A new isoform of interleukin-32 suppresses IL-8 mRNA expression in the intestinal epithelial cell line HT-29

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Abstract. Interleukin (IL)-32 plays a role in the pathophysiology of inflammatory bowel disease (IBD). We isolated a new isoform of the IL-32 transcript in the process of cloning the full-length IL-32 gene from human colonic subepithelial myofibroblasts (SEMFs). The expression of mRNA in the samples was assessed by RT-PCR and real-time PCR analyses. The PCR products from the IL-32 genes were ligated into the expression vector pIRESneo2. The new isoform of the IL-32 transcript (336 nucleotides) completely lacked exon 4 of the IL-32γ gene, and was 60 bp shorter than IL-32α. TNF-α induced the mRNA expression of the new IL-32 isoform in a dose- and time-dependent manner. Stable transfection of this new isoform significantly decreased TNF-α-induced IL-8 mRNA expression in HT-29 cells, but the expression of the IL-32α gene had no effect. The mRNA expression of this new isoform was significantly elevated in the inflamed mucosa of IBD patients. A new isoform of the IL-32 transcript may play an anti-inflammatory role in the inflamed mucosa of IBD.

Introduction

Interleukin (IL)-32 is a recently-described cytokine produced by T lymphocytes, natural killer cells, monocytes and epithelial cells (1,2). 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 (3). The gene encoding IL-32 is located on human chromosome 16p13.3, and is organized into eight exons (4). There are four splice variants (IL-32α, IL-32β, IL-32δ and IL-32γ). In addition, two more isoforms (IL-32ε and ζ) have been reported, but these have not been found to be abundantly expressed in cell types besides activated T cells (5). Although cytokine gene splicing events are unusual, they exist in other cytokines, such as IL-1F7 and vascular endothelial growth factor (6,7). Of the six isoforms, IL-32γ is the longest and exhibits the highest biological activity in vitro and in vivo (8). IL-32 exhibits several properties typical of proinflammatory cytokines (1,2); for example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-6 and IL-8 by the activation of NF-κB and p38 mitogen-activated protein kinases (MAPKs) (1,2).

Ulcerative colitis (UC) and Crohn’s disease (CD), two common forms of inflammatory bowel disease (IBD), are chronic relapsing inflammatory disorders of the gastrointestinal tract (9-11). IBD result from the inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora (9-11). Previously, Netea et al reported that IL-32 augments the production of IL-1β and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction from bacteria, by means of nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism (2). NODs are a family of intracytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF-κB activation (12). Mutations in NOD2 have been implicated in the pathogenesis of CD (13,14), and CD patients homozygous for the frameshift 3020insC mutated allele have defective responses to MDP in terms of cytokine production (9,15,16). Recently, it has been shown that the NOD2 mutation in CD patients potentiates NF-κB activity and IL-1β processing (17). Thus, these findings suggest a pivotal role for IL-32 in the pathophysiology of IBD (18,19), and in particular CD.

Several studies have correlated IL-32 expression with IBD (18-20), and we previously reported that IL-32 expression by epithelial cells is increased in the inflamed mucosa of IBD patients (18). In our further investigation of IL-32 expression in the intestinal mucosa, we found a new isoform of the IL-32 transcript (336 nucleotides) through the process of cloning the full-length IL-32 gene from human colonic subepithelial myofibroblasts (SEMFs). Stable transfection of this new isoform of the gene into the intestinal epithelial cell line HT-29 suppressed TNF-α-induced inflammatory responses.

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**Materials and methods**

**Reagents.** Recombinant human IL-6, IFN-γ and TGF-β were purchased from PeproTech (Rocky Hill, NJ, USA), and all other cytokines were obtained from R&D Systems (Minneapolis, MN, USA). Anti-human IL-32 antibodies were purchased from R&D Systems. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Culture of human colonic subepithelial myofibroblasts (SEMFS) and colonic epithelial cell lines.** Primary colonic SEMF cultures were prepared according to the method reported by Mahida et al (21). The cellular characteristics and culture conditions were as described in our previous report (22). The human colon cancer cell line HT-29 was cultured as previously described (23).

**Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR.** The expression of mRNA in the samples was assessed by RT-PCR and real-time PCR analyses. RT-PCR was performed according to methods described in our previous report (24). The oligonucleotide primers used in this study are shown in Table I. The different pairs of primers for specific IL-32 splice variants were designed using a particular intron and exon border, where a distinct splicing event takes place in each isoform (8,25). Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan). PCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Data were normalized to β-actin for human IL-32.

**Cloning of the new IL-32 isoform and IL-32γ and transfection into HT-29 cells.** Each PCR product for the new IL-32 isoform (336 bp), IL-32α (396 bp) and IL-32γ (705 bp) was ligated into the BamHI and EcoRI restriction sites of the expression vector pIRESneo2 (Clontech Laboratories, Mountain View, CA). The expression vectors were transfected to HT-29 cells using FuGene HD (Roche Diagnostics K.K., Tokyo, Japan), and were selected by neomycin.

**Tissue samples.** The diagnosis of IBD was based on conventional clinical and endoscopic criteria. Biopsied specimens from 18 patients with UC (7 active, 11 inactive) and 15 patients with CD (10 active, 5 inactive) were used with informed consent. The ethics committee of the Shiga University of Medical Science approved the study. During the sample collection period, all patients were clinically and endoscopically active based on the colitis activity index for UC (26) and the Crohn’s disease activity index (27). Histological examina-

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**Table I. Primers used in this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>IL-32α, γ, new isoform (primers for cloning)</td>
<td>TTGGCTCTTGAACCTTTTGG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCAAAGGTTGCGTGTCAGTATG&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-32 new isoform</td>
<td>AAGCTGAGAAGCCCCGAGATGGT</td>
<td>GGCTCCGTCAGACTTGTCAC</td>
</tr>
<tr>
<td>IL-8</td>
<td>TGATTGAGATTGGACCACAC</td>
<td>CCAAGAATCTTGATGTCAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGACCCAGATCATGTTTGGACACTT</td>
<td>CCACGTACACACTTCATGATGAGGAG</td>
</tr>
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<sup>a</sup> From -25 to -6 of the IL-32γ sequence; <sup>b</sup> from 726 to 727 of the IL-32γ sequence.

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**Figure 1.** (A) Nucleotide sequence of the new isoform of IL-32, IL-32α and IL-32γ (8,25). Underscore indicates the PCR primers for the IL-32 new isoform. (B) Schematic representation of the structures of the new isoform of IL-32 and previously reported IL-32 isoforms. Boxes indicate the exons of the IL-32 gene.
tions were performed in macroscopically and microscopically non-affected and affected areas from each patient.

**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**

We isolated a new isoform of the IL-32 transcript (336 nucleotides) through the process of cloning the full-length IL-32 gene from human colonic SEMFs. The nucleotide sequences of this new isoform of the IL-32 transcript, IL-32α and IL-32γ, are shown in Fig. 1A (3,25). The new isoform completely lacks exon 4, and is 60 bp shorter than IL-32α (Fig. 1B).

Previous studies have demonstrated that inflammatory stimuli induce IL-32 expression in various cell types (1,18,28,29). To investigate the molecular mechanisms participating in the induction of the new isoform of the IL-32 gene, we evaluated the effects of various cytokines on the mRNA expression of the new isoform. TNF-α exerted a strong effect on the induction of the new isoform transcript in HT-29 cells (Fig. 2A). IFN-γ also had modest, but significant, effects. The effects of TNF-α occurred in a dose- and time-dependent manner (Fig. 2B and 2C).

Previous studies have suggested that IL-32 plays a role as a cytoplasmic protein. To investigate the role of this new isoform of IL-32 in inflammatory responses, we constructed three different vectors expressing the new isoform of IL-32, IL-32α or il-32γ, and investigated the effects of these constructs on TNF-α-induced IL-8 mRNA expression in HT-29 cells. As shown in Fig. 3, the empty pIRES vector had no effects, and TNF-α induced a marked increase in IL-8 mRNA expression in HT-29 cells transfected with the empty pIRES vector. Transfection with the expression vector containing the new isoform of IL-32 significantly decreased TNF-α-induced IL-8 mRNA, but transfection with the IL-32α gene had no effects. Transfection with the expression vector containing the IL-32γ gene significantly reduced TNF-α-stimulated IL-8 mRNA expression, but its effects were much weaker than those induced by transfection with the new isoform of the IL-32 gene.

To evaluate the mRNA expression of the new isoform of IL-32 in the mucosa, mRNA expression for the new transcript was analyzed by real-time PCR in IBD mucosa. As shown in Fig. 4, there was a significant increase in the mRNA expression of the new isoform in samples from the active and inactive lesions of CD patients, as compared with samples from normal and inactive IBD mucosa.

**Discussion**

Based on sequence analysis, IL-32 does not belong to any established cytokine family, and many details of its biology remain elusive (3). Four isoforms of IL-32 exist due to alterna-
Figure 3. Effects of the stable transfection of the new IL-32 isoform gene on TNF-α-induced IL-8 mRNA expression in HT-29 cells. Three different vectors expressing the new isoform of IL-32, IL-32α or IL-32γ, were transfected into HT-29 cells. The cells were then stimulated with TNF-α (100 ng/ml) for 12 h, and their IL-8 mRNA expression was determined by real-time PCR. Values are expressed as the means ± SD (n=5). *P<0.05; significant difference compared to the values for medium alone.

Figure 4. New IL-32 isoform mRNA expression in the IBD mucosa. Total RNA was extracted from biopsy samples, and the IL-32 isoform mRNA expression was evaluated by real-time PCR analyses. The data from the real-time PCR were normalized versus β-actin for the IL-32 isoform. The lower and upper margins of the box represent the 25th and 75th percentiles, with the bars representing the 10th and 90th percentiles, respectively. H/C, healthy control; CD, Crohn’s disease; UC, ulcerative colitis. *P<0.05.

We have identified a new isoform of the IL-32 transcript. Since the intracellular expression of this gene showed inhibitory effects on TNF-α-induced IL-8 expression, it is possible that this new IL-32 isoform may have different intracellular and extracellular biological functions. Future studies on this new IL-32 isoform should provide further insights into the precise role of this novel transcript in the regulation of inflammatory responses.
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