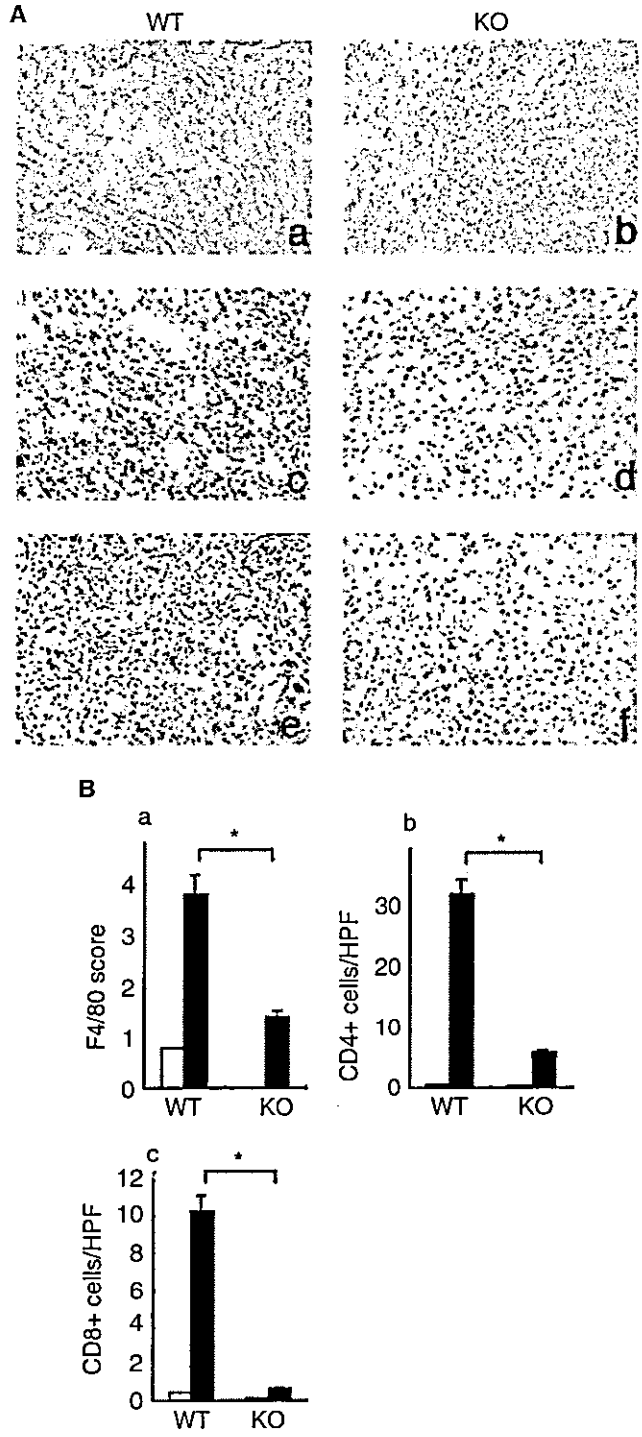

Fig. 4. Reduced renal inflammation in the obstructed kidney of Smad3(−/−) mice after unilateral ureteral obstruction (UUO). (*A*) Representative pictures showing immunohistochemical staining with anti-F4/80 (a and b), CD4 (c and d), and CD8 (e and f) antibodies of the obstructed kidney sections from Smad3(+/+) (a, b, and c) or Smad3(−/−) (d, e, and f) mice 14 days after UUO. (*B*) Quantitative analysis of immunohistochemical staining with anti-F4/80, CD4, and CD8 antibodies. (a) Scoring of the F4/80-positive cells of the left obstructed (■) or nonobstructed right kidney (□) of the mice 14 days after UUO was determined as described in the **Methods** section. (b) and (c) The number of CD4 or CD8-positive interstitial cells of the left obstructed (■) or nonobstructed right kidney (□) of the mice 14 days after UUO was counted in six random fields under high performance fields. Wild-type (WT), Smad3(+/+) mice; knockout (KO), Smad3(−/−) mice ($N = 6$). $^*P < 0.05$.

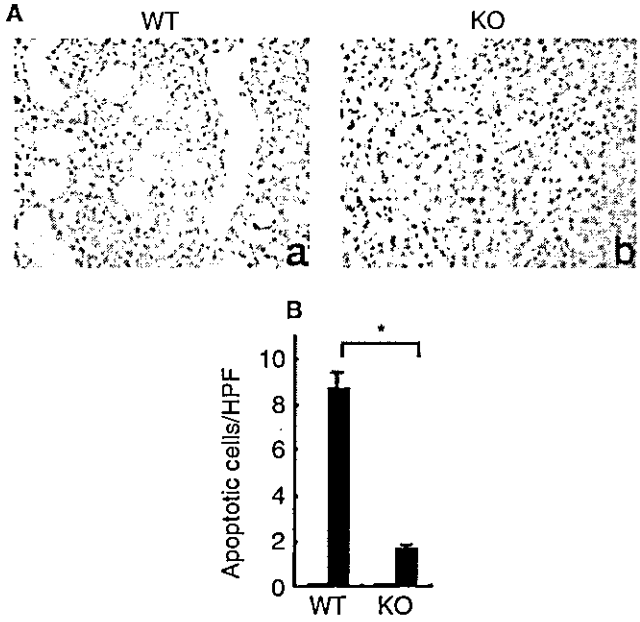

Fig. 5. Reduced tubular apoptosis in the obstructed kidney of Smad3(−/−) mice. (*A*) Representative pictures showing terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining of the obstructed kidney of Smad3(+/+) mice (a) and Smad3(−/−) mice (b) 14 days after unilateral ureteral obstruction (UUO). (*B*) Quantitative analysis of tubular apoptotic cells. The number of TUNEL-positive cells was counted in six random high power fields of the left obstructed (■) or nonobstructed right kidney (□) of Smad3(+/+) mice [wild-type (WT)] and Smad3(−/−) mice [knockout (KO)] 14 days after UUO ($N = 6$). $^*P < 0.05$.

kidney sections of the mice 14 days after UUO (Fig. 5). The obstructed kidney of Smad3(+/+) mice showed significant higher tubular apoptosis when compared with nonobstructed kidney of the mice as previously described [8]. Similar to the findings in renal fibrosis and inflammation, the obstructed kidney of Smad3(−/−) mice showed significantly lower tubular apoptosis than that of Smad3(+/+) mice.

Activation of the Smad pathway after UUO

Since the data described above suggested that the Smad pathway was crucial for the development of UUO, we asked whether the Smad pathway was indeed activated in the obstructed kidney after UUO. Because of limited availability of antibodies against phosphorylated Smad3 suitable for immunohistochemical staining, we performed immunohistochemical analysis with antibody against phosphorylated Smad2, another marker for activation of TGF-β signaling. As shown in Figure 6, the

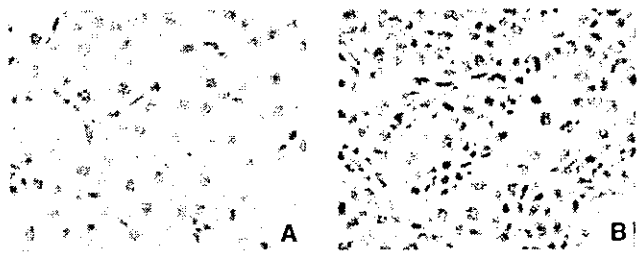

Fig. 6. Increased phosphorylated Smad2-positive cells in renal interstitial tissue after unilateral ureteral obstruction (UUO). Immunohistochemical staining with antiphosphorylated Smad2 antibody was performed in the right nonobstructed kidney (*A*) and left obstructed kidney (*B*) of wild-type mice 14 days after UUO.

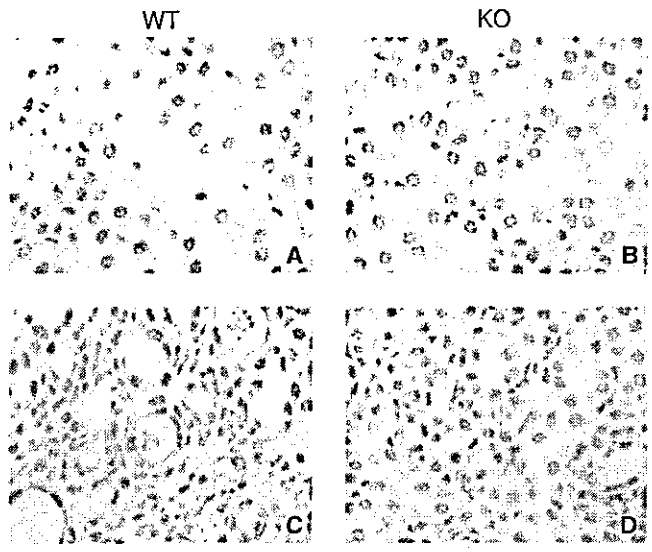

Fig. 7. Increased phosphorylated Smad2/3-positive cells in renal interstitial area after unilateral ureteral obstruction (UUO). Immunohistochemical staining with antibody cross-reacted with phosphorylated Smad2 and phosphorylated Smad3 was performed in the right nonobstructed kidney (*A* and *B*) and left obstructed kidney (*C* and *D*) of Smad3(+/+) mice [wild-type (WT)] and Smad3(−/−) mice [knockout (KO)] 14 days after UUO.

immunoreactivity of phosphorylated Smad2 was prominent in the nucleus of cells in the interstitial area of the kidney after UUO. Morphologic examination suggested that the positive staining cells observed after UUO induction were mostly infiltrated inflammatory cells and fibroblast-like cells. Thus, UUO-dependent activation of Smad2 appeared to occur largely in the cells located or infiltrated in renal interstitial area. Interestingly, renal tubular cells appeared to be constitutively positive for phosphorylated Smad2 staining independent of UUO induction. To further ascertain these results, we performed immunohistochemical analysis with antibody against phosphorylated Smad2/3 and had essentially similar results (Fig. 7). These findings indicated that activation of the Smad pathway indeed occurred in renal interstitial cells after UUO.

DISCUSSION

In this study, we demonstrated that Smad3 deficiency remarkably attenuated renal fibrosis, inflammation, and apoptosis induced by UUO (Figs. 1 to 5). In addition, we showed that endogenous Smad2/3 pathway in renal interstitial area was indeed activated after UUO (Figs. 6 and 7). Thus, we concluded that Smad3 was a key mediator for the development of UUO.

Previous in vitro studies suggested that Smad3 was critical for important aspects of TGF-β–mediated fibrotic, inflammatory, and apoptotic responses, including ECM production from fibroblasts [25], differentiation from fibroblasts to myofibroblasts [25], and chemotaxis of macrophages [27] and induction of cellular apoptosis [28], all of which were down-regulated in the obstructed kidney of Smad3(−/−) mice after UUO (Figs. 1 to 5). Our findings thus suggested that Smad3 mediated these activities in vivo as well as in vitro. It is most likely that Smad3 deficiency attenuated pathologic changes in the kidney after UUO through suppression of these cellular and molecular events.

The role of Smad3 in the differentiation of myofibroblasts (α-SMA–positive cells) is yet controversial; one paper suggested Smad3 as critical for α-SMA expression [26], other papers suggested Smad2 [29] or Smad-independent pathways [13] as important for myofibroblast differentiation. Because we showed that the number of myofibroblast in the obstructed kidney after UUO was significantly reduced in Smad3(−/−) mice (Fig. 3), it should be determined in future study whether the reduction of myofibroblast number was the direct or indirect effect of Smad3 deficiency on myofibroblast differentiation.

Renal interstitial inflammation was remarkably suppressed in the obstructed kidney of Smad3(−/−) mice as demonstrated by hematoxylin-esoin and periodic acid-Schiff staining and the number of infiltrating macrophages and T cells into the kidney (Figs. 2 and 4). Because Smad3 was critical for macrophage/monocyte chemotaxis [27, 30], the inhibition of macrophage migration into the obstructed kidney was most likely due to reduced macrophage/monocyte chemotaxis in the absence of Smad3. Although the role of Smad3 in T-cell chemotaxis remains unclear, it is possible that reduction of T-cell migration occurred through reduced production of cytokines/chemokines from macrophages involved in T-cell chemotaxis.

TUNEL assay showed clear reduction of tubular apoptosis in the obstructed kidney of Smad3(−/−) mice (Fig. 5). Renal tubular apoptosis after UUO was thought

to contribute to the progressive loss of renal function and accumulation of ECM surrounding the lost cells [3]. Our findings clearly showed that Smad3 was involved in renal tubular apoptosis in vivo. However, recently, Dai, Yang, and Liu [31] reported that TGF-β potentiated renal tubular epithelial cell death by a mechanism independent of Smad signaling. It should be therefore determined whether Smad3 was directly or indirectly involved in induction of renal tubular apoptosis in vivo.

We found that endogenous Smad2 or Smad2/3 was phosphorylated in cells located or infiltrated in renal interstitial area upon UUO induction in wild-type mice (Figs. 6 and 7). Thus, UUO-dependent activation of Smad2 appeared to occur largely in the cells in renal interstitial area. These results were consistent with upregulation of TGF-β in renal interstitial tissue after UUO as previously described [32]. Mizuno, Matsumoto, and Nakamura [32] reported that interstitial cells positive for TGF-β (e.g., macrophages, spindle-shaped fibroblast-like cells) were noted in the interstitial area of the mouse kidney after UUO. In addition, we noted that renal tubular cells appeared to be constitutively positive for phosphorylated Smad2 staining regardless of UUO induction (Fig. 6). Future studies should thus determine the biologic significance of constitutive endogenous Smad2 activation in renal epithelial cells as well as that of activation of endogenous Smad2 in renal interstitial area after UUO.

Lan et al [33] recently showed that gene transfer of Smad7, an inhibitor of TGF-β/Smad signaling, into the kidney inhibited renal fibrosis induced by UUO in rats. Most recently, Sato et al [34] reported that Smad3 deficiency protected against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction associated with blockade of epithelial-mesenchymal transition (EMT) as judged by α-SMA staining, abrogation of monocyte influx and collagen accumulation, which was essentially same findings as ours. Taken together with our findings, Smad3 is most likely to be a key mediator for renal fibrosis induced by UUO.

CONCLUSION

We clearly showed that Smad3 deficiency remarkably attenuated renal fibrosis, inflammation, and apoptosis induced by UUO. Modulation of Smad3 expression/or activity may therefore become potential therapeutic target for efficient therapy for obstructed kidney diseases or renal fibrosis.

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Induction of RANTES by TWEAK/Fn14 Interaction in Human Keratinocytes

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TNF-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) family, is a multifunctional cytokine that regulate cellular proliferation, angiogenesis, inflammation, and apoptosis. In this study, we investigated the effect of TWEAK on human keratinocytes. Primary cultured normal human keratinocytes constitutively expressed a TWEAK receptor, fibroblast growth factor-inducible 14 (Fn14), and produced regulated on activation, normal T expressed and secreted (RANTES) upon TWEAK stimulation in a concentration-dependent manner. The TWEAK-induced RANTES production was abrogated by anti-Fn14 antibody, and synergistically augmented by simultaneous stimulation with transforming growth factor-β. In addition, human keratinocytes differentiated *in vitro* with high Ca^{2+}-containing medium showed enhanced production of RANTES upon TWEAK stimulation. Furthermore, TWEAK induced rapid phosphorylation of IκB-α in human keratinocytes. Collectively, TWEAK acts on human keratinocytes as an inducer of RANTES via Fn14. Because RANTES has been implicated in inflammation, TWEAK/Fn14 interaction in human keratinocytes may be involved in the pathophysiology of inflammatory skin disorders.

Key words: keratinocytes/RANTES/TGF-β/TWEAK
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TNF-like weak inducer of apoptosis (TWEAK) was first identified as a new member of the tumor necrosis factor (TNF) superfamily which induced cell death in some tumor cell lines (Chicheportiche *et al*, 1997; Schneider *et al*, 1999; Nakayama *et al*, 2000). The human TWEAK gene is expressed in many different cell types and encodes a ~30 kDa type II transmembrane protein that can be cleaved to generate a ~18 kDa soluble factor with biological activity. Recently, Wiley *et al* (2001) have identified fibroblast growth factor-inducible 14 (Fn14) as a TWEAK receptor with physiological affinity. Fn14 is a type I transmembrane protein composed of only one cysteine-rich domain in the extracellular region and a short cytoplasmic region containing a TNF receptor-associated factor (TRAF)-binding motif. Saitoh *et al* (2003) recently reported that TWEAK stimulates two nuclear factor-κB (NF-κB) signaling pathways: IκB-α phosphorylation and p100 processing via TRAF molecules. These findings have suggested that TWEAK has other biological functions associated with NF-κB activation than regulating cell death.

Recent evidence indeed reveals that TWEAK is a multifunctional cytokine that regulated cellular proliferation, angiogenesis, and inflammation (Wiley and Winkles, 2003). Lynch *et al* (1999) reported that TWEAK induced proliferation of endothelial cells and angiogenesis. We also reported that TWEAK upregulated cell surface expression of adhesion molecules and induced secretion of chemokines such as interleukin (IL)-8 and monocyte chemotactic protein (MCP)-1 in human umbilical vein endothelial cells (HUVEC) (Harada *et al*, 2002). Furthermore, TWEAK induced several cytokines/chemokines such as IL-8, inducible protein (IP)-10, and regulated on activation, normal T expressed and secreted (RANTES) in human dermal fibroblasts and synoviocytes (Chicheportiche *et al*, 2002). Furthermore, TWEAK induced several cytokines/chemokines such as IL = 8, IP = 10, and RANTES in human dermal fibroblasts and synoriocytes (Chicheportiche *et al*, 2002).

In this study, we investigated Fn14 expression on human keratinocytes and examined the effect of TWEAK on chemokine production by human keratinocytes. We found that TWEAK stimulated human keratinocytes to produce a CCL chemokine, RANTES, via Fn14. Thus, TWEAK acts on human keratinocytes as an inducer of RANTES. Because RANTES is a potent chemoattractant for leukocytes and has been associated with a wide range of inflammatory disorders (Homey and Zlotnik, 1999; Zlotnik and Yoshie, 2000; Appay and Rowland-Jones, 2001), TWEAK/Fn14 interaction in human keratinocytes may be involved in pathophysiology of certain inflammatory skin diseases.

Abbreviations: Fn14, fibroblast growth factor-inducible 14; IFN-γ, interferon-γ; IL, interleukin; NF-κB, nuclear factor-κB; RANTES, regulated on activation, normal T expressed and secreted; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; TWEAK, TNF-like weak inducer of apoptosis

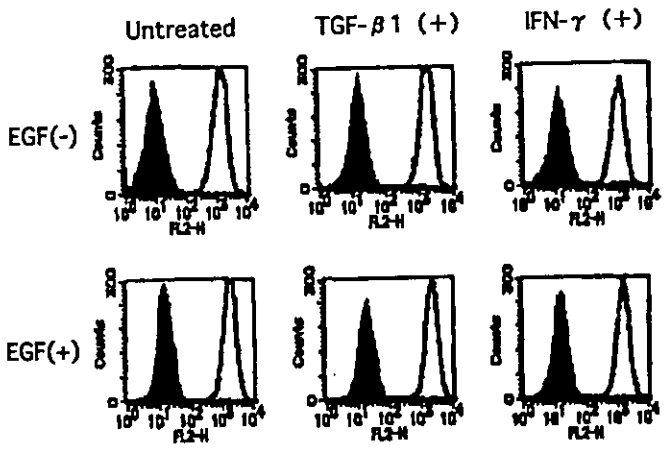

Figure 1
Cell surface expression of Fn14 on human keratinocytes. Cultured human keratinocytes were untreated or treated with 10 ng per mL of TGF-β1 or 10 ng per mL of IFN-γ for 24 h in EGF(−) or EGF(+) medium. Then, cells were stained with biotinylated anti-human Fn14 mAb (open histograms) or control Ig (filled histograms), followed by PE-labeled avidin, and then analyzed by flow cytometry. Representative of three repeated experiments with similar results were shown.

Results

Human keratinocytes constitutively express Fn14 To determine whether TWEAK may act on keratinocytes, we first examined the expression of Fn14 on cultured human keratinocytes. Flourescence activated cell sorting (FACS)-analysis using anti-Fn14 mAb showed that human keratinocytes expressed a high level of Fn14 on the cell surface, which was not affected by the presence or absence of EGF in the culture medium (Fig 1). Since Fn14 expression in endothelial cells and fibroblasts has been reported to be regulated by several growth factors and cytokines (Nakayama *et al*, 2000; Donohue *et al*, 2003; Wiley and Winkles, 2003), we next determined whether the Fn14 expression on human keratinocytes was regulated by certain cytokines. As shown in Fig 1, we found that the expression of Fn14 on keratinocytes was not affected by IFN-γ or TGF-β1.

TWEAK induces RANTES production by human keratinocytes Since cultured human keratinocytes constitutively expressed Fn14, we then investigated whether TWEAK could stimulate human keratinocytes through Fn14. Previous studies have shown that human keratinocytes could produce several chemokines that were associated with inflammatory skin diseases (Ying *et al*, 1995; Giustizieri *et al*, 2001; Horikawa *et al*, 2002). We thus examined the effect of recombinant TWEAK on IL-8, MCP-1, thymus and activation-regulated cytokine (TARC), Eotaxin, and RANTES production by human keratinocytes. As shown in Fig 2, TWEAK induced RANTES production in a concentration-dependent manner, which was almost completely inhibited by blocking the TWEAK/Fn14 interaction with anti-Fn14 mAb (Fig 2). In contrast, IL-8, MCP-1, TARC, and Eotaxin were not significantly produced in the presence or absence of TWEAK (data not shown). These findings indicated that TWEAK stimulated human keratinocytes to produce RANTES via Fn14.

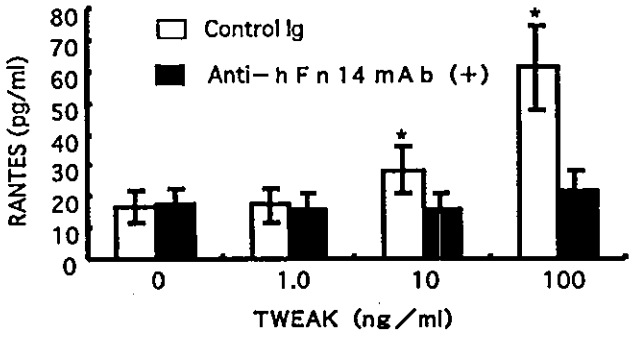

Figure 2
TWEAK induces RANTES production by human keratinocytes via Fn14. Cultured human keratinocytes were stimulated with the indicated doses of recombinant human TWEAK in the presence or absence of anti-Fn14 blocking mAb (5 μg per mL). for 48 h. Then, human RANTES concentration in the culture supernatants was measured by ELISA. Data are indicated as the mean ± SD of triplicate samples. *$p<0.05$, significantly different from the mean value of the corresponding control response. Similar results were obtained in three repeated experiments.

TGF-β synergistically augments TWEAK-induced RANTES production by human keratinocytes TGF-β has been implicated in the regulation of inflammation (Wahl, 1992) and is constitutively expressed in the human epidermis (Quan *et al*, 2002). We were thus interested in examining the effect of TGF-β on TWEAK-induced RANTES production by human keratinocytes. TGF-β1 alone modestly induced RANTES production by human keratinocytes (Fig 3). Notably, TGF-β1 showed a synergistic effect on the TWEAK-induced RANES production by human keratinocytes in a concentration-dependent manner (Fig 3). These findings indicated that TGF-β and TWEAK acted synergistically for RANTES production by human keratinocytes.

Differentiated human keratinocytes produce more RANTES upon TWEAK and TGF-β stimulation We next examined the effects of TWEAK and/or TGF-β1 on more differentiated keratinocytes. The human keratinocytes cultured in the presence of 1.2 mM Ca^{2+} for 2 d ceased proliferation, stratified and cornified as previously described (Stanley and Yuspa, 1983). As compared with the undifferentiated keratinocytes that were maintained in the culture medium containing 0.05 mM Ca^{2+}, the differentiated keratinocytes produced a comparable amount of RANTES in response to TWEAK alone but more RANTES in response to TGF-β1 alone (Fig 4). Moreover, the differentiated keratinocytes produced significantly increased amounts of RANTES in response to the combination of TWEAK and TGF-β1 (Fig 4).

Phosphorylation of IκB-α in TWEAK-stimulated human keratinocytes To explore the mechanisms by which TWEAK stimulated human keratinocytes, we investigated IκB-α phosphorylation in TWEAK-stimulated human keratinocytes. Saitoh *et al* (2003) showed that TWEAK induced phosphorylation of IκB-α, resulting in NF-κB activation in fibroblasts . As shown in Fig 5*A*, we found that TWEAK induced phosphorylation of IκB-α at 10 min after the stimulation in cultured human keratinocytes. Moreover, TWEAK and TGF-β1 showed an additive increase of

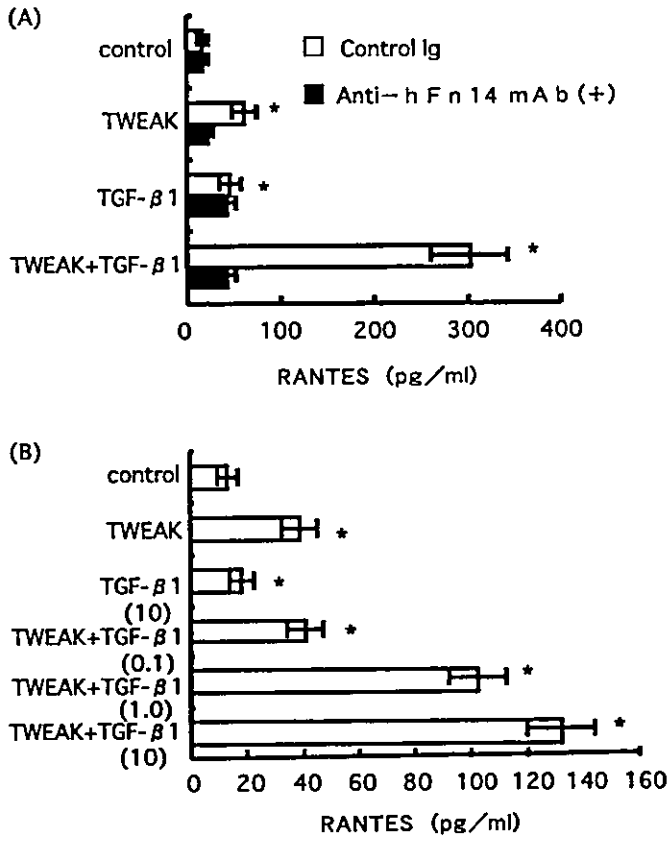

Figure 3
TGF-β1 synergizes with TWEAK for RANTES production by human keratinocytes. Cultured human keratinocytes were stimulated with 100 ng per mL of TWEAK and/or 10 ng per mL of TGF-β1 in the presence or absence of 5 μg per mL of anti-Fn14 blocking mAb (*A*) or stimulated with 100 ng per mL of TWEAK and 0.1, 1.0, or 10 ng per mL of TGF-β1 (*B*) for 48 h. Then, human RANTES concentration in the culture supernatants was measured by ELISA. Data are indicated as the mean ± SD of triplicate samples. *$p < 0.05$, significantly different from the mean value of the corresponding control response. Similar results were obtained in three repeated experiments.

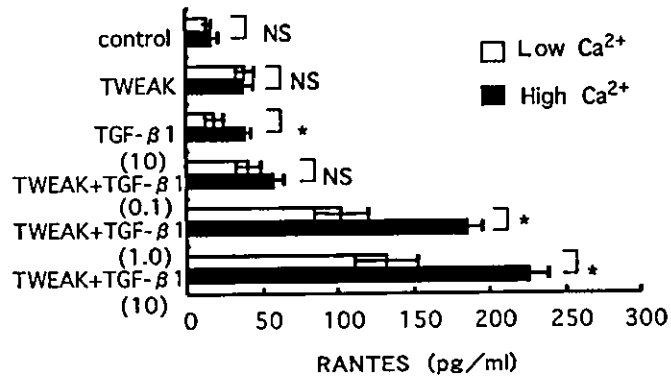

Figure 4
Differentiated keratinocytes show augmented RANTES production in response to TWEAK and TGF-β1. Human keratinocytes were cultured in the presence of 0.05 mM Ca^{2+} (white bars) or 1.2 mM Ca^{2+} (black bars) for 2 d and then stimulated with 100 ng per mL of TWEAK and 0.1, 1.0, or 10 ng per mL of TGF-β1 for 48 h. Then, human RANTES concentration in the culture supernatants was measured by ELISA. Data are indicated as the mean ± SD of triplicate samples. *$p < 0.05$, significantly different from the mean value of the corresponding control response. Similar results were obtained in three repeated experiments.

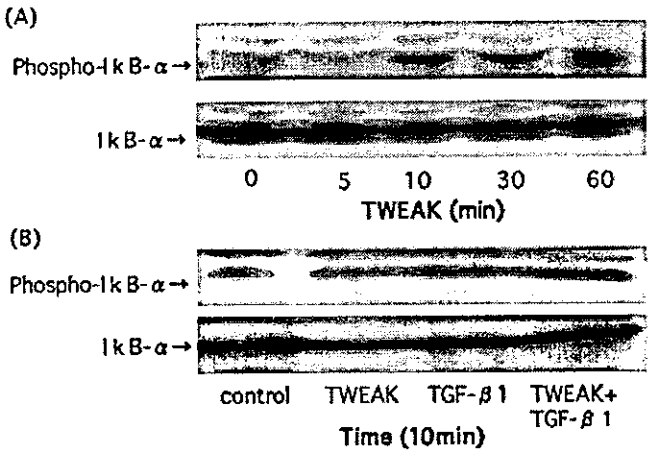

Figure 5
Phosphorylation of IκB-α in TWEAK-stimulated human keratinocytes. Cultured keratinocytes were stimulated with 100 ng per mL of TWEAK for the indicated time periods (*A*) or with 100 ng per mL of TWEAK and/or 10 ng per mL of TGF-β1 for 10 min (*B*). Then, the cell lysates were immunoblotted with anti-phosphorylated IκB-α (Ser32) antibody (upper panels) or anti-IκB antibody (lower panels). Representative of three repeated experiments with similar results.

phosphorylation of IκB-α (Fig 5*B*), which might be at least in part responsible for their synergistic action on the RANTES production.

Discussion

In this study, we demonstrated that cultured human keratinocytes expressed Fn14, which was responsible for TWEAK-induced RANTES production (Figs 1 and 2). TWEAK and TGF-β1 synergized to induce RANTES production by keratinocytes, and differentiated keratinocytes produced more RANTES in response to TWEAK and TGF-β1 (Figs 3 and 4). Because RANTES is a pro-inflammatory chemokine mediating the trafficking and homing of T cells, monocytes, eosinophils, natural killer cells, and mast cells (Homey and Zlotnik, 1999; Zlotnik and Yoshie, 2000; Appay and Rowland-Jones, 2001), these results suggest that TWEAK/Fn14 interaction in human keratinocytes may be involved in the pathogenesis of inflammatory skin disorders through RANTES production.

Cultured human keratinocytes constitutively expressed Fn14 on the cell surface (Fig 1). Because some growth factors or mitogens, including fibroblast growth factor (FGF)- and phorbol myristate acetate (PMA), regulated Fn14 expression in endothelial cells and fibroblasts (Meighan-Mantha *et al*, 1999; Donohue *et al*, 2003), we examined the influence of EGF in the keratinocyte culture and found that the Fn14 expression on human keratinocytes was not changed. We also found that IFN-γ or TGF-β1 did not affect the cell surface Fn14 expression on cultured human keratinocytes. Thus, it appeared that Fn14 expression on human keratinocytes was relatively stable as compared with endothelial cells and fibroblasts, although other growth factors or cytokines should be tested for possible regulation of Fn14 in human keratinocytes.

TWEAK induced RANTES, but not IL-8, MCP-1, TARC, and Eotaxin production in human keratinocytes (Fig 2 and

data not shown). We and others have previously shown that TWEAK induces several cytokines/chemokines; for example, IL-8 and MCP-1 from HUVEC (Harada *et al*, 2002), TNF-α from certain tumor cell lines (Nakayama *et al*, 2002), IL-8, IP-10, and RANTES from human dermal fibroblasts and synoviocytes (Chicheportiche *et al*, 2002). Thus, it seemed that the pattern of cytokines/chemokines induced by TWEAK varied depending on the cell types.

Because TGF-β has been implicated in the regulation of inflammation (Wahl, 1992) and is constitutively expressed in the human epidermis (Quan *et al*, 2002), we were interested in examining the effect of TGF-β on TWEAK-induced RANTES production by human keratinocytes. We found that TGF-β augmented TWEAK-induced RANTES production in human keratinocytes (Figs 3 and 4). Since previous studies showed that NF-κB activation resulted in upregulation of RANTES (Ebnet *et al*, 1997; Moriuchi *et al*, 1997; Ray *et al*, 1997), the findings that TWEAK and TGF-β1 showed an additive increase of phosphorylation of IκB-α (Fig 5*B*) might at least in part explain their synergistic action on the RANTES production. Alternatively, it is possible that TWEAK and TGF-β1 synergistically upregulate RANTES through their unique transcriptional factors. Indeed, synergistic effect of IFN-γ and TNF-α for RANTES production was reported in mouse fibroblasts by Ohmori *et al* (1997). They showed that IFN-γ and TNF-α synergistically upregulated RANTES through signal transducer and activator of transcription 1 (STAT1) and NF-κB, which was most likely mediated by their independent interaction with one or more components of the basal transcriptional complex.

The differentiated keratinocytes produced significantly increased amounts of RANTES in response to TGF-β1 or the combination of TWEAK and TGF-β1 as compared with undifferentiated keratinocytes (Fig 4). Thus, it appeared that differentiated keratinocytes were more susceptible to TGF-β action. Altered responses to TGF-β in undifferentiated and differentiated keratinocytes were also reported on the regulation of extracellular matrix gene expression (Vollberg *et al*, 1991). The mechanisms underlying these findings currently remain completely unclear and should be investigated in future studies.

In summary, we showed a novel biological activity of TWEAK on human keratinocytes. Because RANTES is a proinflammatory chemokine, induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes may be involved in pathophysiology of certain inflammatory skin disorders.

Materials and Methods

Reagents Recombinant human TWEAK was purchased from Alexis Biochemicals (California), and recombinant human transforming growth factor-β1 (TGF-β1) and recombinant human interferon-γ (IFN-γ) were purchased from R&D Inc (Minnesota). Anti-human Fn14 monoclonal antibodies (mAb) (ITEM-1 and ITEM-2) were generated in our laboratory as previously described (Nakayama *et al*, 2003). ITEM-2 is a blocking antibody preventing the interaction between TWEAK and Fn14 (Nakayama *et al*, 2003).

Keratinocyte culture Normal human keratinocytes from infant foreskins were cultured in serum-free keratinocyte growth medium, HuMedia-KG2 (Kurabo Industries, Osaka, Japan), containing epidermal growth factor (EGF) (0.1 ng per mL), insulin (10 μg per mL), hydrocortisone (0.5 μg per mL), gentamycin (50 μg per mL), amphotericin B (50 ng per mL), and bovine brain pituitary extract (0.4%, vol/vol) at 37°C in humidified atmosphere in presence of 5% CO_2. The experiments were carried out using third- or fourth-passage keratinocytes. Induction of keratinocyte differentiation was achieved by culturing the cells for 48 h in the presence of 1.2 mM Ca^{2+} in the culture medium as previously described (Stanley and Yuspa, 1983). Keratinocyte differentiation was confirmed by morphological change.

Flow cytometric analysis for Fn14 expression Cells (1×10^6) were incubated with biotinylated anti-human Fn14 mAb (ITEM-1) for 1 h at 4°C, followed by PE-labeled avidin (BD Pharmingen, San Diego, California). After washing with PBS, the cells were analyzed on a FACSCalibur (BD Pharmingen), and the data were analyzed using the CellQuest program (BD Pharmingen).

Chemokine ELISA The amounts of IL-8, MCP-1, TARC, Eotaxin, and RANTES in the supernatant of human keratinocytes culture (1×10^6 cells/well) were determined using the ELISA kits (R&D) according to the manufacturer's instruction.

Western blot Cell lysates of cultured human keratinocytes were taken after stimulation with recombinant human TWEAK (100 ng per mL) for the indicated times and were electrophoresed on a 10% sodium dodecylsulfate–polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Billerica, Massachusetts). Immunoblotting was performed using the phosphoPlus IκB-α (Ser32) antibody kit (Cell Signaling Technology, Massachusetts) according to the manufacturer's instruction.

Data analysis Data are summarized as mean ± SD. Statistical analysis was performed using the unpaired Student's *t* test. $p < 0.05$ was considered to be significant.

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Exogenous Smad3 Accelerates Wound Healing in a Rabbit Dermal Ulcer Model

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Exogenous administration of transforming growth factor-β (TGF-β) improves wound healing by affecting cellular and molecular events involved in tissue repair. But mice with a deficiency of a key TGF-β signaling intermediate, Smad3, paradoxically showed accelerated cutaneous wound healing, suggesting that endogenous Smad3 had inhibitory effect on cutaneous wound healing. Here we investigated the effect of exogenous expression of Smad3 in dermal fibroblasts on cutaneous wound healing. Subcutaneous injection of adenovirus-containing Smad3 complementary DNA (AdCMV-Smad3) targeting mainly dermal fibroblasts accelerated tissue repair following full-thickness dermal round wounds in rabbit ear as judged by the size of granulation tissue area, number of capillaries, and re-epithelialization rate of the wounds. Expressions of α-smooth muscle actin (α-SMA), vascular endothelial growth factor (VEGF), and fibroblast growth factor receptor were upregulated in the wounded area injected with AdCMV-Smad3. Consistent with the *in vivo* findings, overexpression of Smad3 induced α-SMA, VEGF, and TGF-β1 expression and augmented chemotactic response in cultured dermal fibroblasts. Therefore, exogenous administration of Smad3 targeting dermal fibroblasts accelerated tissue repair in a rabbit dermal ulcer model by affecting fibroblast responses associated with wound healing. The results suggest that Smad3, when over-expressed in dermal fibroblasts, can promote wound healing.

Key words: adenovector/fibroblast/Smad7
J Invest Dermatol 123:229–236, 2004

The wound healing is a multi-step complex process consisting of inflammation, granulation tissue formation, angiogenesis, re-epithelialization, and wound contraction (Singer and Clark, 1999). When a full-thickness dermal wound is made and filled by a fibrin clot, inflammatory cells first migrate into the plasma clot and release local growth factors, such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), which stimulate fibroblasts from the adjacent intact dermis to migrate to the wounded site. The migrating fibroblasts, along with newly formed vessels, fill the wound, which results in the formation of granulation tissue. Activated fibroblasts differentiate into myofibroblasts, a subpopulation of specialized fibroblasts that express α-smooth muscle actin (α-SMA), which elaborate collagen and other matrix components and which eventually contract the newly formed connective tissue to bring together the edges of the wound, followed by re-epithelialization and wound closes.

TGF-β is a multi-functional cytokine involved in cellular proliferation, survival, differentiation, migration, and extracelluar matrix production (Massague, 1990). Numerous evidence suggested that exogenous administration of TGF-β into the skin accelerated wound healing, either directly or indirectly, by stimulating recruitment of inflammatory cells, the production of extracellular matrix production, the formation of new blood vessels, and wound contraction (Sporn *et al*, 1983; Roberts, 1995). For instance, the injection of TGF-β directly to the wound at the time of wounding increased the healing rate accompanied by an increased influx of mononuclear cells and fibroblasts and by marked increases in collagen deposition at the site of application of TGF-β in rats (Mustoe *et al*, 1987).

Recent investigation has revealed that the action of TGF-β is mediated mainly by the Smad family of proteins (Heldin *et al*, 1997; Attisano and Warana, 2000; Massague, 2000). Activated TGF-β receptors phosphorylate the cytosolic receptor-activated Smad2 and Smad3, which form heteromeric complexes with Smad4, and enter the nucleus, bind to DNA, and regulate gene transcription in cooperation with DNA binding cofactors. Inhibitory Smads, Smad6 and Smad7, block TGF-β signal transduction, in part, by preventing the interaction of Smad2/3 with the activated TGF-β type I receptor.

In contrast to the prediction based on therapeutic effect of ectopic TGF-β on wound healing, Smad3-deficient mice showed accelerated wound healing associated with reduction of the influx of inflammatory cells and increased re-epithelialization (Ashcroft *et al*, 1999), suggesting that

Abbreviations: α-SMA, α-smooth muscle actin; cDNA, complementary DNA; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor

endogenous Smad3 functioned inhibitory for wound healing. In this study, we investigated the effect of exogenous expression of Smad3 in dermal fibroblasts on cutaneous wound healing using a rabbit dermal ulcer model.

Results

Adenovector-mediated *in vivo* gene transfer to the skin To determine whether exogenous Smad3 regulated wound healing, we examined the effect of exogenous Smad3 or Smad7 for comparison on tissue repair in a rabbit dermal ulcer model by using adenoviral-mediated skin gene delivery. We have previously demonstrated the feasibility of *in vivo* gene transfer to the skin mediated by replication-deficient adenoviral vectors (Setoguchi *et al*, 1994b).

Consistent with the previous report, subcutaneous injection of the adenovirus-containing lacZ cDNA (AdCMV-lacZ) into the rabbit ear showed significant expression of exogenous lacZ in the skin as judged by *in situ* β-galactosidase staining 2 days after the injection (Fig 1*A*). In higher magnification of the skin, the staining was observed largely in cells with spindle morphology (fibroblasts) in the rabbit dermis (Fig 1*B*).

Immunohistochemical studies with anti-Smad3 or anti-Smad7 antibody showed that the expression of Smad3 or Smad7 was detected on day 8 post-wounding after subcutaneous injection of AdCMV-Smad3 or AdCMV-Smad7 (Fig 2). It should be noted that the antibodies used for immunohistochemical detection of Smad3 or Smad7 failed to detect endogenous Smad3 or Smad7, but detected only overexpressed Smad3 or Smad7 after adenovirus infection in the tissues.

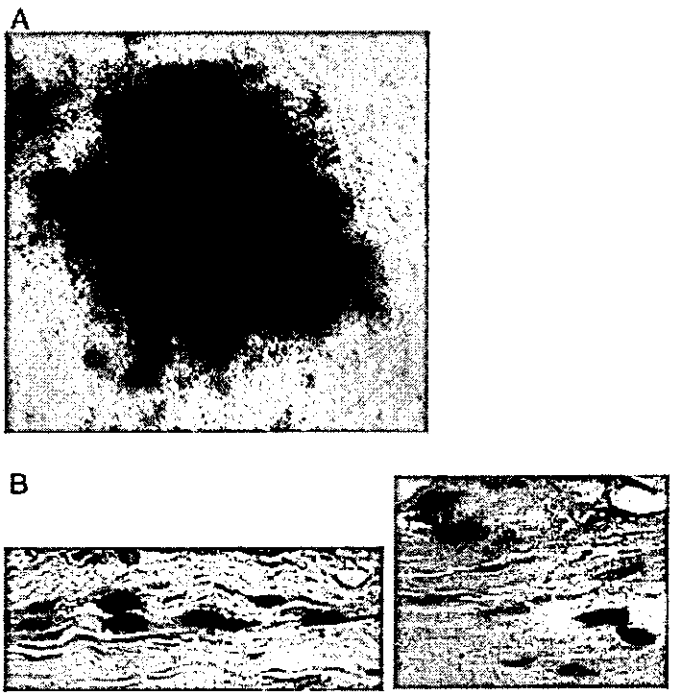


Figure 1
Expression of exogenous lacZ in rabbit skin. AdCMV-lacZ (5×10^8 pfu) was injected subcutaneously in the inner side of the ear. Two days after the injection, the skin was evaluated for the presence of the lacZ product (blue staining, β-galactosidase) using X-gal stain. Macroscopic (*A*) and microscopic (*B*) pictures of the skin subcutaneously injected with AdCMV-lacZ were shown. Please note that βgalactosidase expression (blue staining) was observed in the injected skin area (*A*) and interstitial spindle-like cells (*B*).

Exogenous Smad3, but not Smad7, accelerates cutaneous wound healing in rabbits We then examined the effect of *in vivo* gene transfer and expression of Smad3 or Smad7 on tissue repair after full-thickness wounding on rabbit ear. The amount of granulation tissue area, number of capillary lumens in the granulation tissue, and degree of re-epithelialization 8 days after the wounding were significantly enhanced in the wounded skin injected with AdCMV-Smad3 when compared with those in the wounded skin injected with AdCMV-lacZ (Fig 3). Interestingly, the injection of AdCMV-Smad3 showed a level of the wound healing equivalent to the application of total 1.5 μg of TGF-β1 (+AdCMV-lacZ) into the wounded skin. In contrast, the number of capillary lumens in the granulation tissue and degree of re-epithelialization 8 days after the incisional wounding were significantly reduced in the wounded skin injected with AdCMV-Smad7 when compared with those in the wounded skin injected with AdCMV-lacZ (Fig 3).

Because histological evaluation revealed that the injection of AdCMV-Smad3 accelerated tissue repair in a rabbit ulcer model, we next examined the effect of exogenous Smad3 on cellular and molecular events involved in wound healing. As shown in Fig 4, the number of α-smooth muscle (α-SMA) positive cells (myofibroblasts) and expression of VEGF and FGF receptors were strongly enhanced in the wounded skin injected with AdCMV-Smad3 when compared with the wounded skin injected with AdCMV-lacZ or AdCMV-Smad7. Thus, accelerated healing of dermal ulcer in a rabbit ear injected with AdCMV-Smad3 could be attributed, at least in part, to increased number of myofibroblast and increased expression of VEGF and FGF receptor in the skin.

Overexpression of Smad3 induces α-SMA, VEGF, and TGF-β1 expression and increases chemotactic response in human dermal fibroblasts *in vitro* Because exogenous administration of Smad3 affected granulation tissue formation, angiogenesis, and re-epithelialization *in vivo*, we examined the effect of Smad3 overexpression on several functions of dermal fibroblasts involved in wound healing.

Human dermal fibroblasts were infected with AdCMV-lacZ, AdCMV-Smad3, and AdCMV-Smad7 as previously described (Sumiyoshi *et al*, 2003) and evaluated for α-SMA expression, VEGF and TGF-β1 expression, and fibroblast chemotaxis (Figs 5–7). We found that overexpression of Smad3, but not lacZ or Smad7, strongly induced α-SMA expression as shown by western blotting and immunofluorescence studies (Fig 5). Overexpression of Smad3 also induced VEGF production (Fig 6*A*) and TGF-β1 expression (Fig 6*B*). In addition, fibroblast chemotaxis was augmented in human dermal fibroblasts overexpressing Smad3 when compared with the cells overexpressing lacZ or Smad7 (Fig 7). These results suggested that overexpression of Smad3 was sufficient to express α-SMA, VEGF, and TGF-β1 and to induce chemotactic response in dermal fibroblasts.

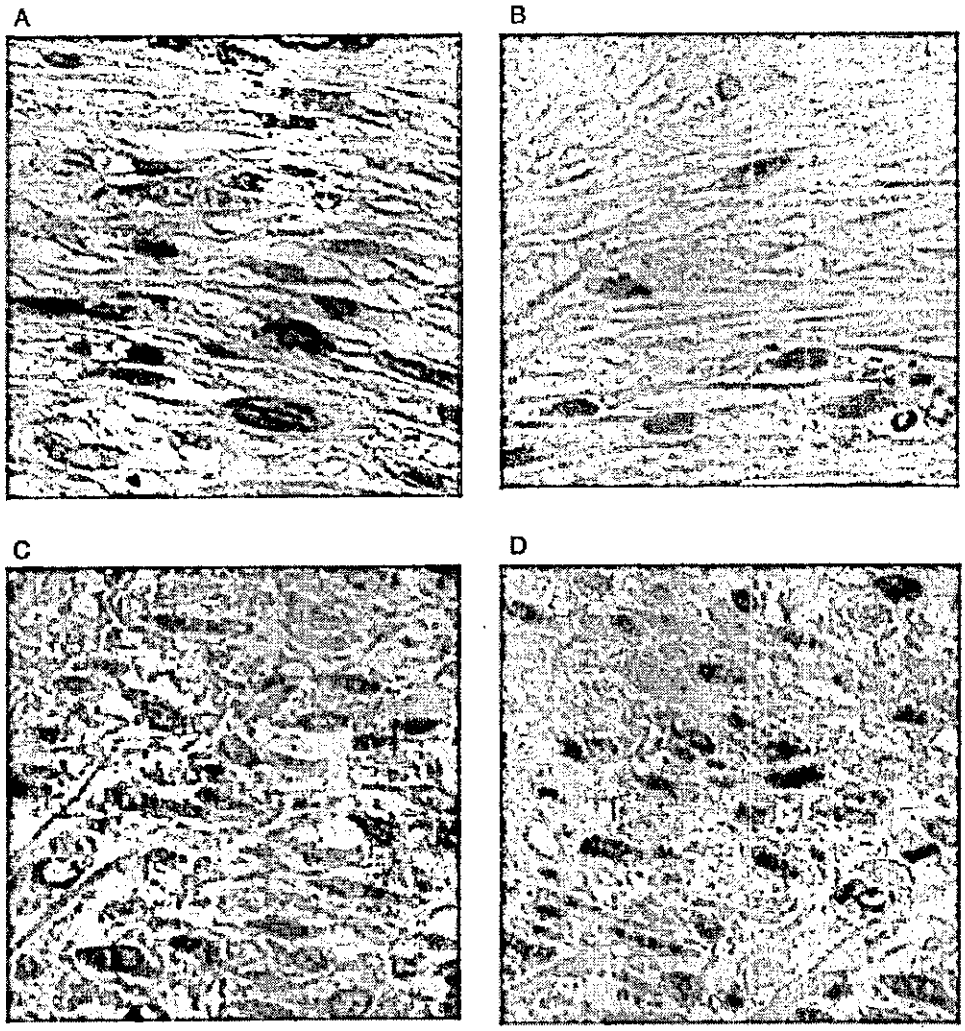


Figure 2
Expression of exogenous Smad3 or Smad7 in wounded skin. AdCMV-Smad3 (*A* and *B*) or AdCMV-Smad7 (*C* and *D*) (5×10^8 pfu) was injected subcutaneously in the inner side of the ear. Two days after the injections, full-thickness dermal round wounds were made in the injected area. On 8 days after the wounding, skin sections were immunohistochemically evaluated for the presence of Smad3 or Smad7 using antibodies against these Smads. Pictures after immunohistochemical stainings with specific antibodies against Smad3 (*A* and *C*) or against Smad7 (*B* and *D*) were shown. Brown color indicates positive staining. Please note that anti-Smad3 antibody showed immunoreactivity in the skin sections injected with AdCMV-Smad3 (*A*), but not with AdCMV-Smad7 (*C*). Please also note that anti-Smad7 antibody showed immunoreactivity in the skin sections injected with AdCMV-Smad7 (*D*), but not with AdCMV-Smad3 (*B*).

Discussion

In this study, we showed that subcutaneous injection of adenovirus-containing Smad3 cDNA into the rabbit ear accelerated tissue repair following full-thickness dermal wounds based on improvement of histological and cellular/molecular parameters (Figs 3 and 4). Because subcutaneous injection of adenoviruses-containing Smads appeared to target mainly dermal fibroblasts in our system (Figs 1 and 2, Setoguchi *et al*, 1994b), the accelerated wound healing by AdCMV-Smad3 could be, at least in part, attributed to the effect of exogenous Smad3 on several fibroblast functions involved in wound healing; that is, myofibroblast differentiation (α-SMA expression), VEGF and FGF receptors, and TGF-β1 expression, and chemotactic response as shown in Figs 5–7.

Ashcroft *et al* (1999) reported that Smad3-deficient mice showed accelerated cutaneous wound healing, suggesting that endogenous Smad3 had inhibitory effect on cutaneous wound healing. They suggested that downregulation of inflammation and acceleration of re-epithelialization of keratinocytes might contribute to the accelerated wound healing in Smad3-deficint mice. In contrast, we found that exogenous Smad3 resulted in increased granulation tissue formation and re-epithelialization (Fig 3). In Smad3-deficient mice, not only fibroblasts but also keratinocytes and inflammatory cells are Smad3-deficient and they observed net results of Smad3 deficiency in cutaneous wound healing. In our study, however, main target cells were dermal fibroblasts (Figs 1 and 2). Therefore, we think that this study emphasizes the activity of TGF-β/Smad3 on dermal fibroblast responses in the process of wound healing and the difference of target cells may explain different results in these studies. Alternatively, endogenous deficiency of Smad3 throughout the development may affect the cell function that is not attributed to the molecule itself or even alter the function of cells that do not express the molecule. This may also explain the different results in these studies. In addition, it should be noted that re-epithelialization in the whole wounded areas is usually complete between days 15 and 20 post-wounding in this dermal ulcer model in the rabbit ear and this study evaluate early phase of cutaneous wound healing regarding re-epithelialization.

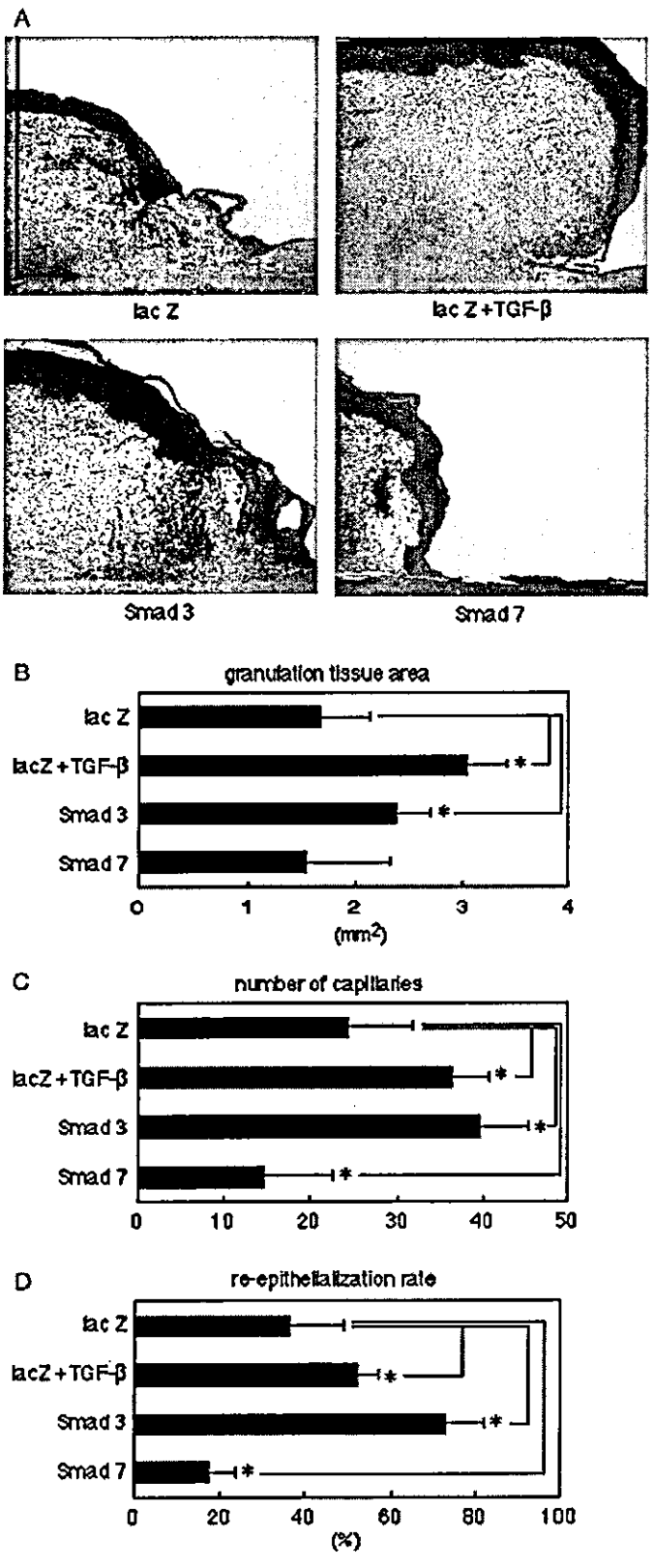
Previous studies reported that dermal fibroblasts expressed α-SMA, VEGF, and FGF receptors in response to TGF-β (Kikuchi *et al*, 1992; Desmouliere *et al*, 1993; Pertovaara *et al*, 1994). Because *in vivo* expression of α-SMA, VEGF, and FGF receptors after AdCMV-Smad3 injection appeared to be confined to the interstitial fibroblast-like cells (Fig 4) and overexpression of Smad3 induced these molecules in human dermal fibroblasts

in vitro (Figs 5 and 6), it is likely that, although TGF-β activates multiple intracellular signaling pathways (Massague, 2000), "Smad3" is sufficient for mediating TGF-β-induced expression of α-SMA, VEGF, and FGF receptors in dermal fibroblasts both *in vitro* and *in vivo*.

TGF-β is a potent chemoattractant for human dermal fibroblasts (Postlethwaite *et al*, 1987). Our findings that overexpression of Smad3 in human dermal fibroblasts enhanced chemotaxis (Fig 7) and increased granulation tissue formation after AdCMV-Smad3 injection (Fig 3) suggested that Smad3 mediated fibroblast chemotaxis both *in vitro* and *in vivo*. It remains unclear, however, whether Smad3, either directly or indirectly, stimulates fibroblast chemotaxis *in vivo*.

We speculate that exogenous expression of Smad3 may have some advantages over that of TGF-β for treatment of cutaneous wounds. First, because we found that the effect of AdCMV-Smad3 on cutaneous wound healing was comparable with the effect of 1.5 μg exogenous TGF-β1 (Figs 3 and 4), exogenous expression of Smad3 may be useful for treatment of cutaneous wounds without unfavorable systemic effect of TGF-β toward other organs or systems such as tissue fibrosis and immune suppression. Secondly, recent evidence suggests that chronic wounds become unresponsiveness to growth factors including PDGF and TGF-β (Hasan *et al*, 1997; Agren *et al*, 1999), resulting in delayed or incomplete wound healing. For instance, Kim *et al* (2003) reported that fibroblasts from chronic wounds showed decreased TGF-β type II receptor expression with their lack of response to TGF-β. Thus, direct activation of intracellular TGF-β signaling by overexpression of Smad3 may be a useful way for treatment of chronic cutaneous wounds even if dermal fibroblasts in wounded areas have altered TGF-β receptor expression and lack of TGF-β response.

In summary, we showed that exogenous overexpression of Smad3 in dermal fibroblasts promoted cutaneous wound healing. Our findings suggest that modulation of Smad3 expression in dermal fibroblasts may have therapeutic potential for the treatment of cutaneous wounds.



Materials and Methods

Adenovirus vector construction and virus purification The recombinant E1-deleted adenoviral vectors carrying mouse Smad3, Smad7, or lacZ complementary DNA (cDNA) under cytomegarovirus promoters, AdCMV-Smad3, AdCMV-Smad7, or AdCMV-lacZ, were generated, purified, and transfected as previously described (Setoguchi *et al*, 1994a; Fujii *et al*, 1999).

***In situ* β-galactosidase staining** *In situ* β-galactosidase staining was performed using *in situ* β-galactosidase staining kit (STRATAGENE) according to the manufacture's instruction.

The rabbit ear dermal ulcer model Wounding design and sample preparation were performed according to the previously reported method (Tsuboi *et al*, 1995). Female white rabbits, 2.5–3.0 kg (Shiraishi Laboratory Animals, Tokyo, Japan) were anesthetized

Figure 3
Acceleration of cutaneous wound healing by exogenous Smad3, but not Smad7. AdCMV-lacZ, AdCMV-Smad3, or AdCMV-Smad7 was subcutaneously injected into rabbit skin. Two days after the injections, full-thickness dermal round wounds were made in the injected area with or without application of TGF-β1 as indicated. Eight days after the wounding, skin sections were microscopically evaluated by HE staining (*A*) and by quantitative analysis of granulation tissue area (*B*), the number of capillaries (*C*), and re-epithelialization rate (*D*) as described in the Materials and Methods. Please note that injection of AdCMV-Smad3 enhanced granulation tissue area (*B*), the number of capillaries (*C*), and re-epithelialization rate as compared with injection of AdCMV-lacZ. The arrow in figure A lacZ picture indicates the wounded area in rabbit skin.

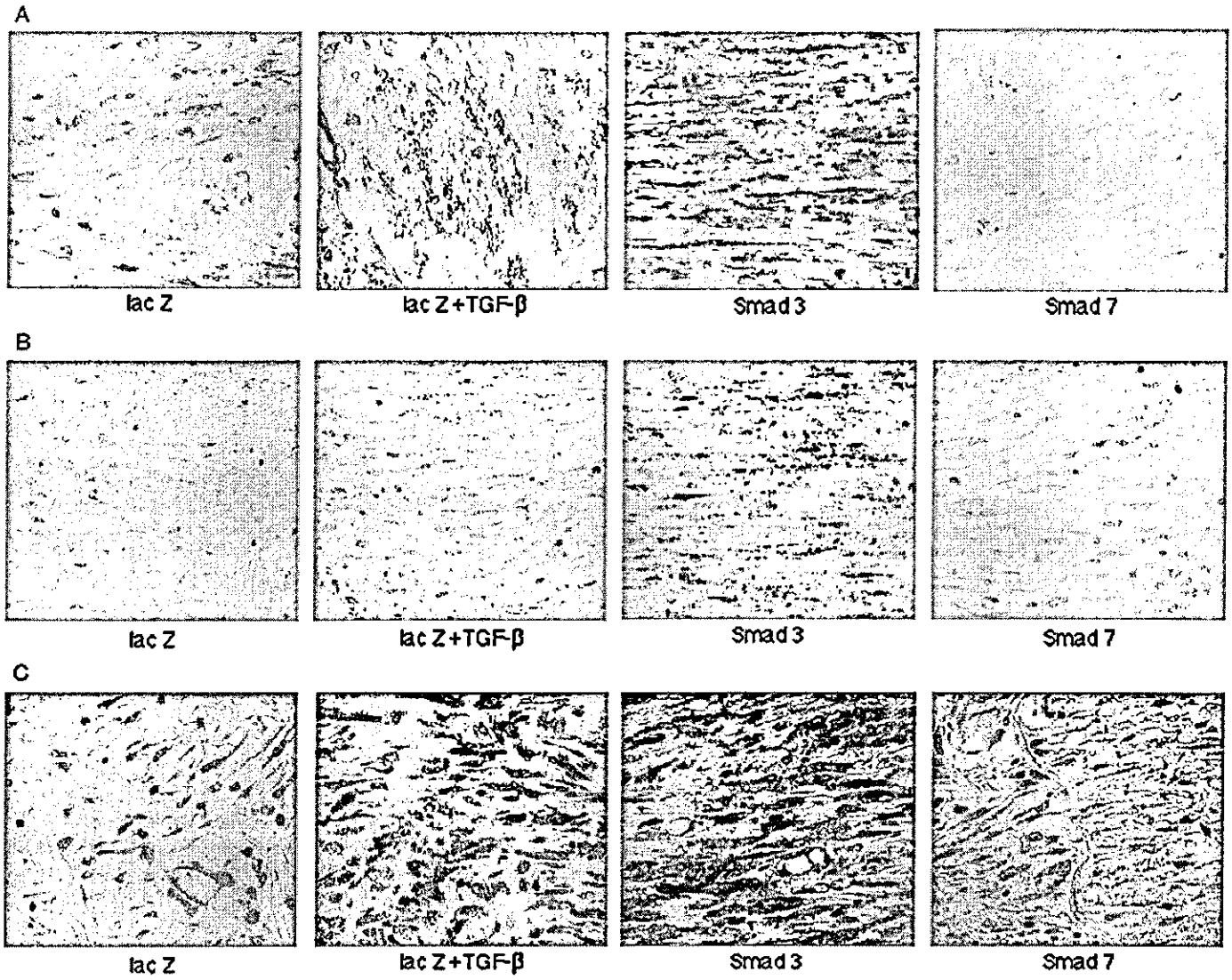


Figure 4
Induction of α-SMA, VEGF, and FGF receptor expression by exogenous Smad3 in wounded skin. AdCMV-lacZ, AdCMV-Smad3, or AdCMV-Smad7 was subcutaneously injected into rabbit skin. Two days after the injections, full-thickness dermal round wounds were made in the injected area with or without application of TGF-β1 as indicated. Eight days after the wounding, skin sections were immunohistochemically stained with antibodies specific for α-SMA (*A*), VEGF (*B*), and FGF receptor (*C*). Brown color indicates the positive staining. Please note that injection of AdCMV-Smad3 enhanced α-SMA (*A*), VEGF (*B*), and FGF receptor (*C*) expression (brown color) in the wounded skin.

with xylazine hydrochloride solution (10 mg per kg) (Bayer Co., Leverkusen, Germany) and ketamine hydrochloride solution (30 mg per kg) (Sankyo Co., Tokyo, Japan). After sterilization with iodine and alcohol, 5×10^8 plaque-forming units (pfu) of AdCMV-lacZ, AdCMV-Smad3, or AdCMV-Smad7 were injected subcutaneously in the inner side of the rabbit ear. The doses and timing of the adenovirus injection were determined according to our previous study (Setoguchi *et al*, 1994b). Forty-eight hours after the injections, four full-thickness round wounds were prepared on the injected area using a calibrated 6 mm trephine (Acu Punch, Acuderm Inc., Lauderdale, Florida) under sterile conditions (day 1). The perichondrium was kept undamaged. The wounds were covered with a sterilized transparent dressing (Tegaderm, 3M, Tokyo, Japan), and 20 μL PBS or 20 μL recombinant human TGF-β1 (R&D, Minnesota) solution (25 ng per μL: 500 ng per one ulcer) was applied to each wound using a syringe once every other day until the 5th day (on day 1, 3, and 5; total 1.5 μg per one ulcer). The ear was bandaged and kept covered throughout the experiment. The rabbits were killed on the 8th day after the wounding by intravenous administration of pentobarbital solution. The wounded areas were excised and fixed in 10% buffered formalin solution. All animal experiments were performed according to the approved manual of the Institutional Review Board of Juntendo University.

Histological evaluation After overnight fixation, the tissue was trimmed and cut through at the widest margin. The tissue was embedded in paraffin and sectioned in 5 μm increments. The sections were made perpendicular to the anterior–posterior axes and perpendicular to the surface of the wound. Three sections were placed on a slide, and stained with hematoxylin and eosin. Of the three sections on any one slide, the section with the widest original wound margin was used for assessment. The parameters measured were degree of granulation tissue area, number of capillaries, and re-epithelialization as previously described (Tsuboi *et al*, 1995). Each of the parameters was graded numerically as described below.

Granulation tissue area The amount of granulation tissue was quantified by measuring the area of granulation tissue (mm^2) in the section perpendicular to the surface of the wound. Granulation tissue was traced by a computerized morphometric analysis (KS-400, Carl Zeiss Inc., Gotingen, Germany).

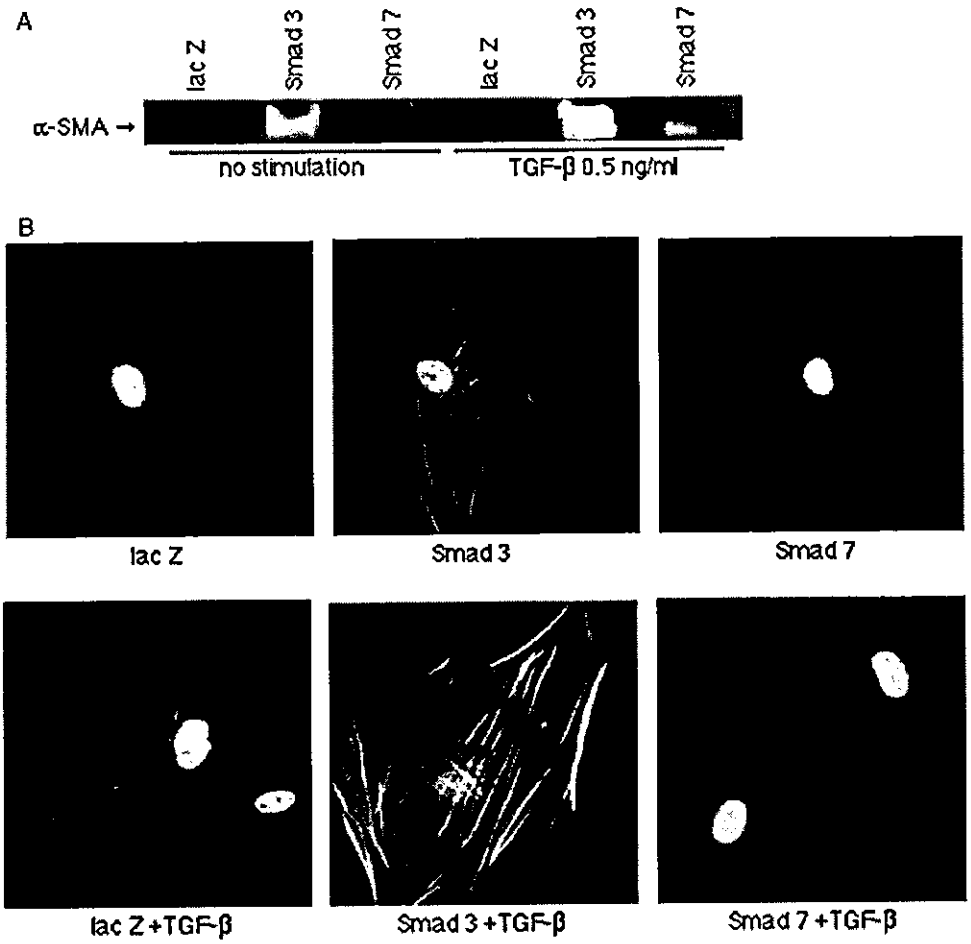


Figure 5
Induction of α-SMA by overexpression of Smad3 in human dermal fibroblasts. Cultured human dermal fibroblasts infected with adenoviruses carrying lacZ, Smad3, or Smad7 cDNA were incubated with 0.5 (*A*) or 10 ng per mL (*B*) of TGF-β1 for 24 h. Then, western blot analysis (*A*) or immunofluorescence anlayisis with anti-α-SMA antibody followed by incubation with Alexa488-conjugated secondary antibody (Green staining) (*B*) was performed. Nucleus was stained with DAPI solution (Blue staining). Similar results were obtained in at least three independent experiments. Please note that overexpression of Smad3 in dermal fibroblasts enhanced α-SMA expression as judged by western blotting (*A*) and immunofluorescence study (*B*).

Number of capillaries The number of capillary lumens in the granulation tissue was counted in the complete wound cross-section at × 100 magnification.

Re-epithelialization The degree of re-epithelialization was measured by a computerized morphometric analysis (KS-400) and was given a value by percentage; 0% was equivalent to no closure and 100% was equivalent to complete wound closure.

Immunohistochemistry Skin paraffin sections were deparaffinized and then preincubated with normal mouse or goat serum (1:20) for 20 min. The slides were incubated overnight at 4°C with mouse monoclonal antibody against vascular endothelial growth factor (VEGF) (05-443, Upstate Biotechnology, Lake Placid, New York 1:50) or mouse monoclonal antibody against FGF receptor (MAB125, CHEMICON, Temecula, California 1:150), goat polyclonal antibody against Smad3 (sc-6202, Santa Cruz Biotechnology, Inc., Santa Cruz, California 1:100), mouse monoclonal antibody against α-SMA (U7033, DAKO EPOS, 1:1), or goat polyclonal antibody against Smad7 (sc-7004, Santa Cruz Biotechnology, Inc., 1:100). Sections were then incubated for 60 min at room temperature with an Envision labeled polymer reagent (K1490, DAKO, Glostrup, Denmark). To increase their sensitivity, sections were then incubated for 10 min at room temperature with a Tyramide signal amplification (TSA) Biotin System (NEN life science products, Boston, Texas, 1:50). Sections were then incubated with a streptavidin (DAKO, 1:400) for 30 min. Between steps, the slides were rinsed for 10 min in PBS. All sections were lightly counterstained with hematoxylin.

Cell culture Primary human dermal fibroblasts were cultured as previously described (Lee *et al*, 1996). Briefly, fibroblasts were obtained from the outgrowth of infant foreskin and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 μg per mL streptomycin. All experiments were carried out using fibroblasts in passage 4–8th. Infection of recombinant adenoviruses was performed at a multiplicity of infection (m.o.i.) of 2×10^2 pfu per cell in 1 mL of serum-free DMEM for 60 min and overexpression of exogenous Smad3 or Smad7 in the cultured human dermal fibroblasts after the transfection was previously reported (Sumiyoshi *et al*, 2003). Dermal fibroblasts infected with the adenovirus solutions were cultured in DMEM supplemented with 10% FCS for 48 h and thereafter used for the following experiments.

Immunoblot Immunoblotting with anti-α-SMA antibody (American Research Product, Belmont, Massachusetts 1:500) was performed as previously described (Nakao *et al*, 1997).

Immunofluorescence microscopy Dermal fibroblasts infected with the adenovirus solutions were grown on rat tail collagen type I-coated eight well culture slides (Beckton Dickinsons Labware, Bedford, Massachusetts) in the absence or presence of TGF-β1 (10 ng per mL) (R&D, Minnesota) for 24 h, then washed with PBS and fixed with 4% paraformaldehyde. Following permeabilization, slides were stained with anti-α-SMA antibody (American Research Product, 1:3) diluted in PBS 90 min at room temperature. After extensive washing, slides were incubated with Alexa488-conjugated goat anti-mouse antibody (1:200) for 1 h at room temperature. After extensive washing, slides were incubated with DAPI solution (100 ng per mL) for 5 min. Fibroblasts were mounted in fluorescent mounting medium (DAKO), and images were acquired using a confocal microscopy (ECLIPSE E800, Nikon, Japan).

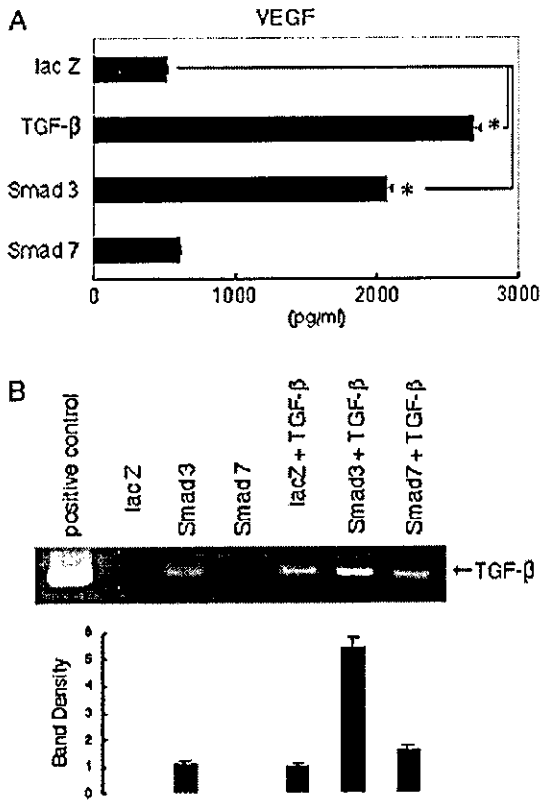


Figure 6
Induction of VEGF and TGF-β1 by overexpression of Smad3 in human dermal fibroblasts. Cultured human dermal fibroblasts infected with adenoviruses carrying lacZ, Smad3, or Smad7 cDNA were incubated with 10 ng per mL of TGF-β1 for 72 h. Then, human VEGF concentration in the culture supernatants was measured by ELISA (*A*) or mRNAs were taken for detection of TGF-β1 mRNA by RT-PCR (*B*). The relative density of the each PCR band from three separate experiments was estimated by using a one-dimensional image analyzer and was indicated as a bar graph. Data are indicated as the mean ± SD. Please note that overexpression of Smad3 in dermal fibroblasts enhanced VEGF (*A*) and TGF-β1 (*B*) expression.

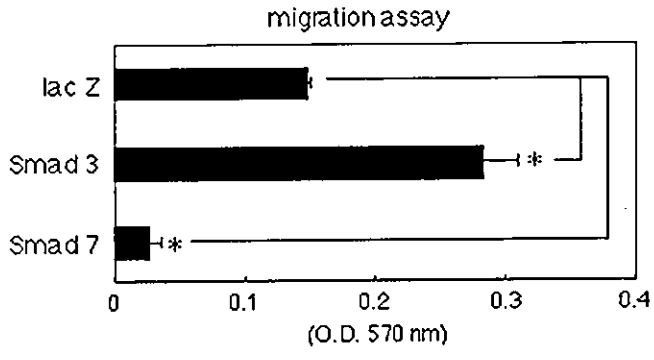


Figure 7
Enhanced migration of human dermal fibroblasts by overexpression of Smad3. Cultured human dermal fibroblasts infected with adenoviruses carrying lacZ, Smad3, or Smad7 cDNA were subjected to migration assay as described in the Materials and Methods. Data are indicated as the mean ± SD of triplicate samples. *$p<0.05$, significantly different from the mean value of the corresponding control response. Similar results were obtained in at least three independent experiments. Please note that overexpression of Smad3 in dermal fibroblasts enhanced their migration.

Migration assay Migration assay was performed using quantitative cell migration assay kit (ECM500, Chemicon International Inc.) according to the manufacture's instruction.

Cytokine ELISA The amount of VEGF in the culture supernatant of fibroblasts was determined using human VEGF ELISA kit (R&D, Minnesota).

RT-PCR Total RNA was prepared from cultures of dermal fibroblasts 72 h after the infection of adenoviruses carrying lacZ, Smad3, and Smad7, as recommended by manufacture's instructions for Isogen solution (Nippon Gene, Japan). cDNA was synthesized from 3 μg of total RNA using first strand cDNA synthesis kit (Ready To Go) (Amersham Pharmacia, Piscataway, New Jersey). PCR amplification (95°C for 1 min, 58°C for 2 min, and 72°C for 2 min; 25 cycles) was performed in a DNA thermal cycler (Perkin-Elmer, Wellesley, Massachusetts). The PCR products were size-fractionated by agarose gel electrophoresis using 2.0% agarose, and stained with 0.5 μg per mL ethidium bromide. As positive controls, we used expression constructs of human TGF-β1 cDNA. Primers used in this study were as follows: TGF-β1 (5′-AGTATGGACACAGGCTCTCC-3′ and 3′-GTCTTATGGCTTCC-GTCTG-5′), and hypoxanthine phosphoribosyltransferase (HPRT) (5′-TTCTTTGCTGACCTGCTG-3′ and 3′-TTTCTACCAGTTCCAG-CG-5′).

Data analysis Data are summarized as mean ± SD. Statistical analysis was performed using the unpaired Student *t* test. $p<0.05$ was considered to be significant.

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Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration

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Abstract

Oxidative stress is a characteristic of chronic inflammatory diseases. The reactive oxygen intermediate hydrogen peroxide (H_2O_2) is an important signaling molecule that modulates gene expression. We have demonstrated that H_2O_2 significantly enhanced cytokine production in BEAS-2B cells, with a maximal effect at 4 h. This did not result from enhanced NF-κB activation, but through decreased activity of histone deacetylase (HDAC)2. This results in increased inflammatory gene expression following acetylation of specific histone residues. Decreased HDAC2 activity was associated with tyrosine nitration status. Peroxynitrite and SIN-1, a peroxynitrite generator, were also able to reduce HDAC2 activity via tyrosine nitration. Our data suggest that oxidative stress contributes to worsening inflammation via reduction of HDAC2 activity through HDAC2 nitration. This novel mechanism of inflammation may be important in increasing the severity and chronicity of inflammatory diseases.

Keywords: Oxidative stress; Histone acetylation; Gene expression; Inflammation; Peroxynitrite

Oxidative stress is characteristic of chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, and inflammatory bowel disease [1,2]. Elevated intracellular reactive oxygen species (ROS) are generated under various physiological and pathological conditions, including inflammation, ischemia and reperfusion, and sepsis. They can be derived from sources as different as activated inflammatory cells, oxidized lipoproteins, cigarette smoke, and structural cells [3]. Major ROS are superoxide anions (O^{2-}), hydroxyl radicals ($^{\cdot}OH$), and H_2O_2. In addition, peroxynitrite is a potent radical formed from a rapid interaction between superoxide anions (O^{2-}) and nitric oxide (NO) [4]. ROS mediate some biological responses, such as cytokine (IL-6, IL-8, and TNFα) induction by IL-1β or TNFα [4,5]. This is reported to be due to enhanced NF-κB activation in response to H_2O_2 [4–6].

Chromatin structure and binding of proteins to DNA can be modulated by reversible acetylation of lysine residues within the N-terminal tails of core histones. In the resting cell, DNA is tightly compacted to prevent transcription factor accessibility. During activation of the cell, this compact inaccessible DNA is made available to transcription factors through histone acetylation. This chemical modification is carried out by histone acetyltransferases (HAT) and counteracted by histone deacetylases (HDAC) [7] and [8] many transcriptional co-activators, such as CREB-binding protein (CBP), have intrinsic HAT activity. IL-1β and TNFα can both stimulate the binding of NF-κB (p65 subunit) to CBP, increase HAT activity, and induce histone acetylation, thus leading to increased inflammatory gene (such as GM-CSF and IL-8) transcription [9,10]. Hydrogen peroxide (H_2O_2) and other ROS can also induce enhanced inflammatory mediator release from cells, a process that is associated with changes in histone acetylation [11].

Several reports have shown that HDACs 1–3 can also be associated with inactive p65 and play a role in the regulation of NF-κB-mediated gene transcription without altering the degree of DNA binding [9,12–14]. Thus, changes in HDAC activity associated with p65 can enhance or repress NF-κB-mediated gene expression [12].

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We have previously reported that HDAC2 expression and activity is decreased in smokers, COPD subjects, and mild asthma patients and that there is a good correlation between cytokine production and HDAC activity in alveolar macrophages from smokers and nonsmokers [11]. Reduced HDAC2 may be a key factor in the development of inflammation in airway obstructive disease. There is abundant evidence for increased oxidative stress and peroxynitrite formation in COPD and severe asthma [2]. We show that nitration of HDAC2 following oxidative stress may account for the reduced HDAC activity seen in cells from patients with oxidant stress related diseases.

Materials and methods

Materials. Thirty percentage of H_2O_2 and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (Sigma, Poole, UK); IL-1β was from R&D (Abingdon, UK); DCFH-DA and SIN-1 were from Molecular Probes (Leiden, Netherlands); and peroxynitrite was from Cayman Chemicals (Ann Arbor, MI, USA). Anti-p65 (sc-109, sc-7151), anti-phosphotyrosine (sc-508), anti-HDAC2 (sc-7899), and anti-HDAC1 (sc-6298) antibodies were obtained from Santa Cruz Biotec (Santa Cruz, California, USA), and anti-nitrotyrosine (1A6) was obtained from Upstate (Charlottesville, VA, USA).

Cell culture. BEAS-2B cells were grown to 50% confluence in keratinocyte conditioned medium (Gibco, Paisley, UK). Before experimentation, cells were serum-starved for 24 h in medium without EGF and bovine pituitary extracts. Cells were stimulated by IL-1β (1 ng/ml) in the presence or absence of H_2O_2 (100 μM) or SIN-1 (500 μM).

Cytokine ELISA. Determination of GM-CSF and IL-8 expression was measured by sandwich ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

DCF assay for oxidative stress. Oxidative stress was detected by the modified method described by Wang and Joseph [15]. Viable cells were plated into 96-well culture plates 1 day before the experiments. On the day of the experiments, after removing the medium, the cells in the plates were washed with Krebs–Ringer–Hepes–glucose–glutamine buffer (KRH buffer) and then incubated with 100 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the loading medium in 5% CO_2/95% air at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with KRH buffer with SIN-1 (500 μM) or H_2O_2 (100 μM) and the fluorescence of the cells from each well was measured and recorded. The excitation filter was set at 485 nm and the emission filter was set at 530 nm.

Nuclear extraction. Cells were collected and resuspended in mild lysis buffer [10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, and complete protease inhibitor cocktail (Boehringer–Mannheim)] for 10 min. Nuclei were collected by microcentrifugation (10,000 rpm, 5 min, 4 °C) and resuspended in Tris-based high salt buffer (10 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol, and complete protease inhibitor cocktail). After 30 min, supernatant was collected and diluted with Tris-based, no salt buffer (10 mM Tris–HCl, pH 8.0, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol, and complete protease inhibitor cocktail) to 40 mM NaCl final concentration.

Immunoprecipitation. Extracts were prepared using 100 μl of modified RIPA buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 0.5% NP-40, 0.1% SDS, 0.5% deoxycholate, and complete protease inhibitor cocktail (Boehringer–Mannheim)] as previously described [9]. For the HDAC assay, immunoprecipitates were washed twice with HDAC buffer (10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 0.25 mM EDTA, and 10 mM of 2-mercaptoethanol) and for Western blotting the buffer was aspirated completely and resuspended in Laemmli buffer.

Western blotting. Immunoprecipitates or nuclear extractions were analyzed by SDS–PAGE and Western blot analysis using ECL as previously described [9].

NF-κB activation. NF-κB activation was measured with TransAM NF-κB kit (Active Motif, California, USA).

Histone deacetylase assay. HDAC assays were performed as previously described [9].

Immunocytochemistry. BEAS-2B cells (0.5×10^6) were cultured in 8-well slide chambers with IL-1β (1 ng/ml) in the presence or absence of H_2O_2 and immunocytochemistry for p65 performed as previously described [9]. Stained cells were observed by confocal microscopy. Confocal scanning laser microscopy images were collected with a Leica confocal microscope, equipped with a 488/514 nm dual band argon ion laser. An oil-immersion objective was used and images were collected using TCSNT software.

Chromatin immunoprecipitation (ChIP) assay. BEAS-2B cells pretreated with H_2O_2 (4 h) or trichostatin A (TSA, 10 min) were treated with IL-1β (1 ng/ml) as described above. After a 0.5-h incubation, protein–DNA complexes were fixed by formaldehyde (1% final concentration) and treated as previously described [9]. Acetylated H4 or NF-κB–p65 binding IL-8 promoter (−121 to +61) was quantified by real-time PCR using a QuantiTect SYBR Green PCR kit (Qiagen) on a Rotor-Gene 3000 (Corbett Research, NSW, Australia).

Statistics. Results are expressed as means ± standard error of the mean (SEM). A multiple comparison was made between the mean of the control and the means from each individual treatment group by Dunnett's test using SAS/STAT software (SAS Institute, Cary, NC, USA). All statistical testing was performed using a two-sided 5% level of significance.

Results

H_2O_2 enhances IL-1β-stimulated cytokine expression

Pretreatment of human airway epithelial cells (BEAS2B) with H_2O_2 (100 μM) for 4 h slightly enhanced basal IL-8 production but markedly potentiated IL-1β-stimulated IL-8 production (2497 ± 226 ng/ml versus 1066 ± 64) (Fig. 1A) without affecting cell survival (data not shown). This enhancement by pretreatment of H_2O_2 was maximal at 4 h (Fig. 1B). Four hour pretreatment of H_2O_2 also enhanced GM-CSF production as well as IL-8 (211 ± 22 vs 138 ± 36). This effect was blocked by the anti-oxidant *N*-acetyl-L-cysteine (NAC, 10 mM, Fig. 1C) (IL-1β only 1066 ± 64, IL-1β + H_2O_2, 2497 ± 226, and IL-1β + H_2O_2 + NAC 1334 ± 09 ng/ml).

H_2O_2 does not enhance NF-κB nuclear translocation

IL-1β-induced p65 nuclear translocation was rapid (detectable at 10 min), peaked at 30 min, and remained elevated for at least 2 h (data not shown). H_2O_2 (100 μM) induced some p65 nuclear translocation but this was minimal compared to that seen with IL-1β and peaked at 2 h (Fig. 2A). H_2O_2 did not significantly enhance IL-1β-induced p65 nuclear translocation (Fig. 2B). These results were confirmed by immunocytochemistry (Fig. 2C). Furthermore, H_2O_2 did not

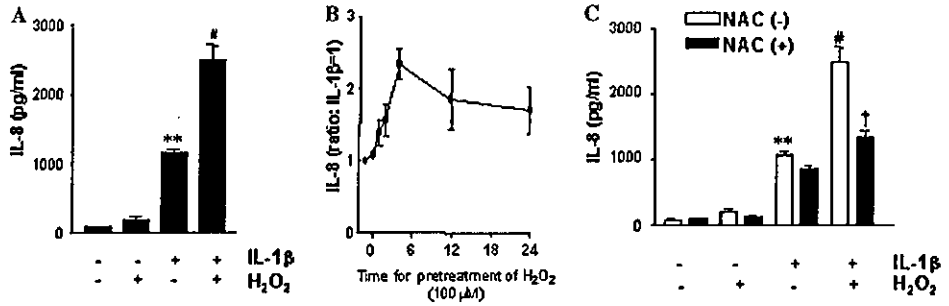

Fig. 1. Effect of H_2O_2 on IL-1β-induced inflammatory cytokine production. (A) BEAS-2B cells were stimulated by IL-1β (1 ng/ml) for 24 h in the presence or absence of H_2O_2 (100 μM) and IL-8 was measured by ELISA. (B) Effect of different pretreatment periods with H_2O_2 on IL-1β-induced IL-8 production. (C) The effect of *N*-acetyl-L-cysteine (10 mM) added 10 min before H_2O_2 pretreatment. Cells were stimulated with IL-1β 4 h after treatment and IL-8 was measured after overnight incubation. Results are expressed as means ± SEM ($n = 3$–5), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control; $^{\#}p < 0.05$ compared with IL-1β-stimulated; and $^{\dagger}p < 0.05$ compared with IL-1β plus H_2O_2.

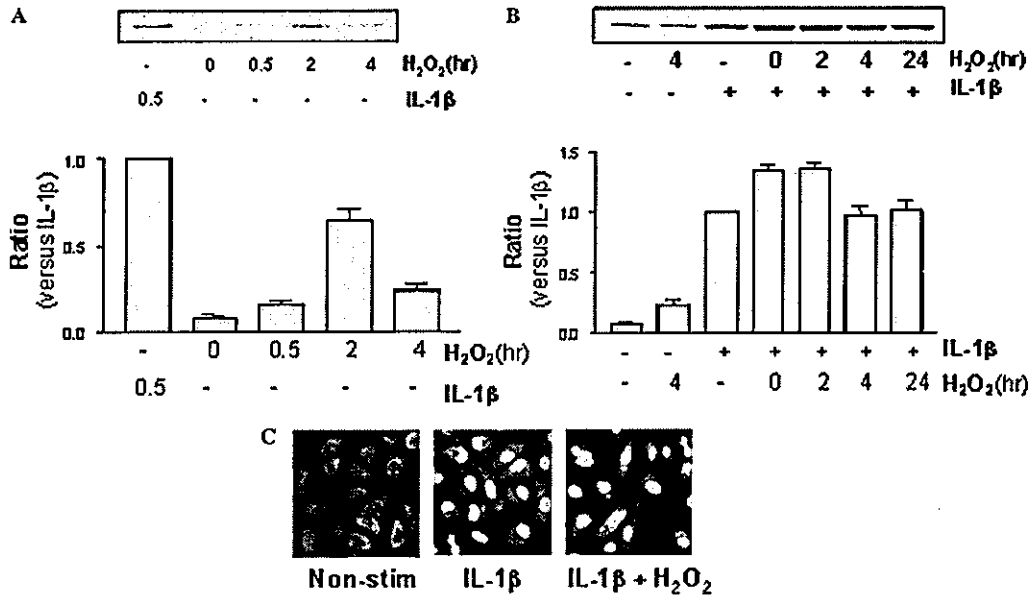

Fig. 2. H_2O_2 does not markedly enhance IL-1β-induced NF-κB nuclear translocation. (A) Representative Western blot analysis of NF-κB p65 subunit nuclear translocation in BEAS-2B cells. The cells were treated with IL-1 (1 ng/ml) or H_2O_2 (100 μM) and collected at the indicated time (h). Densitometric analysis of each band is plotted below. Results are expressed as means ± SEM as the ratio versus IL-1β alone, $n = 3$ independent experiments. (B) Representative Western blot analysis of NF-κB p65 subunit nuclear translocation in BEAS-2B cells treated with IL-1β (1 ng/ml) in the presence of H_2O_2 (100 μM). H_2O_2 was pretreated for indicated time. Cells were collected 30 min after IL-1β stimulation. Densitometric analysis of each band is plotted below. Results are expressed as means ± SEM as the ratio versus IL-1β alone. (C) Immunocytochemistry of p65 in BEAS-2B cells. Cells were pre-treated with H_2O_2 for 4 h and fixed 30 min after IL-1β stimulation.

significantly enhance IL-1β induced p65 activation measured with TransAM kit (absorbance: basal, 0.21 ± 0.045; IL-1β, 1.43 ± 0.16; and IL-1β + H_2O_2, 1.58 ± 0.16). Trichostatin A (10 ng/ml), a histone deacetylase inhibitor, also enhanced IL-1β-induced IL-8 production (2821 ± 171 vs. 1066 ± 64 ng/ml) without activation of NF-κB (data not shown). Pathways, other than NF-κB activation, must therefore be responsible for the marked elevation of IL-1β-stimulated cytokine release.

H_2O_2 enhances GM-CSF promoter-associated histone acetylation

We analyzed the effect of H_2O_2 (100 μM) on IL-1β-induced increase in histone 4 acetylation associated with the IL-8 promoter in BEAS-2B cells by quantitative chromatin immunoprecipitation. Following IL-1β treatment p65 immunoprecipitates showed a marked enrichment of IL-8 promoter (−121 to +61) DNA (Fig. 3A). Immunoprecipitation with an antibody