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IL-13 promotes the proliferation of rat pancreatic stellate cells through the suppression of NF-κB/TGF-β_1 pathway

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ABSTRACT

In chronic pancreatitis, pancreatic stellate cells (PSCs) play a central role in tissue fibrogenesis. Transforming growth factor β_1 (TGF-β_1) and the Th2 lymphokines such as interleukin (IL)-13 are major profibrogenic cytokines in many organs. Activated PSCs produce various inflammatory cytokines including TGF-β_1. In this study, we investigated whether IL-13 affects pancreatic fibrogenesis by modulating the functions of PSCs. IL-13 promoted PSCs proliferation without activation through the suppression of autocrine TGF-β_1. IL-13 enhanced Stat6 phosphorylation in PSCs but Stat6 was not involved in the suppression of TGF-β_1. IL-13 inhibited the transcriptional activity of NF-κB, and the expression of mutant I-κB reproduced the suppression of autocrine TGF-β_1 and promoted PSCs proliferation. Taken together, we demonstrated that IL-13 promotes PSCs proliferation through the suppression of the transcriptional activity of NF-κB, resulting in the decrease of autocrine TGF-β_1. This finding provides an unequivocal evidence of IL-13 participation in pancreatic fibrosis, illustrating a new strategy for chronic pancreatitis.

Introduction

Tissue fibrosis is a well-documented consequence of Th2 cytokine-dominated inflammatory responses. Th2 inflammation plays a central role in the pathogenesis of a variety of other fibrotic disorders including systemic sclerosis, idiopathic pulmonary fibrosis, asthma, hepatic fibrosis and ulcerative colitis [1–5]. This is probably because Th2 cytokines induce monocytes and macrophages to produce TGF-β_1, a potent regulator of extracellular matrix formation and tissue remodeling *in vivo* [6]. IL-13, secreted predominantly by activated Th2 cells, has been proposed as one of the central mediators in Th2 cytokine pathologies [7]. IL-13 was also reported to promote organ fibrosis by increasing the TGF-β_1 expression levels [8,9] and has been recognized as a profibrotic cytokine in liver fibrosis induced by schistosomiasis as well as in pulmonary fibrosis caused by asthma via allergic mechanisms [7]. Furthermore, IL-13 induces B lymphocytes to produce IgE, and this mechanism (termed class switching) causes the onset of asthma and allergic inflammatory changes.

The receptor system for IL-13 is composed of IL-4Rα and IL-13Rα1. IL-13 binds to IL-13Rα1, heterodimerizing with IL-4Rα, thus leading to the activation of the signal transducer and activator of transcription protein 6 (Stat6) signaling pathway. Both receptors are expressed in fibroblasts [7,10]. IL-4 shares the same receptor system and intracellular signal transduction pathway with IL-13 [10].

The main pathological feature of chronic pancreatitis is tissue fibrosis caused by sustained pancreatic inflammation. Recent studies have identified, isolated and characterized pancreatic stellate cells (PSCs) [11]. These cells play a central role in pancreatic fibrogenesis. In a stable, non-inflammatory state of the pancreas, PSCs are quiescent and are located in peri-acinar region. After the onset of inflammation, PSCs are rapidly activated and transform into myofibroblast-like cells. Rapidly proliferating activated PSCs produce various inflammatory cytokines including IL-1β, IL-6, and TGF-β_1 and migrate to the injured area of pancreas, resulting in the expansion of pancreatic fibrosis [12–14]. TGF-β_1 is one of the major profibrogenic cytokines associated with organ fibrosis, and it is well known to promote pancreatic fibrogenesis [15].

As a model system to study the involvement of IL-13 in pancreatic fibrosis, we used rat cultured PSCs and examined whether IL-13 affects pancreatic fibrogenesis by modulating the function of PSCs, which play a central role in the pathogenesis of chronic pancreatitis. In the present study, we examined the expressions

Abbreviations: α-SMA, α-smooth muscle actin; ELISA, enzyme-linked immunosorbent assay; HRP, Horseradish peroxidase; HSC, hepatic stellate cell; Ig, immunoglobulin; IL, interleukin; NF-κB, nuclear factor-kappaB; PI3K, phosphatidylinositol 3-kinase; PSC, pancreatic stellate cell; Stat, signal transducer and activator of transcription protein; TGF-β_1, transforming growth factor β_1.

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of IL-13 in the pancreas and the various effects of IL-13 on culture-activated PSCs in order to elucidate its function. Clarifying the molecular mechanisms of the lymphokine that regulates PSCs will therefore enable us to develop a new therapeutic strategy for the treatment of pancreatic fibrosis.

Materials and methods

Materials. Recombinant rat IL-13 and anti-Stat6 antibody were purchased from R&D Systems (Abington, UK). Pronase, Nycodenz, and anti-α-SMA antibody were from Sigma (St. Louis, MO). DNase-1 was from Roche (Basel, Switzerland). Collagenase P was from Boehringer Mannheim (Mannheim, Germany). Anti-α-tubulin antibody was from Santa Cruz (Santa Cruz, CA) and anti-phospho-Stat6 (Y641) antibody was from Upstate (Billerica, MA). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated donkey anti-goat IgG, and Cy3-conjugated donkey anti-rabbit IgG were from Jackson Immuno Research (West Grove, PA).

Isolation and culture of rat pancreatic stellate cells. The rat PSCs were isolated as described previously [11]. Briefly, the rat pancreas was resected and digested in Gey's balanced salt solution with a mixture of 0.05% collagenase P, 0.02% pronase, and 0.1% DNase-I. After filtration through nylon mesh, the cells were centrifuged in a 13.2% Nycodenz gradient at 1400*g* for 20 min. PSCs in the band just above the interface of the Nycodenz solution were collected, washed, and resuspended in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. PSCs were cultured at 37 °C in a humidified environment of 95% air and 5% CO_2 atmosphere. We used PSCs between passages two and four as culture-activated PSCs in the experiments. All rat experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Jichi Medical University.

Real-time quantitative PCR. Total RNA was obtained from PSCs with ISOGEN (Wako, Tokyo, Japan). First-stranded cDNA was synthesized from total RNA by using ReverTra Ace system (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Real-time quantitative PCR for rat TGF-β_1 and β-actin was carried out with the following primers; (a) rat TGF-β_1: sense 5′-AAGAAGTCACC CGCGTGCTA-3′, anti-sense 5′-TGTGTGATGTCTTTGGTTTTGTCA-3′, probe 5′-TGGTGGACCGCAACAACGCAATC-3′; (b) rat β-actin: sense 5′-AGGCCAACCGTGAAAAGATG-3′, anti-sense 5′-CACAGCCTGGATG GCTACGT-3′, probe 5′-AGATCATTGCTCCTCCTGAGCGCAAGT-3′. The reactions were conducted as follows using ABI PRISM 7900HT (Applied Biosystems): denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s.

Western blotting. Western blotting was performed as described previously [16]. For gel electrophoresis, 10 μg of protein were loaded on each lane of a 10% sodium dodecyl sulfate–polyacrylamide gel. Enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) was used to visualize the secondary antibody.

Measurement of DNA synthesis. DNA synthesis was evaluated by measuring [^{3}H]thymidine incorporation into cells. [^{3}H]thymidine was added to the culture medium and incubated for 2 h. The [^{3}H]thymidine incorporation was then measured as described previously [17].

Measurement of TGF-β_1 peptide secretion. The secretion of TGF-β_1 peptides was measured by determining their concentrations in the culture medium using a commercial ELISA kit (DRG International, Mountainside, NJ), according to the manufacturer's instructions.

Expression plasmids and Transient transfection. A plasmid combining human TGF-β1 promoter (−1362/+55 bp flanking the transcription start site) with luciferase reporter gene was kindly provided by Dr. Seong-Jin Kim (phTG1) [18]. The expression plasmid of Stat6 (STAT6WT) was kindly provided by Ohmori [19]. For transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. Luciferase assays were performed using Dual-luciferase reporter assay system (Promega, Madison, WI).

Nuclear extract preparation and NF-κB transcription factor assay. We extracted the nuclear protein of PSCs using a commercial Nuclear Extract Kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. After the nuclear extraction, NF-κB transcription factor assay was performed using a TransAM NF-κB p65 Chemi kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

Adenovirus infection. Recombinant adenovirus of mutant I-κB (AdI-κBm) was kindly provided by Dr. Yuji Iimuro (Hyogo Medical Collage). We used an adenovirus expressing β-galactosidase (AdLacZ) as an infection control. The cells were infected with a recombinant adenovirus at a dose of 10 plaque-forming units (pfu) per cell.

Statistical analysis. One-way ANOVA was used to determine statistical significance. A *p*-value of <0.05 was considered to be significant.

Results

IL-13 promotes the proliferation of PSCs without activation

We first tried to elucidate the expressions of IL-13 in human and rat pancreas. Immunohistochemistry study was carried out using anti-IL-13 antibody on normal pancreas and chronic pancreatitis tissues. Although normal human and rat pancreas did not show any IL-13 signals, a high degree of IL-13 signals were detected in human and rat chronic pancreatitis tissues, corresponding to the fibrotic region indicated by azan staining (Supplementary Fig. S1). We presumed that PSCs might secrete IL-13 during pancreatic fibrogenesis and analyzed the mRNA expression and secretion of IL-13 by RT-

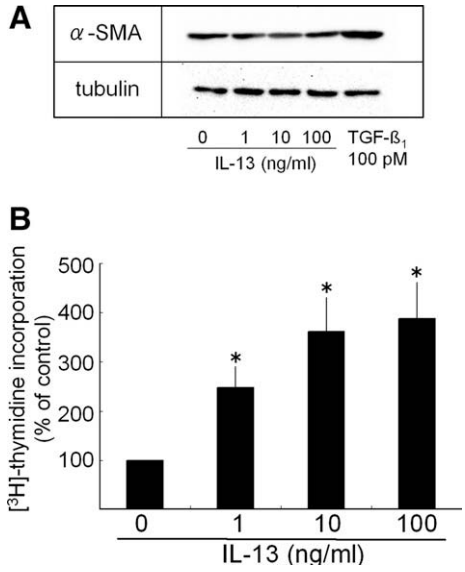

Fig. 1. The effect of IL-13 on the activation and proliferation of PSCs. (A) PSCs were incubated with the indicated amount of IL-13 and TGF-β_1 for 48 h. Total cellular homogenates were prepared and aliquots of 10 μg of protein were subjected to electrophoresis. A Western blotting analysis was carried out using anti-α-SMA and anti-tubulin monoclonal antibodies. The representative figures are shown in three independent experiments. (B) The cells were incubated in a serum-free medium with the indicated amount of IL-13 for 72 h. The medium was changed and fresh IL-13 was added every 24 h. After the incubations, the proliferation of PSCs was examined by determining [^{3}H]thymidine incorporation into cells. The results are indicated as the percentage of the controls. The values are expressed as means ± SE of three independent experiments. $^*p < 0.05$.

PCR and ELISA. However, PSCs neither express nor secrete IL-13 (data not shown). We subsequently assessed whether PSCs have a receptor system for IL-13 using RT-PCR and immunocytochemistry. The receptor system for IL-13 is a heterodimer of IL-4Rα and IL-13Rα1. The RT-PCR experiment showed the expressions of IL-4Rα and IL-13Rα1 at the mRNA level and the immunocytochemistry study also showed the existence of the receptor components in activated PSCs (Supplementary Fig. S2).

We examined whether IL-13 activates PSCs by measuring the levels of α-SMA expression. Recombinant IL-13 was added to the culture medium for 48 h, and Western blotting was subsequently performed. As shown in Fig. 1A, IL-13 did not affect the α-SMA expression level (in contrast to TGF-β_1). We then examined the effect of IL-13 on the proliferation of PSCs. Recombinant IL-13, ranging from 1 to 100 ng/ml, enhanced DNA synthesis in PSCs in a dose-dependent manner (Fig. 1B).

IL-13 promoted PSCs proliferation by decreasing autocrine TGF-β_1 via the suppression of promoter activity and mRNA expression

We tried to elucidate the molecular mechanism of IL-13-enhanced PSCs proliferation. It is well known that TGF-β_1 decreases PSCs proliferation in an autocrine fashion [20], and we hypothesized that IL-13 promotes PSCs proliferation by decreasing the secretion of autocrine TGF-β_1. To examine this hypothesis, we quantified TGF-β_1 secreted from PSCs by ELISA. As shown in Fig. 2A, IL-13 decreased the secretion of TGF-β_1 in a dose-dependent manner. To determine the step at which IL-13 inhibited the secretion of TGF-β_1, we evaluated the promoter activity of TGF-β_1 by luciferase assay and the mRNA level by real-time quantitative PCR. IL-13 decreased the promoter activity and mRNA expression of TGF-β_1 (Fig. 2B and C). Autocrine TGF-β_1 has been reported to inhibit the proliferation of PSCs [20]. Therefore, we suggest that IL-13 enhances PSCs proliferation by decreasing autocrine TGF-β_1 at the transcriptional level.

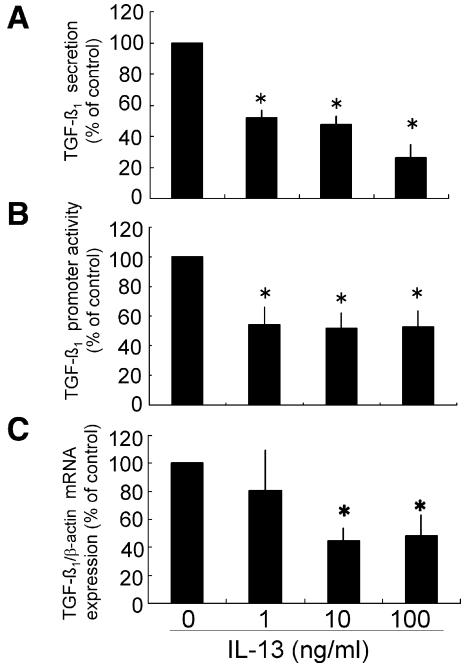

Fig. 2. IL-13 effects on the TGF-β_1 system in activated PSCs. (A) IL-13 effect on the TGF-β_1 secretion from activated PSCs. The cells were incubated for 48 h with the indicated amount of IL-13. The concentration of TGF-β_1 secreted from PSCs into the culture medium was determined by ELISA. (B) IL-13 effect on the TGF-β_1 promoter activity. Lipofection with a TGF-β_1 reporter plasmid (phTG1) and a reference plasmid (phFBG) was conducted as described in the methods section. After 24 h, the indicated amount of IL-13 was added. We incubated the PSCs for a further 48 h, and a luciferase assay was subsequently performed on cellular extracts. (C) IL-13 effect on TGF-β_1 mRNA expression in activated PSCs. PSCs were incubated with IL-13 for 48 h and the expression of TGF-β_1 mRNA was determined by real-time quantitative RT-PCR compared with β-actin expression as a reference. The results are indicated as the percentage of the controls. The values are expressed as means ± SE of three independent experiments. $^*p < 0.05$.

Stat6 was not involved in IL-13-induced TGF-β_1 suppression

We next investigated the intracellular signal transduction pathway through which IL-13 decreases TGF-β_1 secretion. Stat6 is a latent cytoplasmic transcriptional factor that plays a central role in IL-13-regulated signal transduction and gene expression [10]. We examined whether Stat6 was involved in IL-13-induced suppression of autocrine TGF-β_1. The activation of Stat6 in PSCs was examined by Western blotting using anti-Stat6 and anti-phospho-Stat6 antibodies. IL-13 enhanced Stat6 phosphorylation in PSCs and the maximum amount of enhancement was observed in 5 min (Fig. 3A). However, a luciferase assay showed that plasmid-mediated Stat6 over-expression did not decrease TGF-β_1 promoter activity (Fig. 3B). These data suggest that IL-13 inhibits the TGF-β_1 promoter activity via a pathway other than Stat6.

IL-13 suppressed autocrine TGF-β_1 at the transcriptional level by inhibiting NF-κB activity

We next assessed NF-κB participation in the IL-13 suppression of autocrine TGF-β_1. NF-κB plays an important role in inflammation and immune responses in other cell types, and it is activated by exposure to inflammatory cytokines or infections [21]. IL-13 decreases NF-κB activity in a rat lung model [22] and NF-κB up-regulates TGF-β_1 transcription in a rat mesangial cell model [23]. Therefore, we hypothesized that IL-13 might inhibit the TGF-β_1 system through the decrease of NF-κB activity. The transcriptional

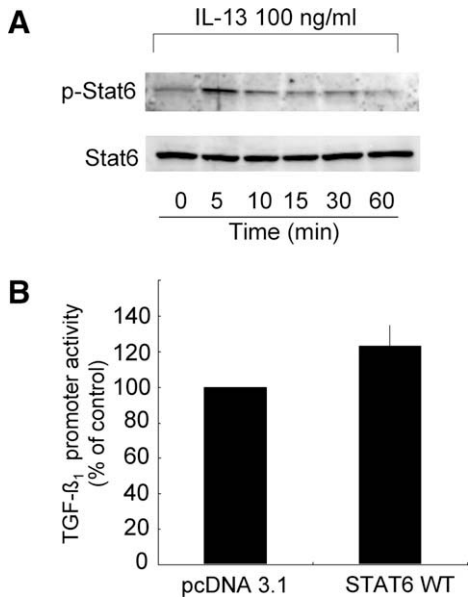

Fig. 3. The effect of IL-13 on Stat6 activation and the effect of Stat6 on TGF-β_1 promoter activity. (A) The cells were incubated with 100 ng/ml IL-13 for the indicated times. The activation of Stat6 was then determined by Western blotting using anti-phosphorylated Stat6 antibody (upper panel) and anti-Stat6 antibody as an internal control (lower panel). (B) The promoter activity was evaluated by luciferase assay. Lipofection with phTG1 and STAT6WT was conducted as described in the methods section. After 24 h, a luciferase assay on cellular extracts was carried out. A plasmid of pcDNA3.1 was used as a control. The results are indicated as the percentage of the controls. The values are expressed as means ± SE of three independent experiments.

activity of NF-κB was elevated in activated PSCs as compared to quiescent PSCs (Fig. 4A). As shown in Fig. 4B, IL-13 decreased the NF-κB activity through a 24 h period. Aware of the participation of NF-κB in TGF-β_1 transcription, we used an adenovirus system to over-express mutant I-κB in PSCs (AdI-κBm) and an adenovirus to express LacZ as a control (AdLacZ). Adenovirus-mediated mutant I-κB expression decreased NF-κB activity by about 40% in the activated PSCs (Data not shown). We then examined whether mutant I-κB over-expression affects TGF-β_1 mRNA expression and peptide secretion. As shown, PSCs infected with AdI-κBm decreased TGF-β_1 mRNA expression (Fig. 4C) and peptide secretion (Fig. 4D). To confirm this finding, we examined the PSCs proliferation in response to an infection of mutant I-κB adenovirus. AdI-κBm adenovirus infection increased PSCs proliferation (Fig. 4E). These data indicate that IL-13 decreases autocrine TGF-β_1 at the transcriptional level through the suppression of NF-κB activity. Consequently, IL-13 promotes PSCs proliferation by decreasing autocrine TGF-β_1.

Discussion

In this study, we demonstrated that IL-13 promotes PSCs proliferation through the reduction of NF-κB activity and the suppression of autocrine TGF-β_1.

In 1989, IL-13 was first reported as "p600" produced by Th2 lymphocytes. Inflammation is primarily modulated by Th1 cytokines, such as interferon-γ or IL-12, and fibrosis is mainly controlled by Th2 cytokines, such as IL-13 or IL-4 [7]. IL-13 shares many functional activities with IL-4 because both cytokines use the same receptor component (IL-4Rα)-Stat6 signaling pathway [7]. However, studies of IL-13 transgenic and knock out mice have demonstrated that they also have unique and non-redundant roles in host immunity [24,25]. IL-13 studies have been focused on allergic mechanisms, including increments of B lymphocyte proliferation and class switching [26]. IL-13 plays central roles in bronchial hypersensitivity and mucosal secretion [26] and the over-expression of IL-13 induces allergic reactions *in vivo* [24]. IL-13 has also been shown to participate in various aspects of organ fibrosis and was thought to be a primary regulator of the fibrotic response [7,27]. In a mouse lung model, the over-expression of IL-13, although not IL-4, induced subepithelial fibrosis [24]. In schistosomiasis, the typical Th2 cytokines were associated with chronic parasite egg-induced liver fibrosis, and IL-13 inhibitor (soluble IL-13Rα2 fusion protein) decreased collagen deposition by 85% without inflammatory inhibition [8].

HSCs play a central role in hepatic fibrogenesis [28,29]; likewise, PSCs promote pancreatic fibrogenesis [15]. In liver fibrogenesis, IL-13 promotes fibrosis via TGF-β_1-independent mechanisms [30]. In a mouse model, the over-expression of IL-13 promotes lung

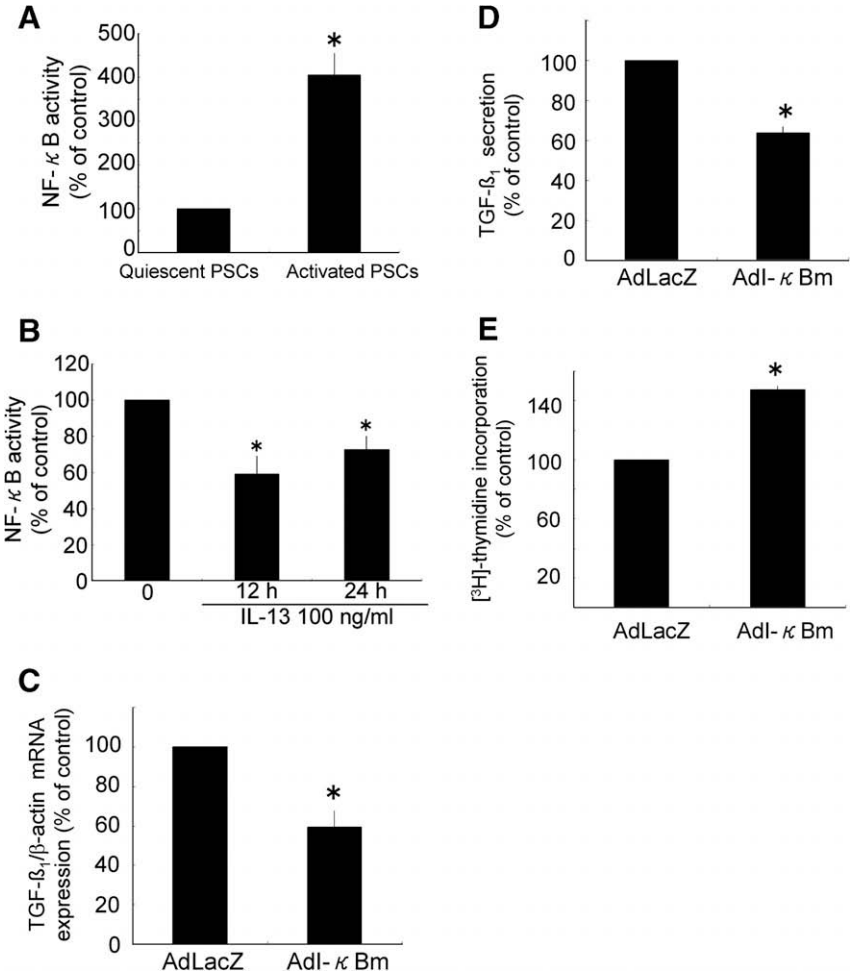

Fig. 4. The effect of IL-13 on NF-κB activity in activated PSCs. (A) NF-κB activity in quiescent and activated PSCs. NF-κB activity was measured in fresh PSCs (24 h after the isolation) and activated PSCs (96 h after the isolation) as described in method section. (B) The effect of IL-13 on NF-κB activity. The cells were incubated with 100 ng/ml IL-13 for the indicated time and NF-κB activity was measured. (C–E) The effect of mutant I-κB expression on TGF-β_1 mRNA expression (C), TGF-β_1 secretion (D), and PSCs proliferation (E). The cells were incubated for 48 h after the infection of mutant I-κB-expressing adenovirus (AdI-κBm) and control adenovirus (AdLacZ). All the results are indicated as the percentage of the controls. The values are expressed as means ± SE of three independent experiments. $^*p < 0.05$.

fibrosis by increasing TGF-β_1, and TGF-β_1 inhibitor alleviated the fibrosis [9]. In THP-1, a macrophage cell line, IL-13 enhanced the TGF-β_1 promoter activity cooperating with TNF-α [31]. Therefore, we first presumed that IL-13 increases the production of TGF-β_1 in PSCs. However, we clarified that IL-13 decreased the promoter activity, the mRNA expression, and the secretion of TGF-β_1, which resulted in the induction of PSCs proliferation (Figs. 1, 2, and 4). Sugimoto et al. reported that IL-13 activated Stat6 in HSCs and suppressed cellular proliferation [32]. In PSCs, IL-13 similarly activated Stat6 but Stat6 did not negatively regulate the TGF-β_1 promoter activity (Fig. 3). Although, Stat6 is a well known transcription factor in IL-13 signaling, Stat6-independent signaling pathways have been reported using mice models with null mutations of Stat6 or IL-4Rα [33,34]. Actually, there is no Stat6 binding motif (TTCnnnnGAA) [19] in the promoter region of rat and human TGF-β_1. Lentsch et al. reported that IL-13 reduced nuclear localization of NF-κB by reducing the I-κB degradation in a rat lung model [22] and in a rat mesangial model the increased activity of NF-κB was shown to promote TGF-β_1 transcription [23]. We then examined whether IL-13 affects the NF-κB activity and revealed that IL-13 inhibits autocrine TGF-β_1 at the transcription level through the suppression of NF-κB activity. This growth promotional effect of IL-13 on PSCs might lead to the fibrogenesis of pancreas.

As to the effect of IL-13 in PSCs activation, we showed that IL-13 did not change α-SMA expression in spite of a 70% decrement of autocrine TGF-β_1, which has been known to enhance PSCs transformation (Figs. 1A and 2A). Other inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and Activin A, might influence the PSCs transformation.

In our preliminary immunohistochemical study, we could not detect the IL-4 signals in pancreatitis specimens (data not shown). IL-4 shares the receptor component with IL-13 and in fact, IL-4 also promotes the proliferation of rat cultured PSCs (data not shown). We have to enlarge the number of clinical samples and further studies are needed to uncover the involvement of Th2 lymphokines in pancreatic fibrosis.

In conclusion, we herein demonstrated that IL-13 promotes PSCs proliferation by decreasing autocrine TGF-β_1 through the suppression of NF-κB activity. This provides a new insight to better understand IL-13 participation in pancreatic fibrogenesis and also to develop a new strategy for its treatment.

Conflicts of Interest

All of the authors disclose no conflicts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.078.

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