Interleukin-32α expression in human colonic subepithelial myofibroblasts

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Received August 22, 2010; Accepted October 19, 2010

DOI: 10.3892/ijmm.2010.575

Abstract. Interleukin (IL)-32 is a recently described proinflammatory cytokine, characterized by induction of nuclear factor (NF)-κB activation. We studied IL-32α expression in human colonic subepithelial myofibroblasts (SEMFs). Colonic SEMFs were isolated from normal human colon tissue. IL-32α protein expression was evaluated by Western blot analyses, and IL-32α mRNA expression was analyzed by real-time PCR. IL-32α mRNA was weakly expressed without a stimulus, and its expression was markedly enhanced by IL-1β and TNF-α. IL-1β and TNF-α enhanced intracellular accumulation of IL-32α protein, but IL-32α was not detected in supernatants. Each cytokine dose- and time-dependently induced IL-32α mRNA expression. An inhibitor of phosphatidylinositol 3-kinase (LY294002) significantly suppressed IL-1β- and TNF-α-induced IL-32α mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blotting. Blockade of NF-κB activation by an adenovirus expressing a stable mutant form of IκBa markedly suppressed IL-1β- and TNF-α-induced IL-32α mRNA expression. Human colonic SEMFs expressed IL-32α in response to IL-1β and TNF-α. IL-32α mRNA expression depends on the phosphatidylinositol 3-kinase and the NF-κB system.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tract. IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora (1-3). This aberrant response is most likely facilitated by defects in both the barrier function of the intestinal epithelium and the mucosal immune system. The investigation of the immunological responses in the intestinal mucosa is a fundamental approach to the understanding of the pathophysiology of IBD.

Interleukin (IL)-32 is a recently described cytokine that is produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells (4,5). Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that epithelial cells are the dominant and widespread source (6). 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-32δ and IL-32γ), 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 (4). IL-32 exhibits several properties typical of proinflammatory cytokines (4,5). For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-6 and IL-8 by means of the activation of NF-κB and p38 mitogen-activated protein kinases (MAPKs) (4,5).

Netea et al recently demonstrated that IL-32 augments the production of IL-1β and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism (5). NODs are a family of intracytoplasmic bacterial sensors, and recognition of bacterial peptidoglycans subsequently induces NF-κB activation (8). Mutations in NOD2 have been implicated in the pathogenesis of CD (9,10), and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in cytokine production (11,12). Recently, it has been shown that the NOD2 mutation in CD patients potentiates NF-κB activity and IL-1β processing (13). Thus, these data suggest a pivotal role of IL-32 in the pathophysiology of IBD and in particular of CD.

In this study, we investigated the expression of IL-32α in human colonic subepithelial myofibroblasts (SEMFs). Colonic SEMFs are α-smooth muscle actin (α-SMA)-positive...
mesenchymal cells and are located subjacent to the basement membrane of the small and large intestines. These cells belong to a family of α-SMA positive fibroblast-like cells, such as the cells of the hepatic stellate (Ito) renal mesangial/tubulointerstitial, the lung interstitial contractile cells, and the pancreatic stellate cells (14-16). Colonic SEMFs act to mediate information flow in both directions to and from the intestinal epithelium and the immune and the other mesenchymal and neural elements of the lamina propria, and play an important role in ECM metabolism affecting the growth of the basement membrane in the intestinal mucosa. Current observations suggest a role of colonic SEMFs in the pathophysiology of IBD via IL-32 expression.

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-32 antibodies were purchased from R&D Systems. All other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Culturing of human colonic SEMFs and colonic epithelial cell lines. Primary colonic SEMF cultures were prepared according to a method reported by Mahida et al (17). The cellular characteristics and culture conditions have been described in our previous report (18).

Real-time-polymerase chain reaction (PCR). 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’AGCTGGAGGACGACTCTCAA (nucleotides 192-212, GenBank accession No. BC018782) (19) and 3’AGGGTG GTGTCAGTATCTTCA (642-623). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. PCR was conducted using a SYBR-Green PCR Master mix (Applied Biosystems, Foster City, CA). Data were normalized versus β-actin for human IL-32.

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 (20). Biotinylated anti-human IL-32 antibodies were purchased from R&D Systems and peroxidase-conjugated secondary antibodies were purchased from Amersham (Arlington Heights, IL).

Adenovirus-mediated gene transfers. We used a recombinant adenovirus expressing a stable mutant form of IκBα (Ad-IkBΔN) (21), a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) (22) and a recombinant adenovirus containing bacterial β-galactosidase cDNA (Ad-LacZ). The stable mutant form of IκBα (IkbAN) lacks 54 NH2-terminal amino acids of wild-type IκBα, and is neither phosphorylated nor proteolyzed 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 3-122 of wild type c-Jun, but it retains the DNA-binding domain. In preliminary experiments, Ad-LacZ infections of colonic myofibroblasts with a multiplicity of infection (MOI) of 10 showed a maximal expression (85% positive) of β-galactosidase. The recombinant adenovirus was transferred into the cells, and cells were made quiescent for 48 h before being assessed for the effects of the transferred gene.

Statistical analysis. Statistical significances of differences were 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

To investigate the regulatory mechanisms underlying IL-32α induction in colonic SEMFs, cells were stimulated with various cytokines for 12 h and IL-32α mRNA expression was assessed by real-time PCR analyses (Fig. 1A). In these cells, IL-32α mRNA was weakly expressed without any stimulus, and IL-1β and TNF-α markedly enhanced IL-32α mRNA expression. IFN-γ weakly induced IL-32α mRNA expression.

Similar results were observed at the protein level. Cells were stimulated for 24 h with IL-1β 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 (5). Stimulation with IL-1β and TNF-α enhanced intracellular accumulation of IL-32α protein (Fig. 1B). However, we could not detect the secreted forms of IL-32α in supernatants.

The effects of IL-1β and TNF-α on IL-32α mRNA expression were more closely examined. Colonic SEMFs were incubated for 12 h with increasing concentrations of IL-1β and TNF-α, and the IL-32α mRNA expression was analyzed by real-time PCR. As shown in Fig. 2, these cytokines dose-dependently up-regulated IL-32α mRNA expression. The IL-1β effect was detected at a concentration as low as 0.01 ng/ml, and reached a maximum at 1.0 ng/ml. The TNF-α effect was observed at a concentration as low as 0.1 ng/ml, and reached a maximum at 100 ng/ml.

The kinetics of IL-1β- and TNF-α-induced IL-32α mRNA expression were evaluated (Fig. 3). Cells were stimulated with IL-1β (10 ng/ml) or TNF-α (100 ng/ml) for predetermined times, and IL-32α mRNA expression was sequentially analyzed by real-time PCR analyses. Data are expressed as IL-32α mRNA expression relative to β-actin mRNA expression (mean ± SD of 4 different experiments; **P<0.01.

In human pancreatic myofibroblasts, the induction of Akt phosphorylation by IL-1β and TNF-α was evaluated by Western blotting. As shown in Fig. 5, IL-1β and TNF-α induced the Akt phosphorylation as early as 5 min after the stimulation. These data indicate that Akt, a protein kinase recruited by...
Figure 4. Effects of MAPK inhibitors and a PI3K inhibitor on IL-32α mRNA expression. Cells were stimulated with each cytokine [IL-1β (10 ng/ml) or TNF-α (100 ng/ml)] in the presence or absence of MEK inhibitors [PD98059 (20 μM) and U0126 (12.5 μM)], a p38 inhibitor [SB203580 (25 μM)], and a PI3K inhibitor [LY294002 (25 μM)] for 12 h, and then IL-32α mRNA expression was determined by real-time PCR. Data are expressed as IL-32α mRNA expression relative to β-actin mRNA expression (mean ± SD of 4 different experiments); **P<0.01.

Figure 5. Kinetics of Akt activation in human colonic SEMFs. Cells were stimulated with cytokines [IL-1β (10 ng/ml) or TNF-α (100 ng/ml)], and phosphorylated (phospho-) and total Akt were sequentially detected by Western blotting.

Figure 6. Effects of NF-κB and/or AP-1 inhibition on IL-32α mRNA expression. Cells were infected with an adenovirus expressing the IκBαΔN or DN-c-Jun, and after 48 h of infection the cells were stimulated with IL-1β (10 ng/ml) or TNF-α (100 ng/ml) for 12 h. IL-32α mRNA expression was determined by real-time PCR analyses. Data are expressed as IL-32α mRNA expression relative to β-actin mRNA expression (mean ± SD of 4 different experiments); **P<0.01. Adenovirus expressing LacZ was used as a negative control.
PI3K activation, is rapidly activated by IL-1β and TNF-α in colonic SEMFs.

To assess the role of the transcription factors NF-κB and AP-1, we evaluated the effects of a recombinant adenovirus containing a stable mutant form of IkBo (Ad-IkBAN) and a dominant negative mutant of c-Jun (Ad-DN-c-Jun) on cytokine-induced IL-32α mRNA expression. As shown in Fig. 6, cells were infected with a recombinant adenovirus, cultured for 48 h and stimulated for 12 h with IL-1β (10 ng/ml) or TNF-α (100 ng/ml), and the expression of IL-32α mRNAs was determined by real-time PCR. Ad-IkBAN inhibited the effects of both IL-1β and TNF-α on IL-32α mRNA expression, but Ad-DN-c-Jun did not suppress the effects of IL-1β or TNF-α. Inhibitory effects were not induced by the Ad-LacZ gene, which was used as a negative control. These results suggest that NF-κB plays a role in IL-1β- and TNF-α-induced IL-32α mRNA expression.

**Discussion**

A previous study showed that intestinal epithelial cells are a local site for IL-32α expression (5,28), and that IL-32α expression is enhanced in the inflamed mucosa of patients with IBD (28). In this study, we evaluated the IL-32α expression in colonic SEMFs. This suggests that colonic SEMFs also contribute to the elevation of IL-32α expression in the IBD mucosa. Since IL-32 acts as a proinflammatory cytokine, which is characterized by induction of the release of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and chemokines) through the NF-κB- and p38 MAPK-activation pathways (4,6), IL-32α derived from colonic SEMFs stimulates infiltrating immune cells to secrete proinflammatory cytokines and contributes to the deterioration of mucosal inflammation.

The molecular mechanisms underlying IL-32α induction remain unclear. Previous studies have demonstrated that proinflammatory cytokines such as IL-1β, IL-12, IL-18 and IFN-γ are stimulators for IL-32 expression (4,28). In the present study, we demonstrated that IL-1β and TNF-α are potent inducers of IL-32 mRNA expression in human colonic SEMFs. IL-1β- and TNF-α-induced IL-32α mRNA expression was suppressed by a PI3K-inhibitor (LY294002), but not by p42/44 MAPK-inhibitors (PD98059 and U0126) or a p38 MAPK-inhibitor (SB203580). Furthermore, in these cells IL-1β and TNF-α induced the phosphorylation of Akt, a protein kinase immediately recruited by PI3K activation (29). These observations indicate that the PI3K/Akt pathway contributes to IL-1β- and TNF-α-mediated IL-32α mRNA induction in colonic SEMFs.

Many cytokine-inducible responses are mediated by 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), suggesting an involvement of NF-κB activation in IL-1β- and TNF-α-induced IL-32α mRNA expression. To confirm this possibility, we used a recombinant adenovirus expressing a stable mutant form of IkBo (Ad-IkBAN) (21). As shown in Fig. 6, pretreatment with Ad-IkBAN blocked IL-1β- and TNF-α-induced IL-32α mRNA expression. These data indicate that NF-κB activation plays a role in IL-32α mRNA induction in colonic SEMFs. In addition, recent studies indicate that the PI3K/Akt pathway regulates activation of transcription factors, such as NF-κB (27,30-33), suggesting cross-talk between the PI3K/Akt pathway and NF-κB activation in cytokine-induced IL-32 mRNA expression.

Whether IL-32 exerts its biological effects as a secretory cytokine remains unclear, since the IL-32 protein does not possess a typical hydrophobic signal peptide in its N-terminus which is a typical feature of secreted cytokines (7). In Cos7 cells transfected with IL-32α cDNA, intracellular IL-32α levels were approximately 7-fold higher compared to secreted IL-32α levels (4). In contrast, in Cos7 cells transfected with IL-32α cDNA, the abundance of IL-32α was comparable in supernatants and lysates (4). In this study, IL-32α was detected in cell lysate, but was not detected in supernatants. Although it is unclear which of the IL-32 isoforms is effectively secreted from particular cell types, it may be that IL-32α plays a role as a cytoplasmic protein. Recently, Goda et al demonstrated that overexpression of intracellular IL-32α induced apoptosis in HeLa cells (34). These data suggest a role for cytoplasmic IL-32 in cell turnover. Damaged cells are deleted and tissue architecture is restored through apoptosis, and IL-32α may induce apoptosis in damaged cells at inflammatory sites such as IBD.

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

**References**


