingival fibroblasts (5). To further analyze the cross-talk between the MAPK pathways and Smad3, we utilized adenoviral gene delivery of constitutively active MEK1 (RAdMEK1CA), an upstream activator of the ERK1/2, together with recombinant adeno-viruses for wild-type p38α (RAdp38α), constitutively active MKK3b (RAdMKK3bE), Smad3 (RAdSmad3), or Smad4 (RAdSmad4) or with empty control virus RAd66 and incubated for 24 h. Total cellular RNA was analyzed with Northern blot hybridizations for the expression of MMP-13, pro-α1(I) collagen, PAI-1, and GAPDH mRNAs. B–D, human gingival fibroblasts were infected with recombinant adeno-viruses RAdp38α, RAdMKK3bE, constitutively active MEK1 (RAdMEK1CA), RAdSmad3, or with empty control virus RAd66, as indicated (MOI 500). After 24 h of incubation, conditioned media were analyzed with Western blotting for the production of pro-MMP-13, pro-MMP-1, and TIMP-1 (B and C), and the cell layers were analyzed for the levels of activated p38 MAPK (p-p38) and ERK1/2 (p-ERK1/2) and total p38 and ERK1/2 (D) with corresponding antibodies.

To exclude the possibility that adeno-viral expression of Smad3 could alter the activation of p38 and ERK1/2 MAPKs, we analyzed the levels of activated p38 and ERK1/2 in the same cells by Western blotting. As shown in Fig. 4D, infection of cells with adeno-viruses for constitutively active MKK3b and MEK1 resulted in potent activation of p38 MAPK and ERK1/2, respectively, but simultaneous expression of Smad3 had no effect on the phosphorylation of endogenous p38, adeno-virally expressed p38α, or ERK1/2 (Fig. 4D).

The Induction of MMP-13 Expression by Constitutively Active MKK3b and MKK6b Is Mediated by p38α—Next, we utilized gene delivery with recombinant adeno-viruses for dominant negative p38α (RAdp38AF), together with adeno-viruses for constitutively active MKK3b, Smad3, and Smad4. In accordance with the observations above, activation of p38α by MKK3b and simultaneous expression of Smad3 and Smad4 resulted in induction of MMP-13 production in the absence of TGF-β (Fig. 5A). Infection of fibroblasts with RAdp38AF significantly (by 70%) inhibited the production of MMP-13, and treatment of cells with p38 inhibitor SB203580 (10 μM) abrogated the production of MMP-13 (Fig. 5A), indicating that p38α mediates the induction of MMP-13 gene expression. The production of
Smad3 Regulates Collagenase-3 Expression

We have previously reported that MMP-13 is expressed by fibroblasts during human gingival and fetal skin wound repair and that the expression of MMP-13 in human gingival and fetal skin fibroblasts is induced by TGF-β via p38 pathway (5, 6). In the present study, we have constructed recombinant replication-deficient adenoviruses coding for wild-type HA-tagged Smad2, Smad3, and Smad4 and utilized these in dissecting the role of Smad signaling in the regulation of MMP-13 gene expression by TGF-β in human gingival fibroblasts. Our results show that adenovirus-mediated gene transfer of Smad3 potently enhances the TGF-β-elicited induction of MMP-13 production and that the effect of TGF-β can be blocked by dominant negative Smad3. We also provide evidence for a novel type of cross-talk between the p38 MAPK pathway and Smad3 in the context of the induction of MMP-13 gene expression.

Our findings using adenovirus-mediated gene delivery of wild-type Smads show that Smad3, but not Smad2, is involved in regulating the TGF-β-elicited induction of MMP-13 expression in human gingival fibroblasts. In addition, adenoviral expression of dominant negative Smad3 inhibited the TGF-β-elicited induction of MMP-13 production by human dermal fibroblasts (42). Our results are also supported by a recent study showing that Smad3 mediates the up-regulatory effect of TGF-β on human MMP-13 gene expression. Our observations show that although Smad2 and Smad3 share close structural similarity, they play distinct roles in mediating the signals triggered by TGF-β. This is consistent with previous findings indicating differential roles for Smad2 and Smad3 in TGF-β signaling (12, 43, 44). Our results are also supported by a recent study showing that the TGF-β induction of MMP-13 expression by human osteoarthritic chondrocytes involves Smad proteins (45).

TGF-β-responsive genes. For instance, the TGF-β-elicited up-regulation of fibronectin gene expression requires activation of the c-Jun N-terminal kinase pathway independently of the Smad pathway (41). In addition, there is recent evidence for the role of p38 MAPK in mediating up-regulation of type I collagen gene expression by dermal fibroblasts (42).

We have previously reported that MMP-13 is expressed by fibroblasts during human gingival and fetal skin wound repair and that the expression of MMP-13 in human gingival and fetal skin fibroblasts is induced by TGF-β via p38 pathway (5, 6). In the present study, we have constructed recombinant replication-deficient adenoviruses coding for wild-type HA-tagged Smad2, Smad3, and Smad4 and utilized these in dissecting the role of Smad signaling in the regulation of MMP-13 gene expression by TGF-β in human gingival fibroblasts. Our results show that adenovirus-mediated gene transfer of Smad3 potently enhances the TGF-β-elicited induction of MMP-13 production and that the effect of TGF-β can be blocked by dominant negative Smad3. We also provide evidence for a novel type of cross-talk between the p38 MAPK pathway and Smad3 in the context of the induction of MMP-13 gene expression.

Our findings using adenovirus-mediated gene delivery of wild-type Smads show that Smad3, but not Smad2, is involved in regulating the TGF-β-elicited induction of MMP-13 expression in human gingival fibroblasts. In addition, adenoviral expression of dominant negative Smad3 inhibited the TGF-β-elicited induction of MMP-13 production, providing further evidence that Smad3 mediates the up-regulatory effect of TGF-β on human MMP-13 gene expression. Our observations show that although Smad2 and Smad3 share close structural similarity, they play distinct roles in mediating the signals triggered by TGF-β. This is consistent with previous findings indicating differential roles for Smad2 and Smad3 in TGF-β signaling (12, 43, 44). Our results are also supported by a recent study showing that the TGF-β induction of MMP-13 expression by human osteoarthritic chondrocytes involves Smad proteins (45). The results in that study showed that in chondrocytes Smad2 is phosphorylated in response to TGF-β and that Smads are found in complexes with AP-1 proteins (45). However, no evidence for phosphorylation of Smad3 was provided; nor were Smad proteins in AP-1 complexes identified. Therefore, it could not be concluded which member of the Smad family actually is involved in up-regulation of MMP-13 expression in chondrocytes.

Here, we also studied the effect of the adenoviral overexpression of Smad4 together with Smad3. We hypothesized that co-expression of Smad4 and Smad3 would further enhance the
Effect of TGF-β on the expression of MMP-13. However, in our model, co-expression of Smad4 together with Smad3 did not augment the effect of TGF-β on the expression of MMP-13, as compared with Smad3 adenovirus-infected gingival fibroblasts. It is likely that the endogenous levels of Smad4 in these cells are high enough to match the levels of adenovirally expressed Smad3 for achieving maximal stimulation of the expression of the endogenous MMP-13 gene. In addition, the negative regulatory mechanisms may take place in our model with adenoviral overexpression of wild-type Smads, since the expression of Smad7 is induced by TGF-β via Smad3 and Smad4 (46–48). It is therefore possible that Smad7 expression is induced in response to activation of Smad3 and Smad4 in our model, resulting in autoinhibition of the effect of Smad3 and Smad4.

Activation of p38α by constitutively active mutants of its upstream activators MKK3b and MKK6b and simultaneous co-expression of Smad3 resulted in potent induction of MMP-13 expression in the absence of TGF-β. In addition, activation of endogenous or adenovirally delivered p38α induced nuclear translocation of Smad3, providing evidence that p38α activates Smad3 either directly or via some endogenous mediator. In this context, there is recent evidence for cross-talk between the MAPK and Smad signaling pathways. For instance, activation of MAPK pathways has been shown to stimulate nuclear translocation of Smad2 (18, 49), and MEKK-1, a MAPK kinase in the c-Jun N-terminal kinase pathway, has been reported to selectively activate Smad2-dependent transcription independently of TGF-β in endothelial cells (18). It was shown that MEKK-1 phosphorylates Smad2 at a site distinct from the C-terminal SSXS motif usually phosphorylated by the type I TGF-β receptor. It is also likely that in our model the activation of Smad3 by p38α involves phosphorylation of Smad3 outside the SSXS motif, since phosphorylated Smad3 could not be detected in the presence of p38 activation (not shown). This could be explained by the fact that the phosphoantibody used was raised against phosphorylated Smad1 and may not recognize the putative phosphorylated serine residues outside the SSXS motif of the Smad3 molecule. However, it is also possible that the levels of phosphorylated Smad3 in this model are low and that the phospho-Smad1 antibody is not sensitive enough to detect low levels of phosphorylated Smad3.

Smads regulate the transcription of their target genes by binding directly to the promoter regions of the respective genes or by associating with other transcription factors or co-activators/repressors. Various Smad-responsive genes have Smad-binding elements in their promoter regions, consisting of sequences like GTCT, AGAC, GACA, and CAGA (46, 50, 51). The promoter region of human MMP-13 contains several sequences resembling putative Smad binding sequences (e.g. GACA, CAGA, or GTCT) (52). In a recent study, the role of these putative Smad-binding element-like elements in the human MMP-13 promoter was examined, but no evidence was found for their role in regulating MMP-13 gene transcription by TGF-β (45). However, since DNA binding is not a prerequisite for Smad-mediated transcriptional activation, induction of human MMP-13 gene transcription may involve interaction of Smads with other transcription factors. The 5′-flanking region of the human MMP-13 gene promoter contains a functional AP-1 motif (52), and the induction of MMP-13 expression by TGF-β requires the presence of functional AP-1 dimers (5). AP-1 DNA-binding sites have also been demonstrated to be essential for TGF-β-elicited induction of other genes (53, 54). Furthermore, Smad3 is able to directly associate with the c-Jun-c-Fos AP-1 transcription factors and to synergize with c-Jun in transcriptional activation of artificial promoters (55–58). Since dominant negative c-Jun potently suppressed the induction of MMP-13 expression by TGF-β (5), it is possible that Smad3 associates with AP-1 transcription factors and stimulates the activity of the human MMP-13 promoter together with AP-1. It is also possible that p38-dependent activation of MMP-13 expression involves mRNA stabilization, as we have recently noted with respect to p38-mediated enhancement of MMP-1 and stromelysin-1 (MMP-3) expression in dermal fibroblasts (40).

In conclusion, the results of the present study together with our previous observations (5, 6) show that TGF-β-induced expression of endogenous MMP-13 gene in human gingival fibroblasts involves activation of two distinct signaling pathways (i.e. p38α and Smad3) (Fig. 6). In addition, our results provide evidence for novel type of cross-talk between these two TGF-β-activated signaling cascades (Fig. 6). It is likely that induction of human MMP-13 expression by interplay between Smad3 and p38α may play a role in situations in which MMP-13 expression in fibroblasts is induced by TGF-β, such as gingival and fetal skin wound repair (5, 6). It is also conceivable that this p38α-mediated activation of Smad3 may serve as a novel target for inhibiting p38-dependent induction of MMP-13 expression by TGF-β (e.g. in squamous carcinoma cells) (59).
Smad3 Regulates Collagenase-3 Expression

30. Johansson, N., Westermark, J., Leppa, S., Hakkinen, L., Koivistbo, L., Lopez-Otin, C., Peltonen, J., Heino, J., and Kahari, V.-M. (1997) Cell Growth Differ. 8, 243–250
31. Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A., and Eisen, A. Z. (1996) J. Biol. Chem. 271, 6600–6605
32. Makela, J. K., Raassina, M., Virta, A., and Vuorio, E. (1988) Nucleic Acids Res. 16, 349
33. Keski-Oja, J., Raghow, R., Sawdye, M., Loskutoff, D. J., Postlethwaite, A. E., Wang, W., and Luo, K. (1999) J. Biol. Chem. 274, 33412–33418
34. von Gerderoff, G., Fusenig, N. E., Chen, Y. (1999) Mol. Cell. Biol. 19, 821–830
35. Dedhar, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L., and Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., Oeda, N., Reunanen, N., Li, S.-P., Ahonen, M., Foschi, M., Han, J., and Ka¨ lis¨e, J. K., Raassina, M., Virta, A., and Vuorio, E. (1988) J. Cell Sci. 96, 10917–10923
36. Liberatori, N. T., and Datto, M. B., Liberati, N. T., Li, J. M., and Wang, X. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4844–4849
37. Hua, X., Miller, Z. A., Wu, G., Shi, Y., and Lodish, H. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13130–13135
38. Pendas, A. M., Balbin, M., Llano, E., Jimenez, M. G., and Lopez-Otin, C. (1997) Genomics 40, 222–233
39. Jin, G., and Howe, P. H. (1997) J. Biol. Chem. 272, 26620–26626
40. Reunanen, N., Li, S.-P., Ahonen, M., Foschi, M., Han, J., and Kahari, V.-M. (2002) J. Biol. Chem. 277, 32360–32368
41. Hocevar, B. A., Brown, T. L., and Howe, P. H. (1999) EMBO J. 18, 1345–1356
42. Sato, M., Shegogue, D., Gore, E. A., Smith, E. A., McDermott, P. J., and Trojanowska, M. (2002) J. Invest. Dermatol. 118, 704–711
43. Nagarajan, R. P., Liu, J., and Chen, Y. (1999) J. Biol. Chem. 274, 31229–31235
44. Werner, F., Jain, M. K., Feinberg, M. W., Sibenga, N. E., Pellacani, A., Wessel, P., Chin, M. T., Topper, J. N., Perrella, M. A., and Lee, M. E. (2000) J. Biol. Chem. 275, 36653–36658
45. Tardif, G., Rebole, P., Dupuis, M., Geng, C., Duval, N., Pelletier, J. P., and Martel-Pelletier, J. (2001) J. Rheumatol. 28, 1631–1639
46. Nagarajan, R. P., Zhang, J., Li, W., and Chen, Y. (1999) J. Biol. Chem. 274, 33412–33418
47. Stepa, M., Anhuf, D., Terstegen, L., Gatsios, P., Gessner, A. M., and Dooley, S. (2000) J. Biol. Chem. 275, 29808–29817
48. von Gerderoff, G., Fusenig, N. E., Reunanen, N., Li, J. M., and Wang, X. F. (1999) Mol. Cell. Biol. 19, 821–830
49. Johansson, N., Ala-aho, R., Uitto, V.-J., Gre´ s, R., Gre´ nna, E., Fuesung, N. E., Lopez-Otin, C., and Kahari, V.-M. (2000) J. Cell Sci. 113, 227–235
Smad3 Mediates Transforming Growth Factor-β-induced Collagenase-3 (Matrix Metalloproteinase-13) Expression in Human Gingival Fibroblasts: EVIDENCE FOR CROSS-TALK BETWEEN Smad3 AND p38 SIGNALING PATHWAYS
Suvi-Katri Leivonen, Andrew Chantry, Lari Häkkinen, Jiahuai Han and Veli-Matti Kähäri

J. Biol. Chem. 2002, 277:46338-46346.
doi: 10.1074/jbc.M206535200 originally published online September 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206535200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 40 of which can be accessed free at http://www.jbc.org/content/277/48/46338.full.html#ref-list-1