Induction of Collagenase-3 (MMP-13) Expression in Human Skin Fibroblasts by Three-dimensional Collagen Is Mediated by p38 Mitogen-activated Protein Kinase*

Laura Ravanti†‡, Jyrki Heino§‡, Carlos López-Otín¶§, and Veli-Matti Kähäri†‡‡****

From the †Department of Dermatology, Turku University Central Hospital and the §Department of Medical Biochemistry, and MediCity Research Laboratory, University of Turku, FIN-20520 Turku, Finland, the ¶Department of Biological and Environmental Science, University of Jyväskylä, FIN-40351 Jyväskylä, Finland, and the **Departmento Bioquímica y Biología Molecular, Universidad de Oviedo, 33006 Oviedo, Spain

Collagenase-3 (matrix metalloproteinase-13, MMP-13) is a recently identified human MMP with an exceptionally wide substrate specificity and restricted tissue-specific expression. Here we show that MMP-13 expression is induced in normal human skin fibroblasts cultured within three-dimensional collagen gel resulting in production and proteolytic activation of MMP-13. Induction of MMP-13 mRNAs by collagen gel was potently inhibited by blocking antibodies against α1 and α5 integrin subunits and augmented by activating antibody against β1 integrin subunit, indicating that both α1β1 and α5β1 integrins mediate the MMP-13-inducing cellular signal generated by three-dimensional collagen. Collagen-related induction of MMP-13 expression was dependent on tyrosine kinase activity, as it was abolished by treatment of fibroblasts with tyrosine kinase inhibitors genistein and herbimycin A. Contact of fibroblasts to three-dimensional collagen resulted in simultaneous activation of mitogen-activated protein kinases (MAPKs) in three distinct subgroups: extracellular signal-regulated kinase (ERK)1 and ERK2, Jun N-terminal kinase/stress-activated protein kinase, and p38. Induction of MMP-13 expression was inhibited by treatment of fibroblasts with a specific p38 inhibitor, SB 203580, whereas blocking the ERK1,2 pathway (Raf/MEK1,2/ERK1,2) by PD 98059, a selective inhibitor of MEK1,2 activation potently augmented MMP-13 expression. Furthermore, specific activation of ERK1,2 pathway by 12-O-tetradecanoylphorbol-13-acetate markedly suppressed MMP-13 expression in dermal fibroblasts in collagen gel. These results show that collagen-dependent induction of MMP-13 in dermal fibroblasts requires p38 activity, and is inhibited by activation of ERK1,2. Therefore, the balance between the activity of ERK1,2 and p38 MAPK pathways appears to be crucial in regulation of MMP-13 expression in dermal fibroblasts, suggesting that p38 MAPK may serve as a target for selective inhibition of collagen degradation, e.g. in chronic dermal ulcers.

*This work was supported by grants from the Academy of Finland, the Sigrid Juselius Foundation, the Cancer Research Foundation of Finland, Turku University Central Hospital, and the Turku University Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed: MediCity Research Laboratory, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland. Tel.: 358-2-3337025; Fax: 358-2-3337000; E-mail: veli-matti.kahari@utu.fi.

1 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; TGF-β, transforming growth factor-β; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, Jun N-terminal kinase/stress-activated protein kinase; MEK, MAPK/ERK kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; SCC, squamous cell carcinoma; FAK, focal adhesion kinase; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; bp, base pair(s); kb, kilobase pair(s); DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
synovium (9, 11, 12), chronic cutaneous ulcers (13), intestinal ulcerations (14), and periodontitis (15), as well as in malignant tumors, i.e., breast carcinomas (4, 16, 17), squamous cell carcinomas (SCCs) of the head and neck (18), cutaneous basal cell carcinomas (19), and chondrosarcomas (20). In cell culture conditions, the expression of MMP-13 is also restricted as compared with MMP-1 and has so far been reported in human fetal and osteoarthritic chondrocytes (6, 8), and in transformed keratinocytes and fibroblasts, osteosarcoma, and chondrosarcoma cells (17, 20–22).

Controlled degradation of fibrillar collagens of type I and III apparently plays an important role in ECM remodeling in cutaneous wound repair. However, the expression of MMP-13 and MMP-1 in human cutaneous wounds is clearly different, as MMP-1 is expressed in migrating keratinocytes (23, 24) and in dermal fibroblasts in acute and chronic wounds (13, 25). In contrast, our recent observations show that MMP-13 is not expressed by keratinocytes in wound repair, although it is expressed in dermal fibroblasts in chronic cutaneous ulcers, but not during acute wound repair (13). We also noted that normal human skin fibroblasts express MMP-13 mRNAs when cultured in three-dimensional collagen gel, but not when grown in monolayer (13). In this study, we show that collagen-specific induction of MMP-13 expression by dermal fibroblasts is mediated via α1β1 and α5β1 integrins and requires tyrosine kinase activity. We also show that the contact of dermal fibroblasts with three-dimensional collagen coordinate activates three distinct classes of mitogen-activated protein kinases (MAPKs), i.e., ERK1,2, JNK, and p38. The activity of p38 MAPK is essential for MMP-13 expression by fibroblasts in collagen gel, whereas specific inhibition of ERK1,2 pathway potently augments induction of MMP-13 expression. These results provide evidence that activity of p38 MAPK pathway plays an essential role in the activation of MMP-13 expression in fibroblasts and it may serve as a target for selective inhibition of collagen degradation, e.g., in chronic dermal ulcers.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Human recombinant tumor necrosis factor-α (TNF-α) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma. C2-merceramid, genistein, herbimycin A, Ro-31-8220, bisindolylmaleimide, SB 203580, and PD 98059 were obtained from Calbiochem (San Diego, CA). Interleukin-1β (IL-1β) was obtained from Boehringer Mannheim (Mannheim, Germany). Bovine TG-β1 was kindly provided by Dr. David R. Olsen (Celtrix Co., Santa Clara, CA). Activating antibody against β1 integrin subunit (mAb13; Ref. 26) was kindly provided by Dr. Kenneth Yamada (NIDR, Bethesda, MD). Blocking antibody against α1 integrin subunit (SR-84; Ref. 27) was a kind gift from Dr. Wolfgang Retting (Boehringer Ingelheim, Germany) and a blocking antibody against α5 integrin subunit (mCA743) was obtained from Sero-tech (Oxford, United Kingdom).

**Cell Cultures—**Normal human skin fibroblast cultures were established from a punch biopsy obtained from a healthy male volunteer (age 27 years). Fibroblast cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories, Irvine, United Kingdom) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin.

**Collagen Gels—**Collagen gels were prepared from bovine dermal collagen, which contains 95% type I collagen and 5% type III collagen (Cellon, Strassen, France). Briefly, eight volumes of Collen were mixed with one volume of 10× concentrated DMEM and one volume of 10× concentrated NaOH (0.05 M) in Heps buffer (0.2 M) and kept on ice. Cells were trypsinized, resuspended in DMEM supplemented with 1% FCS, mixed into neutralized Collen solution, transferred into six-well plates and incubated at 37 °C for 2 h for collagen polymerization. Thereafter, CM was supplemented with 1% or 10% FCS, depending on the experimental conditions, was added, the gels were detached from the sides of the wells and the incubations continued for different periods of time. As controls, fibroblasts were plated on plastic as monolayer and cultured under similar conditions as the corresponding fibroblasts in collagen gel. In the experiments involving cytokines, growth factors, C2-merceramid, or TPA, fibroblasts were first incubated for 24 h as monolayer or inside collagen gels, after which the modulators were added and the incubations continued for an additional 24 h. In experiments involving tyrosine kinase inhibitors, PKC inhibitors, MAPK inhibitors, or functional antibodies against different collagen-binding integrin subunits, these were added to the cell suspension prior to mixing it with neutralized collagen solution or plating the cells on plastic, and the incubations were continued for 48 h. In experiments involving two-dimensional collagen, cell culture dishes were coated with rat tail type I collagen (5 μg/cm²) (Sigma).

**Northern Blot Hybridizations—**Fibroblasts were released from collagen gels by brief treatment with 0.5 mg/ml collagenase (type II, Sigma) in 1× PBS containing 0.1% bovine serum albumin, 0.1 M NaCl, 10 mM Tris (pH 7.4) with 1 μM CaCl₂. Total cellular RNA was isolated from cells using the single-step method (28). Aliquots of total RNA (5–20 μg) were fractionated on 0.8% agarose gel containing 2.2 M formaldehyde, transferred to Zetaprobe filter (Bio-Rad) by vacuum transfer (VacuGene XL, LKB, Bromma, Sweden), and immobilized by heating at 80 °C for 30 min. The filters were prehybridized for 2 h and subsequently hybridized for 20 h with cDNAs labeled with [α-32P]dCTP using random priming. For detection of MMP-13 mRNAs, we used three cDNA fragments covering altogether 1637 bp of human MMP-13 cDNA (21) and for collagenase-1 (MMP-1) mRNA, we used a 2.0-kb human cDNA (29). In addition, a 1.5-kb human stromelysin-1 (MMP-3) cDNA (30), a 2.7-kb human gelatinase-A (MMP-2) cDNA (31), a 0.7-kb human MT1-MMP (MMP-14) cDNA (32), a 0.7-kb human MMP-11 cDNA (33) and a 1.3-kb rat (5) and a 1.0-kb mouse phosphatase dehydrogenase (GAPDH) cDNA (34) were used. The [32P]cDNA/mRNA hybrids were visualized with autoradiography, quantified with densitometry, and corrected for the levels of GAPDH mRNA for each sample.

**RT-PCR—**The levels of MMP-13 mRNAs were also determined by reverse-transcription PCR (RT-PCR) using the Gene Amp RNA PCR kit (Perkin-Elmer/Roche, Branchburg, NJ). The RT reactions were performed with random hexamer primers using 1 μg of total RNA from fibroblasts cultured on plastic or in collagen gel for 48 h as a template. Subsequently, a 300-bp fragment of human MMP-13 cDNA (nucleotides 534–833) (4) was amplified by PCR with 40 cycles of denaturation (95 °C, 1.5 min), annealing (65 °C, 1.5 min), and extension (72 °C, 2 min) using primers oligonucleotides (5′-GTTTAGGGTTGGGGTCTTCATCTGATGGGCCCTCTGGCCTG-3′) and antisense oligonucleotide (5′-GGTGGGTTGGGGTCTTCATCTC-3′), and the PCR products were electrophoretically fractionated on 2% agarose gel. Total RNA (1 μg) from HaCaT keratinocytes treated with TG-β1 (5 ng/ml) for 24 h was used as a positive control (21).

**Assay of MMP-13 Production—**Human skin fibroblasts were seeded inside collagen gels or on plastic and maintained in DMEM supplemented with 1% FCS for 48 h. Equal aliquots of the conditioned media were concentrated and analyzed for the amount of MMP-13 by Western blotting, as described previously (21) using a rabbit antiserum against human recombinant MMP-13 (4) in dilution 1:1000 followed by enhanced chemiluminescence detection of bound primary antibodies (Amersham Corp., United Kingdom).

**Assay of MAPK Activation—**The activation of ERK1,2, JNK/SAPK, and p38 MAPK was determined by Western blotting using antibodies specific for phosphorylated, activated forms of the corresponding MAPKs (New England Biolabs, Beverly, MA). Fibroblasts were seeded in collagen gels in DMEM with 0.5% FCS, released from gels at various time points, as described above, and lysed in 100 μl of Laemmli sample buffer. The samples were then sonicated, fractionated by 10% SDS-PAGE, and transferred to Hybond ECL membrane (Amersham Corp.). Western blotting was performed as described previously (35), with phosphospecific antibodies for ERK1,2, JNK, and p38 in dilution 1:1000. Specific binding of antibodies was detected with peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (ECL) detection system (Amersham).

**RESULTS**

**Collagenase-3 (MMP-13) Expression in Dermal Fibroblasts Is Induced by Contact to Three-dimensional Collagen—**We have recently noted that MMP-13 is expressed by fibroblasts in chronic human cutaneous ulcers but not in normally healing acute dermal wounds (13). In addition, we observed that MMP-13 mRNAs are expressed by human skin fibroblasts cultured in type I collagen gel but not by fibroblasts in monolayer cultures (13). In the present study, we have elucidated the signaling mechanisms mediating the collagen-dependent induction of MMP-13 expression in human skin fibroblasts.
Collagen-dependent Induction of MMP-13 Is Mediated by p38

Initially, we cultured fibroblasts for 48 h on plastic, on type I collagen-coated dishes, or in collagen gels and determined the expression of MMP-13 mRNAs by Northern blot hybridizations. Marked expression of two MMP-13 transcripts of 2.0 and 2.5 kb was noted in dermal fibroblasts cultured inside collagen, whereas no MMP-13 mRNA could be detected in fibroblasts maintained in monolayer cultures on plastic or on two-dimensional collagen (Fig. 1A). In comparison, MMP-1 mRNA abundance was slightly enhanced by contact to 2-dimensional collagen and markedly up-regulated (36.5-fold) in cells maintained inside collagen (Fig. 1A). Examination of the time dependence of MMP-13 induction showed marked expression of MMP-13 mRNAs in dermal fibroblasts 24 h after seeding the cells in collagen gel, and the induction was still detected after 72 h (data not shown).

To confirm the observation that expression of MMP-13 is specifically induced in human skin fibroblasts inside collagen we determined the levels of MMP-13 mRNA by RT-PCR. As shown in Fig. 1B, a specific 300-bp fragment of MMP-13 cDNA was amplified when RNA from fibroblasts cultured in collagen gel for 48 h was used as a template. The same 300-bp MMP-13 cDNA fragment was amplified in RT-PCR reactions using RNA from TGF-β-treated HaCaT keratinocytes known to express high levels of MMP-13 mRNAs (21) (Fig. 1B). In contrast, a very low level of the same MMP-13 cDNA fragment was amplified by PCR using RNA from fibroblasts cultured on plastic indicating that under these conditions normal human skin fibroblasts express very low levels of MMP-13 mRNA (Fig. 1B).

Next, we assayed MMP-13 production by human skin fibroblasts cultured in collagen gel or on plastic for 48 h by Western blot analysis. No detectable amounts of immunoreactive MMP-13 were produced by fibroblasts cultured in monolayer on plastic, supporting the observations above that in dermal fibroblasts in monolayer cultures the expression of MMP-13 mRNAs is very low. In contrast, a specific band with an apparent molecular mass of 52 kDa was detected in conditioned media of fibroblasts cultured in collagen gel (Fig. 1C). Interestingly, this immunoreactive MMP-13 was clearly smaller in size than the 62-kDa latent MMP-13 produced by TNF-α-treated HaCaT keratinocytes, indicating that it had been proteolytically processed to active form (Fig. 1C). No latent MMP-13 was detected in the conditioned media, indicating that all MMP-13 produced by fibroblasts in collagen gel was activated.

Expression of MMP-13 by Dermal Fibroblasts in Collagen Gel Is Down-regulated by TGF-β but Not Altered by IL-1β or TNF-α—In chronic dermal ulcers, fibroblasts are exposed to cytokines and growth factors, which have the ability to regulate the expression of several MMPs including MMP-1 (see Refs. 1 and 2). To examine the regulation of MMP-13 expression by cytokines and growth factors, dermal fibroblasts were cultured in collagen gel for 24 h to allow induction of MMP-13 expression, and were subsequently treated with IL-1β, TNF-α, or TGF-β for another 24 h. Interestingly, treatment of fibroblasts in collagen gels with IL-1β (5 units/ml) or TNF-α (20 ng/ml) did not markedly alter MMP-13 mRNA levels, whereas treatment with TGF-β1 (5 ng/ml) decreased MMP-13 mRNA levels (by 60%), as compared with the levels of untreated cells in collagen gel (Fig. 2, A and B). No MMP-13 mRNAs were detected in fibroblasts cultured in monolayer after a 24-h treatment with TNF-α, IL-1β, or TGF-β1 (Fig. 2A). In comparison, MMP-1 mRNA levels were enhanced up to 1.8-fold by IL-1β and up to 3.2-fold by TNF-α, whereas stromelysin-1 (MMP-3) mRNA abundance was up-regulated 6.1-fold by both IL-1β and TNF-α in fibroblasts inside collagen gel (Fig. 2, A and B). Interestingly, TGF-β1 had no marked effect on the expression of MMP-1 mRNA by fibroblasts inside collagen gels and it even slightly enhanced the abundance of MMP-3 mRNA (Fig. 2, A and B).

In addition to MMP-13 and MMP-1, the mRNA levels of MMP-2 (72-kDa gelatinase), MMP-3, and MT1-MMP (MMP-14) were clearly up-regulated in fibroblasts cultured inside collagen gels, as compared with cells cultured in monolayer (Fig. 2A). In accordance with our previous observations (32), the levels of MT1-MMP mRNA in fibroblasts cultured on plastic were not markedly altered by any of the treatments although a slight up-regulation by IL-1β and TNF-α was detected.

**Fig. 1.** Collagenase-3 (MMP-13) expression in dermal fibroblasts is induced by three-dimensional collagen. A, confluent human skin fibroblasts were cultured in monolayer (Plastic), on type I collagen-coated dishes (2-d), or within collagen gel (3-d) in DMEM with 10% FCS for 48 h. MMP-13, MMP-1, and GAPDH mRNA levels were determined by Northern blot hybridizations of total RNA (10 μg). B, total RNA (1 μg) from human skin fibroblasts cultured in monolayer (Plastic) or in collagen gel (Collagen) for 48 h was used as a template to amplify a 300-bp fragment of MMP-13 cDNA by RT-PCR with specific oligonucleotide primers, as described under “Experimental Procedures.” In parallel RT-PCR reactions, total RNA (1 μg) from HaCaT keratinocytes treated with TGF-β (5 ng/ml) from HaCaT keratinocytes known to express positive control. Aliquots of RT-PCR reactions were fractionated on 2% agarose gel and DNA fragments visualized by ethidium bromide. The sizes of molecular mass markers (in bp) are shown on the left.
Collagen-dependent Induction of MMP-13 Is Mediated by p38

**A** Plastic Collagen

**B**

![Expression of MMP-13 by dermal fibroblasts cultured in collagen gel is down-regulated by TGF-β, but not altered by IL-1β or TNF-α. A. Human skin fibroblasts were cultured in monolayer (Plastic) or within collagen gel (Collagen) for 24 h and then treated with IL-1β (5 units/ml), TNF-α (20 ng/ml), or TGF-β (5 ng/ml) for another 24 h. MMP-13, MMP-1, MMP-2, and MT1-MMP (MMP-14) mRNA levels were determined by Northern blot hybridizations of total RNA (12 μg). B. densitometric quantitation of MMP-13, MMP-1, MMP-2, MMP-3, and MT1-MMP (MMP-14) mRNA levels of dermal fibroblasts cultured in collagen gel and treated with IL-1β, TNF-α, or TGF-β, as indicated in A. The values for each MMP are shown relative to untreated control cells in collagen gel (1.00).**

in fibroblasts cultured inside collagen gel (Fig. 2, A and B).

**Induction of MMP-13 Expression in Dermal Fibroblasts by Three-dimensional Collagen Is Mediated by α1β1 and α2β1 Integrins**—Up-regulation of MMP-1 expression in human osteosarcoma cells and dermal fibroblasts in collagen gel, as well as the induction of MMP-1 expression in epidermal keratinocytes cultured on two-dimensional collagen is mediated via collagen receptor α2β1 integrin (36–38), whereas collagen-specific inhibition of type I collagen gene expression in fibroblasts and osteosarcoma cells is mediated via α1β1 integrin (36, 37, 39). To investigate the roles of these two distinct collagen receptors in the collagen-dependent induction of MMP-1 expression, we added blocking antibodies against α1 or α2 integrin subunits to fibroblasts prior to seeding them inside collagen gels. Interestingly, addition of anti-α2 integrin antibody potently reduced MMP-13 mRNA levels (by 52%) of the levels in control cells treated with rat IgG (Fig. 3A). Previous observations show that most antibodies against integrin α subunits do not alone completely inhibit the signaling function of the corresponding receptor (37). Here, addition of the anti-α1 integrin antibody had no marked effect on enhancement of MMP-1 mRNA abundance, whereas marked (80%) down-regulation of pro-α1(I) collagen mRNA levels in fibroblasts in collagen gel was entirely abrogated by anti-α1 integrin antibody, indicating that in the concentration used (1 μg/ml) this antibody entirely blocks the signaling function of α1β1 integrin (Fig. 3A). In comparison, addition of the anti-α2 integrin antibody resulted in dose-dependent inhibition of the collagen-induced MMP-13 expression, the maximal inhibition (90%) noted with the concentration 5 μg/ml (Fig. 3B). Together, these observations show that the induction of MMP-13 expression in fibroblasts by three-dimensional collagen involves activation of signaling via both α1β1 and α2β1 integrins. However, α2β1 integrin appears to play a more important role, as inhibiting signaling via α2β1 integrin results in nearly complete inhibition of collagen-elicted induction of MMP-13 expression by fibroblasts.

To further elucidate the role of the β1 integrins in collagen-dependent induction of fibroblast MMP-13 expression, we used a monoclonal antibody (mAb13) against β1 integrin subunit, shown to activate β1 integrin-mediated signaling in keratinocytes resulting in enhanced 92-kDa gelatinase (MMP-9) expression (40). Interestingly, addition of mAb13 to fibroblasts before seeding them in collagen gel potently (4.7-fold) augmented the induction of MMP-13 mRNA levels, as compared with cells treated with rat IgG (Fig. 3C). In contrast, treatment with mAb13 did not induce MMP-13 expression by fibroblasts plated on plastic, and it had no effect on MMP-1 mRNA levels in fibroblasts cultured on plastic or inside collagen (Fig. 3C). In accordance with a previous study (37), mAb13 also inhibited contraction of collagen gel by fibroblasts (data not shown). These observations show that induction of MMP-13 expression in dermal fibroblasts is mediated via activation of β1 integrin subunit, but only when cells are in contact with three-dimensional collagen.

Triggering the signaling via β1 integrin subunit by a multivalent ligand or by an activating antibody may require integrin clustering, which results in subsequent activation of downstream signaling pathways (41, 42). To examine the role of β1 integrin clustering in the induction of MMP-13 expression, we utilized Fab fragments of mAb13, which bind to β1 integrins and activate their signaling without inducing their clustering. Interestingly, treatment of fibroblasts with Fab13 prior to seeding in collagen gel enhanced MMP-13 mRNA expression even more potently (8.3-fold) than the intact mAb13, indicating that β1 integrin-mediated induction of MMP-13 expression does not require clustering of β1 integrins (Fig. 3C). However, treatment of fibroblasts plated in monolayer on plastic with Fab13 did not induce the expression of MMP-13 mRNA (Fig. 3C). Furthermore, addition of a secondary antibody (anti-rat IgG) together with mAb13 to further increase β1 integrin clustering did not induce the expression of MMP-13 mRNA in fibroblasts in monolayer, indicating that clustering and activation of β1 integrins is not alone sufficient to induce expression of MMP-13 in fibroblasts in monolayer culture (Fig. 3C).

**Induction of MMP-13 Expression in Fibroblasts Cultured in Collagen Gel Is Dependent on Tyrosine Kinase Activity**—One of the initial events following the ECM ligand binding and activation of β1 integrins is the association of the cytoplasmic domain of β1 integrin with focal adhesion kinase (FAK), a cytosolic non-receptor tyrosine kinase, leading to the phosphorylation of FAK (41, 42). Accordingly, tyrosine phosphorylation has been shown to be involved in the signal transduction triggered by contact of fibroblasts to three-dimensional collagen (43) and it has been shown that contraction of collagen gel by fibroblasts requires protein-tyrosine phosphorylation (44). To elucidate the signaling mechanisms mediating the collagen-de-
Collagen-dependent Induction of MMP-13 Is Mediated by p38
p38-dependent induction of MMP-13 expression, we first added two different tyrosine kinase inhibitors to fibroblasts before seeding them in collagen gel. Interestingly, induction of MMP-13 mRNA expression in fibroblasts within collagen gel was entirely inhibited by genistein (50 μM) and herbimycin A (1 μM), indicating requirement for tyrosine kinase activity (Fig. 4). In accordance with previous observations (37) genistein and herbimycin A also potently inhibited the induction of MMP-1 gene expression by three-dimensional collagen (Fig. 4). In contrast, MMP-13 induction was not altered by two specific PKC inhibitors, bisindolylmaleimide (5 μM) and Ro-31-8220 (1 μM), indicating that PKC activity is not required for induction of MMP-13 expression in collagen gel (data not shown).

Activation of ERK1,2, JNK/SAPK, and p38 in Dermal Fibroblasts by Collagen Gel—Activation of tyrosine kinase-dependent signaling by β1 integrin stimulation can result in subsequent activation of Ras, which in turn can activate downstream signaling cascades including MAPKs (42, 45). In this context, we first examined the activation of ERK1,2 by Western blot analysis of cellular proteins from dermal fibroblasts at various time points after seeding them in collagen gel using an antibody against the active, phosphorylated forms of ERK1 and ERK2 (p44 and p42 MAPK, respectively). The levels of activated ERK2 were 2-fold increased 2 h after seeding the cells inside collagen and a marked activation (5-fold) was detected at 3- and 6-h time points (Fig. 5, A and B). The activated form of ERK1 was also detected at 3- and 6-h time points, although at a markedly lower level, as compared with activated ERK2 (Fig. 5, A and B). The activation of ERK2 and ERK1 occurs rapidly after the formation of collagen gel, which takes place approximately 2 h after neutralizing the collagen solution and seeding the cells inside it.

In addition to ERK1,2 pathway, Ras can activate two additional subgroups of MAPKs, i.e. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK, e.g. via small GTPases Rac and Rho (see 42, 45). In the next set of experiments, we examined the activation of JNK and p38 utilizing antibodies specific for phosphorylated JNK or p38, respectively. Contact of dermal fibroblasts to three-dimensional collagen resulted in a marked activation (9-fold) of JNK1 (p46 SAPK), noted 3 h after seeding the cells inside collagen and the activation gradually decreased by 12 and 24 h of incubation (Fig. 5, A and B). A lower abundance activated JNK with molecular mass of 48 kDa was also detected at 3- and 6-h time points, possibly representing an alternatively spliced form of JNK1 (Fig. 5A).

Fig. 3. Induction of MMP-13 expression by fibroblasts in collagen gel is mediated by α1β1 and α2β1 integrins. A and B, human skin fibroblasts were cultured in monolayer (Plastic) or within collagen gel (Collagen), as indicated, for 48 h and total RNA was extracted. Prior to seeding in collagen gel, rat IgG (10 μg/ml), or blocking antibodies against A, α1 (anti-α1; SR-84, 1 μg/ml), or B, α2 (anti-α2; MCA743, 1 or 5 μg/ml) integrin subunits were added to cell suspension. Ctl, no antibodies added. Aliquots of total RNA (7 or 15 μg) were analyzed for expression of MMP-13, MMP-1, pro-α(1) collagen, and GAPDH mRNAs by Northern blot hybridizations. A and B, lower panels, densitometric quantitation of MMP-13 (3), MMP-1 (8), and pro-α(1) collagen (4) mRNA levels in dermal fibroblasts cultured in monolayer on plastic (pl) or in collagen gel (Collagen) treated with rat IgG, anti-α1 (a-a1), or anti-α2 (a-a2) integrin antibodies, as indicated. The values for each mRNA are shown relative to the levels in untreated control cells in collagen gel (ctl)/MMP-13), or on plastic (pl) (1.00) (pro-α(1) collagen) after correction for GAPDH mRNA levels. C, before plating dermal fibroblasts in monolayer (Plastic) or in collagen gel (Collagen), rat IgG (10 μg/ml), activating antibody against β1 integrin subunit (mAb13; 1 μg/ml), or the Fab fragments of mAb13 (Fab13; 1 μg/ml), or a combination of mAb13 and anti-rat IgG (anti-rat) (1 μg/ml each) were added to cell suspension. Cells were cultured for 48 h, and aliquots of total RNA (7 μg) were analyzed for expression of MMP-13, MMP-1, and GAPDH mRNAs by Northern blot hybridizations.

Fig. 4. Induction of fibroblast collagenase-3 (MMP-13) expression in collagen gel is dependent on tyrosine kinase activity. Normal human skin fibroblasts were cultured in monolayer (Plastic) or within type I collagen gel (Collagen) and incubated for 48 h without (Ctl) or with tyrosine kinase inhibitors genistein (Genist., 50 μM), or herbimycin A (Herb. A, 1 μM), added to cell suspension before seeding in collagen gel. Total RNA (10 μg) was analyzed for levels of MMP-13, MMP-1, and GAPDH mRNA by Northern blot hybridizations.

Fig. 5. Activation of ERK1,2, JNK/SAPK, and p38 in dermal fibroblasts in collagen gel. A, human skin fibroblasts were seeded in collagen gel and incubated in DMEM supplemented with 0.5% FCS for different periods of time, as indicated. The levels of activated ERK1 and ERK2 (ERK1-P, ERK2-P), JNK (JNK1-P), and p38 (p38-P) were determined by Western blot analysis using phosphospecific antibodies for the corresponding MAPKs. The migration positions of the molecular size markers (Mr × 10−3) are shown on the left. B, the levels of activated ERK1 and ERK2 (ERK1,2, □), JNK/SAPK (●), and p38 (□) were quantitated by scanning densitometry and are shown relative to the levels at 1 h of incubation (1.00).
A marked (4.5-fold) activation of p38 MAPK was also detected in human skin fibroblasts, as early as 2 h after seeding the cells inside collagen with the maximal activation (7-fold) noted at 3 h, followed by a gradual decrease in the cellular levels of the activated p38 by 12 h (Fig. 5, A and B). The activation of p38 MAPK was quite persistent, as elevated (3.5-fold) levels of activated p38 were still detected after 24 h of incubation (Fig. 5, A and B). Together, these results show that contact of fibroblasts to three-dimensional collagen results in coordinate activation of MAPKs in three distinct subgroups, i.e. ERK1 and ERK2, JNK1, and p38 MAPK, all capable of activating the expression of c-Jun and c-Fos, components of the classical AP-1 dimer (45).

Collagen-dependent Induction of MMP-13 Is Mediated by p38 MAPK

To elucidate the roles of ERK1,2 and p38 MAPKs in mediating the collagen-dependent induction of MMP-13 expression in dermal fibroblasts, we used selective inhibitors and activators of these MAPKs. First, to block the activity of ERK1,2 kinases MEK1,2, or with SB 203580 (10 μM), a selective inhibitor of p38 MAPK, both added to cell suspension prior to seeding in collagen gel. Total RNA was extracted and 20-μg aliquots analyzed for levels of MMP-13, MMP-1, and GAPDH mRNA by Northern blot hybridizations. B and C, human skin fibroblasts were cultured in monolayer (Plastic) or within type I collagen gel (Collagen), as indicated, and incubated for 48 h with or without PD 98059 (40 μM), a specific inhibitor of ERK1,2 kinases MEK1,2, or with C2-ceramide (C2-cer; 50 μM) for another 24 h (C). Total RNA was extracted and 10-μg aliquots analyzed for expression of MMP-13, MMP-1, and GAPDH mRNAs by Northern blot hybridizations. A-C, lower panels, densitometric quantitations of MMP-13 (u) and MMP-1 (f) mRNA levels corrected for GAPDH mRNA levels are shown relative to the levels of untreated control cells in collagen gel (1.00).
Collagen-dependent Induction of MMP-13 Is Mediated by p38

ERK1,2 pathway (Raf/MEK1,2/ERK1,2), we added PD 98059, a specific inhibitor of the activation of MEK1 and MEK2 (46, 47), to fibroblasts immediately before seeding them inside collagen. Surprisingly, PD 98059 (40 μM) potently (7.3-fold) augmented the induction of MMP-13 mRNA as compared with untreated fibroblasts in collagen gel (Fig. 6A). In contrast, addition of selective p38 inhibitor SB 203580 (10 μM) to fibroblasts before seeding them inside collagen entirely abrogated the induction of MMP-13 mRNA expression by collagen (Fig. 6A). In the same cells, collagen-dependent induction of MMP-1 mRNA levels was slightly (by 40%) reduced by treatment of cells with PD 98059 and somewhat more potently (by 65%) by SB 203580 (Fig. 6A). These results show that the activity of p38 is essential for MMP-13 expression in collagen-embedded dermal fibroblasts, whereas induction of MMP-1 is only partially inhibited by blocking p38 or ERK1,2 pathways.

Augmentation of MMP-13 expression by dermal fibroblasts inside collagen obtained by blocking the ERK1,2 pathway by PD 98059 (Fig. 6A) provides evidence that ERK1,2 MAPKs play an inhibitory role in MMP-13 expression. To further examine this possibility, we cultured dermal fibroblasts inside collagen gel for 24 h to allow induction of MMP-13 expression and subsequently treated them for 24 h with phorbol ester TPA, which selectively activates ERK1,2, but not p38 in dermal fibroblasts (50) (data not shown). As shown in Fig. 6B, treatment with TPA markedly (76%) reduced the levels of MMP-13 mRNAs in fibroblasts, providing further evidence for the inhibitory role of ERK1,2 pathway in the regulation of MMP-13 expression. In comparison, the abundance of MMP-1 mRNA in fibroblasts in collagen gel was not markedly altered by treatment with TPA, indicating differential regulation of MMP-13 and MMP-1 expression by ERK1,2 MAPKs (Fig. 6B).

To further investigate the role of distinct MAPK pathways in the regulation of MMP-13 expression, dermal fibroblasts cultured for 24 h in collagen gel were incubated for another 24 h in the presence of C2-ceramide (50 μM), which coordinately activates ERK1,2, JNK/SAPK, and p38 in these cells (35). Interestingly, treatment with C2-ceramide further enhanced (2-fold) the levels of MMP-13 mRNAs in fibroblasts cultured in collagen gel, whereas MMP-1 mRNAs were only slightly (1.2-fold) increased (Fig. 6C). In accordance with our previous observations (35) C2-ceramide potently enhanced the levels of MMP-1 mRNA in dermal fibroblasts cultured on plastic, but did not induce their MMP-13 mRNA expression (Fig. 6C).

DISCUSSION

Culturing fibroblasts in collagen gel is a well established model for studying the formation of ECM in vivo under conditions resembling three-dimensional collagen matrix surrounding fibroblasts in vitro (see 51). The floating, contracting collagen gel is suggested to mimic the cellular environment in dermal layer of normal skin or scar, and the anchored gel simulates granulation tissue (51). In the present study we show for the first time that the expression of collagenase-3 (MMP-13) in normal human skin fibroblasts is specifically induced by three-dimensional type I collagen via both α1β1 and α2β1 integrins and that this requires tyrosine kinase activity. We also show that contact of fibroblasts to three-dimensional collagen results in coordinate activation of three distinct subgroups of MAPKs, i.e. ERK1,2, JNK/SAPK, and p38, and that collagen-dependent induction of MMP-13 expression requires p38 MAPK activity and is in turn potently augmented by blocking the ERK1,2 MAPK pathway (Fig. 7).

Contact of human skin fibroblasts with three-dimensional collagenous matrix has profound effects on their morphology, growth factor response, and matrix gene expression (51). In fibroblasts, collagen receptors α1β1 and α2β1 integrins play an important role in regulating ECM remodeling, as shown by observations that α1β1 integrin mediates signals responsible for down-regulation of type I collagen gene expression and α2β1 integrin activates signaling pathways mediating induction of MMP-1 expression (36, 37, 39). The results of the present study show that collagen-dependent induction of MMP-13 expression in dermal fibroblasts is partially prevented by blocking antibody against α1 integrin subunit and nearly completely abrogated by anti-α2 integrin antibody, indicating that collagen-dependent activation of MMP-13 expression involves activation of signaling pathways via both α1β1 and α2β1 integrins. Thus, in contrast to regulation of MMP-1 and type I collagen expression mediated by specific integrin α subunits, our results show that common β1 subunit is more important in regulation of MMP-13 expression. This notion is further supported by our finding that induction of fibroblast MMP-13 expression by three-dimensional collagen is potently augmented by an activating antibody against β1 integrin (mAb13) and by Fab fragments of the same antibody (Fab13), indicating that collagen receptor-mediated induction of MMP-13 expression involves direct activation of signaling via β1 integrin subunit and does not require clustering of β1 integrins. However, induction of MMP-13 expression by both collagen and anti-β1 integrin antibody was dependent on the presence of three-dimensional collagen matrix, indicating that activation of β1 integrin subunit alone is not sufficient for triggering signaling pathways mediating MMP-13 induction. It is apparent that ligand binding sites of α1β1 and α2β1 integrins are fully occupied when fibroblasts are in three-dimensional type I collagen recognized by specific I domains of α1 and α2 integrin subunits (52, 53). In contrast, mAb13 binds to the β1 integrin subunit and may cause conformation changes resulting in further stimulation of signaling via β1 integrin.

Previous observations have shown that seeding fibroblasts in collagen gel results in tyrosine phosphorylation of a 125-kDa protein corresponding to FAK (43) and that down-regulation of TGF-β receptor by collagen contact is dependent on the activity

![Fig. 7. Signaling pathways mediating regulation of MMP-13 expression in dermal fibroblasts by three-dimensional type I collagen.](http://www.jbc.org/)

**Fig. 7.** Signaling pathways mediating regulation of MMP-13 expression in dermal fibroblasts by three-dimensional type I collagen. Contact of dermal fibroblasts to type I collagen via α1β1 and α2β1 integrin collagen receptors results in activation of ERK1,2 and p38 MAPKs. Induction of MMP-13 expression by three-dimensional collagen is inhibited by tyrosine kinase inhibitors (genistein, herbimycin A). Activity of p38 is also required for MMP-13 expression, whereas blocking the ERK1,2 pathway augments induction of MMP-13 expression. In addition, activation of ERK1,2 cascade by phorbol ester (TPA) inhibits MMP-13 expression indicating that ERK1,2 MAPKs mediate inhibitory signals on MMP-13 expression. MEK, MAP/ERK kinase; MKK, MAPK kinase; MAPKK, MAPKK kinase; SB 203580, a specific inhibitor of p38 MAPK; PD 98059, a specific inhibitor of MEK1,2.
fibroblast MMP-13 expression in dermal fibroblasts (Fig. 7). 

Our results show that contact of fibroblasts to three-dimensional collagen results in activation of an atypical PKC, PKC-γ, the activity of which is required for induction of MMP-1 in this model (56). However, in our study, induction of MMP-13 expression in fibroblasts in collagen gel was not altered by specific PKC inhibitors bisindolylmaleimide and Ro-31-8220, indicating that PKC activity is not essential for the activation of MMP-13 expression.

The expression of MMP-13 in fibroblasts in monolayer culture is not inducible by TNF-α, IL-1β, TPA, or C2-ceramide, all of which are capable of stimulating expression of MMP-1 under similar conditions (see Refs. 1 and 2). Furthermore, once turned on by contact to three-dimensional collagen matrix, the expression of MMP-13 remains relatively unresponsive to modulation, as it is not stimulated by TNF-α and IL-1β, which enhance the expression of MMP-1 and MMP-3 by dermal fibroblasts in collagen gel. In contrast, the expression of MMP-13 in fibroblasts within collagen gel was somewhat inhibited by TGF-β. The different modulation of fibroblast MMP-13 and MMP-1 expression by TNF-α and IL-1β may be one explanation for distinct localization of fibroblasts expressing MMP-13 and MMP-1 in chronic dermal ulcers (13). Three-dimensional collagen contact also induces fibroblast expression of stromelysin-1 (MMP-3), 72-kDa gelatinase (MMP-2), and MT1-MMP (MMP-14), all capable of activating latent MMP-13 (5, 7). It is therefore not surprising that all MMP-13 detected in the culture media of dermal fibroblasts in collagen gel is in active form. Thus, contact to three-dimensional collagen matrix most likely plays an important role in stimulating the proteolytic capacity of fibroblasts in ECM remodeling during normal wound repair and in excessive degradation of ECM in chronic ulcers (13, 14, 25). In analogy, the expression of MMP-2 and MT1-MMP in endothelial cells is also induced by three-dimensional collagen and this may play an important role in angiogenesis during wound repair (58). Similarly, we have recently observed that expression of MMP-13 by periodontal ligament cells is induced by contact to two-dimensional collagen, which may be an important factor in inducing MMP-13 expression by these cells during chronic inflammation of oral mucosa in vivo (15).

The expression of MMP-13 in vivo and in cultured cells is clearly more restricted than the expression of most other MMPs (see Refs. 1, 2, and 22). In addition to chronic dermal and intestinal ulcers, and periodontal inflammation (13–15), the only situations in which the expression of MMP-13 has been detected in fibroblasts in vivo are SCCs of the head and neck, and breast carcinomas (17, 18). The expression of MMP-13 has been recently documented in monolayer cultures of human immortalized fibroblasts of embryonal origin (KMST cells), in which its expression is stimulated by IL-1α and TGF-β (17, 59). Together these observations are in accordance with a recent study, in which expression of MMP-13 mRNA was detected in several transformed human fibroblast lines, whereas the expression in normal human skin fibroblasts was low (22). As it has been shown that stromal fibroblasts derived from SCCs can also modulate the phenotype of tumor cells (60), it is possible that the stromal fibroblasts in SCCs and breast carcinomas expressing MMP-13 represent a dedifferentiated fibroblast phenotype, in which MMP-13 expression is regulated differently as compared with normal human skin fibroblasts. This notion is supported by our observations that the expression of MMP-13 in normal dermal fibroblasts is inhibited by TGF-β and not altered by IL-1α, whereas both clearly enhance MMP-13 expression in KMST fibroblasts (17, 59).

The ability to degrade type I collagen is essential for migration of epidermal keratinocytes (38). However, human epidermal keratinocytes do not express MMP-13 under any conditions in culture or in vivo (13, 21), indicating that MMP-1 is the collagenase of choice for restricted cleavage of type I collagen required for keratinocyte migration. In contrast, the ability of MMP-13 to degrade several ECM components is obviously beneficial for invading transformed keratinocytes, e.g. SCC cells (18). It is possible that degradation of ECM components in chronic dermal ulcers by MMP-13 alters the cell-matrix interactions of both epidermal keratinocytes and fibroblasts and may affect their migration capacity (61). In this context, identification of p38 MAPK as an essential signaling pathway in the induction of MMP-13 in normal human skin fibroblasts suggests p38 MAPK as a target for selective inhibition of degradation of collagenous ECM in chronic dermal ulcers.

Acknowledgements—We gratefully acknowledge the expert technical assistance of Ulla Paasio and Marita Potila. We also thank Drs. E. Bauer, M. Kurkinen, G. Goldberg, and P. Fort for plasmids.

REFERENCES
Induction of Collagenase-3 (MMP-13) Expression in Human Skin Fibroblasts by Three-dimensional Collagen Is Mediated by p38 Mitogen-activated Protein Kinase
Laura Ravanti, Jyrki Heino, Carlos López-Otín and Veli-Matti Kähäri

doi: 10.1074/jbc.274.4.2446

Access the most updated version of this article at http://www.jbc.org/content/274/4/2446

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 61 references, 26 of which can be accessed free at http://www.jbc.org/content/274/4/2446.full.html#ref-list-1