Phosphatidylinositol 3-kinase/Akt signaling mediates interleukin-32α induction in human pancreatic periacinar myofibroblasts

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INTERLEUKIN (IL)-32 is a recently described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes, and epithelial cells (20, 23). Although IL-32 was first reported as a transcript in IL-2-activated NK and T cells, it appears that tissue cells are a dominant and widespread source for IL-32 (11). The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons (7). There are four splice variants (IL-32α, IL-32β, IL-32γ, and IL-32δγ), and IL-32α is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)-γ in lung epithelial cells and monocytes (20).

IL-32 exhibits several properties typical of proinflammatory cytokines (20, 23). For example, it stimulates the secretion of IL-1β, TNF-α, IL-6, and IL-8 by means of the activation of nuclear factor (NF)-κB and p38 mitogen-activated protein kinases (MAPKs) (20, 23). Recently, Netea et al. (23) demonstrated that IL-32 augments the production of IL-1β and IL-6 induced by muramyl dipeptide, a peptidoglycan fraction of bacteria, by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism. NODs are a family of intracytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF-κB activation (27).

IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis (5, 11, 18, 32), mycobacterium tuberculosis infections (21, 24), and inflammatory bowel disease (31). However, IL-32 expression in cells of pancreas origin remains unclear. Furthermore, precise molecular mechanisms controlling IL-32 expression also remain unclear. In this study, we investigated IL-32α expression in human pancreatic periacinar myofibroblasts, which are located in the periacinar regions of normal human pancreas (29). These cells are characterized by the expression of extracellular matrixes (ECMs) and α-smooth muscle actin (α-SMA). They play important roles in the regulating ECM metabolism and inflammatory responses in the pancreas (17, 30).

MATERIALS AND METHODS

Reagents. Recombinant human IL-1β, IL-17, and IFN-γ were purchased from R&D Systems (Minneapolis, MN), and other cytokines were obtained from PeproTech (Rocky Hill, NJ). Anti-human IL-32α antibodies were purchased from R & D Systems. All other reagents were purchased from Sigma Chemical (St. Louis, MO).

Human pancreatic myofibroblast cultures. Primary cultures of pancreatic periacinar myofibroblasts were isolated according to the methods previously described (29). Cells were cultured in DMEM containing 10% FBS. All culture media were supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. More than 98% of the cells were positive for α-SMA. Studies were performed on passages 2–6 of myofibroblasts isolated from six resection specimens. The study design was approved by the ethics committee of Shiga University of Medical Science. Written, informed consent was obtained from all patients prior to sample collection.

Real-time polymerase chain reaction. Expression of human IL-32α mRNA in samples was assessed by real-time PCR analyses. Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied
Science, Tokyo, Japan) with the following primers specific for human IL-32α: 5′-AGCTGGAGACGTCTGTCG (nucleotides 192–211, GenBank accession no. BC018782) (34) and 5′-AGGTGGTTGCTAG- TATCTGCT (nucleotides 642–623). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. PCR was conducted using a SYBRgreen PCR Master Mix (Applied Biosystems, Foster City, CA). Data were normalized vs. β-actin for human IL-32α.

Northern blot analyses. Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (8). Northern blots were performed according to a previously described method (2). Hybridizations were performed with 32P-labeled human probes, generated by a random primed DNA labeling kit (Amersham, Arlington Heights, IL), and evaluated by autoradiography.

Western blot analyses. For analysis of IL-32α protein expression, cells were exposed to cytokines for predetermined periods of time. Cells were then washed with PBS and lysed in SDS sample buffer containing 100 μM orthovanadate. For Western blotting, 10 μg of protein from each sample was subjected to SDS-PAGE on a 4–20% gradient gel under reducing conditions (30). Biotinylated anti-human IL-32α antibodies were purchased from R&D Systems and peroxidase-conjugated streptavidin was purchased from Dako Japan (Kyoto, Japan). Subsequently, detection was performed using the enhanced chemiluminescence Western blotting system (Amersham).

For Akt phosphorylation analyses, cells were exposed to cytokines for predetermined periods of time. Antibodies directed against phosphorylated and total Akt were purchased from Cell Signaling Technology (Beverly, MA), and peroxidase-conjugated second antibodies were purchased from Amersham.

Adenovirus-mediated gene transfers. We used a recombinant adenovirus expressing a stable mutant form of IκBα (Ad-IκBΔN) (25), a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-cJun) (38), and a recombinant adenovirus containing bacterial β-galactosidase cDNA (Ad-LacZ). The stable mutant form of IκBα (IκBΔN) lacks 54 NH2-terminal amino acids of wild-type IκBα and is neither phosphorylated nor proteolysed in response to signal induction but fully inhibits NF-κB activation. The dominant negative mutant c-Jun (TAM67) lacks the transactivational domain of amino acids 3 to 122 of wild-type c-Jun but retains the DNA-binding domain. In preliminary experiments, Ad-LacZ infections of colonic cell lines markedly enhanced IL-32α mRNA expression. TNF-α and IFN-γ effects were stronger than those induced by IL-1β. In contrast, we could not detect secreted forms of IL-32α in supernatants.

RESULTS

Induction of IL-32α in human pancreatic myofibroblasts. To investigate regulatory mechanisms underlying IL-32α induction in human pancreatic periacinar myofibroblasts, cells were stimulated with various cytokines for 12 h and IL-32α mRNA expression was detected by Northern blot analyses (Fig. 1A). In these cells, IL-32α mRNA was weakly expressed without any stimulus, and IL-1β, IFN-γ, and TNF-α markedly enhanced IL-32α mRNA expression. TNF-α and IFN-γ effects were stronger than those induced by IL-1β.

Similar results were observed at the protein level. Cells were stimulated for 24 h with IL-1β, IFN-γ, and TNF-α, and IL-32α protein expression was analyzed by Western blots. IL-32α was detected as a molecular weight protein of 25 kDa, which is comparable with a previous report (23). Stimulation with IL-1β, IFN-γ, and TNF-α enhanced intracellular accumulation of IL-32α protein (Fig. 1B). As observed at mRNA level, TNF-α, and IFN-γ effects were stronger than those induced by IL-1β. In contrast, we could not detect secreted forms of IL-32α in supernatants.

Fig. 1. IL-32α mRNA and protein expression in human pancreatic myofibroblasts. A: IL-32α mRNA expression. Cells were stimulated with cytokines [IL-1β (50 ng/ml) and other cytokines (200 ng/ml)] for 12 h. IL-32α mRNA expression was analyzed by Northern blotting. Control, cells cultured in medium alone. B: Intracellular IL-32α protein expression. Cells were stimulated with each cytokine [IL-1β (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (100 ng/ml)] for 48 h and then lysed with lysis buffer. IL-32α protein was analyzed by Western blotting. C: Combined effects of cytokines on IL-32α mRNA expression. Cells were stimulated with IL-1β (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (100 ng/ml), and combinations of these cytokines for 12 h, and then IL-32α mRNA expression was determined by Northern blotting.

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Next, we tested the effects of combinations of IL-1β, IFN-γ, and TNF-α (Fig. 1C). Northern blot analyses showed that combinations of IL-1β plus TNF-α, IL-1β plus IFN-γ, and/or TNF-α plus IFN-γ synergistically enhanced IL-32α mRNA expression.

Effects of IL-1β, TNF-α, and IFN-γ. Effects of IL-1β, TNF-α, and IFN-γ on IL-32α mRNA expression were examined more precisely. Human pancreatic myofibroblasts were incubated for 12 h with increasing concentrations of IL-1β, TNF-α, and IFN-γ, and the IL-32α mRNA expression was analyzed by Northern blotting. As shown in Fig. 2, A–C, these cytokines dose dependently upregulated IL-32α mRNA expression. The IL-1β effect was detected at as low as 0.01 ng/ml and reached a maximum at 10 ng/ml. The TNF-α effect was observed at as low as 0.1 ng/ml and reached a maximum at 100 ng/ml. The IFN-γ effect was also detected as low as 0.1 ng/ml and gradually increased to 500 ng/ml.

The kinetics of IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression were evaluated (Fig. 2, D–F). Cells were stimulated with IL-1β (10 ng/ml), TNF-α (100 ng/ml), or IFN-γ (100 ng/ml), and sequential changes in IL-32α mRNA expression were determined by Northern blotting. IL-1β induced an increase in the accumulation of IL-32α mRNA, and this reached a maximum 12 h after stimulation. Thereafter, the induced-IL-32α mRNA levels decreased. TNF-α and/or IFN-γ also induced a gradual but continuous increase in the accumulation of IL-32α mRNA for 24 h.

Effects of MAPK inhibitors and PI3K inhibitors. The MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are implicated in cytokine signaling in various cell types. To investigate molecular mechanisms underlying IL-32α induction in human pancreatic myofibroblasts, we evaluated the effects of following inhibitors: p42/44 MAPK inhibitors (PD98059 and U0216) (1, 13), a p38 MAPK inhibitor (SB203580) (9) and a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) (12). Real-time PCR demonstrated that treatment with MEK inhibitors (PD98059 and U0216) or a p38 MAPK inhibitor (SB203580) had no effect on IL-1β-, TNF-α-, and/or IFN-γ-induced IL-32α mRNA (Fig. 3, A–C). Contrary to these findings, a PI3K inhibitor, LY294002 (35), significantly blocked the effect of IL-1β-, TNF-α-, and/or IFN-γ on IL-32α mRNA expression (Fig. 3, A–C). The effects of LY294002 were confirmed by Northern blotting (Fig. 3D), and wortmannin (12), another PI3K inhibitor, also blocked IL-1β-, TNF-α-, and/or IFN-γ-induced IL-32α mRNA expression (Fig. 3E). These results suggest that PI3K activation is involved in IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression in human pancreatic myofibroblasts, although MEK and p38 MAPK pathways are dispensable.

Akt activation in response to cytokines. In human pancreatic myofibroblasts, the induction of Akt phosphorylation by IL-1β, TNF-α, and IFN-γ was evaluated by Western blotting. As shown in Fig. 4, IL-1β, TNF-α, and IFN-γ induced the Akt phosphorylation as early as 5 min after the stimulation. These data indicate that Akt, a protein kinase recruited by PI3K activation, is rapidly activated by IL-1β, TNF-α, and IFN-γ in human pancreatic myofibroblasts.

Effects of inhibition of NF-κB and AP-1 signaling. Promoter sequences analyzed by the UCSC Genome Browser created by the Genome Bioinformatics Group of UC Santa Cruz (the University of California, Santa Cruz, CA) showed consensus binding sites for NF-κB (at bp −638 to −649) and AP-1 (at bp −230 to −242) in promoter regions of human IL-32α gene. To assess the role of transcription factors NF-κB and AP-1, we assessed the role of transcription factors NF-κB and AP-1, we...
evaluated the effects of a recombinant adenovirus containing a stable mutant form of IκBα (Ad-IκBΔN) and a dominant negative mutant of c-Jun (Ad-DN-c-Jun) on cytokine-induced IL-32α mRNA expression. As shown in Fig. 5, cells were infected with recombinant adenovirus and were cultured for 48 h. Cells were stimulated for 12 h with IL-1β (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (100 ng/ml), and the expression of IL-32α mRNA was determined by Northern blotting. Ad-IκBΔN inhibited the effects of both IL-1β and TNF-α on IL-32α mRNA expression, and Ad-DN-c-Jun also suppressed the effects of IL-1β, TNF-α, and IFN-γ on IL-32α mRNA expression. Inhibitory effects were not induced by the Ad-LacZ gene, which was used as a negative control. These suggest that NF-κB and AP-1 play a role in IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression.

Effects of PI3K-inhibitor (LY294002) on NF-κB/AP-1 activation. The PI3K/Akt pathway is an upstream activator for NF-κB and AP-1 in various cell types (19, 22, 26, 28, 35). To investigate the possibility that in human pancreatic myofibroblasts the PI3K/Akt pathway contributes to NF-κB/AP-1 activation, we investigated the effects of LY294002 on NF-κB and AP-1 activation in these cells. As shown in Fig. 6A, EMSAs showed that PI3K inhibition by LY294002 suppressed both IL-1β- and TNF-α-induced NF-κB activation. The specificity of this reaction was confirmed by the addition of cold oligo-DNA, which abolished the reactive band (Fig. 6C). The addition of antibodies directed against a 50-kDa subunit (p50) and a 65-kDa subunit (p65) of NF-κB induced supershifts of the binding complexes, indicating that this binding complex was a heterodimer consisting of p50 and p65 subunits (Fig. 6C).

Similarly, LY294002 blocked IL-1β-, TNF-α-, and IFN-γ-induced AP-1 activation (Fig. 6B). The specificity of this reaction was confirmed by the addition of cold oligo-DNA, which abolished the reactive band (Fig. 6D). The addition of antibodies directed against Fos/Jun induced supershifts, indicating that this binding complex was a heterodimer consisting of p50 and p65 subunits (Fig. 6D).

In human pancreatic myofibroblasts IL-1β-, TNF-α-, and IFN-γ-induced activation of PI3K/Akt, NF-κB, and AP-1 pathways contributes to IL-32α mRNA induction.

DISCUSSION

IL-32 is a recently reported cytokine, expressed by T lymphocytes, NK cells, monocytes, and epithelial cells (20, 23). However, IL-32 expression by mesenchymal cells has not been identified. Furthermore, IL-32 expression by cells of pancreatic origin remains unclear. In the present study, we demonstrate several findings: 1) human pancreatic myofibroblasts are a source of IL-32α; 2) IL-1β, TNF-α, and IFN-γ are potent stimulators for IL-32α induction; and 3) PI3K/Akt pathway-dependent NF-κB/AP-1 activation plays a crucial role in IL-32α induction.

Previous studies defined that proinflammatory cytokines such as IL-1β, IL-12, IL-18, and IFN-γ are stimulators for IL-32 expression (20, 31). However, these observations are limited in genetically engineered cells or in transformed cells, and molecular mechanisms underlying IL-32α induction remain unclear. In the present study, we showed that in human pancreatic myofibroblasts IL-1β, TNF-α, and IFN-γ are potent
inducers of IL-32α mRNA expression. To address the molecular mechanism contributing to IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression, we evaluated the effects of MAPK inhibitors and PI3K inhibitor on IL-32α induction. Although we have previously shown that MAPKs play a crucial role in inducing proinflammatory cytokines such as IL-6 and IL-8 in human pancreatic and colonic myofibroblasts (3, 16, 30), p42/44 MAPK-inhibitors (PD98059 and U0216), and p38 MAPK inhibitor (SB203580) did not affect IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression. In contrast, a PI3K inhibitor (LY294002) effectively suppressed IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression. A similar suppression was also confirmed by another PI3K inhibitor, wortmannin. Wortmannin and LY294002 have different structures and bind to different PI3K epitopes, and inhibitory effects observed with both wortmannin and LY294002 provide a good indication for PI3K involvement (36). Furthermore, in these cells IL-1β, TNF-α, and IFN-γ induced phosphorylation of Akt, a protein kinase immediately recruited by PI3K activation (6). These observations indicate, for the first time, that the PI3K/Akt pathway contributes to proinflammatory cytokine-induced IL-32α mRNA expression in human pancreatic myofibroblasts. Recent studies showed that the PI3K/Akt pathway plays an important role in pancreatic regenerative responses (37), acinar cell functions (15), and endocrine pancreas (39). In addition, the PI3K/Akt pathway regulates trypsinogen activation during acute pancreatitis (33). Besides these functions, our findings suggest a new aspect of the PI3K/Akt pathway in inflammatory and immune responses in the pancreas.

Many cytokine-inducible responses are mediated by the important DNA binding proteins such as NF-κB and AP-1. The promoter region of the human IL-32α gene has consensus binding sites for NF-κB (at bp −638 to −649) and AP-1 (at bp −230 to −242), suggesting an involvement of NF-κB and AP-1 activation in IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression. To confirm this possibility, we used a recombinant adenosine expressing a stable mutant form of IκBα (Ad-IκBΔN) (25) and a recombinant adenosine expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) (38). Successful infection of Ad-IκBΔN and/or Ad-DN-c-Jun fully inhibits NF-κB and AP-1 activation. As shown in Fig. 6, pretreatment with Ad-IκBΔN blocked IL-1β- and TNF-α-induced IL-32α mRNA expression, and treatment with Ad-DN-c-Jun also suppressed IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression. These data indicate that NF-κB and AP-1 activation play a role in IL-32α mRNA induction in our system.

Recent studies indicate that the PI3K/Akt pathway regulates activation of transcription factors, such as NF-κB and

![Fig. 4. Kinetics of Akt activation in human pancreatic myofibroblasts. Cells were stimulated with cytokines [IL-1β (10 ng/ml; A), TNF-α (100 ng/ml; B), and IFN-γ (100 ng/ml; C)], and phosphorylated (P-) and total Akt were sequentially detected by Western blotting.](image-url)

![Fig. 5. A: effects of NF-κB and/or activated protein-1 (AP-1) inhibition on IL-32α mRNA expression. Cells were infected with an adenovirus expressing the IκBΔN or DN-c-Jun, and after 48 h after infection cells were stimulated with IL-1β (10 ng/ml), TNF-α (100 ng/ml), or IFN-γ (100 ng/ml) for 12 h. IL-32α mRNA expression was determined by Northern blot analyses. B: adenovirus expressing LacZ were used as negative controls.](image-url)
AP-1, in some cell types (19, 22, 26, 28, 35). These studies implicated several mechanisms of transcription factor regulation by PI3K, which may act in a cell-specific manner. On the basis of this notion, we assumed cross talk between the PI3K/Akt pathway and NF-κB/AP-1 activation in cytokine-induced IL-32 mRNA expression. EMSAs showed that IL-1β and TNF-α induced NF-κB and AP-1 activation, and IFN-γ stimulated AP-1 activation in human pancreatic myofibroblasts. LY294002 potently suppressed NF-κB and/or AP-1 activation, indicating a role for the PI3K/Akt pathway in IL-1β-, TNF-α-, and IFN-γ-induced NF-κB and AP-1 activation. Thus, combined with inhibitory effects of LY294002 on IL-1β-, TNF-α-, and IFN-γ-induced IL-32α mRNA expression, it is likely that the sequential activation of PI3K/Akt-induced NF-κB/AP-1 pathways may be crucial in cytokine-induced IL-32α mRNA induction in human pancreatic myofibroblasts.

Experiments using recombinant IL-32α suggest that IL-32α is a proinflammatory cytokine that is characterized by inducing the release of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and chemokines) through NF-κB and p38 MAPK activation pathways (11, 20). However, it remains unclear whether IL-32α exerts its biological effects as a secretory cytokine, since the IL-32α protein does not possess a typical hydrophobic signal peptide in its NH2-terminus, which is a typical feature of secreted cytokines (7). In Cos7 cells transfected with IL-32α cDNA, intracellular accumulation of IL-32α was approximately sevenfold as abundant as amounts of secreted IL-32α (20). In the aforementioned model, there is a possibility that IL-32α in supernatants was released by apoptotic cells or during cell disruption. In this study, we could not detect IL-32α secretion by immunoprecipitation in human pancreatic myofibroblasts. This was also confirmed in human colonic myofibroblasts (data not shown). In contrast to IL-32α, in Cos7 cells transfected with IL-32β cDNA, the abundance of IL-32β was comparable in supernatants and lysates (20). Although it is unclear which of the IL-32 isoform is effectively secreted from particular cell types, it may be that IL-32α plays a role as a cytoplasmic protein. Recently, Goda et al. (14) demonstrated that overexpression of intracellular IL-32β induced apoptosis in HeLa cells, which was blocked by interference of IL-32α transcription. These data suggest a role for cytoplasmic IL-32 in cell turnover. Apoptosis functions to delete damaged cells and restore tissue architecture, and IL-32α may induce apoptosis in damaged cells at inflammatory sites such as pancreatitis. IL-32α might function as a mediator bridging apoptosis and inflammation.

In conclusion, we demonstrated that IL-32α is expressed in human pancreatic myofibroblasts. IL-32α was induced by IL-1β, IFN-γ, and TNF-α and was mediated by interactions between the PI3K/Akt-pathway and the NF-κB/AP-1 system. Interestingly, IL-32α was not secreted by human pancreatic myofibroblasts. The role of the cytoplasmic accumulation of IL-32α in human pancreatic myofibroblasts should be further investigated.
REFERENCES


