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Expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts is induced by transforming growth factor-β via p38 mitogen-activated protein kinase

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ABSTRACT

Human collagenase-3 (MMP-13) is characterized by wide substrate specificity and limited tissue specific expression. We have previously noted that human collagenase-3 (MMP-13) is expressed by gingival fibroblasts in culture and during gingival wound repair characterized by minimal scarring. Here we show that human MMP-13 is expressed by dermal fibroblasts during early wound repair in fetal skin grafted on SCID mice. The expression of MMP-13 by fetal skin fibroblasts in monolayer culture was enhanced by transforming growth factor β1 (TGF-β1) and TGF-β3, whereas MMP-13 expression was not detected in neonatal skin fibroblasts. Treatment of fetal skin fibroblasts with TGF-β1 potently activated p38 mitogen-activated protein kinase (MAPK). Induction of MMP-13 expression by TGF-β1 was blocked by p38 MAPK inhibitor SB203580, and by adenovirally delivered dominant negative form of p38α. These observations demonstrate a remarkable difference in the regulation of collagenolytic capacity between fetal and neonatal skin fibroblasts, which suggests a role for MMP-13 in rapid turnover of collagenous matrix during repair of fetal cutaneous wounds, which heal without scar.

Key words: matrix metalloproteinase • collagenase • fibroblast

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-dependent, extracellular endopeptidases collectively capable of degrading essentially all extracellular matrix (ECM) components. MMPs are implicated in ECM remodeling in many physiological situations, such as in ECM remodeling and keratinocyte migration during wound repair, or in pathological situations characterized by excessive ECM degradation(e.g., in chronic dermal ulcers. To date, the MMP family includes 20 human

members, most of which can be divided into subgroups of collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs) based on their substrate specificity and structure (see 1–3). Members of the collagenase subfamily—collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13)—have the unique ability to cleave native fibrillar collagens of type I, II, and III collagens at a specific site generating 3/4 N-terminal and 1/4 C-terminal fragments, which denature at 37°C and are further degraded by other MMPs, such as gelatinases (see 1–3).

Compared with other MMPs, MMP-13 has a wide substrate specificity. In addition to collagens type I, II, and III, MMP-13 degrades gelatin, type IV, X, and XIV collagens, large tenascin C, fibronectin, fibrillin, versican, and aggrecan core protein (4–8). The expression of human MMP-13 has been detected only in physiological situations characterized by rapid remodeling of collagenous ECM, including fetal bone development, postnatal bone remodeling, and normal gingival wound repair (9–11). In contrast, MMP-13 expression is detected *in vivo* in pathological situations characterized by excessive degradation of collagenous ECM, such as osteoarthritic cartilage (5), rheumatoid synovium (10), chronic cutaneous ulcers (12), intestinal ulcerations (13), chronic periodontal inflammation (14), atherosclerotic plaques (15), and abdominal aortic aneurysms (16). The expression of MMP-13 is also detected *in vivo* in invasive malignant tumors; that is, breast carcinomas (17, 18); squamous cell carcinomas of the head, neck, and vulva (19, 20); cutaneous basal cell carcinomas (21); primary and metastatic malignant melanomas (22, 23); chondrosarcomas (24); and transitional cell carcinomas of the urinary bladder (25).

During wound healing, controlled degradation of collagenous ECM is required in reepithelialization, angiogenesis, and remodeling of the granulation tissue (26). MMP-1 expression has been detected in keratinocytes and in dermal fibroblasts in both acute and chronic dermal wounds (12, 27, 28), and it has been shown that MMP-1 is required for keratinocyte migration on native type I collagen (29). In contrast, MMP-13 is not expressed by human keratinocytes in acute or chronic dermal wounds, acute gingival wounds, or in normal epidermal keratinocytes in culture (11, 12, 30). It is interesting that MMP-13 is expressed by fibroblasts in chronic dermal ulcers, but not in normally healing dermal wounds (11). Human skin fibroblasts in culture express MMP-13 only when cultured inside collagen gel, but not when cultured in monolayer on collagen or on plastic (12, 31). We have recently shown that MMP-13 is expressed in fibroblasts *in vivo* during acute gingival wound repair, which is characterized by minimal scarring (11). In addition, human gingival fibroblasts in monolayer culture express MMP-13 when exposed to transforming growth factor β (TGF-β), a potent stimulator of granulation tissue formation during wound healing (11).

In the present study we show, that MMP-13 is expressed by dermal fibroblasts in wounds of normally healing human fetal skin grafted on SCID mice. In addition, in contrast to human adult and neonatal dermal fibroblasts, fetal skin fibroblasts express MMP-13 when cultured in monolayer and exposed to TGF-β1 and TGF-β3. Furthermore, we show, that the expression of human MMP-13 by fetal skin fibroblasts in culture is induced by TGF-β1 via the p38 mitogen-activated protein kinase (MAPK) signaling cascade. In the human body, both oral cavity and fetal skin are privileged sites in which wounds heal with minimal scarring. The results of this study demonstrate a remarkable similarity in the regulation of human MMP-13 expression between fetal skin and gingival fibroblasts, which suggests that MMP-13 plays a role in rapid

remodeling of collagenous ECM during healing of human fetal dermal wounds and contributes to their repair with minimal scarring.

MATERIALS AND METHODS

Reagents

Human recombinant TGF-β1 and TGF-β3 were obtained from Sigma Chemical Co. (St. Louis, Mo.). SB203580 and PD98059 were obtained from Calbiochem (San Diego, Calif.).

Fetal skin wounding protocol

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia in accordance with NIH guidelines. SCID-C57BL6 (Charles River Breeding Laboratories, N. Wilmington, Mass.) mice between 4 and 6 wk of age were housed in cages of four and were fed standard chow and water ad libitum. Human fetal skin of gestational age 16 to 20 wk was obtained from fresh pathological specimens from surgical procedures, trimmed of subcutaneous fat, and then cut into circular grafts measuring 15 to 20 mm in greatest dimension. The human skin-to-SCID mouse skin grafts were performed as previously described (32). Briefly, SCID mice were anesthetized with inhaled Metaphane, and all surgical manipulations were performed under sterile conditions in a laminar flow hood. Full-thickness skin grafts were transplanted into full- thickness size-matched wound beds prepared on each flank of the SCID mice. The grafts were surgically secured by using six interrupted sutures of 6-O polypropylene (U.S. Surgical, Norwalk, Conn.). Each graft was allowed 3–4 wk for stable engraftment prior to experimental manipulation. SCID mice bearing two human skin grafts were surgically wounded as previously described by using a 2-mm trephine (Miltex Instrument Co., Lake Success, N.Y., (33)). The site of the wound was marked its base with India ink to assist in localization of the wound on histologic evaluation, and the wounds were harvested at 24 h and 4 days.

Immunohistochemistry

Wounded human fetal skin grafts were fixed overnight in 10% neutral buffered formalin at 4°C and paraffin-embedded for histologic assessment. Serial sections of 5 μm were taken from each paraffin-embedded tissue block for immunohistochemistry. Before staining, deparaffinized sections were processed with citrate buffer in microwave oven. MMP-13 immunostaining was performed on formalin-fixed paraffin sections by using the biotin-avidin-peroxidase technique (DAKO StreptABComplex/HRP Duet Mouse/Rabbit Kit). 3-amino-9-ethylcarbazole (AEC, Sigma) solution with hydrogen peroxide was used as chromogenic substrate. Mouse monoclonal antibody against human MMP-13 (181-15A12; Calbiochem, San Diego, Calif.), which does not cross-react with mouse MMP-13, was diluted 1:40 in 0.3% BSA-PBS and reacted for 6 h at room temperature. Negative control sections were incubated without primary antibody. No staining was seen in these control sections. Mayer’s hematoxylin was used as counterstain in all immunostainings.

Cell Cultures

Human fetal skin fibroblasts were established from the abdominal skin of a 17-wk-old fetus aborted for health reasons, and neonatal skin fibroblasts from the skin of a newborn with permission of the Joint Ethical Committee of the Turku University Central Hospital and the University of Turku, Turku, Finland. Fibroblasts were cultured in Dulbecco's modification of Eagle's medium (DMEM, Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin-G, and 100 μg/ml streptomycin.

Western blot analysis of MMP-13, MMP-1, and TIMP-1

Fetal skin fibroblasts were maintained in serum-free DMEM for 18 h and incubated without or with TGF-β1 or TGF-β3 (5 ng/ml) for 48 h. Equal aliquots of the conditioned media were analyzed by Western blotting, as described previously (11) by using a mouse monoclonal antibody (181-15A12) against human MMP-13 (Calbiochem) in dilution 1:100, polyclonal rabbit antiserum against human MMP-1 (kindly provided by Dr. H. Birkedal-Hansen, National Institute of Dental Research, Bethesda, Md.) in dilution 1:5000, or polyclonal rabbit antiserum against tissue inhibitor of metalloproteinases (TIMP)-1 (Chemicon International Inc., Temecula, Calif.) in dilution 1:750. For TIMP-1 Western blot, samples were reduced with 5% mercaptoethanol prior to electrophoretic fractionation. Specific binding of antibodies was detected with corresponding peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence detection system (Amersham Corp., Buckinghamshire, U.K.). The levels of immunoreactive MMP-13, MMP-1, and TIMP-1 were quantitated by densitometric scanning of X-ray films.

RNA analysis

Total cellular RNA was isolated from cells by using the single-step method as described previously (11). Aliquots of total RNA (10 μg) were fractionated on 0.8% agarose gel containing 2.2 M formaldehyde, transferred to Zeta probe filter (Bio-Rad, Richmond, Calif.) 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-^{32}P]dCTP by 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 (30). In addition, a 1.3 kb rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (34) was used. The ^{32}P-cDNA/mRNA hybrids were visualized with autoradiography, quantified with densitometry, and corrected for the levels of GAPDH mRNA for each sample.

Analysis of MAPK activation

The activation of ERK1,2 and p38 MAPK was determined by Western blot analysis with antibodies specific for phosphorylated, activated forms of the corresponding MAPKs. Fibroblasts were treated with TGF-β1 in DMEM with 0.5% FCS at various time points, and were lyzed in 100 μl of sample buffer. The samples were sonicated, fractionated by 10% SDS-PAGE, and transferred to Hybond ECL membrane (Amersham). Western blotting was performed as described previously (11, 31), with phosphospecific antibodies for extracellular signal-regulated kinase (ERK)1,2, Jun N-terminal kinase (JNK), and p38 in dilution 1:1000, using ECL (Amersham). To control the loading, the filters were stripped and reprobed by using antibodies

against total ERK1,2 and p38 (both from New England Biolabs, Beverly, Mass.) in dilution 1:1000.

Infection of fibroblasts with recombinant adenoviruses

Recombinant replication-deficient adenovirus RAd*lac*Z (RAd35) (35), which contains the *Escherichia coli* β-galactosidase (*lac*Z) gene under the control of CMV IE promoter, and the empty adenovirus RAd66 (35) were kindly provided by Dr. Gavin W.G. Wilkinson (University of Cardiff, Wales). Recombinant adenovirus for dominant negative Rac1 (RAdN17rac1) (36) was kindly provided by Dr. Toren Finkel (National Heart, Lung, and Blood Institute, Bethesda, Md.). Construction and characterization of recombinant adenoviruses containing the coding regions of mutated, constitutively active human MAPK/ERK kinase (MEK)1 (RAdMEK1CA) (37), MAPK kinase (MKK)6b (RAdMKK6bE) (38), and dominant negative p38α (RAdp38AF) (38) genes driven by CMV IE promoter has been described previously.

Infection of cells with adenoviruses was performed as described previously (11). To determine the infection efficiency of human fetal skin fibroblasts, cells in suspension were mixed with RAd*lac*Z at different multiplicities of infection (MOI), plated, and incubated for 18 h. The cells were then fixed and stained for β-galactosidase activity (11). The maximal infection efficiency was obtained with MOI 500 (not shown). In experiments with adenoviruses for dominant negative Rac1 (RAdN17rac1) and p38α (RAdp38AF) 2×10^5 cells in suspension were mixed with corresponding viruses or with RAd66 at MOI 500, plated, and incubated for 5 h in DMEM with 1% FCS. The medium was then changed to serum-free DMEM without or with TGF-β1 (5 ng/ml), the incubations were continued for 24 h, and conditioned media were analyzed for the levels of MMP-13 and TIMP-1 by Western blot. In experiments with adenoviruses for constitutively active MAPK kinases, 2×10^5, fibroblasts in suspension were mixed with adenoviruses RAd66, RAdMEK1CA, or RAdMKK6bE at MOI 500, plated, and incubated for 18 h in DMEM with 1% FCS. Thereafter, medium was changed to serum-free DMEM, the incubations continued for 24 h, conditioned media were collected and analyzed for the levels of MMP-13, MMP-1, and TIMP-1 by Western blot. Cell layers were harvested and used for determination of ERK1,2 and p38 MAPK activation, as described above.

RESULTS

Human MMP-13 is expressed by dermal fibroblasts in fetal wounds

We have previously noted that MMP-13 is expressed by fibroblasts during normal repair of human gingival wounds, which are characterized by minimal scar formation (11). In addition, MMP-13 is expressed by fibroblasts in chronic human cutaneous ulcers *in vivo* but not in normally healing dermal wounds (12). To elucidate the role and regulation of MMP-13 in human fetal wound repair, also characterized by minimal scar formation (39), we first examined the expression of MMP-13 in a well-characterized model of normally healing incisional wound of human fetal skin grafted on SCID mice (32, 33). For this, human fetal skin of gestational age 16 to 20 wk and obtained from surgical procedures was grafted on SCID mice and allowed 3–4 wk for engraftment. The human fetal skin grafts were then surgically wounded and harvested at 24 h and 4 days postwounding for determining MMP-13 expression with immunostaining. MMP-13

positive fibroblasts were detected within the dermal layer in 4-day-old wounds (Fig. 1A, B). Numerous MMP-13 positive fibroblasts were detected also in the edge of the fetal skin graft, whereas dermal fibroblasts in the adjacent murine skin were negative for MMP-13, which confirms the specificity of the antibody for human MMP-13 (Fig. 1C). No MMP-13 positive fibroblasts were detected in 1-day-old wounds (not shown). These observations demonstrate a remarkable difference in MMP-13 expression between acute fetal and adult cutaneous wounds (12) and suggest that the regulation of MMP-13 expression in adult and fetal human skin fibroblasts is fundamentally different.

Expression of MMP-13 in fetal skin fibroblasts is induced by TGF-β

We have recently shown that, in contrast to adult human skin fibroblasts, human gingival fibroblasts in monolayer culture express MMP-13 when exposed to TGF–β1 (11). As fetal wounds also heal with minimal scarring (39), we wanted to examine the regulation of MMP-13 expression in fetal skin fibroblasts in culture. Fibroblasts from human fetal skin at gestational age of 17 wk and from neonatal skin were treated without or with TGF-β1 (5 ng/ml) for 48 h, and the levels of proMMP-13 in conditioned media were determined by Western blot analysis. Fibroblasts from fetal skin produced proMMP-13 when treated with TGF-β1, whereas neonatal skin fibroblasts in monolayer culture did not produce detectable amounts of proMMP-13 (Fig. 2A), which is in accordance with our previous observations with adult skin fibroblasts (31). In fetal skin fibroblasts, proMMP-1 production was not altered markedly by TGF-β1, whereas in neonatal skin fibroblasts proMMP-1 production was down-regulated by TGF-β1, as in human adult skin fibroblasts (31). TIMP-1 production was enhanced slightly by TGF-β1 in both in fetal and neonatal skin fibroblasts, indicating that these cells respond to TGF–β1 (Fig. 2A). Treatment with both TGF-β1 and TGF-β3 also enhanced the abundance of MMP-13 mRNAs in fetal skin fibroblasts, but not in neonatal skin fibroblasts (Fig. 2B).

TGF-β activates p38 MAPK in fetal dermal fibroblasts

Treatment of human gingival fibroblasts with TGF-β rapidly and transiently activates two MAPKs—ERK1,2 and p38—and p38 MAPK activity is required for induction of MMP-13 expression by TGF-β1 (11). To study the role of MAPK pathways in the regulation of human fetal skin fibroblast MMP-13 expression, we first determined the activation of MAPKs by Western blot analysis of cellular proteins at various time points after exposure to TGF–β1 by using antibodies against the active, phosphorylated forms of these MAPKs. The levels of activated p38 were increased 3.8-fold at 30 min of incubation with TGF–β1, and potent induction (6.1-fold) was still noted at 6 h of incubation (Fig. 3A, B). Treatment with TGF-β1 did not result in activation of ERK1,2 in fetal skin fibroblasts (Fig. 3A, B). In contrast to fetal skin fibroblasts, ERK1,2 was rapidly activated (2.4-fold) at 15 min of incubation with TGF-β1 in neonatal skin fibroblasts (Fig. 3C, D). In addition, p38 was activated maximally at 1 h (2.2-fold) after addition of TGF-β1 (Fig. 3C, D). No activation of JNK was detected in fetal or neonatal skin fibroblasts treated with TGF-β1 (not shown). The total cellular levels of ERK1,2, or p38 in fetal or neonatal skin fibroblasts were not altered by treatment with TGF-β1 (Fig. 3A, C).

Induction of MMP-13 expression in fetal skin fibroblasts

by TGF–β1 is mediated by p38 MAPK

To study the specific roles of MAPKs in mediating the induction of MMP-13 expression by TGF–β1 in fetal skin fibroblasts, we first used selective chemical inhibitors for ERK1,2 and p38 MAPK. Blocking the ERK1,2 pathway (Raf→MEK1,2→ERK1,2) by PD98059 (30 μM), a specific inhibitor of MEK1,2, added to fibroblasts 1 h prior to TGF–β1, had no effect on the induction of proMMP-13 production by TGF-β1 (Fig. 4A). In contrast, addition of selective p38 inhibitor SB203580 (10 μM) to fibroblasts 1 h before TGF–β1 entirely inhibited the induction of proMMP-13 by TGF-β1 (Fig. 4A). Treatment of fibroblasts with TGF-β1 with or without PD98059 or SB203580 had no effect on proMMP-1 production (Fig. 4A). TIMP-1 production was slightly (1.5-fold) enhanced by TGF-β1, but this stimulation was not altered by PD98059 or SB203580 (Fig. 4A).

To further elucidate the role of MAPK signaling pathways mediating the induction of MMP-13 gene expression by TGF–β1, we used recombinant replication-deficient adenoviruses to specifically inhibit and activate endogenous MAPK signaling cascades. Determination of the transduction efficiency of human fetal skin fibroblasts by using adenovirus RAd*lac*Z showed that β-galactosidase gene was delivered to all cells at MOI 500 (not shown), which was then used in experiments.

Next, we infected fetal skin fibroblasts with adenoviruses coding for dominant negative forms of small GTPase Rac1 (RAdN17rac1) involved in the activation of JNK and p38 (40) and p38α (RAdp38AF). Treatment of fetal skin fibroblasts with TGF–β1 resulted in an increase in proMMP-13 production in fetal skin fibroblasts infected with the empty control virus RAd66 (Fig. 4B). Infecting fibroblasts with adenovirus for dominant negative Rac1 (RAdN17rac1) had no marked effect on the induction of proMMP-13 production by TGF-β1, compared with RAd66 infected cells (Fig. 4B). In accordance with the results obtained with p38 inhibitor SB203580, adenovirus-mediated expression of dominant negative p38α (RAdp38AF) markedly (by 76%) reduced induction of proMMP-13 production by TGF–β1, compared with RAd66 infected cells (Fig. 4B), corroborating the role of p38 MAPK in mediating TGF-β1-elicited induction of MMP-13 expression. Production of TIMP-1 was also enhanced by TGF-β1 in cells infected with RAd66 and RAdN17rac1 (2.8- and 1.6-fold, respectively), compared with corresponding untreated cells, and adenovirus-mediated expression of RAdp38AF slightly inhibited enhancement of TIMP-1 production by TGF-β1 (Fig. 4B).

MMP-13 and MMP-1 expression are regulated differentially by activation of MAPKs in fetal skin fibroblasts

To directly examine the role of MAPKs in the regulation of MMP-13 expression, we used adenovirus-mediated gene delivery of constitutively active MEK1 and MKK6b to fibroblasts to activate ERK1,2 and p38 MAPK, respectively. Infection of fibroblasts with adenovirus for constitutively active MEK1 (RAdMEK1CA) resulted in activation of ERK1,2, but not p38 MAPK (Fig. 5A). In parallel, adenovirus-mediated expression of constitutively active MKK6b (RAdMKK6bE) resulted in activation of p38, but not ERK1,2 (Fig. 5A). Expression of constitutively active MEK1 or MKK6b had no effect on the total cellular levels of ERK1,2 or p38 (Fig. 5A).

The levels of proMMP-13, proMMP-1, 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) was not sufficient to induce proMMP-13 production in fetal skin fibroblasts (Fig. 5B). Instead, production of proMMP-1 was induced up to 6.3-fold as a result of ERK1,2 activation by constitutively active MEK1, compared with cells infected with RAd66 (Fig. 5B). In contrast, expression of constitutively active MKK6b was not sufficient to induce the production of proMMP-1 (Fig. 5B). Production of TIMP-1 was up-regulated 2.1-fold in cells expressing constitutively active MEK1 as compared with the cells infected with RAd66. Whereas the expression of constitutively active MKK6b did not alter TIMP-1 production compared with the cells infected with RAd66 (Fig. 5B).

DISCUSSION

In the present study we show that human collagenase-3 (MMP-13) is expressed by dermal fibroblasts during acute wound repair of human fetal skin grafted onto SCID mice. In addition, we show, that fetal skin fibroblasts in culture express MMP-13 and that its expression is induced by TGF-β1 via p38 MAPK signaling pathway. MMP-13 expression has previously been detected *in vivo* by fibroblasts in the early phase of normal gingival wound healing (11). In contrast, MMP-13 is not expressed in normally healing adult dermal wounds, but is detected in fibroblasts in chronic dermal ulcers (12). Expression of MMP-13 mRNAs has been detected in human immortalized embryonal fibroblasts and transformed fibroblasts in monolayer culture, as well as in primary human gingival fibroblasts treated with TGF-β1 (11, 41, 42). However, the expression of MMP-13 in normal human skin fibroblasts in monolayer culture is undetectable but can be induced by culturing these cells within three-dimensional collagen (12, 31). Thus, our results show an interesting similarity between human gingival and fetal skin fibroblasts, both of which express MMP-13 during normal wound repair *in vivo*, and in culture, when exposed to TGF-β1.

Fetal wound repair differs from adult cutaneous wound healing in several ways. In general, fetal wounds regenerate without scar during the first and the second trimester of gestation, after which the dermal wound healing occurs with scarring. However, the wounds of premature infants born during the second trimester heal with scar, which suggests that transfer from uterine environment is sufficient to alter the wound repair from scarless to scar-forming healing (39). Compared with adult dermal wound healing, fetal wound repair is characterized by rapid and well-organized matrix deposition, high levels of hyaluronan (HA), and minimal inflammatory response. In addition, fetal wounds are surrounded by sterile amniotic fluid, rich in HA, growth factors, and fibronectin, which provides further evidence that the uterine environment plays a role in scarless healing (39). The results of the present study show that human fetal skin fibroblasts are also phenotypically different from adult and neonatal skin fibroblasts. In contrast to human neonatal and adult skin fibroblasts, fetal skin fibroblasts in monolayer culture express MMP-13 when treated with TGF-β1, similarly as human gingival fibroblasts. Both gingival and fetal skin wound repair are characterized by formation of minimal collagenous scar, which suggests that MMP-13, as a potent collagenolytic MMP, may play a role in this phenomenon. However, our previous observations show that human MMP-13 is not expressed in intact fetal skin at gestational age of 8 to 17 wk, indicating that MMP-13 is not involved in normal fetal

development of skin (9).

Our results show that, in contrast to human fetal skin fibroblasts, neonatal skin fibroblasts in culture resemble adult skin fibroblasts, as they do not express detectable levels of MMP-13 (12, 31). This finding suggests that the ability to express MMP-13 is characteristic for fetal skin fibroblasts, and that the change in fibroblast collagenolytic phenotype occurs within a short period around the time of birth. Previous studies have also documented other differences between adult and fetal skin fibroblasts. Fetal skin fibroblasts migrate more efficiently into collagen gel than adult fibroblasts (43), display a higher proliferation rate, and express lower amounts of urokinase-type plasminogen activator and MMP-1 compared with adult skin fibroblasts (44, 45). In addition, fetal skin fibroblasts express lower levels of fibroblast growth factor-1, -2, and TGF-β1 (46), and respond differently to TGF-β1, as compared with adult dermal fibroblasts. Specifically, TGF-β1 inhibits migration and HA synthesis of fetal skin fibroblasts, but not of adult skin fibroblasts (47). In addition, migration and HA synthesis of confluent fetal fibroblasts is inhibited by all three TGF-β isoforms, whereas migration and HA synthesis of confluent adult fibroblasts is stimulated by TGF-β3, but not by TGF-β1 and 2 (48).

It is interesting that production of MMP-13 by fetal skin fibroblasts was induced by TGF-β1, a growth factor involved in all phases of cutaneous wound repair. It has been shown, that TGF-β1 is a potent activator of monocyte chemotaxis, fibroblast proliferation, formation of granulation tissue, and angiogenesis (49–51). TGF-β1 induces the expression of several components of ECM in adult dermal fibroblasts *in vitro* and *in vivo* (51), and addition of TGF-β1 into rabbit fetal excisional wounds increases fibrosis and type I collagen expression (52). However, in adult rat wounds, blocking the activity of TGF-β1 and -2 relative to TGF–β3 inhibits scar formation (53, 54). The role of TGF-β3 as a scar-reducing growth factor remains unclear; in rabbit ear model, TGF-β3 does not reduce scarring (55) and, in human lung fibroblasts, TGF-β1 and TGF-β3 are equally potent in increasing deposition of ECM molecules, decreasing MMP-1 secretion and increasing TIMP-1 expression (56). Our results show that TGF-β1 and TGF-β3 induce MMP-13 expression by fetal skin fibroblasts, which suggests that both play a role in up-regulation of MMP-13 expression and rapid turnover of collagen during fetal wound repair.

Our results show that exposure of human fetal skin fibroblasts to TGF-β1 results in more potent and persistent activation of p38 MAPK than in neonatal skin fibroblasts. Inhibition of p38 MAPK activity in human fetal skin fibroblasts by a chemical inhibitor, SB203580, or by adenovirus-mediated expression of dominant negative p38α potently inhibits induction of MMP-13 expression by TGF-β1. Our previous results show that p38 MAPK is required for induction of MMP-13 expression by TGF-β1 in gingival fibroblasts as well as for induction of MMP-13 expression by collagen gel in adult dermal fibroblasts (11, 31). Taken together, these results show that p38 MAPK plays a crucial role in regulation of the expression of MMP-13 in fibroblastic cells. Exposure of human neonatal skin fibroblasts to TGF-β1 resulted in rapid activation of ERK1/2, as noted previously with adult fibroblasts (11). However, TGF-β1 did not activate ERK1/2 in fetal skin fibroblasts, providing further evidence that fetal and neonatal skin fibroblasts differ in their response to TGF-β.

To study the specific roles of MAPKs in endogenous regulation of human MMP-13 expression by fetal skin fibroblasts, we infected these cells with adenoviruses coding for constitutively

active MEK1 or MKK6b, the upstream activators of ERK1,2 and p38, respectively. Adenovirus-mediated expression of constitutively active MEK1 in fetal skin fibroblasts resulted in activation of ERK1,2, and the expression of constitutively active MKK6b specifically activated p38. As in gingival fibroblasts, the expression of constitutively active MEK1 resulted in induction of MMP-1 production. However, in accordance with our results with gingival fibroblasts (11), activation of ERK1,2, or p38 MAPK was not sufficient for induction of MMP-13 expression in human fetal skin fibroblasts. It is therefore likely that induction of MMP-13 expression by TGF-β in fetal skin fibroblasts involves activation of other signaling pathways and transcription factors, for example Smad3, which mediates enhancement of type VII collagen and type I collagen gene transcription by TGF-β in fibroblasts (57, 58). Smad3 in turn has been shown to interact with transcription factor ATF-2, a substrate of p38 MAPK (59), and dimerize with c-Jun and bind to the AP-1 binding site of human MMP-1 promoter (60).

In conclusion, the results of the present study show that the regulation of the collagenolytic capacity in human fetal skin fibroblasts is similar to that of human gingival fibroblasts. In addition, these observations demonstrate a fundamental difference in the regulation of collagenolytic capacity between fetal and neonatal skin fibroblasts, especially in response to TGF-β, a growth factor implicated in ECM accumulation in wound repair and fibrosis. It is therefore possible that as a collagenolytic MMP with a wide substrate specificity, MMP-13 may play an important role in rapid turnover of collagenous ECM of granulation tissue during fetal wound repair, resulting in minimal scar formation.

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Fig. 1

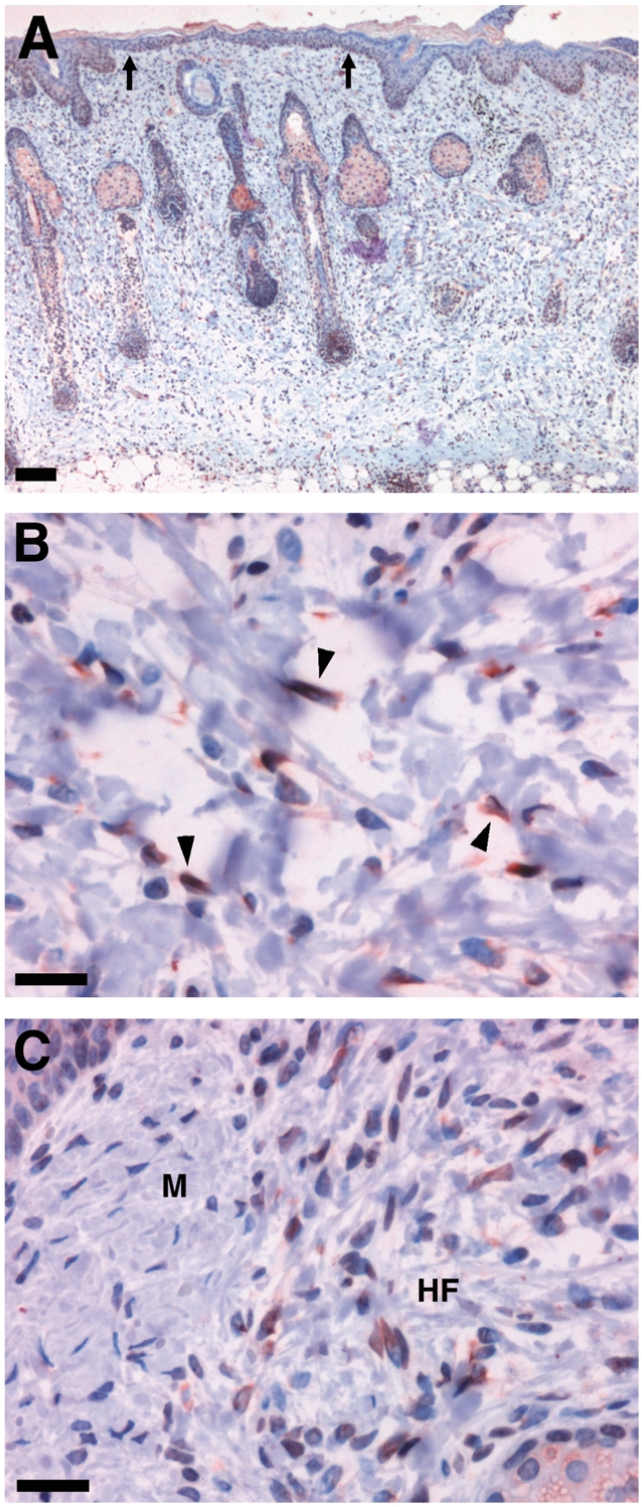

Figure 1. **Expression of human collagenase-3 (MMP-13) by fibroblasts in fetal skin wounds.** Human fetal skin of gestational age 16 to 20 wks was grafted onto SCID mice and wounded as described in Materials and Methods. Wound samples obtained at 4 days were immunostained with anti-MMP-13 antibody, which does not cross-react with murine MMP-13. **(A)** The margins of newly epithelialized wound are marked with arrows; **(B)** MMP-13 positive dermal fibroblasts are indicated by arrowheads; **(C)** MMP-13 positive fibroblasts are detected in human fetal dermal layer (HF) but not in murine dermal layer (M). Bars: **(A)** 100 μM; **(B, C)** 20 μM.

Fig. 2

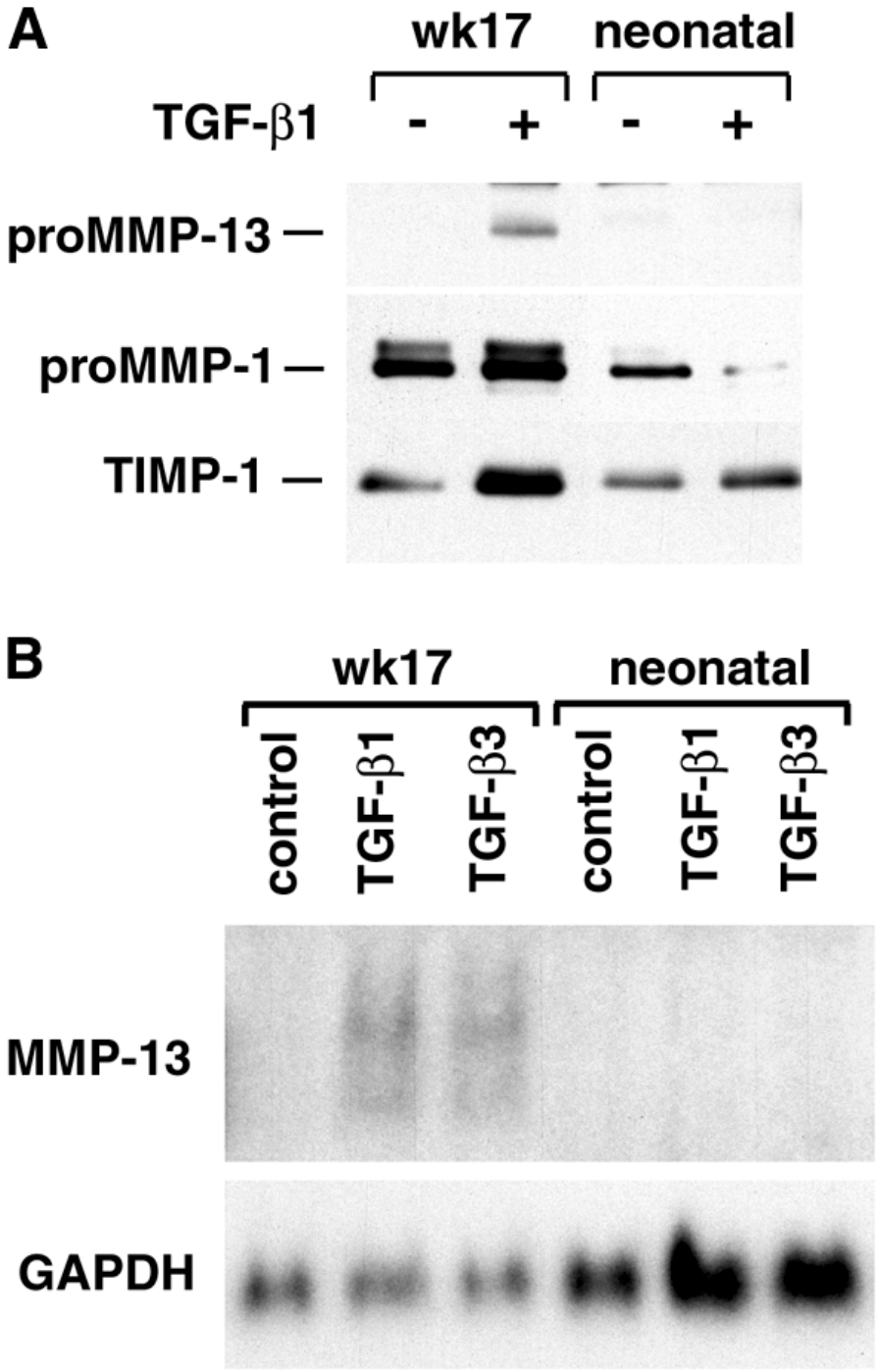

Figure 2. TGF-β induces expression of human collagenase-3 (MMP-13) in fetal skin fibroblasts. **(A)** Human fetal (wk 17) and neonatal skin fibroblasts were cultured in serum-free DMEM for 18 h. Thereafter, the incubations were continued for 48 h without (–) or with (+) transforming growth factor-β1 (TGF-β1; 5 ng/ml). The levels of proMMP-13, proMMP-1, and TIMP-1 in conditioned media of treated fibroblasts were determined by Western blot analysis. **(B)** Human fetal (wk 17) and neonatal skin fibroblasts were cultured in DMEM supplemented with 0.5% FCS for 18 h. The incubations were continued for 24 h with (+) or without (–) TGF-β1 and TGF-β3 (5 ng/ml each). MMP-13 and GAPDH mRNA levels were determined by Northern blot hybridizations of total RNA (10 μg).

Fig. 3

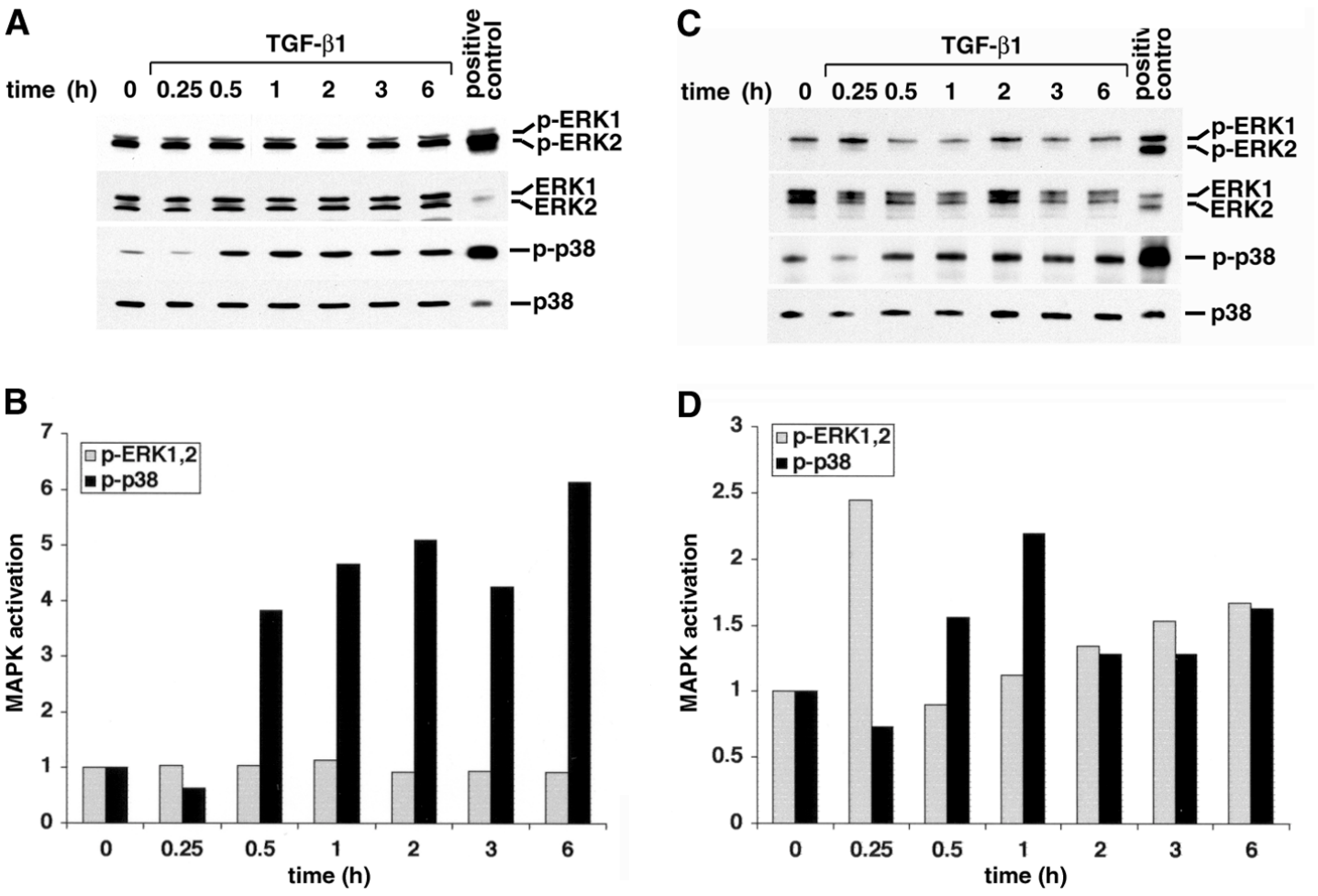

Figure 3. TGF-β activates different MAPKs in fetal and neonatal skin fibroblasts. (A) Human fetal skin fibroblasts (wk 17) and **(C)** neonatal skin fibroblasts were incubated with TGF-β1 (5 ng/ml) in DMEM supplemented with 0.5% FCS for different periods, as indicated. The levels of activated ERK1 and ERK2 (p-ERK1, p-ERK2) and p38 (p-p38) were determined by Western blot analysis by using phosphospecific antibodies for the corresponding MAPKs. As controls, the levels of total ERK1,2 and p38 MAPK were determined by Western blotting by using specific antibodies. Cell lysate from HaCaT cells treated with TNF-α for 20 min was used as a positive control for activated ERK1,2 and p38 MAPK. **(B, D)** The levels of activated ERK1 and ERK2 (p-ERK1,2) and p38 MAPK (p-p38) in fetal **(B)** and neonatal **(D)** skin fibroblasts, quantitated by scanning densitometry and corrected for the levels of total ERK1,2 and p38 MAPK in the same samples, respectively, are shown relative to the levels at time point 0 h (1.00).

Fig. 4

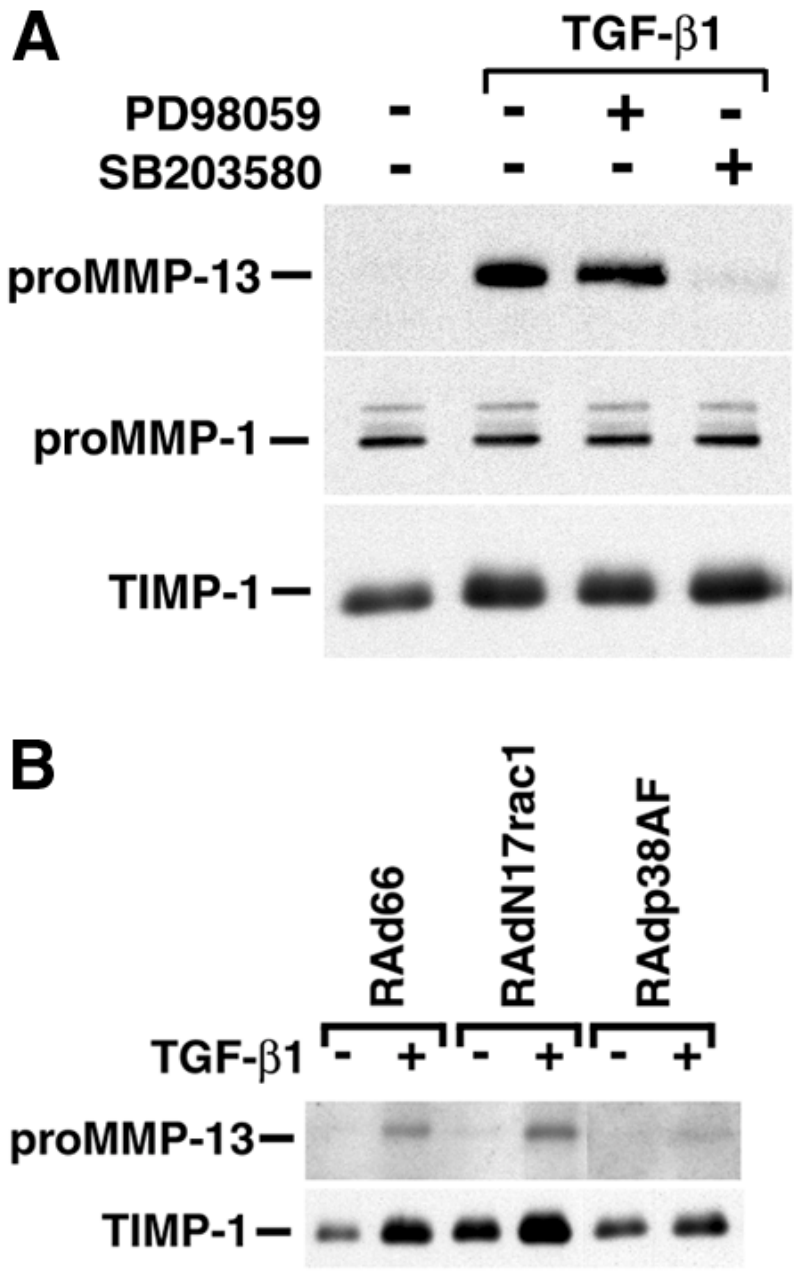

Figure 4. Induction of collagenase-3 (MMP-13) production by fetal skin fibroblasts by TGF-β is dependent on the activity of p38 MAPK. (A) Human fetal skin fibroblasts (wk 17) were incubated with TGF-β1 (5 ng/ml) for 48 h in serum free DMEM. PD98059 (30 μM), a specific inhibitor of ERK1,2 kinases MEK1,2, or SB203580 (10 μM), a selective p38 inhibitor, were added to cultures indicated (+) 1 h prior to TGF-β1. The levels of proMMP-13, proMMP-1, and TIMP-1 in conditioned media of fetal skin fibroblasts were determined by Western blot analysis. **(B)** Human fetal skin fibroblasts (wk 17) were infected at MOI 500 with control adenovirus (RAd66) and with adenoviruses for dominant negative Rac1 (RAdN17rac1) and dominant negative p38α (RAdp38AF), and incubated for 5 h in DMEM with 1% FCS. Thereafter, medium was replaced with serum-free DMEM without (–) or with (+) TGF-β1 (5 ng/ml) and incubations were continued for 24 h. The levels of proMMP-13 and TIMP-1 in the conditioned media were determined by Western blot analysis.

Fig. 5

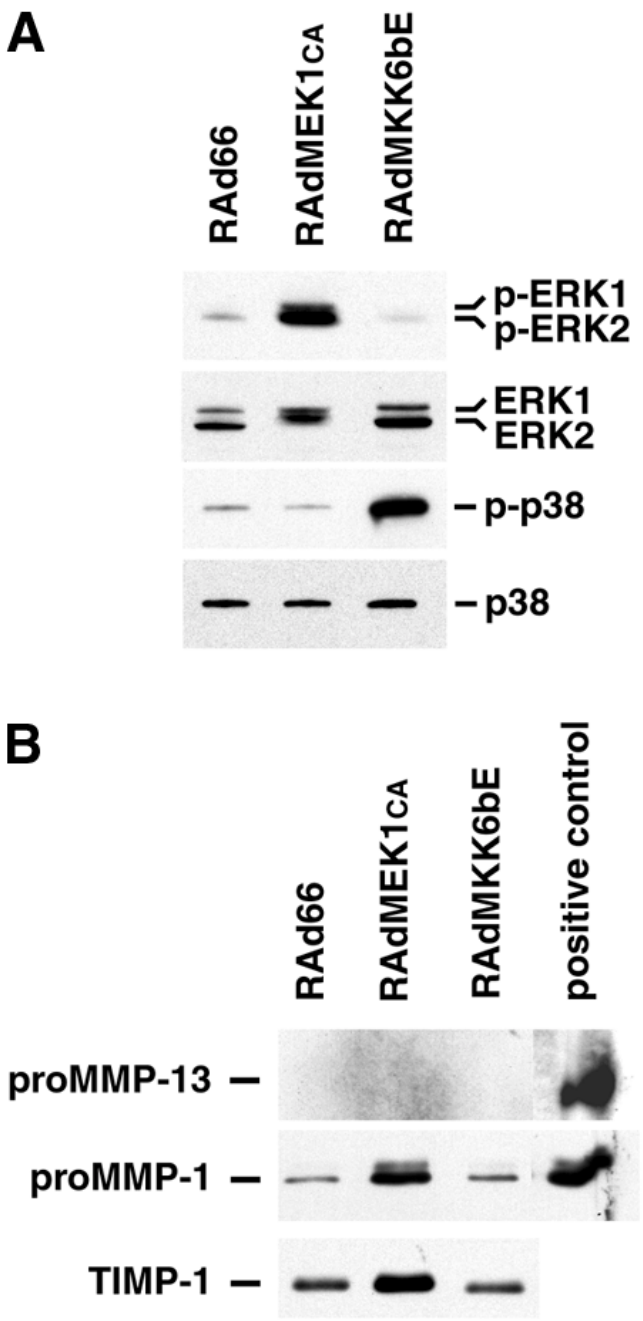

Figure 5. MMP-13 and MMP-1 expression are differentially regulated by activation of MAPKs in fetal skin fibroblasts. (A, B) Human fetal skin fibroblasts (wk 17) were infected with control adenovirus (RAd66), or with adenoviruses coding for constitutively active MEK1 (RAdMEK1cA) or MKK6b (RAdMKK6bE), and incubated for 18 h in DMEM with 1% FCS. Thereafter, the medium was replaced with serum-free DMEM, and the incubations were continued for 24 h. **(A)** The levels of activated ERK1 and ERK2 (p-ERK1, p-ERK2) and p38 (p-p38) in cell lysates were determined by Western blot analysis by using phoshospecific antibodies for the corresponding MAPKs. As controls, the levels of total ERK1,2 and p38 MAPK were determined by using specific antibodies. **(B)** The levels of proMMP-13, proMMP-1, and TIMP-1 in the conditioned media of cells were determined by Western blot analysis. Positive controls: for proMMP-13, conditioned medium of HaCaT cells treated with TNF-α; for proMMP-1, conditioned medium of human skin fibroblasts treated with TNF-α.