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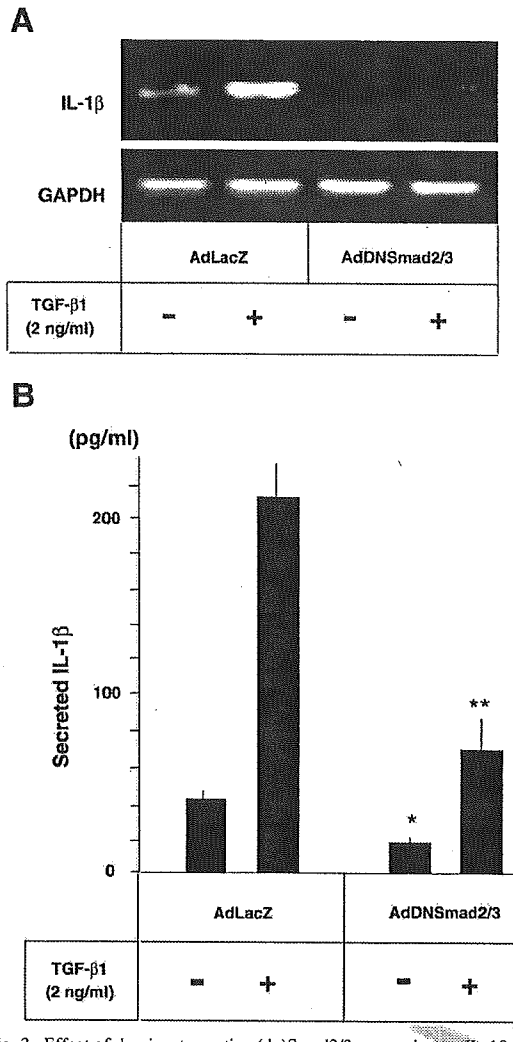

Fig. 3. Effect of dominant-negative (dn)Smad2/3 expression on IL-1β expression and secretion by PSCs. PSCs were infected with 10 plaque-forming units (PFU)/cell of adenovirus expressing β-galactosidase (Ad-LacZ) or dnSmad2/3 adenovirus (Ad-dnSmad2/3) and then incubated for 48 h with or without 2 ng/ml TGF-β_1. *A*: after incubation, IL-1β mRNA expression was determined by RT-PCR using GAPDH mRNA expression as a control. *B*: concentration of IL-1β secreted from PSCs into culture medium during 48-h incubation was determined by ELISA. Values are means ± SE of 3 independent experiments. *$P < 0.05$ vs. first column. **$P < 0.01$ vs. second column.

TGF-β_1 augmented IL-1β secretion from PSCs in a dose-dependent manner (Fig. 2*B*). We next examined exogenous TGF-β_1 effect on IL-1β expression and secretion at earlier time points. As shown in Fig. 2, *C* and *D*, 2 ng/ml TGF-β_1 started enhancing IL-1β mRNA expression and secretion within 3 and 6 h of incubation, respectively. These data imply that TGF-β_1 enhances IL-1β expression and secretion by PSCs.

Autocrine TGF-β_1 enhances IL-1β expression and secretion by PSCs through Smad-dependent pathways. We next examined the intracellular signaling pathway through which TGF-β_1 stimulates IL-1β expression and secretion by PSCs. Because Smad proteins are the major mediators of TGF-β signaling, we investigated Smad-dependent-pathways using adenoviral vectors of Smad2 (Ad-Smad2), Smad3 (Ad-Smad3), and dn-Smad2/3 (Ad-dnSmad2/3). We used an adenovirus expressing β-galactosidase (Ad-LacZ) as an infection control. We previously reported that >98% of PSCs are infected with these adenoviral vectors and expressed each Smad protein sufficiently (25). In addition, the infections of these adenoviruses do not affect TGF-β_1 mRNA expression or peptide secretion by PSCs (25). Therefore, we can observe the effect of these adenoviral infections on IL-1β expression and secretion by PSCs modulated by autocrine TGF-β_1, regardless of the effect of the infection on the amount of autocrine TGF-β_1. When Smad-dependent pathways were blocked by Ad-dnSmad2/3, both basal IL-1β mRNA expression (Fig. 3*A*; compare first and

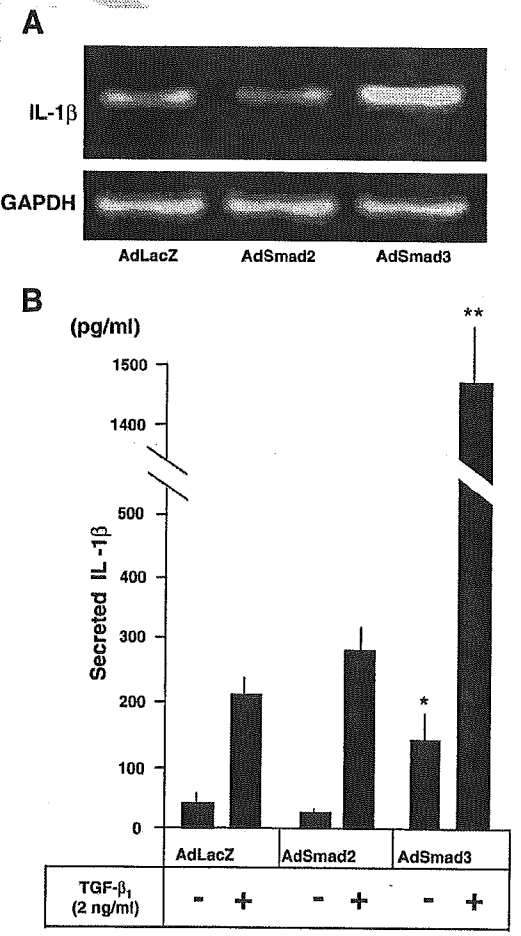

Fig. 4. Effect of Smad2 and Smad3 overexpression on IL-1β expression and secretion of PSCs. PSCs were infected with 10 PFU/cell of Ad-LacZ, Ad-Smad2, or Ad-Smad3. *A*: IL-1β mRNA expression was determined by RT-PCR after 48-h incubation using GAPDH mRNA expression as a control. *B*: concentration of IL-1β secreted from PSCs into culture medium during 48-h incubation with or without 2 ng/ml TGF-β_1 was determined by ELISA. Values are means ± SE of 3 independent experiments. *$P < 0.05$ vs. first column. **$P < 0.01$ vs. second column.

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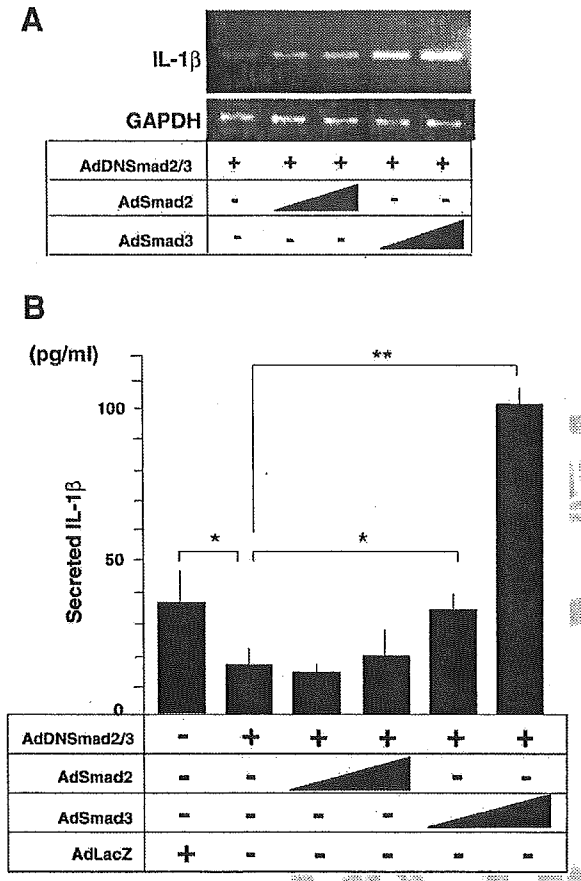

Fig. 5. Effect of coexpression of Smad2 or Smad3 with dnSmad2/3 on IL-1β expression and secretion by PSCs. For double-adenovirus infection experiments, PSCs were infected with the combination of Ad-Smad2 and Ad-dnSmad2/3 or Ad-Smad3 and Ad-dnSmad2/3. Single infection of Ad-LacZ or Ad-dnSmad2/3 was used as a control. *A*: after 48-h incubation, IL-1β mRNA expression was determined by RT-PCR using GAPDH mRNA expression as an internal control. *B*: concentration of IL-1β secreted from PSCs into culture medium during 48-h incubation was determined by ELISA. Values are means ± SE of 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control.

third lanes) and basal peptide secretion (Fig. 3*B*; compare first and third columns) of PSCs were attenuated. Because infection with Ad-dnSmad2/3 does not alter autocrine TGF-β1 secretion from PSCs as reported previously (25), these data indicate that TGF-β1 stimulates IL-1β mRNA expression and peptide secretion in an autocrine manner via a Smad-dependent pathway. Moreover, Ad-dnSmad2/3 attenuated exogenous TGF-β1-enhanced IL-1β expression and secretion (Fig. 3, *A* and *B*), thus reinforcing our suggestion that TGF-β1 stimulates IL-1β expression and secretion via a Smad-dependent pathway.

Smad3-dependent pathway mediates TGF-β1 stimulation to IL-1β expression and secretion by PSCs. We subsequently tried to elucidate whether the Smad2- or Smad3-dependent pathway mediates TGF-β1 stimulation of IL-1β expression and secretion by PSCs. Ad-Smad3, but not Ad-Smad2, increased *1*) basal IL-1β mRNA expression (Fig. 4*A*) and *2*) basal and TGF-β1 stimulated IL-1β secretion by PSCs (Fig. 4*B*). Because both Smad2 and Smad3 competitively inhibit one another at the receptor and *smad4* binding sites, the effect of Smad3 overexpression on IL-1β expression and secretion by PSCs may result from the inhibition of endogenous Smad2 and may not imply the involvement of Smad3-dependent pathway in TGF-β1-enhanced IL-1β expression and secretion. To exclude this possibility, we investigated the specific roles of Smad2 and Smad3 in eliciting autocrine TGF-β1-stimulated IL-1β expression and secretion after coinfection of PSCs with Ad-dnSmad2/3 and either Ad-Smad2 or Ad-Smad3. On the one hand, coinfection with Ad-Smad3 precluded Ad-dnSmad2/3 inhibition of IL-1β expression (Fig. 5*A*) and secretion (Fig. 5*B*). On the other hand, coinfection with Ad-Smad2 did not alter Ad-dnSmad2/3 inhibition of IL-1β expression or secretion (Fig. 5). These data suggest that TGF-β1 increases the expression and secretion of IL-1β by PSCs via a Smad3-dependent, Smad2-independent pathway.

Autocrine IL-1β promotes TGF-β1 secretion from PSCs. Because IL-1β has been reported to modulate PSC function (17, 19), we hypothesized that IL-1β may increase the autocrine secretion of TGF-β1 from PSCs. To test this hypothesis, we first examined the effect of anti-IL-1β antibody, which neutralizes IL-1β bioactivity, on TGF-β1 secretion from PSCs. As shown in Fig. 6, anti-IL-1β antibody added into culture medium attenuated TGF-β1 secretion from PSCs in a dose-dependent manner. In contrast, nonimmune IgG did not affect TGF-β1 secretion from PSCs. These data indicate that autocrine IL-1β stimulated TGF-β1 secretion from PSCs.

IL-1β enhances TGF-β1 mRNA expression and peptide secretion by PSCs through an ERK-dependent pathway. We next examined the effect of exogenous IL-1β on TGF-β1 expression

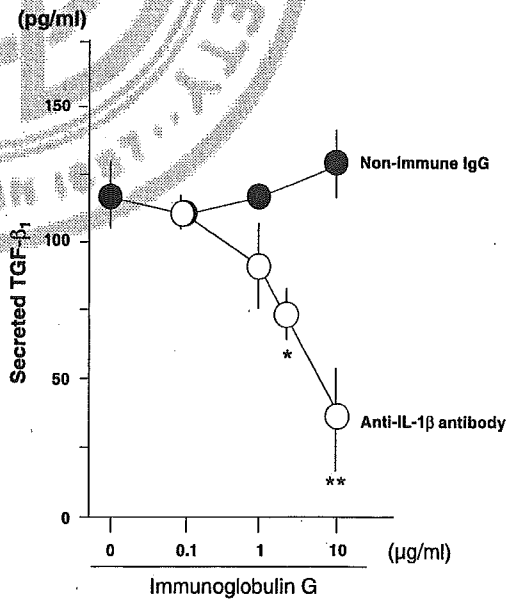

Fig. 6. Effect of anti-IL-1β-neutralizing antibody and nonimmune IgG on TGF-β1 secretion from PSCs. Concentration of TGF-β1 secreted from PSCs into culture medium was determined by ELISA after 48-h incubation with indicated amounts of anti-IL-1β antibody (○) or nonimmune IgG (●). Values are means ± SE of 3 independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control.

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and secretion by PSCs. Because secreted IL-1β concentration in culture medium reaches a maximum after 48 h of incubation, we first examined the effect of IL-1β on TGF-β_1 mRNA expression and peptide secretion by PSCs at this time point. As shown in Fig. 7, *A* and *B*, exogenous IL-1β added to culture medium enhanced TGF-β_1 mRNA expression and peptide secretion in a dose-dependent manner. We next examined IL-1β effect on TGF-β_1 expression and secretion at earlier time points. As shown in Fig. 7, *C* and *D*, 10 ng/ml IL-1β started augmenting TGF-β_1 mRNA and peptide secretion in 3 and 6 h of incubation, respectively. These data reinforce that IL-1β enhances TGF-β_1 secretion from PSCs. Because ERK is one of IL-1β signaling mediators (16), we subsequently examined the participation of the ERK-dependent pathway in IL-1β stimulation of TGF-β_1 expression and secretion by PSCs. To this end, we blocked ERK activation using the MEK1 inhibitor PD-98059. We previously reported that pretreatment of PSCs with 10 nM PD-98059 for 48 h successfully blocks TGF-β_1-induced ERK activation (25). Pretreatment of the cultures with PD-98059 attenuated exogenous IL-1β enhancement of TGF-β_1 mRNA expression in PSCs (Fig. 8*A*; compare second and fourth lanes). Moreover, PD-98059 pretreatment decreased both basal and IL-1β-stimulated TGF-β_1 peptide secretion from PSCs (Fig. 8*B*). Finally, we have confirmed that IL-1β activates ERK in PSCs (Fig. 8*C*). These data indicate that IL-1β stimulates TGF-β_1 expression and secretion by PSCs via an ERK-dependent pathway.

DISCUSSION

In this study, we have demonstrated that anti-TGF-β_1 and anti-IL-1β neutralizing antibodies attenuate IL-1β and TGF-β_1 secretion from activated PSCs, respectively. Furthermore, IL-1β expression and secretion by PSCs are enhanced by TGF-β_1 via a Smad3-dependent pathway. We also found that IL-1β stimulates TGF-β_1 expression and secretion by PSCs via

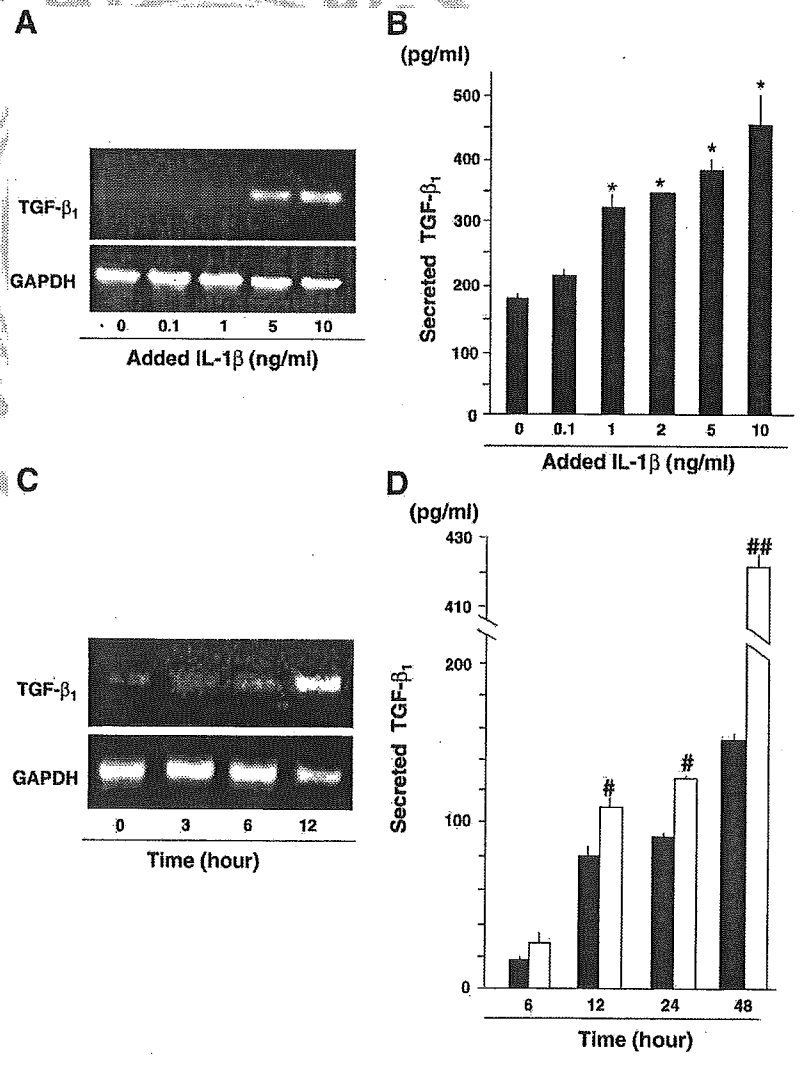

Fig. 7. Effect of IL-1β on TGF-β_1 expression and secretion by PSCs. *A* and *C*: TGF-β_1 mRNA expression was determined by RT-PCR after 48-h incubation with indicated amounts of IL-1β (*A*) or after incubation for indicated times with 10 ng/ml IL-1β (*C*). GAPDH mRNA expression was determined as an internal control. *B* and *D*: concentration of TGF-β_1 secreted into culture medium from PSCs was quantified by ELISA after 48-h incubation with indicated amounts of IL-1β (*B*) or after incubation for indicated times with (open bars) or without (solid bars) 10 ng/ml IL-1β (*D*). Values are means ± SE of 3 independent experiments. $*P < 0.05$ vs. control (*B*). $\#P < 0.05$, $\#\#P < 0.01$ vs. controls without IL-1β (*D*).

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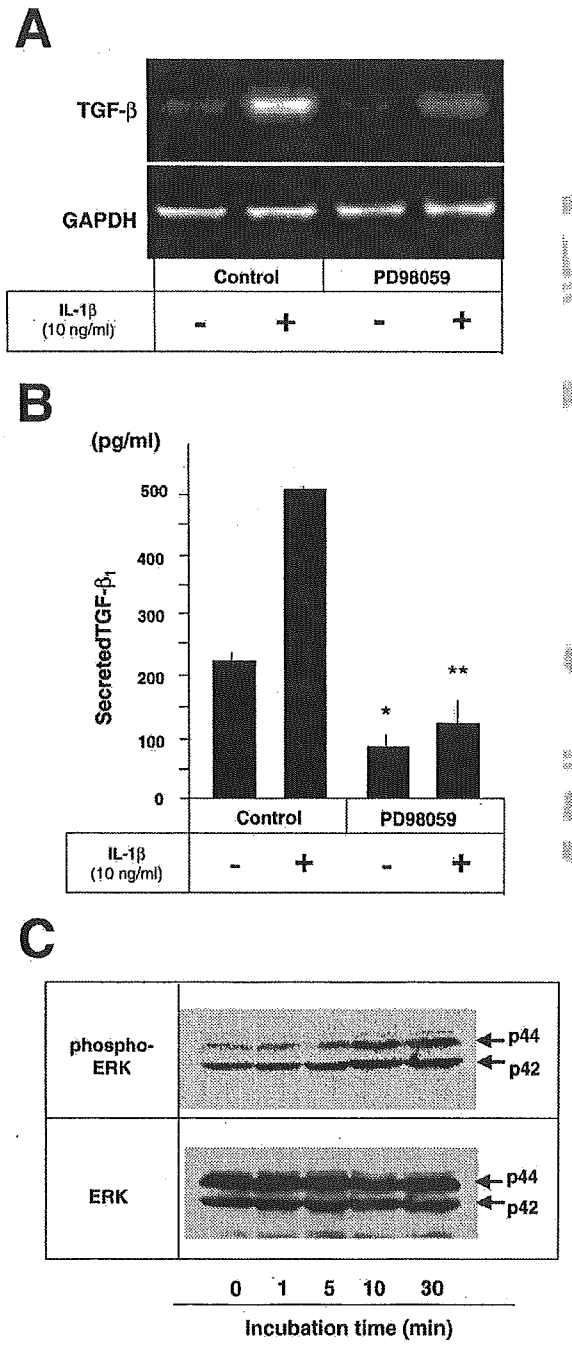
AQ: 7 an ERK-dependent pathway. Accordingly, TGF-β and IL-1β stimulate each other's expression and secretion via Smad3- and ERK-dependent pathways, respectively, indicating the existence of an autocrine loop between IL-1β and TGF-β_1 in activated PSCs. Because both TGF-β_1 and IL-1β are fibrogenic factors, our observations suggest an interaction between TGF-β_1 and IL-1β during pancreatic fibrosis.



IL-1β is a major proinflammatory cytokine and plays a critical role in various inflammatory diseases of a variety organs (10). For example, at the onset of acute pancreatitis, IL-1β is produced in and secreted from pancreatic acinar cells and transmigrated leukocytes and subsequently mediates systemic inflammatory responses, such as systemic circulatory failure and respiratory distress (8, 21). Repeated acute pancreatitis results in chronic pancreatitis with irreversible parenchymal destruction and glandular fibrosis. Pancreatic fibrosis is characterized by disorganized collagen deposition and acinar cell atrophy (8, 29). Although systemic inflammatory responses are readily resolved in chronic pancreatitis, the serum level of IL-1β remains significantly elevated in these patients (7), and IL-1β is known to promote pancreatic fibrosis. Currently, it is assumed that local cytokine sources are more important than distant sources because cytokines function exclusively in their immediate pericellular milieu (11). Therefore, in studies of the mechanism of inflammatory diseases, considerable attention has been focused on both autocrine and paracrine sources of cytokines (11). In acute pancreatitis, numerous inflammatory cells infiltrate pancreas tissues and secrete various cytokines, which induce both local and systemic inflammatory responses (22). Although not as severe as in acute pancreatitis, the infiltration of inflammatory cells into pancreatic tissues also is frequently observed in chronic pancreatitis (9a). Thus inflammatory cells, including macrophages, are assumed to be important sources of proinflammatory cytokines in chronic pancreatitis. In addition to inflammatory cells, activated PSCs also secrete various cytokines. However, the mechanism that regulates cytokines production and secretion by activated PSCs has not been resolved. We designed the present study to examine IL-1β expression and secretion by culture-activated PSCs and to elucidate the molecular mechanism whereby TGF-β_1 regulates this expression.

TGF-β_1 participates in the regulation of a variety of PSC functions. For example, TGF-β_1 *1*) stimulates ECM synthesis, *2*) promotes PSC activation, *3*) attenuates proliferation in an autocrine manner, and *4*) reduces matrix metalloproteinase (MMP)-3 and -9 expression (30). However, the intracellular signaling pathways through which TGF-β_1 exerts these diverse regulatory effects on PSC function have not been demonstrated. The facts that Smad proteins are major mediators of TGF-β_1 signaling and dual Smad2 and Smad3 pathways transduce TGF-β_1 stimuli raise questions concerning which pathway mediates the TGF-β_1 effects on cell function in various organs. However, because Smad2 and Smad3 are competitive with each other at TGF-β_1 receptor and Smad4 binding sites, it

Fig. 8. Effect of MEK1 inhibitor PD-98059 on TGF-β_1 expression and secretion by PSCs. *A* and *B*: after 2-h pretreatment with or without 10 nM PD-98059, cultured PSCs were incubated for 48 h in the presence or absence of 10 ng/ml IL-1β. *A*: after incubation, TGF-β_1 mRNA expression was determined by RT-PCR using GAPDH mRNA expression as an internal control. *B*: TGF-β_1 peptide secreted into culture medium from PSCs during 48-h incubation quantified by ELISA. Values are means ± SE ($n = 3$). *$P < 0.05$ vs. first column. **$P < 0.001$ vs. second column. *C*: effect of IL-1β on ERK activation in PSCs. Cells were incubated with 10 ng/ml IL-1β for indicated times. ERK activation was then determined using Western blot analysis with antiphosphorylated ERK antibody (*top*). Western blot analysis using anti-ERK antibody was performed as an internal control (*bottom*).

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has been difficult to examine their specific roles in TGF-β_1 signal transduction. The targeted homozygous deletion of Smad2 and Smad3 genes in mice is a powerful tool for analyzing their functions in embryogenesis (9, 20 33, 34). However, because Smad2-knockout mice usually die in utero, elucidating the specific roles of Smad2 and Smad3 concurrently in differentiated cells derived from mature animals is difficult (20, 33). We developed a novel method to analyze Smad2- and Smad3-specific roles in TGF-β_1 signal transduction using adenovirus-mediated coexpression of Smad2 or Smad3 with dnSmad2/3 (25). We have demonstrated that TGF-β_1 enhances IL-1β expression and secretion by activated PSCs via a Smad3-dependent pathway.

Our data regarding IL-1β-enhanced TGF-β_1 expression and secretion by activated PSCs are important. IL-1β has been shown to induce TGF-β_1 production in various cell types (1, 28). However, the intracellular signaling pathway through which IL-1β stimulates TGF-β_1 expression is unclear. Because NF-κB is the proinflammatory transcription factor, the role of NF-κB in TGF-β_1 production during inflammatory responses has been studied. Rameshwar et al. (28) reported that IL-1β-induced TGF-β_1 expression in monocytes obtained from patients with idiopathic myelofibrosis is dependent on NF-κB. However, NF-κB participation in TGF-β_1 induction, even that induced by IL-1β, is thought to be indirect because of the absence of NF-κB binding site on the enhanced promoter region of the TGF-β_1 gene (26). To our knowledge, this report is the first to elucidate the intracellular signaling pathway for IL-1β induction of TGF-β_1.

TGF-β_1 is secreted as a latent complex with TGF-β propeptide and latent TGF-β binding protein. After secretion, multiple TGF-β activators, such as matrix MMP-2 and -9, convert latent TGF-β_1 to active TGF-β_1 by proteolytic cleavage of TGF-β_1 propeptide and latent TGF-β binding protein (3). Recently, it was shown that PSCs express and secrete MMP-2 and -9 (27, 30). In addition, their expression and secretion by PSCs are regulated by various cytokines (27, 30). Thus it is an intriguing question whether IL-1β controls autocrine TGF-β_1 activation by modulating the expression and secretion of MMP-2 and -9 by PSCs. A further study of the participation of MMP-2 and -9 in autocrine loop between IL-1β and TGF-β_1 in activated PSCs is warranted.

In conclusion, we have shown the existence of autocrine loop between TGF-β_1 and IL-1β in activated PSCs via Smad3- and ERK-dependent pathways, respectively. These observations provide new insights for understanding the mechanism of pancreatic fibrosis and developing a novel therapeutic strategy for its treatment.

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GRANTS

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