Transforming Growth Factor-β- and Tumor Necrosis Factor-α-mediated Induction and Proteolytic Activation of MMP-9 in Human Skin

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Abstract

Both cytokines and matrix metalloproteinases (MMPs) are active during physiologic and pathologic processes such as cancer metastasis and wound repair. We have systematically studied cytokine-mediated MMP regulation. Cytokine-mediated protease induction and activation were initially investigated in organ-cultured human skin followed by determination of underlying cellular and molecular mechanisms using isolated skin cells. In this report we demonstrate that tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) synergistically induce pro-MMP-9 in human skin as well as isolated dermal fibroblasts and epidermal keratinocytes. Furthermore, TNF-α promotes proteolytic activation of pro-MMP-9 by conversion of the 92-kDa pro-MMP-9 to the 82-kDa active enzyme. This activation occurred only in skin organ culture and not by either isolated fibroblasts or keratinocytes, although the pro-MMP-9 activation could be measured in a cell-free system derived from TNF-α-activated skin. The cytokine-mediated induction of pro-MMP-9 in dermal fibroblasts was evident by increased mRNA. At the transcription level, we examined the cytokine-mediated transactivation of the 5′-region promoter of the human MMP-9 in dermal fibroblasts. The results demonstrated that TNF-α and TGF-β could independently stimulate the 5′-flanking 670-base pair promoter. A TGF-β response element (−474) and an NF-κB-binding site

‡ Supported by National Institutes of Health GM 50967.
¶ Supported by National Institutes of Health Grant GM55081.
(−601) were identified to be the cis-elements for TGF-β or TNF-α activation, respectively. Taken together, these findings suggest a specific mechanism whereby multiple cytokines can regulate MMP-9 expression/activation in the cells of human skin. These results imply roles for these cytokines in the regulation of MMP-9 in physiologic and pathologic tissue remodeling.

Cytokines have been shown to be involved in many physiologic and pathologic processes. Tumor necrosis factor-α (TNFα) is thought to be essential for macrophage-mediated normal wound healing (1,2). Whereas elevated levels of TNF-α have been linked to deficient wound healing (3,4), a lack of TNF-α was found to be associated with hypertrophic scars (5,6). This suggests that misregulated TNF-α results in disordered wound healing. Similarly, transforming growth factor-β (TGF-β) is a component of normal wound healing (7,8). Increased TGF-β is associated with hypertrophic scar and fibrosis (9,10). In addition, accumulating evidence has suggested a role for inflammatory cytokines in promoting tumor metastasis, although the mechanism is not clarified (11,12). The roles for these mediators in the regulation of extracellular matrix may relate to the spectrum of genes they induce and appear to share a common theme of tissue remodeling.

Compelling evidence has documented the role of matrix metalloproteinases in the remodeling of connective tissue during angiogenesis, tumor metastasis, and tissue repair (13). Of the growing family of MMPs, MMP-2 (gelatinase A, 72-kDa type IV collagenase, EC 3.4.24.24) and MMP-9 (gelatinase B, 92-kDa type IV collagenase, EC 3.4.24.35) are unique for their fibronectin-like collagen binding domains (14). MMP-2 and MMP-9 are thought to be responsible for detaching basal keratinocytes from the basement membrane and thus promote their migration to cover exposed connective tissue (15,16). This notion is based on their restricted expression pattern at the wound edge and their substrate preference for basement membrane, type IV collagen. In addition, the type IV collagenases may also degrade type VII collagen, the major collagen component of anchoring fibrils essential for the attachment of the epidermis to the dermis, (17,18). Although the actual functions of the type IV collagenases in normal physiologic processes are not clear, accumulated evidence has linked them to many diseases. Excessive type IV collagenase activity is associated with non-healing chronic wounds where it is thought that type IV collagen is over-digested during re-epithelialization (19,20). Consistent with this fact, low levels of MMP-9 were found in hypertrophic scars where collagen is over-deposited (21).

The expression of pro-MMP-9 is regulated by many soluble mediators such as TNF-α, IL-1β, TGF-β, the ECM, oncogenes, and tumor promoters (22–25). After secretion into the ECM, the activity of MMP-9 can be further regulated by specific tissue inhibitors and by proteolytic activation via removal of the amino-terminal inhibitory domain. Most of the previous investigations on cytokine-mediated MMP-9 expression utilized tumor or transformed cell lines in which the mitogenic signaling and the cell cycle machinery are constitutively active. In chronic wounds and some invasive cancer tissue the 92-kDa pro-MMP-9 is processed into the active 82-kDa form (26–28). Very little is known about the molecular regulation of expression and proteolytic activation of MMP-9 in these pathologic situations.

Because multiple cytokines are coordinately present in wound sites, we have studied the interaction of multiple cytokines on MMP-9 induction and its proteolytic activation in organ-cultured human skin. In the second phase of the study we dissected the cytokine-mediated

1The abbreviations used are: TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; bp, base pair; MMPs, matrix metalloproteinases; DMEM, Dulbecco’s modified Eagle’s medium; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ECM, extracellular matrix; TRE, TGF-β-response element; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; II, interleukin.
MMP-9 regulation at the cellular level. We isolated dermal fibroblasts and keratinocytes from human skin and examined the effect of cytokines on MMP-9 regulation. Finally, we investigated the cytokine-responsive cis-elements in the promoter of the human MMP-9 gene to identify the molecular target sites of such regulation.

In a previous report we demonstrated that exposure of human skin to TNF-α led to activation of the pro-MMP-2, and such proteolytic activation could be reconstructed by embedding the dermal fibroblasts in collagen lattices (29). In the present study we extend our findings by showing that the 92-kDa pro-MMP-9 is induced in human skin by TGF-β, and this induction is additively enhanced by a second signal from TNF-α. In addition, we found that the 92-kDa pro-MMP-9 is converted to the 82-kDa active form when organ-cultured skin is treated with TNF-α. Furthermore, we show here that the TNF-α-mediated pro-MMP-9 activation is caused by an unidentified factor that is tightly associated with skin tissue. Our cellular dissection experiments demonstrate that cytokine-mediated pro-MMP-9 induction in the human skin is due to dermal fibroblasts and epidermal keratinocytes. At the molecular level, we provide evidence showing that these two cytokines target their response elements in the 5′-promoter of the human MMP-9 gene. These findings represent the first demonstration for additive roles of TNF-α and TGF-β on the induction and activation of MMP-9 in human skin.

EXPERIMENTAL PROCEDURES

Materials and Reagents

Vitrogen containing 95% type I collagen was purchased from Cohesion Technologies (Palo Alto, CA). Cytokines were purchased from R & D Systems (Minneapolis, MN). The antibodies against MMP-9 (AB805) were purchased from Chemicon International (Temecula, CA). The Immobilon-P was purchased from Millipore (Bedford, MA). The enhanced chemiluminescence (ECL) was purchased from Amersham Pharmacia Biotech. The gelatin was from Sigma. Gelatin-Sepharose 4B was purchased from Amersham Pharmacia Biotech. RNA was extracted by Trizol from Life Technologies, Inc. The reagents for reverse transcriptase-PCR were purchased from Promega (Madison, WI). The Platinum Taq DNA polymerase and oligodeoxynucleotide primers were from Life Technologies, Inc. KGM and KBM were from Clonetics. Quick-change site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). DNA sequencing was carried out at the University of Southern California/Norris Comprehensive Cancer Center.

Organ Culture and Cytokine Stimulation of Human Skin

Normal human skin was obtained from patients undergoing reconstructive or aesthetic surgery (University of Southern California IRB 999061). The full thickness skin was decontaminated by incubation in 2× antibiotic containing DMEM (200 units/ml penicillin G sodium, 200 units/ml streptomycin sulfate, and 0.5 µg/ml amphotericin B) at 4 °C overnight. Then the skin was cut into equal sizes with 0.5 cm on each side and incubated in DMEM at 37 °C with 5% CO₂ for 8 h. To decrease the effects of endogenous soluble factors in the skin induced by the harvesting process, the medium was changed three times during the 8-h incubation. Finally, the skin piece was immersed in 2 ml of DMEM with specific cytokines and was maintained at 37 °C with 5% CO₂. The conditioned media were sampled at the indicated times for gelatinolytic zymogram assay and Western blot as mentioned below.

Preparation and Culture of Human Dermal Fibroblasts and Keratinocytes

Dermal fibroblasts and keratinocytes were isolated from full thickness human skin (3). The isolated fibroblasts were cultured in DMEM containing 10% fetal bovine serum with antibiotics. The keratinocytes were grown in complete KGM. Before exposure to cytokines the medium was replaced with serum-free DMEM for fibroblasts and KBM, the basal medium,
for keratinocytes. For some experiments the fibroblasts were embedded in collagen lattices (29).

Purification of Pro-MMP-9

The transformed human keratinocytes (kindly provided by Dr. David Woodley, University of Southern California) were cultured in KGM to confluence. The cells were stimulated by 10 ng/ml TNF-α in KBM for 72 h in standard culture condition. In these conditions most of the gelatinase secreted in the medium is the 92-kDa pro-MMP-9. The conditioned media from 10 10-cm dishes were collected, and particulate debris was removed by centrifugation at 4,000 × g for 10 min. The conditioned media were passed through a 1-ml gelatin-Sepharose 4B column followed by washing with 400 mM NaCl in 50 mM Tris, pH 7.5. The bound gelatinase was eluted by 100 mM HCl and immediately neutralized by Tris base to pH 7.5.

Activation of Pro-MMP-9 in Human Skin

The explants of full thickness skin were stimulated with or without TNF-α at 10 ng/ml for 70 h in DMEM at 37 °C with 5% CO₂. To test whether the TNF-α-mediated pro-MMP-9 activation occurred inside the skin tissue or by secreted factors, the explants were washed and recultured in fresh DMEM without cytokine. The original conditioned media from the 70-h stimulation and the conditioned media from the re-culture were incubated at 37 °C for additional 8–20 h and analyzed by gelatinolytic zymography. In another experiment, the TNF-α-treated skin explant was minced and then extracted using a buffer containing 2% Triton X-100, CaCl₂ at 0.2 g/liter, KCl at 0.4 g/liter, MgSO₄ at 0.1 g/liter, NaCl at 6.4 g/liter, and 50 mM Tris, pH 7.5. After a 5-h incubation at room temperature the Triton-soluble and -insoluble fractions were obtained by centrifugation at 12,000 × g for 10 min. Purified pro-MMP-9 was added to these fractions and incubated at 37 °C for 20 h followed by the zymogram analysis.

Gelatinolytic Zymogram

The conditioned media were mixed with SDS-PAGE sample buffer in the absence of reducing agent and electrophoresed in 10% polyacrylamide gel containing 0.1% (w/v) gelatin. Electrophoresis was performed at 4 °C with 120 V for 16 h. After electrophoresis, SDS in the gel was removed by incubation with 2.5% Triton X-100. Gelatinolytic activities were developed in buffer containing 5 mM CaCl₂, 150 mM NaCl, and 50 mM Tris, pH 7.5, for 16 h at 37 °C. The gelatinolytic activities were visualized by staining with Coomassie Blue R-250.

Western Blot

The gelatinase from conditioned media was enriched by binding it to gelatin-conjugated Sepharose 4B. Briefly, 1 ml of conditioned media was incubated with 40 µl of gelatin-Sepharose 4B (Amersham Pharmacia Biotech) for 3 h. The beads were washed four times with 0.4 M NaCl in 50 mM Tris, pH 7.5. The bound protein was eluted with 1× SDS-PAGE sample buffer at the reducing condition. After SDS-PAGE, the protein was transferred to Immobilon-P (Millipore). The protein blot was exposed to anti-human MMP-9 antibodies and followed by horse-radish peroxidase-conjugated secondary antibodies that were detected by enhanced chemiluminescence.

Reverse Transcriptase-Polymerase Chain Reaction

Stimulation—The dermal fibroblasts were grown in 10-cm dishes until subconfluent. To induce quiescence in the cells, the dishes were washed with DMEM and incubated in serum-free DMEM for 4 h. For the titration experiment, the dishes were stimulated with TNF-α (10 ng/ml) and TGF-β (1 ng/ml) individually or in combination in DMEM for 20 h. For the time course experiment the cells were stimulated by a combination of TNF-α with TGF-β and harvested 0, 2, 4, 6, 12, and 20 h later.
**Total RNA Preparation**—After the dishes were stimulated for the indicated time, they were washed with phosphate-buffered saline, and the total RNA was extracted by Trizol. RNA was quantified by measuring the adsorption at 260 nm.

**Reverse Transcription and PCR**—2µg of total RNA was reverse-transcribed using avian myeloblastosis virus-reverse transcriptase with 1 µg of random deoxynucleotide hexamer in the presence of 4 mM MgCl₂ and 2.5 mM dNTP. After completion of the 1-h incubation at 37 °C, the reaction was terminated via heating to 94 °C for 3 min. The annealing temperature for amplification of human MMP-9 and β-actin was 64 °C (ROBOCYCLER, Stratagene). The PCR amplification was performed with platinum *Taq* DNA polymerase for 30 cycles. The product was resolved in agarose gel (1.8%), followed by staining with ethidium bromide, and recorded by digital camera. The relative density of the products was quantitated by the Alpha imaging system. The oligonucleotide primers for PCR were adapted from a previous report (30,31). The predicted PCR product size for β-actin and for MMP-9 is 548 and 479 bp, respectively. The identity of the PCR product for MMP-9 was confirmed by DNA sequencing (Norris Cancer Center, University of Southern California).

### Plasmid Constructs

DNA fragments containing the 5′-region of the human MMP-9 gene and the NF-κB-response elements were inserted into pBLCAT2 (32). The wild type NF-κB reporter plasmid, pNFκB3x-CAT, was constructed by inserting an enhancer element into pBLCAT2 at HindIII/BamHI sites. The insertion fragment consists of a triple tandem repeat of the NF-κB consensus binding site with the sequence of 5′-AGC TTG GGA CTT TCC GGG ACT TTC CGG GAC TTT CCG GAT-3′ (Promega). An NF-κB enhancer mutant, pNFκBm3x-CAT, was constructed by insertion of the adapter (5′-AGC TGT ACA CTT TCC TAC ACT TTCC TAC ACT TTC CG-3′) into pBLCAT2.

The plasmid containing the wild type 670-bp upstream region of human MMP-9 gene was a gracious gift of Drs. Sato and Boyd (33,34). To facilitate reconstruction, the entire 670-bp promoter was inserted into pBLCAT2 and named as pM9–670-CAT. Briefly, the 670-bp promoter was amplified by PCR with the forward primer 5′-AAG CTT GAG GCT ACT GTC CCC-3′ and the reverse primer 5′-TCT AGA GGT GTC TGA CTG CAG GTG-3′. The PCR product was cloned into pCR2.1-TOPO, an intermediate vector (Invitrogen). Finally, the insert from the TOPO vector was inserted into pBLCAT2.

A p65 NF-κB consensus binding site at −601 was characterized previously (35). Scanning the 670-bp promoter for potential transcription factor binding sites, we noticed a consensus p50 NF-κB-binding site located at −328 from the transcription start site (MatInspector version 2.2, GSF-National Research Center for Environment and Health). A p65 NF-κB enhancer deletion construct, pM9–590-CAT, that contains the 590-bp region upstream from the transcription start site was created. This construct was generated by PCR with a forward primer 5′-AAG CTT AGC CTT GCC TAG CAG AGC CCA TTC-3′ and a backward primer 5′-TCT AGA GGT GTC TGA CTG CAG GTG-3′. Another deletion construct, pM9–460-CAT, was generated by deleting the p65 NF-κB and the potential TGF-β-response element (TRE) (−474). This plasmid was constructed by digesting pM9–670-CAT with HindIII and EcoRV, filling-in, and self-ligation. A mutation construct in the TRE, pM9–670-mTRE-CAT, was generated by PCR-based mutagenesis. Briefly, the TRE mutation was generated by PCR using sequencing grade *Taq* DNA polymerase with a forward primer 5′-AAG CTT AGC CTT GCC TAG CAG ACT GCT CTC CCC-3′ and reverse primer 5′-GTC AGA TAT CCT CCC CTG ATC ATC CCT CCC CAC ACT-3′. In this TRE mutant the wild type sequence 5′-AGGTTTGGGGA-3′ was substituted by 5′-TGATCAGGGGA-3′ (the mutant bases are underlined). The PCR product was ligated to pCR2.1-TOPO vector. Then the 200-bp HindIII/EcoRV fragment from the wild type pM9–670-CAT
was replaced by the mutant version. Finally, we created a site-directed mutant at the potential p50 NF-κB-binding region (−328/−319) and named it pM9–670-mp50-CAT. This was accomplished by the QuickChange Site-directed Mutagenesis Kit (Stratagene) with the following primer: 5′-TCA GAC CAA GGG ATG AAG GAT AAC TCC AGC TTC ATC CCC CTC CC-3′ (the mismatched four nucleotides are underlined). The insertion fragments of the wild type, the mutant pM9–670mTRE-CAT, and mutant pM9–670-mp50-CAT were confirmed by DNA sequencing (Norris Cancer Center, University of Southern California).

**Transient Transfection, Cytokine Stimulation, and CAT Assay**

The early passages of human dermal fibroblasts were seeded in 6-cm dishes. Transfection was conducted with 0.5-µg plasmid and LipofectAMINE-PLUS according to the manufacturer’s instructions (Life Technologies, Inc.). After incubation for 3 h the plasmid complex was removed and replaced with 0.5% fetal bovine serum/DMEM, and then cytokines were added (TNF-α at 10 ng/ml and TGF-β 1 ng/ml). For the time course experiment the cells were harvested at 24, 48, and 60 h post-transfection. For promoter analysis experiments most of the transfection times were 62 h. The cells were washed by phosphate-buffered saline and harvested in 400-µl 0.25 M Tris at pH 8.0 buffer. The cells were lysed via three rounds of quick freeze and thaw followed by heating for 10 min at 60 °C. The extracts were briefly centrifuged, and the supernatant was harvested for the CAT assay. The reaction was performed in a 125-µl system with 50 µl of lysate, 1 µl of [14-C]chlor-amphenicol (PerkinElmer Life Sciences, 1.9 MBq/ml), 5 µl of 10 m butyl-CoA (Roche Molecular Biochemicals), and 69 µl of the 0.25 M Tris, pH 8.0. After incubating at 37 °C for 14 h, the lipid phase was extracted by chloroform. The products were resolved by silica gel TLC (Whatman) with 3% methanol and 97% chloroform. The acetylated products were detected by PhosphorImaging (Molecular Dynamics).

**RESULTS**

**Cytokine-mediated Induction and Activation of MMP-9 in Human Skin**

To establish the profile of cytokine-exerted MMP-9 induction and activation, we first utilized organ culture of human skin. Normal human skin, discarded after reconstructive surgery, was cultured in serum-free DMEM and exposed to TNF-α and TGF-β either individually or combined (10 ng/ml for TNF-α and 1 ng/ml for TGF-β). The conditioned media were sampled at the indicated time points. The resultant conditioned media were assayed for type IV collagenase activity by gelatinolytic zymography. In the absence of exogenous cytokine, minimal 92-kDa gelatinolytic activity was present in the conditioned medium after culturing for 72 h (Fig. 1A). Treatment of the skin with TNF-α induced a small increase of the 92-kDa gelatinolytic activity. Remarkably, in the presence of TNF-α, the 92-kDa gelatinolytic activity progressively disappeared with a concomitant increase in the 82-kDa gelatinolytic activity. The 92- and 82-kDa gelatinolytic activities were confirmed as MMP-9 by Western blot (Fig. 1B). In contrast, the exposure of the skin to TGF-β markedly induced the 92-kDa pro-MMP-9. Unlike the response to TNF-α, TGF-β failed to promote MMP-9 proteolytic activation to the 82-kDa form. When both cytokines were applied, a synergistic induction of pro-MMP-9 was detectable at 48 h. Furthermore, simultaneous exposure to the two cytokines led to substantial conversion of the pro-MMP-9 to the active form. After cultivation with the two cytokines for 96 h, most of the pro-MMP-9 was converted to the active 82-kDa active form.

Clinical investigations have outlined a temporal pattern for pro-MMP-9 expression and activation in normal healing cutaneous wound sites. Persistent elevation of MMP-9 was found in association with chronic wounds (36–38). However, causal factors to induce and activate the proteinase in human tissue at wound sites are still obscure. Our findings reported here show
a specific role for the inflammatory cytokines in the induction and activation of MMP-9 in human tissue.

**Characterization of the TNF-α-induced Proteolytic Activation of Pro-MMP-9**

The next question was dissecting the nature of the TNF-α-induced activation of pro-MMP-9 in the organ-cultured human skin and whether activation is performed by a skin-associated factor or a secreted soluble factor. The skin explants were stimulated with or without TNF-α for 70 h, and the conditioned media were collected. The treated explants were washed and re-plated in fresh DMEM without additional cytokine. The previous conditioned medium and the re-plated culture were incubated at 37 °C and sampled at 0, 8, and 20 h (Fig. 2). As shown by zymography, the 82-kDa MMP-9 was generated by direct contact with the skin tissue but not by incubation with the conditioned medium. Next, we attempted to extract the pro-MMP-9 activator from the TNF-α-treated skin. The TNF-α-treated explant was extracted by nonionic detergent, Triton X-100. Purified pro-MMP-9 was added to the Triton-soluble and -insoluble fractions and incubated for 20 h (Fig. 3). The results show that pro-MMP-9 is processed to the 82-kDa form in the Triton-insoluble fraction, which suggests that the unidentified pro-MMP-9 activator is tightly associated with the tissue structure.

**Cytokine-mediated Regulation of MMP-9 in Human Dermal Fibroblasts and Epidermal Keratinocytes**

To understand the cellular mechanism of the cytokine-mediated induction and activation of pro-MMP-9 from intact skin, we studied the MMP profile in isolated dermal fibroblasts and epidermal keratinocytes. The dermal environment was simulated by embedding the fibroblasts in three-dimensional type I collagen lattices. Individual cytokines including TNF-α, TGF-β1, PDGF-AB, EGF, IL-8, and IL-6 were applied at 10 ng/ml in the serum-free culture medium. In another panel, a combination of TGF-β with other cytokines was applied to the cells. After culturing for 68 h, the conditioned media were analyzed for gelatinolytic activity (Fig. 4A). The results show that TNF-α alone could moderately induce the 92-kDa gelatinolytic activity, and the other cytokines in the list did not induce the 92-kDa gelatinolytic activity. Remarkably, the combination of TNF-α with TGF-β led to a synergistic induction of the 92-kDa gelatinolytic activity in the fibroblasts. The identity of the 92-kDa gelatinolytic activity was confirmed to be pro-MMP-9 by Western blot (Fig. 4B). In combination with TGF-β, the other cytokines tested, PDGF-AB, EGF, IL-6 and IL-8, all failed to induce the MMP-9. We also tested the combination of these cytokines with TNF-α and found no induction of MMP-9 (data not show). Taken together, these experiments demonstrated that two signals, TNF-α and TGF-β, are required for maximal induction of the pro-MMP-9 in the dermal fibroblasts.

To complete the dissection we also analyzed the effect of these cytokines on the proteinase from the keratinocytes. The early passages of isolated adult keratinocytes cultured as monolayers were stimulated by TNF-α and/or TGF-β. Similar to the results with fibroblasts, TNF-α alone could induce pro-MMP-9 expression, and this effect was enhanced by TGF-β exposure (Fig. 4C). In contrast to fibroblast cultures where TGF-β alone failed to induce the MMP-9, TGF-β alone was sufficient to induce the proteinase in the keratinocytes.

These experiments demonstrated that the synergistic induction of pro-MMP-9 in human skin by TNF-α and TGF-β could be reproduced by both dermal fibroblasts and keratinocytes. However, neither TNF-α alone nor in combination with other cytokines could promote the proteolytic conversion of pro-MMP-9 to the 82-kDa active enzyme in the isolated primary cells. We then hypothesized that an additional skin cell such as dendritic cells or the interaction of several different skin cells might be required for the pro-MMP-9 activation found in intact skin. We tested the effect of the cytokine on the proteinase activation by monocyte and granuocyte colony-stimulating factor-stimulated dendritic cells (kindly provided by Dr. Jeffery...
Webber, University of Southern California). Neither of these cells activated the pro-MMP-9 by TNF-α stimulation. In addition, we performed co-culture of the keratinocytes and fibroblasts. In all these experiments we failed to reconstruct the proteolytic activation of pro-MMP-9 from the human skin.

As we demonstrated previously, TNF-α stimulated fibroblasts in a collagen environment to convert the 72-kDa pro-MMP-2 into the active 62-kDa MMP-2 (29). In this report, we show that TGF-β can also partially stimulate the activation of pro-MMP-2 (Fig. 4A). Other cytokines such as PDGF, IL-8, IL-6, and EGF had no significant effect on the activation of pro-MMP-2 in this system. Another difference between the fibroblast and keratinocyte responses is that the 72-kDa pro-MMP-2 is constantly expressed in fibroblasts, whereas it is induced by TGF-β in the keratinocyte.

**Time Course of Co-induction Pro-MMP-9**

To investigate the kinetics of pro-MMP-9 induction, we performed a time course experiment using human dermal fibroblasts either embedded in collagen lattices or cultured as monolayers. This experimental design could also delineate the role of collagen in the cytokine-mediated MMP-9 expression and activation. The collagen lattices and monolayers were stimulated with combinations of TNF-α and TGF-β both at 10 ng/ml. The conditioned media were sampled at the indicated time points. Results show that in the absence of cytokine co-stimulation, neither the 92-kDa pro-MMP-9 nor the 82-kDa form was expressed in either culture condition. This result indicates that collagen *per se* does not contribute to MMP-9 induction (Fig. 5). As we reported previously, these data reiterate that the pro-MMP-2 activation, as measured by accumulation of 62-kDa MMP-2, is moderately enhanced by collagen. Exposure of the monolayers or the lattices simultaneously with TNF-α and TGF-β led to an equal and substantial induction of 92-kDa pro-MMP-9. The time course experiment shows that significant amounts of pro-MMP-9 protein are accumulated in the conditioned medium between 6 and 20 h after exposure of the fibroblasts to these cytokines.

**The Minimal Concentration of TNF-α and TGF-β on Pro-MMP-9 Induction**

Because collagen apparently had no significant effect on the cytokine-mediated co-induction of pro-MMP-9, we measured the efficacy of TNF-α and TGF-β on pro-MMP-9 expression in monolayer cultures. Since both cytokines are required for the induction of the proteinase in the fibroblasts, we fixed the concentration of one cytokine at 10 ng/ml and varied the other one to determine the minimal efficacy. The results show that at a fixed concentration of TNF-α at 10 ng/ml, TGF-β stimulated the fibroblasts to express pro-MMP-9 in a dose-dependent manner (Fig. 6). With a sufficient amount of TNF-α, TGF-β1 at concentrations as low as 0.1 ng/ml could significantly induce pro-MMP-9 expression, and vice versa, when the TGF-β concentration was fixed at 10 ng/ml, TNF-α stimulated the fibroblasts with dose dependence. With sufficient TGF-β, TNF-α at 0.1 ng/ml could clearly stimulate the fibroblasts to express pro-MMP-9. These concentrations for the efficacy of TNF-α and TGF-β on MMP-9 induction are important because they are similar to concentrations reported previously of the cytokine required to induce angiogenesis and wound healing (1,39–41).

**TNF-α- and TGF-β-mediated Induction of Pro-MMP-9 Is Regulated at mRNA Level**

To address the mechanism of the cytokine-mediated induction of pro-MMP-9 protein, we measured the levels of MMP-9 mRNA in response to the cytokines individually or combined. Results revealed that the MMP-9 mRNA level was significantly higher after stimulation by TNF-α for 20 h, whereas TGF-β individually only slightly increased the proteinase mRNA (Fig. 7A). Simultaneous stimulation by the cytokines led to an additional increase of MMP-9 mRNA. A time course experiment was performed by co-stimulation of the fibroblasts with these two cytokines. As shown in Fig. 7B, the MMP-9 mRNA is induced at 4 h after stimulation,
and the steady state was reached after stimulation for 6 h (Fig. 7B). As a control, the mRNA level of β-actin, a housekeeping gene, was constitutively expressed and unchanged in response to the cytokines. These experimental results suggest that the cytokine-mediated induction of pro-MMP-9 is up-regulated at the mRNA level. The temporal induction of MMP-9 mRNA level (the half-time for the maximal induction, t½ = 3 h) correlated well with the elevated MMP-9 protein level (t½ = 10 h) as well as with the pro-MMP-9 induction in the organ-cultured human skin (t½ = 24 h). The rapid induction of MMP-9 mRNA by these cytokines suggests that the regulation is likely through the direct activation of the transcriptional machinery for the MMP-9 gene. To support this notion we tested the effects of the transcriptional inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide, on the pro-MMP-9 induction. As expected, the cytokine-mediated induction of pro-MMP-9 was totally attenuated by actinomycin D at 2.5 μg/ml and cycloheximide at 5 μg/ml (Fig. 8).

**TNF-α and TGF-β Activate the Human MMP-9 Gene Promoter**

The 5′-regulatory region of the human MMP-9 gene has been documented (34,35,42). The reporter assay experiment shows that the minimal response elements for TNF-α stimulation are located within −670 bp upstream of the transcription start site. Therefore we tested the cytokine-mediated MMP-9 promoter activation in human dermal fibroblasts that have the cytokine responsiveness of the proteinase at both protein and mRNA levels. The cells were transiently transfected with a plasmid containing a CAT reporter gene driven by a segment of 670 bp from the 5′-promoter region of human MMP-9 gene. After transfection the cells were stimulated with either an individual cytokine or a combination of two cytokines. Results showed that TNF-α or TGF-β individually could activate the 670-bp promoter (Fig. 9A). In fact the promoter activation could be measured after stimulation for 24 h. The combination of the two cytokines led to a minor additional stimulation. The transcriptional activation, as measured by CAT assay, solely depends on the MMP-9 promoter because these cytokines failed to activate the vector plasmid that has the thymidine kinase basal promoter (data not shown). The conditioned media from the transfected cells were also assayed for MMP-9 activities, which may represent the endogenous MMP-9 promoter activation (Fig. 9B). These experiments demonstrate that induction of MMP-9 by TNF-α and TGF-β is regulated at least in part within the 5′-promoter region of the human gene.

**One of the Two Potential NF-κB-responsive Elements in the MMP-9 Promoter Is Essential for TNF-α to Activate the Transcription**

An NF-κB-responsive element, TGGAAATTC, which is located −601 to −592 bp upstream of the transcription start site, was previously defined in tumor cells (35). However, the role of this cis-element in TNF-α-mediated induction of MMP-9 in normal human cells has not been addressed. We scanned the 5′-promoter region of the human MMP-9 for potential transcription factor binding sites and found that another potential NF-κB-response element, GGGGGATCC, is located −328 to −319 bp upstream from the transcription start site. The difference between the two NF-κB sites is that the −600/−591 site matches to the subtype p65 NF-κB-binding site, whereas the −328/−319 site matches to p50 NF-κB-binding site. To clarify the role of these two potential NF-κB elements in the induction of MMP-9 promoter, we tested the −601/−592 NF-κB deletion variant, pM9–590-CAT (which retains the −328/−319 NF-κB site) for cytokine-induced trans-activation. Our data showed that deletion of the −601/−592 NF-κB site led to a failure of response to TNF-α but partially retained the TGF-β responsiveness in the dermal fibroblasts (Fig. 10A). This result also indicates that the −328/−319 NF-κB site is not sufficient to mediate the TNF-α signal for transactivation of the promoter. To verify this notion we generated a site-directed mutant within the −328/−319 element, pM9–670-mp50-CAT, of which four conservative nucleotides were replaced (illustrated in Fig. 10B). The CAT assay results show that this mutant has similar responsiveness to the cytokines as the wild type...
To elucidate whether the TNF-α /TGF-β synergistic induction of MMP-9 is a convergence of the two cytokine-initiated pathways on NF-κB signaling, we engineered a triple NF-κB-responsive element in the CAT reporter plasmid (pNFxB3x-CAT). A control plasmid that has the mutated nucleotides within the NF-κB-response element was also generated (pNFxBm3x-CAT). The dermal fibroblasts were transfected by these plasmids and stimulated with TNF-α and TGF-β individually or simultaneously. The results demonstrate that TNF-α, but not TGF-β, activates the NF-κB pathway (data not shown). This also implied that the TGF-β signaling may directly act on the promoter rather than by cross-talking to synergy in the NF-κB pathway.

**DISCUSSION**

The specific mechanisms whereby the early, inflammatory stages of wound healing progress to the later, synthetic phases are not well understood. TNF-α is part of the early cytokine cascade after skin injury. TGF-β is deposited in wounds by platelets trapped in fibrin clots during hemostasis. MMP-9 is present during normal healing for the first few days after injury and then disappears. The specific role of MMP-9 in normal healing is not known. However, the elevated pro-MMP-9 level and more significantly the accumulated active 82-kDa MMP-9 is associated with non-healing wounds (19,26,27,37). Based on the substrate preference for type IV collagen, the basement membrane matrix, the implications for the dys-regulation of this proteinase are also clear. Poorly healing wounds have increased levels of inflammatory mediators, including TNF-α (45). If normal healing requires the down-regulation of MMP-9 to be successful at a defined spatial and temporal manner, then increased TNF-α and TGF-β in the wound maintains MMP-9 activity, and a potential mechanism for failed wound healing is evident. In this report we demonstrate that an inflammatory cytokine, TNF-α, potentiates
activation of pro-MMP-9 in cultured human skin. Based on the facts that both TNF-α and active MMP-9 are elevated in chronic wounds and invasive tumors, we believe that the presence of this inflammatory cytokine may be the causal factor for the proteolytic activation of the proteinase, which in turn breaks down type IV collagen in the basement membrane and thus leads to abnormal healing or tumor cell invasion.

This report provides the first evidence that cytokines can induce and proteolytically activate MMP-9 in intact human skin. Specifically, we found that TGF-β induces pro-MMP-9 expression, and this induction is enhanced by TNF-α. In addition, in the presence of TNF-α, pro-MMP-9 is proteolytically converted to the 82-kDa active enzyme. This TNF-α-mediated proteolytic activation could not be detected in the isolated fibroblast, keratinocyte, or the dendritic cell cultures. However the data suggest that the unidentified TNF-α-mediated MMP-9 activator is tightly associated to the skin tissue.

Previous reports showed that TGF-β and TNF-α individually could induce MMP-9 (22,46–48). However, these results came mainly from tumor-derived cells or lymphocytes, which are not relevant to the reactions of normal human skin. The synergistic effect of the two cytokines on pro-MMP-9 induction in the organ-cultured skin correlated well with the two cytokine-exerted inductions of the enzyme in fibroblasts and keratinocytes. Based on our data we believe that the collective response of the cytokine-mediated MMP-9 expression in human skin is contributed, in part, from both the dermal fibroblasts and epidermal keratinocytes. Defining the minimal efficacy of TNF-α and TGF-β on pro-MMP-9 induction at their level of sub-nanogram level is important because they are within the physiological or pathological region. Three lines of evidence demonstrated here suggest that the cytokine-mediated pro-MMP-9 induction is regulated at transcriptional control. 1) The MMP-9 mRNA is promptly raised after stimulation. 2) Induction of the enzyme is attenuated by actinomycin D. 3) The MMP-9 promoter is activated by the cytokines.

The molecular mechanism for proteolytic activation of pro-MMP-9 in vivo is not clarified. In vitro experiments show that many proteinases including cathepsin G, trypsin, α-chymotrypsin and stromelysin-1 (MMP-3), collagenase-1, matrilysin, and MMP-2 can activate this enzyme (49–51). Based on our finding of TNF-α-mediated MMP-9 activation in the skin, we have surveyed whether TNF-α can induce MMP-3 (stromelysin-1). Indeed, we found that TNF-α could induce MMP-3 in the skin organ culture. However, in dermal fibroblast culture we also found elevated MMP-3 levels, whereas the pro-MMP-9 failed to be activated. In fact, in the stromelysin-1 (MMP-3) knockout mice, pro-MMP-9 could still be activated, which suggested a potential stromelysin-1-independent pro-MMP-9 activation in mice (52). Thus, the cellular and molecular mechanism for the TNF-α-mediated activation of pro-MMP-9 in the human skin remains to be elucidated.

The regulatory elements in the 5′-flanking region of human MMP-9 gene have been analyzed previously (35,42). The TNF-α-response element was identified within the 670-bp of the 5′-promoter region of the human gene. In this study we confirmed the importance of the −601 p65 NF-κB for the TNF-α-mediated transcriptional activation of the MMP-9 gene in the human dermal fibroblasts. In contrast, the −328 p50 NF-κB site is not essential for the TNF-α-mediated transcription of the MMP-9.

A consensus TGF-β-response element was previously recognized at −474 bp in the promoter of the human MMP-9 gene, although the role of this element was not characterized (35). Our speculation that this might be the TGF-β-response element in the 670-bp promoter was demonstrated by mutation and deletion experiments. These showed the essential role of this

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site in TGF-β-mediated MMP-9 promoter activation. Although it was previously defined as an inhibitory element (43), this element can mediate transcription activation of transglutaminase gene in one cell line and inhibition of the same promoter in another cell type (44). The choice between activation and inhibition of transactivation seems to rely on other cellular factors pre-deposited or activated in the particular cell type. In this study we have demonstrated that TNF-α and TGF-β individually activated their distinct response cis-elements in the 670-bp human MMP-9 promoter. Nevertheless, we did not observe the full synergistic transactivation by simultaneous stimulation of the transient transfected cells with TNF-α and TGF-β, whereas the synergy is overwhelming in the enzyme induction. Such a difference may derive from the additional hierarchic regulatory elements in the endogenous promoter of the MMP-9 gene, which is not included in the 670-bp version of the promoter. Interestingly, similar co-induction by TNF-α and TGF-β was observed for human type VII collagen, in which both NF-κB and SMAD factors were found recruited to the 5′-flanking promoter (53). The signal pathway that regulates MMP-9 expression is not understood despite some information suggesting roles for c-Jun amino-terminal kinase and the extracellular signal-regulated kinase (34).

This is the second report from our laboratory indicating that the activity of a particular inflammatory cytokine, TNF-α, is a part of a complex regulatory system for matrix metalloproteinases. Because this cytokine is present during the early period after injury, these findings suggest a role for this mediator in the initial induction and activation of proteinases in the initial stages of healing. The specific role of the MMPs in wound healing is, as yet, not fully understood. Type IV collagen is a substrate for the MMP-9 and an important component of the epidermal basement membrane (54). We speculate that properly induced and activated MMP-9 may play a positive role in tissue repair by promoting keratinocyte detachment from the basement zone through controlled digestion of the type IV collagen. Changing the structure of this matrix protein may make it easier for epidermal cells to detach and begin migrating along the wound margin. Control of this process would be essential, since continued dissolution of the basement membrane would prevent a normal epidermal layer from developing. In the healing wound, TNF-α and TGF-β are seldom both present beyond the first few days after injury. Because there are response elements for both cytokines in the promoter of MMP-9, transcription of this proteinase is more tightly controlled. Validating this hypothesis will be the focus of future experiments.

There are several unanswered questions from the current literature. What is the specific role of MMP-9 in the initial phase of normal wound healing? What does MMP-9 do in chronic wounds to prevent healing to progress? Will blocking TNF-α decrease the activation of MMP-9 in chronic wounds? More importantly, will blocking TNF-α cause chronic wounds to heal? Although TGF-β also appears to be involved in the up-regulation of MMP-9, it is usually present in multiple phases of wound healing. This fact makes us conclude that manipulation of TGF-β concentration is less likely to affect MMP-induced abnormal wound healing. These are questions that will require significant further study.

In conclusion, our results provide integrated studies of multiple cytokines on MMP-9 induction and activation initiated from human skin and extended to the isolated skin cells. The analysis of the cytokine-response elements in the promoter of human MMP-9 gene gives us enriched information for future analysis of abnormal MMP-9 regulation in cancer and aberrant wound healing. These findings may provide an in-depth knowledge of type IV collagenase expression and activation in wound healing or tumor cell metastasis where cytokines play a central role to orchestrate a multilevel of ECM and MMP gene expression, ECM deposition, and degradation.
Acknowledgments

We thank Dr. Peter Laird for valuable editorial assistance and Dr. Susan Downey for supplying of the human skin discarded after reconstructive procedures. We also thank Drs. Nimni and Han for sharing their equipment.

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FIG. 1. Cytokine-mediated induction and activation of MMP-9 in organ-cultured human skin
Normal full thickness human skin was cultured in serum-free DMEM and stimulated by TNF-α (10 ng/ml) and TGF-β (1 ng/ml) either individually or combined. Skin samples from eight distinct individuals were examined for cytokine-mediated induction and activation of MMP-9. All showed similar results. A, conditioned media were sampled at the indicated time points and analyzed for gelatinolytic activities by zymogram. The 92- and 82-kDa gelatinolytic activities are indicated by arrows. B, identities of the 92- and 82-kDa gelatinolytic activities as pro-MMP-9 and active MMP-9 were determined by Western blot. The total gelatinase from 500-µl conditioned media derived from 72-h culture was enriched by gelatin-conjugated Sepharose 4B matrix. The bound protein was resolved by SDS-PAGE and detected by Western blot using polyclonal anti-MMP-9 antibodies.
FIG. 2. TNF-α-mediated pro-MMP-9 activation occurs in the skin tissue but not in the medium
Human skin was cultured in DMEM with or without TNF-α. After 70 h of incubation the conditioned media were collected. The skin tissues were washed and replanted in the fresh DMEM. The original conditioned media and the replanted skin tissue in DMEM were incubated at 37 °C, and media were sampled at the indicated time points. MMP-9 activities were measured by gelatinolytic zymogram.
FIG. 3. Proteolytic activation of pro-MMP-9 in the cell-free skin extract

Adult human skin was stimulated with TNF-α (10 ng/ml) in DMEM for 70 h. The tissue was washed and minced following extraction using 2% Triton X-100 (see “Experimental Procedures”). The detergent-soluble and -insoluble fractions were separated by centrifugation. Purified pro-MMP-9 was added or not to these fractions and incubated at 37 °C for 20 h. MMP-9 activities were measured by gelatinolytic zymogram.
FIG. 4. Cytokine-mediated induction of pro-MMP-9 in human dermal fibroblasts and epidermal keratinocyte
A, dermal fibroblasts were isolated from the adult human skin, and early passages were embedded into type I collagen. The cells were cultured in serum-free DMEM and stimulated with TNF-α, TGF-β, PDGF-BB, EGF, IL-8, and IL-6 at 10 ng/ml. In another panel the cells were stimulated with a combination of TGF-β with TNF-α, PDGF, EFG, IL-8, or IL-6, all at 10 ng/ml. After cultivation for 68 h the conditioned media were analyzed for gelatinolytic zymography. The 92-kDa MMP-9 and 72- and 62-kDa MMP-2 are indicated. B, the identity of the 92-kDa gelatinase activity was confirmed as MMP-9. The total gelatinase from 500-µl conditioned media was enriched by gelatin-conjugated Sepharose 4B matrix. The bound protein was resolved by SDS-PAGE and detected for MMP-9 by Western blot. C, the keratinocytes were isolated from the epidermal portion of adult human skin and the early passages were used for the experiment. The confluent monolayers were stimulated by TNF-α and TGF-β individually or combined in basal medium (KBM) for 70 h. A typical zymography is presented.
FIG. 5. Time course of co-induction of pro-MMP-9 by TNF-α and TGF-β

The early passages of human dermal fibroblasts were cultured as monolayers or embedded in type I collagen. The cells were stimulated by a combination of TNF-α (10 ng/ml) and TGF-β (10 ng/ml) in DMEM. A, the fibroblast monolayers were stimulated by the combined cytokines, and conditioned media was sampled at the indicated time points and analyzed for gelatinolytic activities. B, the fibroblasts embedded in collagen lattices were stimulated by the cytokines, and conditioned media were assayed for gelatinolytic activities.
FIG. 6. Concentration dependence of the cytokine-mediated induction of pro-MMP-9
The human dermal fibroblasts were cultured as monolayers. One set of six wells was stimulated with TNF-α at 10 ng/ml and with varying concentrations of TGF-β as indicated. Another set of six wells was stimulated by TGF-β at 10 ng/ml and varying concentrations of TNF-α. After stimulation for 48 h the conditioned media were analyzed for gelatinolytic zymography.
FIG. 7. The cytokine-mediated induction of MMP-9 is up-regulated at the mRNA level

A, the human dermal fibroblasts as monolayers were stimulated by TNF-α (10 ng/ml) and TGF-β (10 ng/ml) individually or combined in serum-free DMEM. After stimulation for 20 h the total RNA was extracted. The mRNA levels of MMP-9 and β-actin were determined by RT-PCR. For the β-actin, one-third of PCR product was loaded into the gel as shown. The identity of PCR product for MMP-9 was confirmed by its expected size and DNA sequencing.

B, time course of MMP-9 mRNA co-induction by the two cytokines. The mRNA level of MMP-9 and β-actin was measured at the indicated time points, and the relative amount was quantitated by Alpha-imaging.
FIG. 8. Actinomycin D and cycloheximide attenuates the cytokine-mediated induction of pro-MMP-9 in dermal fibroblasts
The dermal fibroblasts were embedded in collagen and stimulated with or without TNF-α (10 ng/ml) and TGF-β (1 ng/ml) as indicated. Actinomycin D (Act D) and cycloheximide (CHX) were applied at the indicated concentration in the DMEM. After 70-h culture the conditioned media were analyzed for gelatinolytic zymography.
FIG. 9. TNF-α and TGF-β activates the human MMP-9 promoter

The human dermal fibroblasts were transiently transfected by pMMP9–670-CAT, which contains 670 bp of 5’-promoter of the human MMP-9 gene. After a 3-h transfection the cells were stimulated by TNF-α (10 ng/ml) and TGF-β (1 ng/ml) as indicated for an additional 24, 48, and 68 h. The promoter activation was measured by CAT assay. A, the CAT activities expressed in the cells are shown as the percentage of acetylated products measured by PhosphorImaging and are the average of three replicates. B, the conditioned medium of the transient transfected cells was analyzed for gelatinolytic activities, which represent the endogenous MMP-9 induction.
FIG. 10. One of the two potential NF-κB-responsive elements in the MMP-9 promoter is essential for TNF-α-mediated promoter activation
The dermal fibroblasts were transiently transfected with pM9–670-CAT (wild type), pM9–590-CAT (p65NF-κB delete), and pM9–670-mp50-CAT (p50NF-κB site mutant). After transfection for 3 h the cells were stimulated with the cytokines individually or combined. A, the CAT activities expressed in the cells were measured. The values represent the mean of results obtained from three replicate culture wells. B, illustration of the construction used in the experiments. The potential p65 and p50 NF-κB-binding sites, and the mutant version are illustrated.
FIG. 11. TGF-β activates the minimal promoter of the human *MMP-9* through the TRE-response site
The human dermal fibroblasts were transiently transfected by the CAT reporter plasmids, pM9–670-CAT (wild type), pM9–670-mTRE-CAT (TRE site mutant), and pM9–460-CAT (p65 NF-κB and TRE delete). The cells were stimulated by cytokines individually or combined. A, the CAT activities from the cells were measured. The mean value of triplicate experiments is indicated for each reporter plasmid. B, the plasmid constructs and the potential cytokine-response elements are illustrated.