Transforming Growth Factor-β Induces Collagenase-3 Expression by Human Gingival Fibroblasts via p38 Mitogen-activated Protein Kinase*

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Human collagenase-3 (matrix metalloproteinase 13 (MMP-13)) is characterized by exceptionally wide substrate specificity and restricted tissue specific expression. Human skin fibroblasts in culture express MMP-13 only when they are in three-dimensional collagen (Ravanti, L., Heino, J., López-Otin, C., and Kähäri. V.-M. (1999) J. Biol. Chem. 274, 2446–2455). Here we show that MMP-13 is expressed by fibroblasts during normal human gingival wound repair. Expression of MMP-13 by human gingival fibroblasts cultured in monolayer or in collagen gel was induced by transforming growth factor-β1 (TGF-β1). Treatment of gingival fibroblasts with TGF-β1 activated two distinct mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase 1/2 (ERK1/2) in 15 min and p38 MAPK in 1 and 2 h. Induction of MMP-13 expression by TGF-β1 was blocked by SB203580, a specific inhibitor of p38 MAPK, but not by PD98059, a selective inhibitor of ERK1/2 activation. Adenovirus-mediated expression of dominant negative p38α and c-Jun potently inhibited induction of MMP-13 expression in gingival fibroblasts by TGF-β1. Infection of gingival fibroblasts with adenovirus for constitutively active MEK1 resulted in activation of ERK1/2 and JNK1 and up-regulation of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) production, but did not induce MMP-13 expression. In addition, activation of p38 MAPK by constitutively active MKK6b or MKK3b was insufficient to induce MMP-13 expression. These results show that TGF-β1-elicited induction of MMP-13 expression by gingival fibroblasts is dependent on the activity of p38 MAPK and the presence of functional AP-1 dimers. These observations demonstrate a fundamental difference in the regulation of collagenolytic capacity between gingival and dermal fibroblasts and suggest a role for MMP-13 in rapid turnover of collagenous matrix during repair of gingival wounds, which heal with minimal scarring.

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‡‡ Controlled degradation of extracellular matrix (ECM) is essential in physiological situations involving connective tissue remodeling, such as tissue morphogenesis, repair, and angiogenesis. On the other hand, excessive breakdown of connective tissue components plays an important role in destruction of functional tissue architecture, e.g. in rheumatoid arthritis, osteoarthritis, atherosclerosis, periodontitis, autoimmune blistering disorders of skin, and dermal photoaging as well as in invasion and metastasis of tumor cells (see Refs. 1–3). Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases collectively capable of degrading essentially all ECM components, and they are implicated in ECM remodeling in the physiologic and pathologic situations mentioned above. At present, 18 human members of the MMP family have been characterized, and most of them can be divided into subgroups of collagenses, gelatinases, stromelysins, and membrane-type MMPs based on their substrate specificity and structure (1–3).

Collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13) are the principal neutral proteinases capable of degrading native fibrillar collagens in the extracellular space. They all cleave type I, II, and III collagens at a specific site, generating ¼ N-terminal and ¼ C-terminal fragments, which denature in physiological temperature and are further degraded by other MMPs, e.g. gelatinases (see Refs. 1–3). MMP-13 also cleaves type I collagen at N-terminal nonhelical telopeptide (4). MMP-1 cleaves type III collagen and MMP-8 type I collagen most effectively (1–3). MMP-13, in turn, cleaves fibrillar collagens with preference to type II collagen over type I and III collagens and displays 40-fold stronger gelatinase activity than MMP-1 and MMP-8 (5–7). In addition, MMP-13 degrades type IV, X, and XIV collagens, tenascin, fibronectin, and aggrecan core protein (8–9). Apparently due to its exceptionally wide substrate specificity, the physiologic expression of MMP-13 is limited to situations in which rapid and effective remodeling of collagenous ECM takes place, i.e. fetal bone development and adult bone remodeling (10, 11). On the other hand, MMP-13 is expressed at sites of excessive degradation of

The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL, interleukin; EGF, epidermal growth factor; PDGF, platelet derived growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MKK, MAPK/ERK kinase; MOI, multiplicity of infection; pfu, plaque-forming unit; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; kb, kilobase pair(s); MKK, MAPK kinase.
collagenous ECM in osteoarthritic cartilage (7, 12), rheumatoid synovium (11, 13), chronic cutaneous ulcers (14), intestinal ulcerations (15), and periodontitis (16) as well as in malignant tumors (i.e. breast carcinomas (5, 17, 18), squamous cell carcinomas of the head and neck (19, 20) and vulva (21), cutaneous basal cell carcinomas (22), malignant melanomas (23), and chondrosarcomas (24)).

Controlled degradation of collagenous ECM plays an important role in reepithelialization, angiogenesis, and reorganization of granulation tissue ECM during wound repair (see Ref. 25). In human dermal wounds, MMP-1 is expressed by keratinocytes at the migrating front of epidermis (26), and it has been shown that cleavage of native type I collagen is required for migration of epidermal keratinocytes on it (27). In contrast, MMP-13 is not expressed by human epidermal keratinocytes in acute or chronic cutaneous wounds (14) or in culture (28). MMP-13 expression is detected in fibroblasts in chronic dermal ulcers, but not during acute cutaneous wound repair (14), in contrast to MMP-1, which is expressed by dermal fibroblasts in both acute and chronic wounds (14, 29). In addition, normal human skin fibroblasts express MMP-13 mRNAs when cultured in three-dimensional collagen gel but not when grown in monolayer (14, 30). Taken together, these observations provide evidence for a distinct role and differential regulation of MMP-13 and MMP-1 in human cutaneous wound repair.

In the present study, we show that MMP-13 is expressed by fibroblasts in normally healing human gingival wounds. In addition, the expression of MMP-13 by human gingival fibroblasts in monolayer culture is induced by TGF-β1 via p38 mitogen-activated protein kinase (MAPK) signaling cascade and AP-1 complex. These results demonstrate a fundamental difference in the regulation of MMP-13 expression between human gingival and dermal fibroblasts and suggest that MMP-13 plays an important role in rapid remodeling of collagenous ECM during repair of human gingival wounds, which generally heal with minimal scarring.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human recombinant tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), and platelet-derived growth factor-AA (PDGF-AA) were obtained from Sigma. Human recombinant epidermal growth factor (EGF), SB203580, and PD98059 were obtained from Calbiochem. Human recombinant interleukin-1β (IL-1β) was obtained from Roche Molecular Biochemicals (Mannheim, Germany).

**Gingival Wound Specimens**—Gingival wounds were generated for healthy male volunteers by creating V-shaped full thickness wounds (about 1.5 cm long, 2 mm wide) to healthy keratinized palatal gingiva of three human volunteers, as described previously (31). After 3, 7, 14, and 28 days, the wound samples were obtained by punch biopsy, the tissue was rinsed briefly with physiological saline, embedded in Tissue-Tek® and snap frozen in liquid nitrogen, and stored at −80 °C for 30 min. The samples were prehydrated for 2 h and subsequently hybridized for 20 h with cDNAs labeled with [α-32P]dCTP using random priming. For hybridizations, MMP-13 cDNA fragments covering the coding region and part of the 3′-untranslated region of the human MMP-13 cDNA (altogether 1.9 kb) were used (28). In addition, a 2.0-kb human collagenase-1 (MMP-1) cDNA (33), a 1.5-kb human stromelysin-1 (MMP-3) cDNA (34), a 0.7-kb human pro-α(1)I collagen cDNA (35), and a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA (36) were used. The 32P–cDNA/mRNA hybrids were visualized with autoradiography, quantified with densitometry, and corrected for the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA for each sample.

**Induction of MMP-13 Expression by TGF-β Is Mediated by p38**

Human gingival fibroblasts were maintained in serum-free DMEM for 18 h, after which TGF-β1 (5 ng/ml) was added, and the incubations were continued for 24 h. Equal aliquots of the conditioned media were analyzed by Western blotting, as described previously (30, 37) using a mouse monoclonal antibody against human MMP-13 (Calbiochem and Oncogene Research Products, Cambridge, MA) in a 1:100 dilution, polyclonal rabbit antiserum against human MMP-1 (kindly provided by Dr. Henning Birkedal-Hansen, NIDCR, National Institutes of Health, Bethesda, MD) in a 1:5000 dilution, and antibodies for ERK1/2, JNK, and p38 in a 1:1000 dilution, using ECL (Amersham Pharmacia Biotech). Western blotting was performed as described previously (31), with phosphospecific antibodies for ERK1/2, JNK, and p38 in a 1:1000 dilution, using ECL (Amersham Pharmacia Biotech). The levels of immunoreactive MMP-13, MMP-1, MMP-3, and TIMP-1 were quantitated by densitometric scanning of the x-ray films.

**Assay of MAPK Activation**—The activation of ERK1/2, JNK, 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 treated with TGF-β1 in DMEM with 0.5% FCS at various time points and lysed in 100 μl of Laemmli sample buffer. The samples were then sonicated, fractionated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membrane (Amersham Pharmacia Biotech). Western blotting was performed as described previously (31), with phosphospecific antibodies for ERK1/2, JNK, and p38 in a 1:1000 dilution, using ECL (Amersham Pharmacia Biotech). As loading controls, Western blots were also performed using antibodies against total ERK1/2, and p38 (both from New England Biolabs, Beverly, MA) in a 1:1000 dilution, and JNK1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in a 0.5 μg/ml dilution.

**Infection of Fibroblasts with Recombinant Adenoviruses**—Recombinant replication-deficient adenovirus RAdΔ2Z (RAd35) (38), which contains the Escherichia coli β-galactosidase (lacZ) gene under the control of the cytomegalovirus IE promoter and the empty adenovirus RAdΔ06 (38) were kindly provided by Dr. Gavin W. G. Wilkinson (University of Colorado, Denver, CO). Recombinant adenovirus for dominant negative Rac1 (RAdΔN1rac1) (39) was kindly provided by Dr. Toren Finkel (NHLBI, National Institutes of Health, Bethesda, MD), and adenovirus for dominant negative c-Jun (RAdΔTAM67) (40) was kindly provided by Dr. Michael Birrer (NCI, National Institutes of Health, Bethesda, MD). Construction and characterization of replication-deficient adenoviruses containing lacZ or c-Jun were performed by Dr. Henning Birkedal Hansen (NHLBI, National Institutes of Health, Bethesda, MD), Adenovirus for dominant negative c-Jun (RAdΔTAM67) (40) was kindly provided by Dr. Michael Birrer (NCI, National Institutes of Health, Bethesda, MD). Construction and characterization of replication-deficient adenoviruses containing lacZ or c-Jun were performed by Dr. Henning Birkedal Hansen (NHLBI, National Institutes of Health, Bethesda, MD), and adenoviruses for dominant negative c-Jun (RAdΔTAM67) (40) were kindly provided by Dr. Michael Birrer (NCI, National Institutes of Health, Bethesda, MD). Construction and characterization of replication-deficient adenoviruses containing lacZ or c-Jun were performed by Dr. Henning Birkedal Hansen (NHLBI, National Institutes of Health, Bethesda, MD).
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RESULTS

MMP-13 Is Expressed by Fibroblasts in Normal Human Gingival Wounds—We have previously noted that MMP-13 is expressed by fibroblasts in chronic human cutaneous ulcers in vivo but not in normally healing dermal wounds (14). In addition, MMP-13 expression is detected in fibroblasts in intestinal ulcerations (15) and in chronically inflamed periodontal tissue (16). To elucidate the role of MMP-13 in physiological turnover of collagenous ECM, we examined its expression in normally healing human gingival wounds of different ages (3 days to 4 weeks) by immunostaining. Interestingly, MMP-13-positive fibroblasts were detected adjacent to the fibrin clot in 3-day-old wounds and in the vicinity of and within the newly formed granulation tissue at 7 days and also in 2- and 4-week-old wounds (Fig. 1, and data not shown). In contrast, epithelial cells remained negative for MMP-13 in all samples studied (Fig. 1 and data not shown). Fibroblasts in the same areas also stained positive for MMP-1 in all wound samples (Fig. 1), and expression of MMP-1 was also detected in migrating keratinocytes (not shown). These observations demonstrate a remarkable difference in MMP-13 expression between acute gingival and cutaneous (14) wounds, suggesting that the regulation of MMP-13 expression in human dermal and gingival fibroblasts is fundamentally different.

Expression of MMP-13 in Gingival Fibroblasts Is Induced by TGF-β—We have recently noted that human skin fibroblasts in culture express MMP-13 only when they are embedded in collagen gel, and once turned on, the expression remains unre sponsive to modulation by IL-1β and TNF-α and is downregulated by TGF-β (30). To examine the regulation of MMP-13 expression in human gingival fibroblasts, the cells were first cultured for 24 h in monolayer or inside collagen gel and subsequently treated with IL-1β, TNF-α, and TGF-β1 for an additional 24 h, and the expression of MMP-13 mRNAs was determined by Northern blot hybridization. Interestingly, treatment of gingival fibroblasts in monolayer with TGF-β1 (5 ng/ml) markedly (12.1-fold) enhanced MMP-13 mRNA levels, as compared with untreated cells, which expressed low levels of MMP-13 mRNAs (Fig. 2A). Treatment of gingival fibroblasts with TNF-α (20 ng/ml) also enhanced (4.1-fold) MMP-13 mRNA abundance, whereas IL-1β (5 units/ml) had no effect on MMP-13 mRNA levels (Fig. 2A). In contrast, MMP-1 mRNA abundance in cells cultured in monolayer was not markedly altered by TGF-β1, but it was up-regulated by TNF-α and IL-1β (9.8- and 3.5-fold, respectively) (Fig. 2A).

Culturing gingival fibroblasts in collagen gel for 48 h also somewhat (2.4-fold) up-regulated MMP-13 mRNA levels (Fig. 2A). Interestingly, the abundance of MMP-13 mRNAs was also potently (11.7-fold) up-regulated by TGF-β1 in fibroblasts within collagen gel as compared with untreated cells in collagen gels (Fig. 2A). Treatment of cells in collagen gel with TNF-α also enhanced MMP-13 mRNA levels, although less potently than TGF-β1 (3.2-fold), whereas IL-1β had no effect on MMP-13 mRNA levels in cells in collagen gels (Fig. 2A). Interestingly, the expression of stromelysin-1 (MMP-3) mRNA was also enhanced by TGF-β1 (12.0- and 22.3-fold) both in cells cultured in monolayer and cells cultured in collagen gel, respectively, as compared with corresponding untreated cultures (Fig. 2A). Treatment with TGF-β1 up-regulated pro-α1(I) collagen mRNA abundance 8.8-fold in cells in monolayer and 3.5-fold in cells within collagen gel, as compared with the respective untreated cultures (Fig. 2A).

We also examined the regulation of gingival fibroblast MMP-13 expression by two mitogenic growth factors, EGF and PDGF-AA, known to induce MMP-1 expression by fibroblasts (see Refs. 1–3). Treatment of cells with EGF (25 ng/ml) and PDGF-AA (20 ng/ml) for 24 h had no effect on MMP-13 mRNA levels alone, although MMP-1 mRNA abundance was potently up-regulated by EGF (18.6-fold) and PDGF-AA (11.2-fold) (Fig. 2B). However, exposure of cells to EGF in combination with TGF-β1 slightly (1.5-fold) augmented the up-regulation of MMP-13 mRNAs, whereas combination of PDGF-AA and TGF-β1 did not alter MMP-13 expression, as compared with TGF-β1 alone (Fig. 2B). In accordance with previous observations (44, 45), TGF-β1 markedly inhibited enhancement of MMP-1 mRNA abundance by EGF and PDGF-AA (Fig. 2B). Pro-α1(I) collagen mRNA levels were potently up-regulated by TGF-β1 (45.5-fold), and treatment of cells with EGF and TGF-β1 resulted in less potent enhancement in pro-α1(I) collagen mRNA levels (24.2-fold) than with TGF-β1 alone (Fig. 2B).

TGF-β Activates ERK1/2 and p38 MAPK in Gingival Fibroblasts—We have previously shown that contact with three-dimensional collagen activates three distinct MAPKs: ERK1/2, JNK, and p38 in human skin fibroblasts (30). Of these, p38 MAPK activity is required for MMP-13 expression in human skin fibroblasts in collagen gel, whereas activation of the ERK1/2 cascade inhibits MMP-13 expression (30). To study the role of the three MAPK pathways in the regulation of gingival fibroblast MMP-13 expression, we first determined MAPK activation by Western blot analysis of cellular proteins at various time points of exposure to TGF-β1 using antibodies against the active, phosphorylated forms of these MAPKs. As shown in Fig. 3, A and B, the levels of activated ERK1/2 were rapidly and.

Fig. 1. Expression of collagenase-3 (MMP-13) by fibroblasts in acute human gingival wounds. Human gingival wound samples obtained at 3 days (3 d), 7 days (7 d), and 4 weeks (4 wk) were immunostained with anti-MMP-13 and anti-MMP-1 antibodies, as indicated. MMP-13- and MMP-1-positive fibroblasts are indicated by arrowsheads. e, mucosal epithelium; c, fibrin clot. Magnification, 255× (MMP-13 for 3 days) and 408× (MMP-13 for 7 days/2 weeks and MMP-1 for 4 weeks).
transiently increased (5.8-fold) at 15 min of incubation by TGF-β1. In addition, the levels of activated p38 MAPK were increased, maximally 3.1-fold at 1 and 2 h of incubation with TGF-β1 (Fig. 3, A and B). In contrast, treatment with TGF-β1 did not activate JNK in gingival fibroblasts (Fig. 3 A). The total cellular levels of ERK1/2, JNK1, or p38 in gingival fibroblasts were not altered by TGF-β1 (Fig. 3 A).

Induction of MMP-13 Expression in Gingival Fibroblasts by TGF-β1 Requires p38 Activity—To elucidate the specific roles of ERK1/2 and p38 MAPK in mediating the induction of MMP-13 expression by TGF-β1 in gingival fibroblasts, we first used selective chemical inhibitors for these MAPKs. Blocking the ERK1/2 pathway (Raf-MEK1/2-ERK1/2) by PD98059 (30 μM), a specific inhibitor of MEK1 and MEK2 (46, 47), added to fibroblasts 1 h prior to TGF-β1, did not markedly alter the induction of MMP-13 mRNA levels by TGF-β1 (Fig. 4 A). In contrast, the addition of selective p38 inhibitor SB203580 (10 μM) (48, 49) to fibroblasts 1 h before TGF-β1 entirely abrogated the induction of MMP-13 mRNAs by TGF-β1 (Fig. 4 A). The abundance of MMP-1 mRNA was reduced (by 58%) by TGF-β1, and this effect was not markedly altered by PD98059 or SB203580 (Fig. 4 A).

Interestingly, up-regulation of MMP-3 mRNA levels (5.3-fold) by TGF-β1 was also entirely abrogated by SB203580 and also in part (by 47%) inhibited by PD98059 (Fig. 4 A). In contrast,
enhancement of pro-α1(Ⅰ) collagen mRNA abundance by TGF-β1 (15.6-fold) was not altered by SB203580, showing that SB203580 does not serve as general inhibitor of TGF-β signaling (Fig. 4A). Enhancement of pro-α1(Ⅰ) collagen mRNA abundance by TGF-β1 was augmented (2.7-fold) by co-treatment of cells with PD98059 (Fig. 4A).

We also determined the levels of pro-MMP-13 in the conditioned media of human gingival fibroblasts by Western blot analysis. As shown in Fig. 4B, treatment of gingival fibroblasts with TGF-β1 stimulated the production of pro-MMP-13 by 3.6-fold, and this enhancement was totally blocked by SB203580. In contrast, PD98059 had no marked effect on TGF-β-elicited stimulation of MMP-13 expression in human gingival fibroblasts (Fig. 4B). In the same fibroblast cultures, MMP-3 produc-

tion was also enhanced 4.1-fold by TGF-β1, and this enhancement was totally blocked by SB203580 and in part (by 54%) inhibited by PD98059 (Fig. 4B). TIMP-1 production by human gingival fibroblasts remained unaltered by exposure of cells to TGF-β1 alone, but co-treatment of cells with TGF-β1 and SB203580 resulted in 2.3-fold induction in TIMP-1 production (Fig. 4B). Together, these observations show that the TGF-β-elicited induction of MMP-13 and also MMP-3 expression in gingival fibroblasts is dependent on the activity of p38 MAPK.

**Induction of MMP-13 Expression by TGF-β in Gingival Fibroblasts Is Inhibited by Dominant Negative p38α and c-Jun**—To further elucidate the signaling pathways mediating the induction of MMP-13 gene expression by TGF-β1, we utilized recombinant replication-deficient adenoviruses coding for dominant negative forms of small GTPase Rac1 (RAdN17rac1), dominant negative p38α (RAdp38AF), and dominant negative c-Jun (RAdTAM67). First, we determined the transduction efficiency of human gingival fibroblasts with recombinant replication-deficient adenovirus RAdlacZ, which contains the E. coli β-galactosidase (lacZ) gene under the control of the cytomegalovirus IE promoter. The cells were infected at different MOI values, fixed, and stained for β-galactosidase activity, indicated as blue color. Bar, 47 μm. Human gingival fibroblasts were infected at a MOI of 500 pfu/cell with control adenovirus (RAd66) and with adenoviruses for dominant negative Rac1 (RAdN17rac1), dominant negative p38α (RAdp38AF), and dominant negative c-Jun (RAdTAM67) and incubated for 5 h in DMEM with 1% FCS. Thereafter, medium was replaced with DMEM without FCS, TGF-β1 (5 ng/ml) was added, and incubations were continued for 24 h. The levels of pro-MMP-13 and TIMP-1 in the conditioned media were determined by Western blot analysis.

**Fig. 4.** Induction of collagenase-3 (MMP-13) expression in gingival fibroblasts by TGF-β is dependent on the activity of p38 MAPK. A and B, normal human gingival fibroblasts were incubated with TGF-β1 (5 ng/ml) for 24 h in DMEM with 1% FCS. PD98059 (30 μM), a specific inhibitor of ERK1/2 kinases MEK1/2 or SB203580 (10 μM), a selective inhibitor of p38 MAPK, was added to the cultures indicated 1 h prior to TGF-β1. A, aliquots of total RNA (20 μg) were analyzed for MMP-13, MMP-1, MMP-3, and pro-α1(Ⅰ) collagen mRNA levels by Northern blot hybridizations. 28 S rRNA was visualized by ethidium bromide staining. B, the levels of pro-MMP-13, pro-MMP-1, pro-MMP-3, and TIMP-1 in conditioned media of gingival fibroblasts, treated as in A, were determined by Western blot analysis using specific antibodies.

**Fig. 5.** Induction of collagenase-3 (MMP-13) expression by TGF-β in gingival fibroblasts is inhibited by dominant negative p38α and c-Jun. A, Human gingival fibroblasts were infected with replication-deficient recombinant adenovirus RAdlacZ coding for E. coli β-galactosidase at a MOI of 500 pfu/cell and incubated for 18 h in DMEM with 1% FCS. The cells were then fixed and stained for β-galactosidase activity, indicated as blue color. Bar, 47 μm. B, Human gingival fibroblasts were infected at a MOI of 500 pfu/cell with control adenovirus (RAd66) and with adenoviruses for dominant negative Rac1 (RAdN17rac1), dominant negative p38α (RAdp38AF), and dominant negative c-Jun (RAdTAM67) and incubated for 5 h in DMEM with 1% FCS.
Induction of MMP-13 Expression by TGF-β Is Mediated by p38

Up-regulatory effect of TGF-β1 on pro-MMP-13 production was somewhat more potent in cells infected with the empty control virus RAd66 (Fig. 5B). Since activation of JNK and p38 has been shown to involve activation of small GTPases Rac and Rho (50), we infected fibroblasts with adenovirus for dominant negative Rac1 (RAdN17rac1), which inhibited induction of pro-MMP-13 production by TGF-β1 by 60%, as compared with RAd66-infected cells (Fig. 5B). In accordance with the observations with p38 inhibitor SB203580, adenovirus-mediated expression of dominant negative p38C (RAdp38CAF) reduced induction of pro-MMP-13 production by TGF-β1 (by 81%), as compared with RAd66 infected cells (Fig. 5B), corroborating the role of p38 MAPK in mediating TGF-β1-elicited induction of MMP-13 expression. In parallel, infection of gingival fibroblasts with adenovirus for dominant negative c-Jun (RAdTAM67) also potently (by 94%) reduced TGF-β1-elicited induction of MMP-13 production, indicating that functional AP-1 dimers are required for activation of MMP-13 gene expression by TGF-β1 in gingival fibroblasts (Fig. 5B).

In production, TIMP-1 was not markedly altered by TGF-β1 in uninfected cells or in cells infected with RAd66 (Fig. 5B). However, infection of fibroblasts with RAdN17rac1 or RAdp38CAF decreased basal TIMP-1 production by 79 and 77%, respectively, and this down-regulation was inhibited by TGF-β1 (Fig. 5B). In addition, infection of cells with RAdTAM67 slightly reduced their TIMP-1 production (Fig. 5B).

Distinct Roles of ERK1/2, JNK1, and p38 MAPK in Regulation of MMP-13, MMP-1, and MMP-3 Expression by Gingival Fibroblasts—To directly examine the role of ERK1/2 and p38 MAPK in the regulation of MMP-13 expression, we used adenovirus-mediated gene delivery of constitutively active MEK1 and MKK6 to fibroblasts to specifically activate ERK1/2 and p38 MAPK, respectively. As shown in Fig. 6A, infection of fibroblasts with adenovirus for constitutively active MEK1 (RAdMEK1CA) resulted in activation of ERK1/2 but not p38 MAPK. Interestingly, adenovirus-mediated expression of constitutively active MEK1 also resulted in activation of JNK1 in gingival fibroblasts (Fig. 6A). In parallel, adenovirus-mediated expression of constitutively active MKK6 (RAdMKK6bE) alone resulted in activation of p38 but not JNK or ERK1/2 (Fig. 6A). Simultaneous expression of constitutively active MEK1 and MKK6b did not markedly alter the activation of ERK1/2 and JNK1 or of p38, as compared with cells infected with RAdMEK1CA or RAdMKK6bE alone (Fig. 6A). Expression of constitutively active MEK1 or MKK6b alone or in combination had no effect on the total cellular levels of ERK1/2, JNK1, or p38 (Fig. 6A). Infection of cells with control virus RAdlacZ did not activate ERK1/2, JNK, or p38 (Fig. 6A).

The levels of MMP-13, MMP-1, MMP-3, and TIMP-1 were determined in aliquots of conditioned media from the same cultures by Western blot analysis. Infection of cells with adenoviruses for constitutively active MEK1 (RAdMEK1CA) or MKK6b (RAdMKK6bE) alone or in combination did not induce pro-MMP-13 production by gingival fibroblasts (Fig. 6B). However, production of pro-MMP-1 and pro-MMP-3 was induced as a result of ERK1/2 and JNK1 activation by constitutively active MEK1, and co-expression of constitutively active MEK1 and MKK6b augmented the enhancement of pro-MMP-1 (2.2-fold) and pro-MMP-3 production (1.4-fold), as compared with cells infected with RAdMEK1CA alone (Fig. 6B). In contrast, expression of constitutively active MKK6b alone was not sufficient to induce production of pro-MMP-1 or pro-MMP-3 (Fig. 6B). Infection of fibroblasts with RAdlacZ had no effect on the production of pro-MMP-1 or pro-MMP-3 (Fig. 6B). Infection of gingival fibroblasts with adenovirus for constitutively active MKK3b (RAdMKK3bE) alone, or in combination with RAdMEK1CA, did not induce MMP-13 expression (not shown).

Production of TIMP-1 was up-regulated (10.0-fold) in cells expressing constitutively active MEK1, while expression of constitutively active MKK6b alone or in combination with constitutively active MEK1 did not markedly enhance TIMP-1 production (Fig. 6B).

Discussion

In the present study, we show for the first time that collagenase-3 (MMP-13) is expressed by fibroblasts in normally healing human gingival wounds in vivo. We also show that the expression of MMP-13 by human gingival fibroblasts in monolayer cultures and in collagen gel is induced by TGF-β1 and that this induction is mediated by small GTPase Rac1 and requires p38 MAPK activity and the presence of functional AP-1 dimers. The role of human MMP-13 in physiological ECM remodeling appears to be limited, since until the present study, the only normal tissue in which human MMP-13 had been detected was developing fetal bone (10, 11). In addition, the only situations in which the expression of MMP-13 has been detected so far in fibroblasts in vivo (i.e. chronic dermal and intestinal ulcers, periodontal inflammation, squamous cell carcinomas of the head and neck and vulva, and breast carcinomas (14–16, 18–21)) are characterized by loss of normal connective tissue architecture. The results of the present study show that MMP-3 is expressed in vivo in normally healing gingival wounds, in contrast to cutaneous wounds, in which the expression of MMP-13 is not detected during normal repair (14). These observations show that the regulation of MMP-13 in human skin and gingival fibroblasts is fundamentally different and suggest an important role for MMP-13 in the normal repair of gingival wounds.

Wound healing is initiated by aggregation of platelets and formation of a fibrin clot followed by inflammation, cell proliferation and migration, and angiogenesis (25). Wound repair in gingiva is in general similar to cutaneous wound repair (31). In wounds of human oral mucosa, gelatinase B (MMP-9) is expressed in mucusal epithelium and in granulation tissue, and the levels of gelatinase A (MMP-2) produced by fibroblasts and endothelial cells are constant throughout the wound healing (51). In addition, migrating gingival keratinocytes express MMP-1 (52). However, as compared with dermal wound healing, gingival wounds heal more rapidly and with minimal scarring (53, 54). This has been suggested to be due to the presence of different growth factors, e.g. EGF, TGF-α, TGF-β, and vascular endothelial cell growth factor in saliva (55). In addition, it has been proposed that gingival fibroblasts resemble fetal fibroblasts rather than skin fibroblasts in their phenotype (56). Our observations show that human gingival wound repair differs from cutaneous wound healing with respect to the presence of MMP-13, which is expressed by gingival fibroblasts throughout the acute phase of gingival wound repair. Since MMP-13 is not expressed by fibroblasts during acute cutaneous wound repair (14), it is possible that the differential expression of MMP-13 plays an important role in the rapid turnover of granulation tissue ECM during gingival wound repair.

As compared with other collagenases, MMP-1 and MMP-8, MMP-13 has a wider substrate specificity, and its expression in vivo is clearly more restricted than the expression of most other MMPs (see Refs. 1–3). The expression of MMP-13 in cultured cells is also restricted (see Ref. 3). MMP-13 mRNAs have been detected in monolayer cultures of human immortalized embryonal fibroblasts and transformed fibroblasts, whereas expression of MMP-13 in primary human fibroblasts is low or undetectable (57, 58). Our recent observations show that MMP-13 expression is induced in human skin fibroblasts only by contact...
with three-dimensional collagen (30). In addition, once turned on by collagen matrix, the expression of MMP-13 by dermal fibroblasts is not altered by TNF-α and IL-1, and it is down-regulated by TGF-β (30). In the present study, we show for the first time that primary human gingival fibroblasts express MMP-13 in monolayer culture and in collagen gel when exposed to TGF-β1. TGF-β is a potent inducer of ECM accumulation, and it also inhibits turnover of ECM by inhibiting the expression of MMP-1 by dermal fibroblasts (44, 45) and enhancing the expression of TIMP-1 and -3 (59). Abundant expression of TGFβ1 is detected in cutaneous fibrosis (e.g., hypertrophic scars, keloids, and scleroderma (60–62)), and in adult rat wounds blocking the activity of TGF-β1 increases the proteolytic capacity of gingival fibroblasts by enhancing their production of MMP-13 and MMP-3. In addition to TGF-β1, the expression of MMP-13 by gingival fibroblasts was enhanced by TNF-α, which may play a role in induction of fibroblasts MMP-13 expression in vivo during gingival wound repair and in chronic periodontal inflammation (16). Together, these observations show that human gingival fibroblasts are fundamentally different from dermal fibroblasts with respect to the regulation of their collagenolytic activity and that TGF-β plays an important role in stimulating the proteolytic capacity of gingival fibroblasts.

In the present study, exposure of human gingival fibroblasts to TGF-β1 resulted in activation of two distinct MAPKs: ERK1/2 and p38. Our observations show that inhibition of p38 MAPK activity in human gingival fibroblasts either by a chemical inhibitor, SB203580, or by adenovirus-mediated expression of dominant negative p38α potently inhibits induction of MMP-13 expression by TGF-β1. In contrast, blocking the ERK1/2 signaling pathway by PD98059 had no marked effect on induction of MMP-13 expression by TGF-β, indicating that activation of the ERK1/2 pathway is not essential in the enhancement of MMP-13 expression in gingival fibroblasts. This is in contrast to our recent observations showing that in human skin fibroblasts in collagen gel, activation of the ERK1/2 signaling pathway potently inhibits expression of MMP-13 (30). Interestingly, the enhancement of MMP-3 expression by TGF-β1 in gingival fibroblasts is also dependent on p38 MAPK activity and in part on the activation of ERK1/2. Together with our recent observations (30), these results show that the activity of p38 MAPK plays a crucial role in activation of the expression of MMP-13 both in human gingival and skin fibroblasts.

To determine the specific roles of ERK1/2, JNK, and p38 MAPKs in regulation of MMP-13 expression, we utilized adenovirus-mediated gene delivery of constitutively active MEK1 or MKK6b, the upstream activators of ERK1/2 and p38, respectively. In accordance with our recent observations on human skin fibroblasts,2 adenovirus-mediated expression of constitutively active MEK1 in gingival fibroblasts resulted in simultaneous activation of ERK1/2 and JNK1, and the expression of constitutively active MKK6b specifically activated p38. As in dermal fibroblasts,2 the expression of constitutively active MEK1 results in induction of MMP-1 and MMP-3 production. However, activation of ERK1/2 and JNK1 or p38 MAPK alone or simultaneous activation of all three MAPKs was not sufficient for induction of MMP-13 expression in human gingival fibroblasts, indicating that other signaling pathways are also required for induction of MMP-13 expression. It has recently been shown that activation of type VII collagen (64) and type I

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collagen gene transcription (65) by TGFB-β in fibroblasts involves direct binding of Smad3-containing complex to the Smad binding elements in the respective promoters. Interestingly, it has also been shown that transcription factor ATF-2, a substate of p38 MAPK, interacts with Smad3 (66). In addition, it has been shown that Smad3 can dimerize with c-Jun and bind to the AP-1 binding site of human MMP-1 promoter (67). This is interesting in the context of our observations showing that activation of MMP-13 expression by TGFB-β in gingival fibroblasts also involves Smad transcription factors. However, a recent study (68) showed that TGFB-β-elicited up-regulation of fibronectin expression in human fibrosarcoma HT-1080-derived cell line (BAHgp) is Smad4-independent and JNK-dependent, providing evidence for Smad-independent activation of gene expression by TGFB-β.

In conclusion, the results of the present study show for the first time that regulation of the collagenolytic capacity in human gingival fibroblasts is fundamentally different from that of human skin fibroblasts. It is intriguing that in gingival fibroblasts, TGFB-β, a growth factor which plays an important role in cutaneous wound repair, scar formation, and fibrosis, induces the expression of MMP-13, a “super-collagenase” with the capacity to degrade a number of other ECM components in addition to fibrillar collagens. Based on these observations, it is likely that MMP-13 and MMP-1 play an entirely different role in ECM turnover in gingival wound repair. Restricted cleavage of type I and III collagen by MMP-1 may play a role in fibroblast migration, in analogy with the role of MMP-1 in keratinocyte migration (27). MMP-13, in turn, may be involved in rapid turnover of the collagenous ECM being deposited in response to TGFB-β in the vicinity and within the gingival granulation tissue. It is possible that MMP-13 plays an important role in maintaining the delicate balance between deposition and degradation of ECM during gingival wound repair, resulting in minimal scar formation. It is conceivable that unveiling the molecular mechanisms underlying differential regulation of MMP-13 expression in dermal and gingival fibroblasts may help in developing novel therapeutic modalities to combat tissue fibrosis.

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