Transforming growth factor-β1 increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair?

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Lechapt-Zalcman, E., V. Prulière-Escabasse, D. Advenier, S. Galiacy, C. Charrière-Bertrand, A. Coste, A. Harf, M.-P. d’Ortho, and E. Escudier. Transforming growth factor-β1 increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair? Am J Physiol Lung Cell Mol Physiol 290: L1277–L1282, 2006. First published January 13, 2006; doi:10.1152/ajplung.00149.2005.—In vivo, transforming growth factor (TGF)-β1 and matrix metalloproteinases (MMPs) present at the site of airway injury are thought to contribute to epithelial wound repair. As TGF-β1 can modulate MMP expression and MMPs play an important role in wound repair, we hypothesized that TGF-β1 may enhance airway epithelial repair via MMPs secreted by epithelial cells. We evaluated the in vitro influence of TGF-β1 on wound repair in human airway epithelial cells cultured under conditions allowing differentiation. The results showed that TGF-β1 accelerated in vitro airway wound repair, whereas MMP inhibitors prevented this acceleration. In parallel, we examined the effect of TGF-β1 on the expression of MMP-2 and MMP-9. TGF-β1 induced a dramatic increase of MMP-2 expression with an increased steady-state level of MMP-2 mRNA, contrasting with a slight increase in MMP-9 expression. To confirm the role of MMP-2, we subsequently evaluated the effect of MMP-2 on in vitro airway wound repair and demonstrated that the addition of MMP-2 reproduced the acceleration of wound repair induced by TGF-β1. These results strongly suggest that TGF-β1 increases in vitro airway wound repair via MMP-2 upregulation. It also raises the issue of a different in vivo biological role of MMP-2 and MMP-9 depending on the cytokine microenvironment.

Human airway epithelium cells; matrix metalloproteinase-9; wound healing; cell migration

HUMAN AIRWAY EPITHELIUM ACTS as a protective barrier and is subject to injury during exposure to a variety of inhaled toxins or infectious agents. Repair of epithelial injury requires a wound repair process involving multiple distinct steps, starting with spreading and migration followed by proliferation and differentiation of epithelial cells, which are accompanied by remodeling of the extracellular matrix (ECM) (7, 24). After injury, fibroblasts, inflammatory, endothelial, and epithelial cells produce various cytokines and growth factors including transforming growth factor-β (TGF-β) (19, 28). TGF-β exerts a wide spectrum of biological functions contributing to wound repair (13, 15, 19). In vitro, TGF-β1 is known to modulate the composition of the provisional matrix over which the epithelial cells migrate (16) and to trigger behavioral changes in epithelial cells involved in wound repair (2). TGF-β1 can also regulate the synthesis of certain matrix metalloproteinases (14, 20, 25). Matrix metalloproteinases (MMPs) form a family of zinc-dependent endopeptidases that are able to degrade the various macromolecular components of the ECM. Within the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) more specifically hydrolyze denatured collagens (gelatin) and native type IV collagen (29). MMP-2 and MMP-9 play a major role in physiological tissue remodeling processes, such as organogenesis and wound repair (10, 23). On the other hand, abnormal expression of these proteases is known to contribute to diverse disorders, including healing disorders, occurring in chronic inflammatory airway diseases (1, 11, 12). Although MMP-2 and MMP-9 share structural similarities, there is strong evidence that MMP-2 and MMP-9 have overlapping and independent contributions to physiological and pathological processes (5, 26).

We wondered whether TGF-β1 could modulate the wound repair process of airways via MMPs secreted by epithelial cells. We consequently investigated the effect of TGF-β1 on wound repair in relation to gelatinases. In this paper, we report that TGF-β1 increases MMP-2 expression and the steady-state level of MMP-2 mRNA. Furthermore, TGF-β1 and MMP-2 accelerate in vitro airway wound repair, whereas MMP inhibitors prevent this acceleration. These results strongly suggest that TGF-β1 increases in vitro airway wound repair via MMP-2 up-regulation.

MATERIALS AND METHODS

Reagents

Ham’s F-12 nutrient medium (F12) and Dulbecco’s modified Eagle’s nutrient mixture (DMEM) were purchased from Life Technologies (Cergy-Pontoise, France). Penicillin, streptomycin, amphotericin B, fetal calf serum (FCS), trypsin, and UltraGel G were purchased from GIBCO-BRL (Cergy-Pontoise, France). Dithiothreitol, pronase, lactalbumin, and gentamicin were obtained from Sigma Chemical (L’Ille d’Abeau Chêne, France). Human TGF-β1, tissue inhibitor of metalloproteinase (TIMP)-2, and MMP-2 were obtained from Sigma. BB-3103 (hydroxamic acid-based zinc metalloproteinase inhibitor) was a generous gift from British Biotech (Slough, UK).

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Primary Cultures of Human Nasal Epithelial Cells

Human nasal epithelial cells (HNEC) were isolated from nasal mucosa as previously described (21). Nasal tissues were obtained from the inferior turbinate in 23 snorers undergoing inferior turbinatectomy. Turbinates were removed because of vasomotor but noninflammatory swelling of the mucosa, leading to nasal obstruction. Briefly, nasal tissues were immediately placed in DMEM/F-12 supplemented with antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 μg/ml amphotericin B, and 100 mg/ml gentamicin) and transported to the laboratory for processing. Enzymatic digestion [0.1% (wt/vol) pronase in culture medium] was performed for 16 h at 4°C. HNEC (1 × 10^6 cells per well) were then plated in inserts (12-mm Transwell; Costar, Cambridge, MA) with 12-mm-diameter polycarbonate micropore membranes coated with type IV collagen (Sigma) and incubated at 37°C in 5% CO₂. For the first 24 h, cells were incubated with 1 ml of DMEM/F-12-antibiotics with 2% Ultroser G in the lower chamber and DMEM/F-12-antibiotics with 10% FCS in the insert. After 24 h, the medium in the insert was removed to place the cells at an air-liquid interface. Culture medium (DMEM/F-12-antibiotics with 2% Ultroser G) was then changed daily in the lower chamber. The epithelial nature of the cultured cells was confirmed as previously described (21). As HNEC reached a stable differentiated state with detection of ciliated, secretory, and basal cells during the second and third weeks of culture (21), all experiments were performed at the end of the second week.

For all experiments involving TGF-β1 addition, HNEC were cultured in culture medium until day 13. At the time of the experiment, inserts with cell cultures were deprived of Ultroser G and placed in culture medium until day 13. The epithelial nature of the cultured cells was confirmed as previously described (21). As HNEC reached a stable differentiated state with detection of ciliated, secretory, and basal cells during the second and third weeks of culture (21), all experiments were performed at the end of the second week.

Gelatinase Expression

Basal HNEC gelatinase expression by zymogram analysis. To characterize the time-dependent basal gelatinase expression in HNEC cultures, culture medium was collected daily from day 1 to day 20, from the same well. Gelatinase expression was then determined in culture medium by gelatin zymography as previously described (11). Twelve microliters of tested culture medium were loaded without heating or reduction and resolved by 8% SDS-polyacrylamide gel electrophoresis (1 mg/ml gelatin). In separate experiments, we investigated the proteinase inhibition profile by adding one of the following to the incubation buffer: 5 mM EDTA, an MMP inhibitor; 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), a serine protease inhibitor; or 2 mM N-[N-(t-3-trans-carboxyriane-2-carbonyl)-l-leucyl]-agmatine (Ef64), a cysteine protease inhibitor. We also performed an activation experiment by incubating the samples in the presence of aminophenyl-mercuric acetate (APMA). Molecular weights of gelatinolytic bands were estimated with prestained molecular weight markers. After scanning the gels, we quantified gelatinase expression by automated image analysis (Bio imaging system model; GeneGenius, Ozyme, France). Results were expressed in arbitrary units/24 h (2% Ultroser G medium was used as standard).

Analysis of TGF-β1 effect on HNEC gelatinase expression. Culture medium for each well was collected before addition (basal condition) and 24 h after addition (TGF-β1 condition) or without addition (control) of TGF-β1. Media from the various conditions were analyzed for gelatinase expression by zymography. Results were expressed as a percentage of basal gelatinase expression for each well.

Northern blot analysis of MMP-2 gene expression. Because the effect of TGF-β1 was prominent on MMP-2 expression, we investigated TGF-β1 modulation of MMP-2 gene expression by Northern blot analysis. Cells were collected 24 h after incubation with or without TGF-β1 (TGF-β1 condition and control, respectively). The experiment was performed on four independent cultures. Total RNA was extracted from epithelial cell cultures with TRizol reagent (Life Technologies, Cergy-Pontoise, France) according to the manufacturer’s instruction. The integrity of each RNA sample was assessed by electrophoresis of an aliquot on 1% agarose gel with 0.5 μg of ethidium bromide/ml buffer. Northern blot analysis was performed on equal amounts (20 μg) of total RNA. RNA samples were analyzed by electrophoresis on 0.8% denaturing agarose gel containing 6% (wt/vol) formaldehyde, soaked, and transferred to nylon membranes in 20× standard saline citrate (SSC; 1× SSC is 150 mM sodium chloride and 15 mM sodium citrate). Blots were hybridized with random-primed 32P-labeled cDNA (Prime-a-Gen Labeling System; Promega, Madison, WI) overnight at 60°C in 5× SSC, 50 mM phosphate buffer (pH 7), 5× Denhardt, 0.1% SDS, 0.5 mM EDTA, 4% dextran sulfate, and 100 μg/ml of denatured salmon sperm DNA. Blots were then successively washed, briefly and three times for 10 min each, at room temperature in 2× SSC, 0.5% SDS, and 15 min at 0°C in 0.2× SSC, 0.5% SDS. The membranes were then exposed to X-ray films. The autoradiographies were scanned with the GeneLink apparatus (Syngene) and the GeneSnap (Syngene) software. Quantification was then performed using the GeneTools (Syngene) software. The signal intensity was normalized by 18S rRNA DECAprobe (a 1.2-kb fragment of the mouse 18S RNA gene; Ambion, Austin, TX), cDNA probes of rat liver MMP-2, (generously provided by Dr. N. Theret) were prepared as previously described (27).

Wound Repair Analysis

In vitro wound repair model. The in vitro wound repair assay was carried out according to a model of mechanical injury adapted from a previously described method (22). We introduced a linear wound of 10 mm length × 1.4 mm width by scraping the HNEC with a pipette tip, followed by extensive washing to remove cellular debris. To determine the repair rate, time-lapse images were taken at regular intervals with an inverted microscope (Zeiss, Rueil-Malmaison, France) equipped with an ×4 objective and a charge-coupled device video camera (CCD-IRIS, Sony, Japan) over a period of 24–30 h, depending on the time of wound closure. The image of the wound was subsequently captured by an image-analyzing frame-grabber (Video enhancer FA320, FUTEK). The wound areas were then quantified by image analysis software (Scion Image 1.3b).

Effect of TGF-β1 and MMP-2 on wound repair. Closure in the presence of TGF-β1 was compared with closure without TGF-β1 (control). HNEC cultures were deprived of Ultroser G and placed in culture medium until day 13. The epithelial nature of the cultured cells was confirmed as previously described (21). As HNEC reached a stable differentiated state with detection of ciliated, secretory, and basal cells during the second and third weeks of culture (21), all experiments were performed at the end of the second week.

To investigate the effect of MMP-2 on HNEC repair, we added exogenous MMP-2 (10 ng/ml) 1 h after wounding, for 24 h (i.e., until the repair assay), and wounds were followed by time-lapse analysis.

Fig. 1. Zymogram analysis of matrix metalloproteinase (MMP)-2 and MMP-9 expression after addition of transforming growth factor (TGF)-β1 (5 ng/ml). Culture medium for each well was collected before incubation (basal conditions) and 24 h after incubation with or without TGF-β1 (TGF-β1 condition and control, respectively). The results of three independent cultures are shown. Lanes 1, 3, 5, 7, 9, 11: basal conditions; lanes 2, 6, 10: TGF-β1-treated cultures; lanes 4, 8, 12: controls; lane M: culture medium.
TGF-β1 MODULATION OF AIRWAY WOUND REPAIR

Under basal conditions, zymographic analysis of gelatinase activities in the culture medium showed two main forms: a minor 72-kDa band and a major 95-kDa band, corresponding to the molecular mass of latent MMP-2 and latent MMP-9, respectively. In separate experiments, we investigated the protease inhibition profile by adding EDTA, which completely inhibited the activity of all gelatinases, whereas AEBSF and E64 did not. Moreover, incubation of aliquots of HNEC culture medium in the presence of the APMA organomercurial-activated proforms of MMP-2 and MMP-9, which were visible as 88- and 62-kDa bands, confirmed that the 95- and 72-kDa proteins are indeed MMPs. MMP-2 and MMP-9 expression was detectable on day 2, became stable from day 7, and was then 2 and 10 times the MMP-2 and MMP-9 activity detected in 2% Ultroser G medium (standard), respectively (data not shown).

Zymographic analysis of TGF-β1-containing medium showed that MMP-2 and MMP-9 secreted expressions were both increased (Fig. 1). However, total MMP-2 expression was markedly increased (2.78-fold, \(P = 0.0002\)) compared with the slight increase of total MMP-9 expression (1.2-fold, \(P = 0.04\)). The increased amount of MMP-2 secreted into the TGF-β1-containing medium was measured by zymography quantitation (data not shown) and determined to be equivalent to 10 ng/ml of MMP-2. Northern blot analyses revealed a twofold increase in the steady-state level of MMP-2 mRNA, in TGF-β1-treated HNEC wells compared with controls (Fig. 2).

**TGF-β1 Promotes HNEC Wound Repair In Vitro via MMP-2**

**TGF-β1 accelerates wound repair.** Under serum-free conditions, the wounds closed in ~30 h (Fig. 3). Wound areas were plotted vs. time, and a linear regression was calculated that showed a constant repair rate until closure (data not shown).

With TGF-β1, the wounds closed in about 24 h. We therefore decided to measure the repair rate during the first 24 h. As shown in Fig. 4, addition of TGF-β1 (5 ng/ml) significantly increased the closure repair rate in primary HNEC by 28 ± 12% compared with controls \(P < 0.01\). As for basal conditions, wound areas were plotted vs. time, and a linear regression was calculated, demonstrating a constant rate of repair \(R^2 = 0.99 ± 0.01\)

![Fig. 2. Northern blot analysis of MMP-2 after addition of TGF-β1 (5 ng/ml). Total RNA (20 μg) from 3 human nasal epithelial cell (HNEC) cultures were electrophoresed, transferred to nylon membranes, and hybridized with random-primed \(^32\)P-labeled cDNA probes of rat MMP-2. The signal intensity was electrophoresed, transferred to nylon membranes, and hybridized with random-primed \(^32\)P-labeled cDNA probes of rat MMP-2. The signal intensity was normalized with an 18S rRNA DECAprobe (a 1.2-kb fragment of the mouse 18S RNA gene; Ambion, Austin, TX). The results of 3 independent cultures are shown. Lanes 1, 3, 5: controls; lanes 2, 4, 6: TGF-β1-treated cultures.](http://ajplung.physiology.org/)

**RESULTS**

**TGF-β1 Increases MMP-2 Expression in HNEC**

**Function-blocking experiments.** To investigate the involvement of MMPs in wound repair, the effects of various MMP inhibitors on TGF-β1 or MMP-2-modulated wound repair were analyzed. Untreated (control) and TGF-β1- or MMP-2-treated wells were placed immediately after wound creation in a medium that did or did not contain synthetic MMP inhibitors BB-3103 (10 μM) or tissue inhibitor of metalloproteinase (TIMP)-2 (46 μM) and were followed for 24 h by time-lapse analysis.

**Statistical Analysis**

Data are presented as means ± SE or SD when appropriate. Between-group comparisons were performed with the nonparametric Mann-Whitney U-test (Instat 2.00). A \(P\) value of <0.05 was considered to be significant. All experiments were performed in duplicate on at least four independent cultures. In view of our results of the decrease in wound area with time, we tested linear regression using the least-square method of estimation, which was shown to be appropriate.

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> Fig. 3. Representative video micrographs of in vitro HNEC wound healing assay. Freshly isolated HNEC were plated in inserts (12-mm Transwell; Costar, Cambridge, MA) with 12-mm-diameter polycarbonate micropore membranes coated with type IV collagen. When stable differentiation was reached, HNEC were gently scraped with a pipette tip, followed by extensive washing to remove cellular debris. Closure of the wound was observed over a period of 24–30 h, depending on the time of closure. Time-lapse images were taken at regular intervals on an inverted microscope equipped with an ×4 objective and a charge-coupled device video camera. H0, initial wound area; H12, remaining wound area 12 h after scarring; H28, closure of the wound area 28 h after scarring. Arrowhead shows the scar at the time of closure. Bar, 500 μm.
MMP-2 enhances HNEC wound repair. We then examined the ability of MMP-2 (10 ng/ml) to enhance HNEC repair. As shown in Fig. 5, the addition of MMP-2 reproduced the enhanced HNEC repair induced by TGF-β1.

TGF-β1 promotes HNEC repair in vitro via MMPs. To demonstrate whether TGF-β1-increased MMP-2 expression is responsible for the increased HNEC repair induced by TGF-β1, we examined the ability of MMP inhibitors to block HNEC repair. BB-3103 and TIMP-2 had no significant effect on wound repair in controls, whereas BB-3103 (Fig. 6A) or TIMP-2 (Fig. 6B) significantly inhibited increased HNEC wound repair induced by TGF-β1 (P < 0.05). These results indicate that the increased wound repair induced by TGF-β1 is MMP dependent. We checked that the inhibition of the repair rate of HNEC by BB-3103 and TIMP-2 was similar if the HNEC were treated with MMP-2 or TGF-β1 (Fig. 6, A and B).
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DISCUSSION

As a model of in vitro epithelial airway wound repair, we used HNEC cultured under conditions allowing differentiation. HNEC form cohesive cells closely resembling normal airway epithelium within 1 wk and maintain their differentiation pattern for at least 3 wk (21). We then adapted a model of mechanical wound repair that has been widely used to investigate in vitro wound repair in various cell types (9).

A basal level of predominant MMP-9 and minor MMP-2 was observed in our in vitro model of HNEC cultures. Several in vitro studies have shown that primary cultures of epithelial cells from different origins including airway epithelial cells can produce basal levels of MMPs (3, 30). These results are also in accordance with in vivo studies demonstrating weak expression of MMP-2 and MMP-9 in normal airway epithelium where they may contribute to remodeling of basal lamina to maintain cellular homeostasis (11). In the present in vitro model, addition of TGF-β1 induced a dramatic increase of MMP-2 and a slight increase of MMP-9. To our knowledge, our results are the first to indicate that TGF-β1 is able to modulate MMP-2 and MMP-9 expression in airway epithelial cells. Classically, airway epithelial cells preferentially upregulate MMP-9 expression, notably in response to inflammatory molecules (32). It must be stressed that MMP-9 expression is closely regulated at the level of transcription, whereas MMP-2 expression is constitutive and mainly controlled at a posttranscriptional level (26). However, the effect of TGF-β1 on MMP-2 expression seems to be highly cell-type specific. For example, TGF-β1 increases MMP-2 expression in human gingival fibroblasts, keratinocytes, and lens epithelial cells, while it decreases or does not modify MMP-9 expression (14, 20, 25).

We then showed that TGF-β1 promotes repair of primary HNEC cultures, as previously established for keratinocytes (14) and 16HBE 14o bronchial epithelium-derived cells (8). In keratinocytes, the increased repair observed in the presence of TGF-β1 was related to MMP-2 (14). Because TGF-β1 enhanced both wound repair and MMP-2 expression, we hypothesized that the impact of TGF-β1 on wound repair could be related to MMP-2. It has been previously shown that MMP-2 can modulate epithelial airway wound repair in vitro (6). This led us to examine whether MMP-2 enhanced HNEC repair in our model. Addition of exogenous MMP-2 mimicked the increased HNEC repair observed in the presence of TGF-β1. MMP-2 could promote cell migration and facilitate wound repair via various effects, such as clearing of ECM, epithelial-mesenchymal transition, or activation of signaling molecules (17, 26). MMP inhibitors had no significant effect on controls, suggesting that the mechanism of repair involved in TGF-β1-modulated wound repair may be different from that involved in controls. Together, these findings suggest a role for MMP-2 as an effector of TGF-β1 in airway epithelial wound repair. To our knowledge, such a mechanism has not been previously shown in a model of airway wound repair.

Previous studies demonstrated that stromelysins and MMP-9 are responsible for promoting in vitro airway epithelial wound repair, whereas MMP-2 is thought to play a minor role (3, 4). The preferential involvement of MMP-9 could be attributable to experimental procedures (i.e., HNEC obtained from nasal polyps vs. inferior turbinate, collagen I-coated coverslips without air-liquid interface vs. micropore membrane coated with type IV collagen with air-liquid interface, and chemical injury vs. mechanical injury). It has been demonstrated that human bronchial epithelial cells cultured on type I+III collagen exhibit a higher basal expression and an increased upregulation of MMP-9 in response to inflammatory cytokines compared with the same cells cultured on type IV collagen (31). HNEC cultured on type IV collagen could therefore adopt a homeostatic phenotype, close to normal in vivo conditions.

In vivo, normal wound repair is a self-limiting process leading to restoration of structure and function of the tissue. In this setting, TGF-β1 exerts complex functions, with pro- and anti-inflammatory as well as immune-activating and immune-suppressing effects (18). TGF-β1 promotes cell migration and wound healing, whereas it is a potent inhibitor of cell proliferation (8). In addition, TGF-β1 stimulates production of ECM components that facilitate reepithelialization during normal wound repair but can ultimately contribute to fibrosis, a frequent feature of wound healing disorders (19). Abnormal wound repair resulting from repeated damage to the airways observed during chronic inflammatory disorders such as asthma and nasal polyposis maintains the ongoing inflammatory response. The increased MMP-9 expression occurring in this setting could be related to a combination of various inflammatory cytokines such as IL-1β or TNF-α and may not simply depend on TGF-β1 upregulation. In contrast to MMP-9, MMP-2 is not overexpressed in these inflammatory airway disorders (1, 11). In light of this finding and on the basis of the data obtained from the present study, our model may represent a relevant model of normal rapid wound healing. It is likely that an increase of MMP-2 expression occurring in response to TGF-β1 may promote airway repair. MMP-2 could therefore be essential under a condition of homeostasis, whereas MMP-9 could be critical in an inflammatory context characterized by prolonged remodeling processes.

In conclusion, the results of our study indicate that TGF-β1 enhances both in vitro HNEC wound closure and MMP-2 expression. Additional MMP-2 enhanced HNEC repair in vitro, mimicking the effects of TGF-β1, whereas MMP inhibitors prevented the increased HNEC repair induced by TGF-β1 or MMP-2. Together, our results strongly suggest that TGF-β1 increases airway wound repair via MMP-2 upregulation. These results suggest that the MMP mechanisms responsible for airway wound repair may differ between noninflammatory and chronic inflammatory airways, which may have implications for therapeutic strategies in inflammatory airway diseases characterized by remodeling dysregulation, such as asthma and nasal polyposis.

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