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Wound-induced TGF-β1 and TGF-β2 enhance airway epithelial repair via HB-EGF and TGF-α

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ABSTRACT

The abundance of transforming growth factor-beta (TGF-β) in normal airway epithelium suggests its participation in physiological processes to maintain airway homeostasis. The current study was designed to address the hypothesis that TGF-β1 and TGF-β2 might contribute to normal reparative response of airway epithelial cells (AECs). Treatments with exogenous TGF-β1 or TGF-β2 significantly enhanced wound repair of confluent AEC monolayers. Mechanical injury of AEC monolayers induced production of both TGF-β1 and TGF-β2. Wound repair of AECs was significantly reduced by a specific inhibitor of TGF-β type I receptor kinase activity. We investigated whether the TGF-β-enhanced repair required epidermal growth factor receptor (EGFR) transactivation and secretion of EGFR ligands. Both TGF-β1 and TGF-β2 enhanced EGFR phosphorylation and induced production of heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-alpha (TGF-α) in AECs. Moreover, treatment with a broad-spectrum metalloproteinase inhibitor or anti-HB-EGF and anti-TGF-α antibodies inhibited the wound repair and the EGFR phosphorylation by TGF-β1 and TGF-β2, indicating that the TGF-β1 and TGF-β2 effects on wound repair required the release of HB-EGF and TGF-α. Our data, for the first time, have shown that both TGF-β1 and TGF-β2 play a stimulatory role in airway epithelial repair through EGFR phosphorylation following autocrine production of HB-EGF and TGF-α. These findings highlight an important collaborative mechanism between TGF-β and EGFR in maintaining airway epithelial homeostasis.

1. Introduction

The airway epithelium is continuously exposed to inhaled gaseous and particulate components and therefore is subject to injury. A rapid repair after injury is crucial for restoring epithelial barrier function. In response to environmental challenges, bronchial epithelial cells produce a number of growth factors [1–3], including transforming growth factor-beta (TGF-β), which is a multifunctional regulator of cell growth, differentiation, migration, extracellular matrix formation, and immune responses [4,5]. Among the three isoforms of TGF-β family members secreted by mammalian cells, both TGF-β1 and TGF-β2 have been implicated in the early stage of skin wound repair [6]. In reference to the airway, some studies demonstrated that TGF-β2, but not TGF-β1, was produced by damaged airway epithelial cells (AECs) [3,7,8], but others reported that TGF-β1, but not TGF-β2, was expressed in AECs of fibrotic lungs, which promoted the migration of AECs in damaged monolayers [9,10]. Thus, the role of TGF-β1 and TGF-β2 in wound repair of airway epithelium is still controversial.

Epidermal growth factor receptor (EGFR) signaling has been implicated in some effects of TGF-β1, such as accumulation of fibronectin in mesangial cells, regulation of the cell cycle in fetal rat hepatocytes, and migration of smooth muscle cells [11–13]. The main members of EGFR ligands potentially involved in wound repair include epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-alpha (TGF-α), and amphiregulin (AR) [14–16]. All these ligands are synthesized as transmembrane precursors and proteolytically processed to release the biologically active mature protein. An autocrine activation of the EGFR signaling plays a key role in epithelial wound repair by increasing epithelial cell proliferation, migration, differentiation, and survival [15,17]. It have been suggested that the EGFR

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transactivation in response to TGF-β1 may be mediated by the release of a membrane-anchored EGFR ligand [13].

In the present study, we examined the involvement of TGF-β1 and TGF-β2 in airway epithelial repair and the contribution of EGFR transactivation. Our data, for the first time, show that mechanical injury induces secretion of both TGF-β1 and TGF-β2, which enhance EGFR activation and wound repair through production of HB-EGF and TGF-α in an AEC in vitro culture model. These findings suggest that both TGF-β1 and TGF-β2 play an important role in normal airway epithelial repair, which activates EGFR pathway by autocrine HB-EGF and TGF-α.

2. Materials and methods

2.1. Reagents

Recombinant soluble human TGF-β1 and TGF-β2 were from Peprotech (Rocky Hill, NJ, USA). Anti-TGF-β1, anti-phosphorylated EGFR (pY^{845}) antibodies, and neutralizing antibodies of EGF, HB-EGF, TGF-α, and AR were obtained from R&D Systems (Minneapolis, MN, USA). ELISA kits for TGF-β1, TGF-β2, EGF, HB-EGF, TGF-α, and AR were also from R&D Systems. Anti-TGF-β2 and anti-α-tublin antibodies, AG1478, SB431542, and Eagle's minimum essential medium (MEM) were from Sigma Chemicals (St. Louis, MO, USA). GM6001 was from Calbiochem (San Diego, CA, USA). Anti-EGFR antibody was from Upstate Biotechnologies (Lake Placid, NY, USA). Bronchial epithelial growth medium (BEGM) was purchased from Cambrex (East Rutherford, NJ, USA).

2.2. Cell culture

1HAEo$^-$ cells are SV40-transformed normal human airway epithelial cells that have been characterized previously [18]. Primary normal human bronchial epithelial (NHBE) cells were purchased from Cambrex. Cells were grown on collagen I-coated flasks or plates (Asahi Techno Glass, Japan). 1HAEo$^-$ cells were cultured in MEM containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml of streptomycin and 100 U/ml of penicillin G and incubated at 37 °C in 5% CO_2. NHBE cells were cultured in complete BEGM, which consists of bronchial epithelial basal medium (BEBM) supplemented with insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml), triiodothyronine (6.5 ng/ml), epinephrine (0.5 μg/ml), human EGF (0.5 ng/ml), retinoic acid (0.1 ng/ml), gentamycin (50 μg/ml), and bovine pituitary extract (52 μg/ml). Twenty-four hours before and throughout the conduct of each experiment, the 1HAEo$^-$ cells and NHBE cells were cultured in growth factor-free and serum-free medium. Pretreatment with recombinant soluble human TGF-β1 (0.1–100 ng/ml), TGF-β2 (0.1–100 ng/ml), SB431542 (10 μM), AG1478 (1 μM), GM6001 (50 μM), dimethyl sulfoxide (DMSO) as vehicle and neutralizing antibodies of EGF (6 μg/ml), HB-EGF (3 μg/ml), and TGF-α (3 μg/ml) is described in the text.

2.3. Monolayer wound repair assay

We have established this method previously [14]. Briefly, NHBE and 1HAEo$^-$ cells were grown in 6-well plate and then placed upon confluence in the growth factor-free BEBM or serum-free MEM, respectively. A circular wound (~2.0 mm^2) was made in the confluent monolayer using a 20-μl pipette tip (4 wounds per well). In each experiment, one well was used as a negative control with no treatment. The wounds were imaged 0, 12 and 24 h after wound creation using a Nikon Eclipse TE200 inverted microscope equipped with a Nikon Coolpix E995. Corresponding wound areas were determined using ImagePro Plus and the remaining wound areas were calculated as a percentage of area at time 0.

2.4. Preparation of cell lysates and Western blotting

To determine the production of TGF-β1 and TGF-β2 after mechanical injury, confluent monolayers of NHBE in the growth factor-free BEBM and 1HAEo$^-$ cells in the serum-free MEM were subjected to multiple linear injuries (7 × 7 linear scratches in each well) using a 20-μl pipette tip. Monolayers with no scratch wounds were used as the control. In other experiments, to determine the production of EGFR ligands and EGFR phosphorylation, confluent monolayers of NHBE and 1HAEo$^-$ cells were treated with 10 ng/ml of recombinant human TGF-β1 or TGF-β2. Whole cell lysates were prepared at the indicated time points using RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin). Protein concentration was determined by the Bradford assay. Equal amounts of whole cell lysates (20–40 μg) were separated by 8% or 15% SDS–PAGE and blotted onto polyvinylidene difluoride

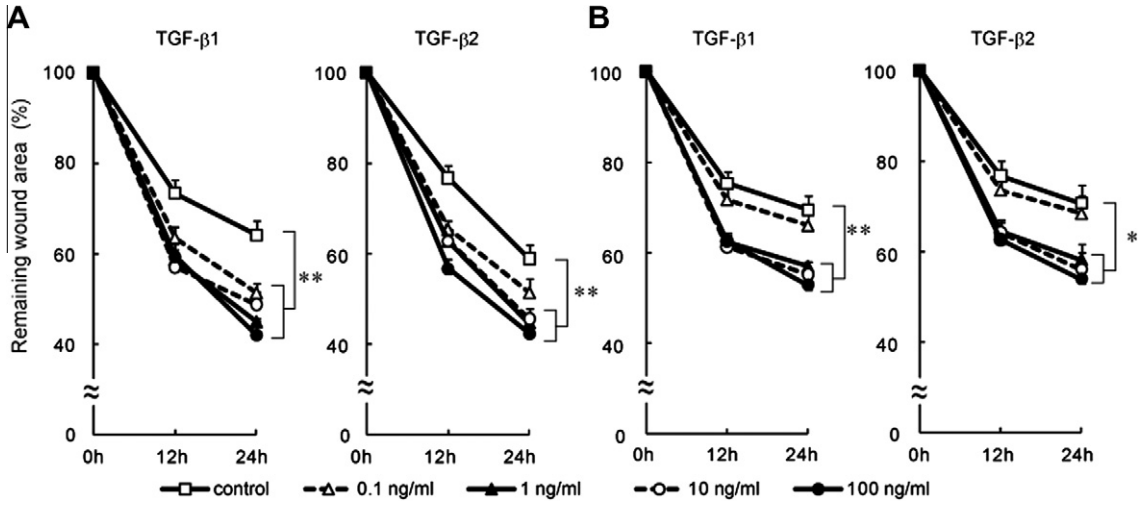

Fig. 1. TGF-β1 and TGF-β2 enhance airway epithelial repair. Injured monolayers of NHBE (A) and 1HAEo$^-$ (B) cells were treated with the indicated concentrations of TGF-β1 or TGF-β2 immediately after the injury, and wound areas were determined at 12 and 24 h. Data are represented as the mean ± SEM of triplicate samples. $^*p < 0.05$, $^{**}p < 0.01$ compared with the absence of TGF-β (control). Similar results were obtained in three independent experiments.

membranes. After blocking with 5% skim milk, blots were incubated overnight with the indicated primary antibodies. Membranes were then incubated with appropriate peroxidase-conjugated secondary antibodies, followed by detection with ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry of Western blot signals acquired with a Fuji LAS-4000 fluorescence imager (Fujifilm Corporation, Tokyo, Japan) with a linearity of 4 orders of magnitude was done using the NIH Image J image analysis software.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Confluent monolayers of NHBE and 1HAEo$^-$ cells were subjected to multiple linear injuries as described above. Injured monolayers were washed to remove cell debris and the medium were replaced with fresh growth factor-free BEBM or serum-free MEM. Culture supernatants were collected at the indicated time points after injury, centrifuged to remove cell debris, and then acidified with 1 M HCl to enable measurement of total TGF-β1 and TGF-β2. After neutralization, TGF-β1 and TGF-β2 levels were measured using sandwich ELISA kits according to the manufacturer's instructions. In other experiments, confluent monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml TGF-β1 or TGF-β2, and culture supernatants were collected at the indicated time points. EGF, HB-EGF, TGF-α, and AR levels were measured using sandwich ELISA kits according to the manufacturer's instructions.

2.6. Statistical analysis

Comparisons between multiple groups were made by ANOVA. Differences were considered to be statistically significant when p values were 0.05 or less.

3. Results

3.1. TGF-β1 and TGF-β2 enhance airway epithelial repair

We first investigated whether exogenous TGF-β1 and TGF-β2 could enhance airway epithelial repair. Injured monolayers of NHBE (Fig. 1A) and 1HAEo$^-$ (Fig. 1B) cells were treated with different concentrations of TGF-β1 or TGF-β2 (0.1–100 ng/ml). Addition of TGF-β1 significantly enhanced the epithelial repair in a dose-dependent manner. This result is consistent with a previous report that TGF-β1 accelerated bronchial epithelial wound repair [9]. Interestingly, TGF-β2 at 1, 10 and 100 ng/ml also significantly enhanced the epithelial repair. These results indicate that not only TGF-β1 but also TGF-β2 can enhance airway epithelial repair.

3.2. Wound-induced TGF-β1 and TGF-β2 enhance airway epithelial repair

To examine whether TGF-β is produced by AECs in response to mechanical injury, whole cell lysates and culture supernatants were collected from injured monolayers of NHBE and 1HAEo$^-$ cells. Levels of TGF-β1 and TGF-β2 in the cell lysates were increased gradually in NHBE (Fig. 2A) and 1HAEo$^-$ (Fig. 2B) cells. Significantly increased secretion of both TGF-β1 and TGF-β2 into the culture supernatants of injured monolayers of NHBE (Fig. 2C) and 1HAEo$^-$ (Fig. 2D) cells was observed at 6–24 h after injury. To determine the contribution of endogenous TGF-β1 and TGF-β2 produced by injured epithelium to the wound repair, injured monolayers of 1HAEo$^-$ cells were treated with SB431542 which is a specific inhibitor of TGF-β type I receptor kinase activity. Addition of SB431542 significantly reduced the wound repair at 24 h after mechanical injury (Fig. 2E). Interestingly, an EGFR-specific tyrosine kinase inhibitor AG1478 also inhibited the wound repair to a similar extent (Fig. 2E). Taken together, these results suggest critical contributions of TGF-β produced by injured airway epithelium and EGFR signaling to the repair.

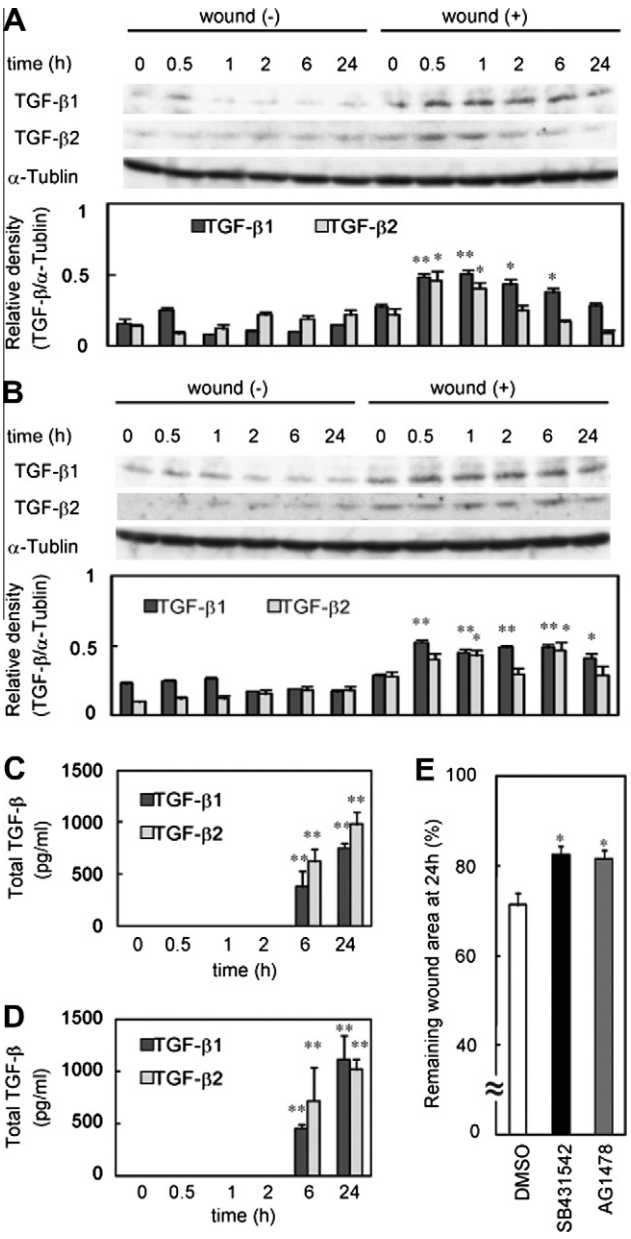

Fig. 2. Induction of TGF-β1 and TGF-β2 production by wound and its involvement in airway epithelial repair. Multiple linear wounds were made on confluent monolayers of NHBE and 1HAEo$^-$ cells. Whole cell lysates and culture supernatants were collected at the indicated time points. TGF-β1 and TGF-β2 in the NHBE (A) and 1HAEo$^-$ (B) cell lysates were examined by Western blot analysis (upper). All membranes were re-probed with anti-α-tublin antibody to confirm equal loading. The density of each band was normalized with α-tubulin and quantified by densitometry using the NIH Image J software (lower). Total TGF-β1 (solid bars) and TGF-β2 (shaded bars) in the culture supernatants of NHBE (C) and 1HAEo$^-$ (D) cells were examined by ELISA. (E) Injured monolayers of 1HAEo$^-$ cells were treated with SB431542 (10 μM), AG1478 (1 μM), or DMSO as vehicle, immediately after the injury, and wound areas were determined at 24 h. Data are represented as the mean ± SEM of triplicate samples. $^*p < 0.05$, $^{**}p < 0.01$ compared with time 0 (A–D) or vehicle. (E) Similar results were obtained in three independent experiments.

3.3. TGF-β1 and TGF-β2 activate EGFR in airway epithelial cells

Next, we examined whether TGF-β could activate EGFR in AECs by estimating the phosphorylation of EGFR. Confluent monolayers

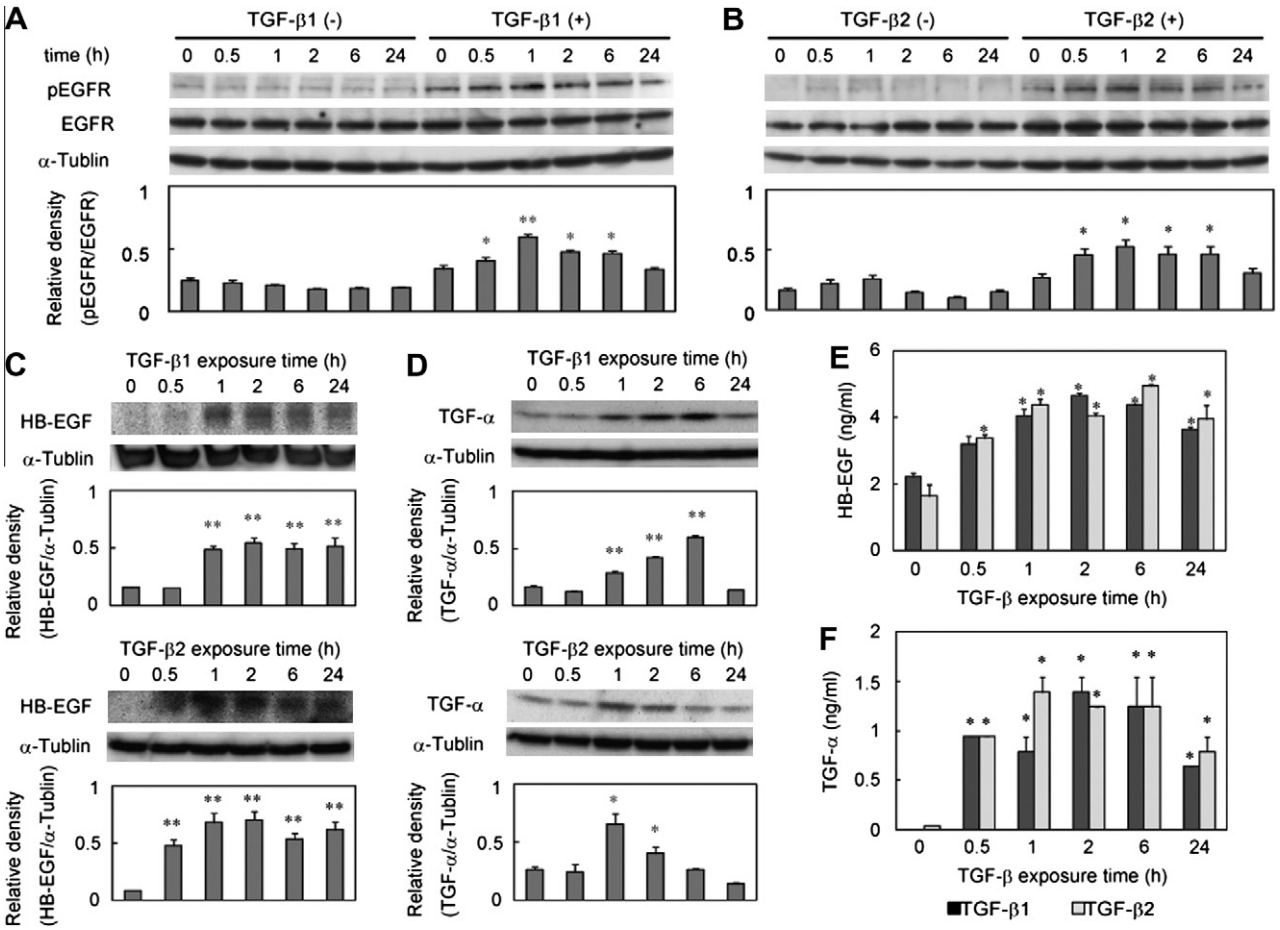

Fig. 3. TGF-β1 and TGF-β2 enhance EGFR phosphorylation and production of HB-EGF and TGF-α by airway epithelial cells. Confluent monolayers of NHBE cells were treated with 10 ng/ml of TGF-β1 (A) or TGF-β2. (B) Whole cell lysates were prepared at the indicated time points after the treatment. Phosphorylation of EGFR was examined by Western blotting with anti-EGFR and anti-pEGFR (pY^{845}) antibodies (upper). Densitometry of pEGFR signal was normalized against total EGFR signal (lower). Confluent monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml of TGF-β1 or TGF-β2. Whole cell lysates and culture supernatants were collected at the indicated time points. HB-EGF (C) and TGF-α (D) in the cell lysates were examined by Western blot analysis (upper). All membranes were re-probed with anti-α-tublin antibody to confirm equal loading. The density of each band was normalized with α-tubulin and quantified by densitometry (lower). HB-EGF (E) and TGF-α (F) in the culture supernatants were examined by ELISA. Data are represented as the mean ± SEM of triplicate samples. $^*p < 0.05$, $^{**}p < 0.01$ compared with time 0. Similar results were obtained in three independent experiments.

of NHBE and 1HAEo$^-$ cells were treated with 10 ng/ml of TGF-β1 or TGF-β2 and phosphorylation of EGFR was detected using anti-pEGFR antibody. Both TGF-β1 and TGF-β2 enhanced the EGFR phosphorylation in NHBE (Fig. 3A and B) and 1HAEo$^-$ (Suppl. Fig. 1A and B) cells after the treatment. These results suggest that both TGF-β1 and TGF-β2 can enhance EGFR phosphorylation in airway epithelium.

3.4. TGF-β1 and TGF-β2 induce production of HB-EGF and TGF-α by airway epithelial cells

The EGFR phosphorylation induced by TGF-β in the absence of an exogenous EGFR ligand suggested that AECs produced some EGFR ligand in response to TGF-β. To address this possibility, confluent monolayers of 1HAEo$^-$ cells were treated with TGF-β1 or TGF-β2, and the levels of EGFR ligands in whole cell lysates and culture supernatants were determined by Western blotting and ELISA, respectively. The levels of HB-EGF in the cell lysates were increased gradually after the TGF-β1 and TGF-β2 treatments (Fig. 3C). The levels of TGF-α in the cell lysates were also increased after the treatments (Fig. 3D). Moreover, 1HAEo$^-$ cells secreted HB-EGF and TGF-α into the culture supernatants after the TGF-β1 and TGF-β2 treatments (Fig. 3E and F). However, TGF-β1 or TGF-β2 did not induce the production of EGF and AR by 1HAEo$^-$ cells (data not shown).

3.5. TGF-β1 and TGF-β2 enhance EGFR phosphorylation and epithelial repair via HB-EGF and TGF-α

Although the above results indicated that TGF-β1 and TGF-β2 induced the production of HB-EGF and TGF-α by AECs, it was not clear whether the HB-EGF and TGF-α were responsible for the TGF-β-enhanced EGFR phosphorylation and wound repair. We thus examined whether the release of HB-EGF and TGF-α was necessary for the TGF-β-induced EGFR phosphorylation. Confluent monolayers of 1HAEo$^-$ cells were treated with TGF-β1 or TGF-β2 with or without a broad-spectrum metalloproteinase inhibitor GM6001 that blocks activation of HB-EGF and TGF-α, neutralizing anti-EGF antibody, neutralizing anti-HB-EGF antibody, neutralizing anti-TGF-α antibody, or a combination of anti-HB-EGF and anti-TGF-α antibodies. The TGF-β1- or TGF-β2-enhanced EGFR phosphorylation in 1HAEo$^-$ cells was abolished by the treatments with GM6001, anti-HB-EGF, anti-TGF-α, or a combination of anti-HB-EGF and anti-TGF-α antibodies (Fig. 4A and B). In contrast, the anti-EGF antibody treatment did not inhibit the TGF-β-induced EGFR phosphorylation. These results indicated that the TGF-β1- and TGF-β2-

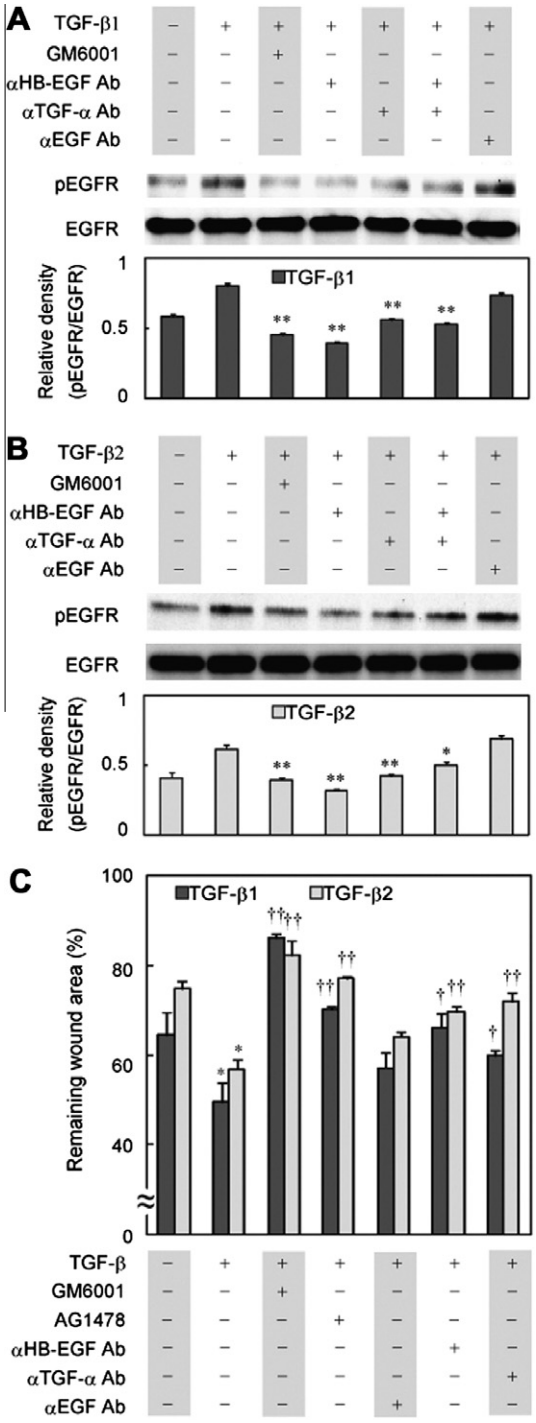

Fig. 4. TGF-β1 and TGF-β2 enhance EGFR phosphorylation and epithelial repair via HB-EGF and TGF-α. Confluent monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml of TGF-β1 (A) or TGF-β2 (B) with or without GM6001 (50 μM), anti-EGF antibody (6 μg/ml), anti-HB-EGF antibody (3 μg/ml), anti-TGF-α antibody (3 μg/ml), or a combination of anti-HB-EGF and anti-TGF-α antibodies. Whole cell lysates were prepared at 1 h after the treatment. Phosphorylation of EGFR was examined by Western blot analysis with anti-EGFR and anti-pEGFR (pY^{845}) antibodies. Densitometry of pEGFR signal was normalized against total EGFR signal. $^*p < 0.05$ compared with TGF-β alone. Similar results were obtained in three independent experiments. (C) Injured monolayers of 1HAEo$^-$ cells were treated with 10 ng/ml of TGF-β1 (solid bars) or TGF-β2 (shaded bars) with or without GM6001 (50 μM), AG1478 (1 μM), anti-EGF antibody (6 μg/ml), anti-HB-EGF antibody (3 μg/ml), or anti-TGF-α antibody (3 μg/ml). Wound areas were determined at 24 h. Data are represented as the mean ± SEM of triplicate samples. $^*p < 0.05$ compared with the untreated culture. $^\dagger p < 0.05$ compared with TGF-β alone. Similar results were obtained in three independent experiments.

enhanced EGFR phosphorylation was mediated by the release of HB-EGF and TGF-α by AECs. We finally examined whether the release of EGFR ligands was necessary for the TGF-β-enhanced wound repair. Wounded monolayers of 1HAEo$^-$ cells were treated with TGF-β1 or TGF-β2 with or without GM6001, AG1478, neutralizing anti-EGF antibody, neutralizing anti-HB-EGF antibody, or neutralizing anti-TGF-α antibody. As shown in Fig. 4C, the TGF-β1- or TGF-β2-enhanced wound repair was abrogated by the addition of GM6001, AG1478, anti-HB-EGF, or anti-TGF-α. These results indicated that the TGF-β-enhanced wound repair was mediated by EGFR signaling triggered by the release of active HB-EGF and TGF-α by AECs in response to TGF-β.

4. Discussion

Autocrine activation of EGFR, at least in part, plays a key role in epithelial wound repair [16,17]. Mechanical damage rapidly induces EGFR phosphorylation in AECs through endogenous mediators. Bronchial epithelial cells produce EGF family, such as HB-EGF and TGF-α [14,19]. Our previous study has revealed that wound-induced HB-EGF enhances airway epithelial repair [14], and also TGF-α has been shown to induce proliferation of AECs [20]. We now showed that mechanical injury induced production of both TGF-β1 and TGF-β2 by AECs. Moreover, our present results showed that both TGF-β1 and TGF-β2 enhanced epithelial wound repair. Previous studies have shown that TGF-β1 inhibits proliferation in normal epithelial cells while it enhances cell migration and shifts integrin expression toward a more migratory phenotype [21,22]. Furthermore, animal studies have suggested that TGF-β either enhances or suppresses epithelialization. It has been reported that TGF-β1- or Smad3-null mice showed accelerated cutaneous wound repair [23,24]. In contrast, others have described over-expression of TGF-β1 enhanced epithelial wound repair [25,26]. These discrepant findings may reflect the complexity of signaling necessary to orchestrate cellular processes in wound repair, emphasizing the importance of a strict spatiotemporal control, in which a small change in the level or timing of any growth factor may have a completely different outcome.

In the present study we found that both TGF-β1 and TGF-β2 enhanced EGFR phosphorylation and wound repair via release of HB-EGF and TGF-α from AECs. A previous study has shown that TGF-β1 induced an increase of metalloproteinases in nasal epithelial cells [27]. Similarly, in our model, addition of a metalloproteinase inhibitor attenuated the TGF-β-induced EGFR phosphorylation and wound repair, suggesting that cleavage of HB-EGF and TGF-α by metalloproteinases was required for the TGF-β-induced EGFR transactivation. We also demonstrated that both TGF-β1 and TGF-β2 induced the synthesis of HB-EGF and TGF-α in AECs. These findings suggested that the TGF-β-induced EGFR transactivation was mediated by not only the enhanced cleavage by metalloproteinases but also transcriptional or translational activation of HB-EGF and TGF-α. TGF-β mobilizes both Smad-dependent and -independent signaling pathways although the actual mechanisms underlying synthesis of HB-EGF and TGF-α in response to TGF-β are not well understood. There is evidence for the Smad-independent alternative pathways, such as various branches of MAP (mitogen-activated protein) kinase pathways [28]. Thus, it will be necessary to study the comprehensive relationship between TGF-β-induced synthesis of EGFR ligands and TGF-β signaling in airway epithelial wound repair. To our knowledge, this is the first study to show that TGF-β1 and TGF-β2 produced by AECs in response to mechanical injury facilitate wound repair through EGFR phosphorylation following temporal synthesis and release of HB-EGF and TGF-α.

Epithelial repair consists of a complex cascade of events that starts immediately after injury and leads to an organized repopulation of epithelial cells. A persistent epithelial damage might be a result of incomplete repair where any component of this cascade does not work properly. It has been shown that disruption of EGFR-mediated epithelial repair led to enhanced release of TGF-β2 by bronchial epithelial cells [3]. We also reported that AECs produced increased amount of IL-13 when EGFR-mediated wound repair was inhibited [14]. These findings suggest that an improper epithelial repair causes a persistent repair phenotype with overproduction of inflammatory mediators and growth factors, which may contribute to the chronic airway changes and remodeling.

In conclusion, we have revealed a novel mechanism of airway epithelial repair. AECs produce both TGF-β1 and TGF-β2 in response to mechanical injury, which promote EGFR-mediated wound repair via autocrine production of HB-EGF and TGF-α. These findings emphasize that collaboration between TGF-β and EGFR plays a critical role in airway epithelial repair and have important implications for understanding the aberrant airway remodeling associated with chronic inflammation such as asthma.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.054.

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