Expression of interleukin-24 and its receptor in human pancreatic myofibroblasts

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Received July 12, 2011; Accepted August 30, 2011

DOI: 10.3892/ijmm.2011.793

Abstract. Interleukin (IL)-24 is a member of the IL-10 family of cytokines. In this study, we investigated IL-24 expression in chronic pancreatitis tissue and characterized the molecular mechanisms responsible for IL-24 expression in human pancreatic myofibroblasts. IL-24 expression in the tissues was evaluated by immunohistochemical methods. IL-24 mRNA and protein expression in the pancreatic myofibroblasts was determined by real-time-PCR and ELISA, respectively. IL-24 was expressed by a-smooth muscle actin-positive myofibroblasts in the chronic pancreatitis tissues. In isolated human pancreatic myofibroblasts, IL-1ß significantly enhanced IL-24 mRNA and protein expression. The p38 MAPK inhibitor SB203580 and the PI3K inhibitor LY294002 significantly reduced the IL-\beta-induced IL-24 mRNA expression. An adenovirus containing a dominant-negative mutant of c-Jun significantly inhibited the effects of IL-1ß on IL-24 mRNA expression, indicating that the IL-1\beta-induced IL-24 mRNA expression was mediated by the activation of transcription factor AP-1. Pancreatic myofibroblasts expressed IL-22R1, IL-20R1 and IL-20R2, and recombinant IL-24 induced the phosphorylation of p42/44, p38, JNK and STAT1/3. IL-24 is expressed in chronic pancreatitis tissue, and may play a role in the pathophysiology of chronic pancreatitis via autocrine pathways.

Introduction

Fibrosis of the pancreas is one of the characteristic histopathologic findings in cases of chronic pancreatitis and the desmoplastic reaction associated with pancreatic cancer. Previous studies have demonstrated that pancreatic myofi-

Key words: MAP-kinase, AP-1, chronic pancreatitis, interleukin-1ß

broblasts play a pivotal role in the progression of pancreatic fibrosis (1-6). Pancreatic myofibroblasts actively proliferate, migrate and produce large amounts of extracellular matrix (ECM) components such as type I collagen and fibronectin. In addition, these cells possess proinflammatory functions characterized by the expression of cytokines, chemokines and cell adhesion molecules (1-6).

Interleukin (IL)-24, a member of the IL-10 family of cytokines (together with IL-10, -19, -20, -22, -26, -28 and -29), was originally termed melanoma differentiation-associated protein 7 (MDA-7) (7), and was renamed IL-24 (8). The human IL-24 gene is located on chromosome 1, within a 195-kb cytokine cluster containing the IL-10, IL-19, IL-20 and IL-24 genes (9). IL-24 shares a 20-30% amino acid homology with IL-10, IL-20 and IL-22, and interacts with two different heterodimeric receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (10-12). Binding to both receptors leads to the activation of signal transducer and activator of transcription 3 (STAT3) (10-13). Immune cells do not express IL-24 receptors (14), suggesting that IL-24 cannot stimulate the acquired immune response (15). In contrast, the restricted expression of the IL-24R components IL-20R1 and IL-22R1 to non-immune tissues suggests that the innate immune response is the selective target of IL-24 (11,14).

IL-24 expression has been identified in certain cell types (14,16), such as cultured melanocytes (7), dermal keratinocytes (15), lipopolysaccharide (LPS)-stimulated monocytes (16) and Th2-polarized T cells (16). Furthermore, treatment with IFN- β plus mezerein was found to induce a transient expression of IL-24 mRNA in some cancer cell lines (9). However, the precise molecular mechanisms of IL-24 induction still remain unclear.

In the present study, we investigated IL-24 expression in chronic pancreatitis tissues. Furthermore, to investigate the molecular mechanisms responsible for IL-24 expression in the pancreas, we analyzed IL-24 expression in human pancreatic myofibroblasts.

Materials and methods

Reagents. Recombinant human cytokines were purchased from R&D Systems (Minneapolis, MN). Inhibitors for the p42/44 MAP-kinases (PD98059 and U0216), and an inhibitor for

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Table I. Oligonucleotides used in this study.

Gene name		Primers	Ref.
IL-24	Sense Antisense	5'-GACTTTAGCCAGCAGACCCTT-3' 5'-GGTTGCAGTTGTGACACGAT-3'	8
IL-22R1	Sense Antisense	5'-CTGTCCGAGATCACCTACTTAGG-3' 5'-GCACATTTGGGTCAGATGTTCTGTC-3'	14
IL-20R1	Sense Antisense	5'-GCTCAGCCTTCTGAGAAGCAGTG-3' 5'-CGCACAAATGTCAGTGGTTCTGAC-3'	20
IL-20R2	Sense Antisense	5'-GCTGGTGCTCACTCACTGAAGGT-3' 5'-TCTGTCTGGCTGAAGGCGCTGTA-3'	20



Figure 1. Representative immunohistochemical staining for IL-24 and α -smooth muscle actin (α -SMA) in chronic pancreatitis tissues. IL-24 protein was clearly detected in the spindle-shaped cells (green fluorescence; left panel). Myofibroblasts were detected as α -SMA-immunopositive cells (red fluorescence; middle panel). α -SMA/IL-24 double-immunopositive cells were detected as yellow, and the α -SMA-immunopositive cells coincided with some of the IL-24-immunopositive cells (right panel). Magnification, x200.

p38 MAPK (SB203580) were purchased from Cell Signaling Technology (Beverly, MA). The procollagen type I C-peptide EIA kit was purchased from Takara Bio (Otsu, Japan).

Antibodies. Goat anti-human IL-24 antibodies (R&D System), mouse anti- α -SMA antibodies (Sigma Chemical Co., St. Louis, MO), goat anti-JAK1 antibodies, anti-MAP kinase, and anti-STAT1 and 3 antibodies (Cell Signaling Technology), goat anti-human c-Jun and c-Fos antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were purchased from commercial suppliers.

Tissue samples and immunochemistry. Human pancreatic tissues were obtained from patients with clinically diagnosed chronic pancreatitis undergoing surgery for intractable back pain, or from patients with significant suspicion of pancreatic cancer. Normal pancreatic tissues were obtained from patients who underwent total gastropancreatectomies due to gastric cancer. The study design was approved by the Ethics Committee of the Shiga University of Medical Science. Informed consent was obtained from all patients prior to sample collection.

Paraffin-embedded tissues were cut into 3 μ m sections. Goat anti-human IL-24 antibodies and anti-human α -SMA antibodies were then applied. Cy2-labeled anti-goat rabbit IgG (Chemicon, Temecula, CA) and Cy3-labeled anti-mouse donkey IgG (Millipore, Billerica, MA) were used as the secondary antibodies. The slides were analyzed for fluorescence using a digital confocal laser scanning microscope (LSM510 ver. 3.0; Carl Zeiss Japan, Tokyo, Japan). Standard immunohistochemical analyses were performed according to the methods described in our previous report (17).

Human pancreatic myofibroblast cultures. Primary cultures of pancreatic myofibroblasts were isolated from normal pancreatic tissues obtained from patients who underwent total gastropancreatectomies due to gastric cancer, according to methods previously described (4). The cells were cultured in DMEM containing 10% FBS. All culture media were supplemented with 50 U/ml penicillin and 50 μ g/ml streptomycin. Over 98% of the cells were immunopositive for α -SMA, a marker for myofibroblasts. The studies were performed on passages 2-6 of myofibroblasts isolated from 4 different resection specimens.

Quantification of human IL-24 and IL-24R. Antigenic IL-24 in all samples was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D systems. The expression of mRNA in the samples was assessed by real-time PCR analysis. The oligonucleotide primers used in this study were described in our previous report (18). Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan). The PCR was performed using a SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA). The oligonucleotide primers used in this study are shown in Table I (7,13,19). The data were normalized versus β -actin for human IL-24. The expression of IL-24R mRNA



Figure 2. IL-24 mRNA expression in human pancreatic myofibroblasts. (A) The cells were stimulated with cytokines (100 ng/ml) or lipopolysaccharide (LPS; 1 µg/ml) for 24 h. IL-24 mRNA expression was then analyzed by real-time PCR. (B) Immunostaining for IL-24 in cultured pancreatic myofibroblasts. The cells were cultured on a chamber slide glass for 24 h in the presence or absence of IL-1 β (10 ng/ml) and immunostained with anti-human IL-24 antibodies. (C) Combined effects of cytokines on IL-24 mRNA expression. The cells were stimulated with IL-1 β (100 ng/ml), TNF- α (100 ng/ml), IFN- γ (100 ng/ml), IL-17 (100 ng/ml) and combinations of these factors for 24 h, and then the IL-24 mRNA expression was determined by real-time PCR. The figures from the real-time PCR were expressed as the IL-24 mRNA expression relative to the β -actin mRNA expression (mean \pm SD from 4 different experiments). **P<0.01 vs. medium only.

was assessed by reverse-transcription (RT)-PCR analyses according to methods described in our previous report (20).

Adenovirus-mediated gene transfers. We used a recombinant adenovirus expressing a dominant-negative mutant of c-Jun (Ad-DN-c-Jun), and a recombinant adenovirus containing bacterial β -galactosidase cDNA (Ad-LacZ). The dominantnegative mutant c-Jun (TAM67) lacks the transactivational domain of amino acids 3 to 122 of the wild type c-Jun (21), but retains the DNA-binding domain. The detailed procedures have been described in our previous report (22).

Statistical analysis. The statistical significance of the differences was determined by the Mann-Whitney U test (Statview version 4.5). Differences resulting in P-values <0.05 were considered to be statistically significant.

Results

IL-24 expression in chronic pancreatitis. To evaluate the expression of IL-24 mRNA in the pancreas, the tissues of chronic pancreatitis patients were double immunostained with anti-human IL-24 antibodies and anti- α -smooth muscle actin (α -SMA; a marker for myofibroblasts) antibodies (Fig. 1). IL-24 protein was clearly detected in the spindle-shaped cells (green fluorescence; Fig. 1, left panel). Myofibroblasts were detected as α -SMA-positive cells (red fluorescence; Fig. 1, middle panel). As shown in Fig. 1 (right panel) the α -SMA/IL-24-double immunopositive cells were detected as yellow, and the IL-24-immunopositive cells. These observations indicated that α -SMA-immunopositive myofibroblasts expressed IL-24 in the chronic pancreatitis tissues.

Regulation of IL-24 expression in pancreatic myofibroblasts. Based on the in vivo expression of IL-24 in chronic pancreatitis tissues, we examined IL-24 expression in isolated human pancreatic myofibroblasts. The cells were stimulated with various cytokines and LPS for 12 h, and then the IL-24 mRNA expression was determined by real-time PCR (Fig. 2A). Very weak IL-24 mRNA expression was detected in the unstimulated pancreatic myofibroblasts, and IL-1ß stimulation significantly enhanced this IL-24 mRNA expression. IL-17, TNF-a and LPS all weakly enhanced IL-24 mRNA expression, but these effects were much weaker than those induced by IL-1β. IL-24 protein expression and its induction by IL-1β were both detected in cultured pancreatic myofibroblasts by immunofluorescent staining (Fig. 2B). The combination of IL-1β and TNF-α markedly enhanced IL-24 mRNA expression, and this was observed as an additive effect (Fig. 2C).

Pancreatic myofibroblasts were stimulated with increasing concentrations of IL-1 β for 24 h, and the IL-24 mRNA expression was determined by real-time PCR. IL-1 β dose-dependently induced IL-24 mRNA expression (Fig. 3A). Looking at the kinetics of IL-24 mRNA expression in response to IL-1 β , the cells were stimulated with IL-1 β (100 ng/ml), and IL-24 mRNA expression was sequentially determined. IL-1 β time-dependently induced IL-24 mRNA expression (Fig. 3B).

Similar effects of IL-1 β were investigated at the protein level. Pancreatic myofibroblasts were stimulated with various concentrations of IL-1 β for 48 h, and then the IL-24 secretion was determined by ELISA. IL-1 β dose-dependently induced IL-24 secretion (Fig. 3C). Furthermore, the cells were incubated with IL-1 β (100 ng/ml) for 48 h, and then the IL-24 protein secretion was analyzed sequentially by ELISA. This IL-24 secretion was observed in a time-dependent manner (Fig. 3D).



Figure 3. IL-24 mRNA expression and IL-24 secretion in human pancreatic myofibroblasts. (A and B) Dose-dependent effects of IL-1 β on IL-24 mRNA expression and IL-24 secretion. Pancreatic myofibroblasts were incubated for 24 h with increasing concentrations of IL-1 β . The levels of IL-24 mRNA expression were then determined by real-time PCR, and the IL-24 protein levels were determined by ELISA. (C and D) Kinetics of IL-24 mRNA expression and IL-24 secretion. The cells were stimulated with IL-1 β (10 ng/ml) for the pre-determined times, and then the IL-24 mRNA expression was determined by real-time PCR, and the IL-24 protein levels were determined times, and then the IL-24 mRNA expression was determined by real-time PCR, and the IL-24 protein levels were determined by ELISA. All values are expressed as means \pm SD (n=4). *P<0.05, **P<0.01; a significant difference from the values for medium alone.



Figure 4. Effects of MAPK, PI3K and AP-1 inhibition on IL-24 mRNA expression. (A) The cells were stimulated with IL-1 β (100 ng/ml) in the presence or absence of MEK inhibitors [U0216 (10 μ M) and PD98059 (10 μ M)], a p38 inhibitor [SB203580 (10 μ M)], and a PI3K inhibitor [LY294002 (10 μ M)] for 24 h. The IL-24 mRNA expression levels were then determined by real-time PCR. (B) The cells were infected with an adenovirus expressing the DN-c-Jun, and at 48 h after infection, the cells were stimulated with IL-1 β (100 ng/ml) for 12 h. The IL-24 mRNA expression was determined by real-time PCR. An adenovirus expressing LacZ was used as the negative control. The values from the real-time PCR were expressed as the IL-24 mRNA expression relative to the β -actin mRNA expression (mean ± SD from 4 different experiments). **P<0.01 vs. absence of inhibitors. NS, not significant.

Role of MAP-kinase and PI3-kinase activation in IL-24 induction. Previously, we demonstrated that IL-1 β induces MAP-kinase activation in pancreatic myofibroblasts (23). To

investigate the role of MAP-kinases in IL-1 β -induced IL-24 mRNA expression in pancreatic myofibroblasts, we evaluated the effects of p42/44 MAPK inhibitors (PD98059 and



Figure 5. (A) RT-PCR analyses for the mRNA expression of IL-20R1, IL-20R2 and IL-22R1 in human pancreatic myofibroblasts. (B) IL-24 induced JAK/ STAT and MAP-kinase activation in pancreatic myofibroblasts. The cells were stimulated with IL-24 (100 ng/ml), and the activation of JAK/STAT and MAPkinases were then evaluated by Western blotting. Antibodies directed against phosphorylated (P)- and total (T)-JAK/STAT and MAP-kinases were used.

U0216) (24,25), a p38 MAPK inhibitor (SB203580) (26) and a PI3-kinase inhibitor (LY294002) (27). The p42/44 MAPK inhibitors did not have any significant effects, while the p38 MAPK inhibitor and PI3K inhibitor significantly reduced the IL- β -induced IL-24 mRNA expression (Fig. 4A). These observations suggest that p38 MAPK and PI3K are involved in IL-1 β -induced IL-24 mRNA expression in pancreatic myofibroblasts.

Role of AP-1 activation in IL-24 induction. Multiple recognition sites for the AP-1 transcription factor are present in the promoter region of the IL-24 gene (28). To assess the role of the transcription factor AP-1, we evaluated the effects of a recombinant adenovirus containing a dominant-negative mutant of c-Jun (Ad-DN-c-Jun) on IL-1 β -induced IL-24 mRNA expression. Pancreatic myofibroblasts were infected with this recombinant adenovirus and were cultured for 48 h (Fig. 4B). The cells were then stimulated for 12 h with IL-1 β (100 ng/ml), and the expression of IL-24 mRNA was determined by real-time PCR. Ad-DN-c-Jun significantly inhibited the effects of IL-1 β on IL-24 mRNA expression. These inhibitory effects were not induced by the Ad-LacZ gene, which was used as a negative control. These findings suggest that AP-1 plays a role in IL-1 β -induced IL-24 mRNA expression.

IL-24 receptor expression in pancreatic myofibroblasts. IL-24 signaling has been reported to be mediated through both IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (10-12), and the expression of IL-20R1 and IL-22R1 is restricted to non-immune cells. However, their expression in pancreatic myofibroblasts remains unclear. RT-PCR studies supported the expression of IL-22R1, IL-20R1 and IL-20R2 in pancreatic myofibroblast (Fig. 5A).

IL-24 induces STAT, Akt and MAP-kinase activation in pancreatic myofibroblasts. To define the biological activities of IL-24 in pancreatic myofibroblasts, we investigated the effects of IL-24 on the activation of p42/44 (extracellular signal-regulated kinases; ERK1/2), p38, JNK, Akt, Jak1 and STAT1/3 in pancreatic myofibroblasts. IL-24 induced the phosphorylation of p42/44, p38, JNK, Jak1 and STAT1/3 as early as 5 min after stimulation (Fig. 5B). These observations indicated that IL-24Rs (IL-20R1/IL-20R2 and IL-22R1/IL-20R2) were functioning actively in pancreatic myofibroblasts.

Discussion

IL-24, a member of the IL-10 family of cytokines (together with IL-10, -19, -20, -22, -26, -28 and -29), shares a 20-30% amino acid homology with IL-10, IL-20 and IL-22. IL-24 interacts with two different heterodimeric receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (10-12). The expression of IL-20R1 and IL-22R1 is restricted to non-immune tissues, indicating that IL-24 targets non-immune immune responses. Recent studies have revealed that when IL-24 is administered via an adenoviral vector, it selectively and efficiently kills cancer cells derived from various tissues (29-33).

The precise molecular mechanisms mediating IL-24 induction remain unclear. In the present study, we investigated IL-24 and its receptor expression in human pancreatic myofibroblasts. We report that i) human pancreatic myofibroblasts are a source of IL-24 and express the IL-24 receptor complex; ii) IL-1 β is a potent inducer of IL-24 expression via a p38 MAPK and PI3K-dependent pathway; iii) pancreatic myofibroblasts express IL-24R complexes; and iv) IL-24 induced the activation of p42/44-/p38-MAPK, JNK1 and STAT1/3 in the pancreatic myofibroblasts.

There are few reports concerning the *in vivo* expression of IL-24 under normal and pathological conditions. Soo *et al* (34) found that IL-24 expression was greatly increased at the edges of cutaneous wounds in an animal model. A recent study by Wolk *et al* (16) showed that IL-24 was expressed by keratinocytes from patients with psoriasis. Recently, we reported that IL-24 expression was up-regulated in the inflamed mucosa of inflammatory bowel disease (35). However, there are no reports concerning IL-24 expression in the pancreas. In the present study, we demonstrated that IL-24 is expressed in the fibrotic tissues of chronic pancreatitis. Furthermore, IL-24-expressing cells coincided with α -SMA-immunopositive cells, suggesting that α -SMA-immunopositive myofibroblasts are the local source of IL-24 in the pancreas.

To investigate the regulatory mechanisms involved in IL-24 expression in the pancreas, we investigated IL-24 induction in human pancreatic myofibroblasts. Among the various cytokines, IL-1 β exerted marked effects on IL-24 induction. TNF- α , IL-17 and LPS all weakly stimulated IL-24 expression, but these were negligible when compared to IL-1 β . Furthermore, the combination of IL-1 β plus TNF- α induced strong IL-24 expression as compared to IL-1 β alone, suggesting that IL-24 may be induced at inflamed lesions where both IL-1 β and TNF- α are expressed.

In the present study, we set out to determine the relevant signaling pathways in IL-1 β -induced IL-24 mRNA expres-

sion. Since the promoter region of the human IL-24 gene has multiple consensus binding sites for AP-1, we evaluated the effects of a recombinant adenovirus expressing a dominant-negative mutant of c-Jun (Ad-DN-c-Jun). The addition of Ad-DN-c-Jun markedly suppressed IL-1 β -induced IL-24 mRNA expression, indicating a role for AP-1 in IL-24 mRNA induction. Furthermore, a p38 MAPK inhibitor (SB203580) and a PI3K-inhibitor (LY294002) significantly inhibited IL-1 β -induced IL-24 mRNA expression. Combined with previous studies reporting that AP-1 acts downstream of evolutionarily conserved signaling pathways, such as MAPK and PI3K (36,37), we concluded that p38 MAPK and PI3K pathway-dependent AP-1 activation plays a role in IL-1 β -induced IL-24 mRNA expression.

The *in vivo* expression of IL-24R remains unclear. We observed that IL-20R1, IL-22R1 and IL-22R2 are expressed by pancreatic myofibroblasts (Fig. 5A). This suggests that IL-24 stimulates pancreatic myofibroblasts in an autocrine fashion. Actually, IL-24 induced a rapid activation of p42/44, p38, JNK and STAT1/3 molecules. It has been reported that the STAT3-mediated activation of the innate immune response contributes to a suppression of inflammatory responses (38-40), whereas the STAT3-mediated activation of the acquired immune response plays a pathogenic role by enhancing the survival of pathogenic T cells (41,42). These suggest that the IL-24-induced selective activation of STAT3 in pancreatic myofibroblasts may contribute to the suppression of inflammatory responses in the pancreas.

In conclusion, we demonstrated that IL-24 is expressed in pancreatic myofibroblasts. Our data suggest that IL-24 plays a role in the pathophysiology of chronic pancreatitis in an autocrine fashion. Recently, a replication-incompetent adenovirus expressing IL-24 has undergone evaluation in a phase I clinical trial for solid tumors and has proven its safety (43). Another study demonstrated that adenovirus-mediated IL-24 suppressed the proliferation of keloid fibroblasts and induced apoptosis (44). Therefore, based on the further characterization of the biological activities of IL-24, this cytokine has potential for clinical applications to regulate the pathophysiology of chronic pancreatitis.

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