Expression Levels of Proinflammatory Cytokines and NLRP3 Inflammasome in an Experimental Model of Oxazolone-induced Colitis

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ABSTRACT

IL-1β and IL-17A are two cytokines with strong proinflammatory activities and are now known to be involved in a number of chronic inflammatory disorders. High-mobility group box 1 (HMGB1) is a nuclear protein regulating the expression of these proinflammatory cytokines. The NLRP3 inflammasome promotes the maturation of the IL-1β and its activation has been shown as a critical mechanism in the pathogenesis of inflammatory bowel disease (IBD). However, underlying mechanisms to modulate their production in IBD are still unclear.

The aim of this study was to investigate the expression levels of mRNA for the NLRP3 inflammasome, HMGB1 and proinflammatory cytokines, IL-1β, IL-17A in the inflamed colon of rats with experimental oxazolone-induced colitis. Experiments were carried out on male wistar rats. IL-1β, IL-17A, HMGB1 and NLRP3 inflammasome mRNA expression were analyzed by real-time reverse transcriptase-polymerase chain reaction.

Our results indicated that the expression levels of IL-1β, IL-17A, NLRP3 and HMGB1 were elevated in the inflamed colon of rats with oxazolone-induced colitis.

Keywords: Colitis; High mobility group box 1 protein; IL-1β; IL-17A; NLR family; Inflammasome

INTRODUCTION

Dysregulation of proinflammatory cytokines is a central feature in the development of inflammation and chronic inflammatory disorders. IL-1 initiates many immunologic responses, including fever, prostaglandin synthesis, neutrophil influx and B-cell activation and antibody secretion as well as T-cell activation, T-cell activation and cytokine production, fibroblast proliferation and collagen formation. IL-1β expression is tightly regulated and most abundantly produced by a subset of professional immune cells—myeloid cells. Classically, inflammatory bowel diseases (IBD) was thought to be primarily mediated by Th1 cells in Crohn's disease (CD) or Th2 cells in ulcerative colitis (UC), but it is now known that Th17 cells and their related cytokines are crucial mediators in both conditions. Th17 cells massively infiltrate the inflamed intestine of IBD patients, where they produce IL-17A and other cytokines, triggering and
amplifying the inflammatory process.\(^2\)

Pro-IL-1β levels are increased in response to TLR activation upon endogenous or exogenous danger signals, and the inflammasome processes pro-IL-1β to mature, active IL-1β via caspase-1 activation. Inflammasomes are multi-protein complexes, containing nucleotide-binding oligomerization domain receptors (NOD)-like receptors (NLRs) pyrin domain containing protein (NLRP1, NLRP3, and NLRC4), an adapter molecule (ASC), and procaspase-1.\(^3\)

Inflammatory stimuli up-regulate the expression of these inflammasome components. The NLRP3 inflammasome activation was identified as a critical mechanism of pathogenesis of IBD and it may serve as a potential target for the development of novel therapeutics for IBD patients.\(^4\)

Many other proteins can induce innate immunity and inflammation, even though they may not strictly be classified as cytokines. Instead, these proteins are generally considered danger-associated molecular patterns (DAMPs) or alarmins. Upon release from the cells, DAMPs such as High Mobility Group Box 1 protein (HMGB1) can bind to multiple PRRs and trigger inflammation.\(^5\) During infection or injury, activated immune cells and damaged cells release HMGB1 into the extracellular space, where HMGB1 functions as a pro-inflammatory mediator and contributes to the pathogenesis of inflammatory diseases.\(^6\)

Therefore the aim of this study was to investigate the mRNA expression levels of IL-1β, IL-17A, NLRP3 and HMGB1 during experimental oxazolone-induced colitis in rats.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

Eight-month-old male Wistar rats weighing 120–150 g at the beginning of the experiment were obtained from the Institute of Molecular Biology and Genetics (National Academy of Science of Ukraine, Kyiv) and then housed individually in a temperature controlled room (22±1°C) with a 12 hour light-dark cycle beginning at 8:00 am and controlled humidity (60–80%). Food and water were freely available. All animal experiments were performed according to international principles of "the European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 18.03.1986) and "General ethical principles of animal research" (Ukraine, 2001). Animals were fasted overnight and sacrificed by cervical dislocation after receiving an overdose of ether for the isolation of gut tissue. For macroscopic observation, the colon was dissected from rats. The intestine was opened longitudinally, washed with saline solution to remove fecal material, and laid out on a board with the mucosa uppermost for macroscopic scoring.

**Fixation**

After removal of the colon, the tissue was flushed with cold phosphate buffered saline and segments were placed into Bouin’s solution for 4 to 5 hours. After fixation, the samples were dehydrated, incubated in xylene, embedded in paraffin, and sectioned using standard histological protocols. The ages of the fixed tissues analyzed ranged from 10 to 12 months. The Bouin’s solution used during that period was traditionally prepared in the laboratory, rather than using a commercially manufactured one. RNA was extracted from 15 μm thick sections of Bouin’s fixed tissues, as reported previously.\(^7\)

**Oxazolone -induced Colitis**

Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one) was obtained from Sigma-Aldrich (St. Louis, MO). In order to presensitize rats, a 2 x 2 cm field of the abdominal skin was shaved, and 200 μl of a 3% (w/v) solution of oxazolone in 100% ethanol was applied. Seven days after pre-sensitization, rats were re-challenged intrarectally with a dose of 1.5 mg/kg body weight of 0.1% oxazolone in 50% ethanol under general anesthesia with ketamine, ~8 cm proximal to the anal verge of rats using a catheter (1 mm diameter) (Sherwood, St. Louis, MO).\(^8\) Rats were kept in a head-down position for 30 s and then returned to their cages. Ethanol (40%) was used in order to help hapitens go through the intestinal epithelial barrier.\(^9\)

**Animal Groups**

Forty rats were randomly allocated into the following groups: group 1: control (rectal challenge with 50% ethanol only and killed on days 6) (n=20); group 2: rats with oxazolone-induced colitis (killed on
days 6) (n=20).

**Macroscopic and Histological Scores**

The distal colon was removed, opened longitudinally and the mucosal damage was scored on a 0-10 scale according to the criteria of Bobin-Dubigeon et al. Histological examination was performed using a sample of colon tissue located precisely 3 cm above the anal canal in rats from all groups. The colon tissue samples were fixed in Bouin’s solution and embedded in paraffin for histological analysis. Five micrometer sections were deparaffinized with xylene and stained with hematoxylin and eosin using routine techniques. Tissues were scored semi-quantitatively from 0 to 5 (grade 0 indicates no changes; grade 1 indicates minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2 indicates mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3 indicates mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4 indicates marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5 indicates marked transmural inflammation with severe ulceration and loss of intestinal glands) in a blinded fashion according to previously described criteria.

**Deparaffinization**

For this study, paraffin-embedded tissue blocks were cut with a disposable microtome blade into 15μm sections and placed in Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Tissues were deparaffinized by incubation in two consecutive baths of xylenes for 5 minutes each, then in two consecutive baths of 100% ethanol for 5 minutes each. After deparaffinization and centrifugation, the pellets were air-dried.

**Analysis of mRNA by Real-Time RT-PCR**

Total RNA was extracted from ileal tissue by Trizol RNA Prep 100 (Isogen, Russia), according to the manufacturer’s instructions. RNA was re-suspended in RNase free water, quantified and subjected to RT-PCR reaction using RT-PCR kit; RT (Syntol, Russia), RT-PCR was performed on the final volume of 25 μl containing 10 μl ready 2.5X reaction mixture, 11 μl of ddH₂O, 1 μl of Random-6 primers, 1 μl of reverse transcriptase and 2 μg of RNA. Reverse transcription was performed at 45°C for 45 minutes, followed by heating for 5 min at 92°C. For real-time RT–PCR with gene-specific primers, we used an CFX96™ Real-Time PCR Detection Systems (Bio-Rad Laboratories, Inc., USA) according to the manufacturer’s recommendations, with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) for detection. The Master Mix includes Maxima Hot Start Taq DNA Polymerase and dNTPs in an optimized PCR buffer. Samples were amplified in a volume of 25 μl reaction mix, with a concentration of 0.3 μM of forward and reverse primers, 12.5 μl of Maxima SYBR Green/ROX qPCR Master Mix (2X), Template DNA ≤500 ng/reaction, nuclease-free water to 25 μl. All primers were designed by using Primer-BLAST design software (NIH, USA) and were synthesized by Metabion (Germany). The primers that we used are listed in Table 1. All PCR was performed using the following parameters. After initial denaturation for 10 min at 95°C, 45 to 50 cycles of sequential steps denaturation was performed at 95°C for 15 s, annealing at 55 to 64°C for 1 min, extension at 72°C for 30 s, followed by a final incubation at 72°C for 7 minutes. Each sample was tested in triplicate, and results were normalized using amplification of the same cDNAs with mouse reference genes GAPDH using ΔΔCt calculations. Real-time PCR analysis of IL-1β, IL-17A, NLRP3 and HMGB1 were expressed as the relative normalized expression of the indicated mRNA.

**Statistical Analysis**

Results were statistically treated with Student’s t-test using STATISTICA 6.0 (StatSoft Inc. 2001, USA) and presented as mean±SEM. p<0.05 was considered significant.

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Table 1. The design of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Temperature melting (°C)</th>
<th>Product length (bp)</th>
<th>Exon junction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>F: 5´-TCTTTGAAGAAGCGGCCCAGCC-3´</td>
<td>60.0</td>
<td>48</td>
<td>354/355</td>
</tr>
<tr>
<td></td>
<td>R: 5´-GGTCCTGCGATCCACACCAG-3´</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17A</td>
<td>F: 5´-CTGGACCTCTGAGGCCGAA-3´</td>
<td>61.1</td>
<td>58</td>
<td>297/298</td>
</tr>
<tr>
<td></td>
<td>R: 5´-TGCTCCCAGACTACAGAAG-3´</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NLRP3</td>
<td>F: 5´-AGCTAAGAGGACAGGAG-3´</td>
<td>59.1</td>
<td>40</td>
<td>713/714</td>
</tr>
<tr>
<td></td>
<td>R: 5´-CGTGCATGACATTTCCACT-3´</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGB1</td>
<td>F: 5´-CGGATGCTTCGTCACCTT-3´</td>
<td>62.0</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5´-CACCTCCTGACAGCTTCTT-3´</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5´-GCCTGGAGAAACCTGCAAG-3´</td>
<td>61.3</td>
<td>52</td>
<td>825/826</td>
</tr>
<tr>
<td></td>
<td>R: 5´-GCCTTACCACCTTTCT-3´</td>
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</tr>
</tbody>
</table>

IL-1β, interleukin-1β; IL-17A, interleukin-17A; NLRP3, NOD-like receptor family, pyrin domain containing 3; HMGB1, High Mobility Group Box 1 protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 1. Relative IL-1β (A), IL-17A (B), NLRP3 (C) and HMGB1 (D) mRNA expression within the colon of rats with oxazolone-induced colitis compared to uninflamed control rats. Target transcription factor were normalized to GAPDH. Data expressed as means ± SEM, * p<0.05, vs. control.
RESULTS

Assessment of Oxazolone-induced Colitis

Animals treated with oxazolone rapidly developed colitis marked by weight loss and diarrhea peaking at the second day following oxazolone administration and resulted in about 40% rat death on day 4. Microscopic inspection of the tissues revealed abscessions of the mucous membrane epithelial cells, erosion of the mucous membrane and ulcer formation. Control rats treated with 50% ethanol alone did not develop wasting disease and exhibited a healthy appearance. Table 2 shows the severity of colitis assessed macroscopically and microscopically on days 6.

The mRNA Expression Levels of IL-1β, IL-17A, NLRP3 and HMGB1 in Colon Samples of Rats with Oxazolone-induced Colitis and Controls

Firstly, we determined that the expression levels of the proinflammatory cytokine IL-1β and IL-17A were increased in colon samples of rats with oxazolone-induced colitis. IL-1β expression was increased 3.5-fold (p<0.05) in inflamed colon compared to uninfamed control tissue while IL-17A expression was increased 8-fold (p<0.05) (Figures 1A and 1B). In addition, we examined NLRP3 expression in these tissues. RT-PCR analysis demonstrated a 71-fold (p<0.05) increased in mRNA expression of NLRP3 in colon tissues of studied samples (Figure 1C). Finally, we found no significant changes in the expression of HMGB1 in inflamed colon tissues (Figure 1D).

DISCUSSION

This study indicated that the expression levels of IL-1β, IL-17A, NLRP3 and HMGB1 mRNA expression levels were elevated in the inflamed colon of rats with oxazolone-induced colitis.

Although it is generally accepted that IBD is an idiopathic, chronic relapsing, inflammatory condition mediating by immunological responses, its etiology has not been clear yet. At present, even the mechanisms controlling the development of ileitis in transgenic or gene-targeted mouse models are unclear.

Activation of lymphocytes, monocytes, macrophages, enterocytes and endothelial cells occur as a consequence of inflammation, and proinflammatory cytokines such as IL1β are increased in mucosal tissue of Crohn's patients. Bersudsky M. et al. studied the differential role of the IL-1 agonists, IL-1α, which is mainly cell-associated versus IL-1β, which is mostly secreted, in colon inflammation, demonstrated that IL-1β is involved in repair of IECs and reconstitution of the epithelial barrier during the resolution of colitis. In the current study, the expression of IL1β was increased ~4-fold in inflamed ileal tissue compared to normal tissue, suggesting that alterations in IL1β plays a role in the pathophysiology of Crohn's disease.

The cytokines IL-1β and IL-18 appear early in intestinal inflammation and their pro-forms are processed via the caspase-1-activating multiprotein complex, the NLRP3 inflammasome. However, the role of inflammasomes in acute intestinal inflammation remains controversial, and the involvement of inflammasomes and inflammasome-mediated IL-1β in chronic colitis is poorly understood.

Because of its potential in inducing proinflammatory response and to cause damage to the host, IL-1β production is tightly controlled at multiple levels. Production of mature IL-1β requires at least two signals: the first signal is initiated by TLR ligands or endogenous molecules that induce pro-IL-1β gene expression; the second signal includes very diverse stimuli that activate inflammasome, leading to IL-1β maturation. Inflammasome is a multimolecular complex, composed of a NLR protein, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1, which controls processing of proinflammatory cytokine IL-1β and IL-18. While several studies show that mice deficient for inflammasome components including NLRP3, ASC, and caspase 1 are highly susceptible to

Table 2. Clinical parameters of oxazolone-induced colitis in rats (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Macroscopy score</th>
<th>Histology score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>130.3 ± 14.2</td>
<td>1.00±0.71</td>
<td>1.00±0.71</td>
</tr>
<tr>
<td>Oxazolone-induced colitis</td>
<td>20</td>
<td>105.0 ± 10.9*</td>
<td>6.60±0.55*</td>
<td>14.00±1.22*</td>
</tr>
</tbody>
</table>

*p<0.05, vs. control group
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acute colitis induced by dextran sodium sulfate (DSS), one study demonstrates the opposite results showing that defective in NLRP3 inflammasome protect mice from DSS-induced acute colitis. Several studies reported that mice deficient for inflammasome components including NLRP3 exhibit enhanced DSS-induced colitis compared with WT mice.

The apparent contradiction about the role of inflammasomes in mucosal immunity may be heavily influenced by gut microflora, though further studies are needed to resolve this issue. Since IBD is a heterogeneous disease, it is possible that in some IBD patients, genetic mutations or environmental factors may cause reduced inflammasome activation and IL-1β production, which compromise the integrity of the epithelial cell barrier against microflora, leading to intestinal inflammation. While in other IBD patients, genetic factors and commensal microflora may cause prolonged or unregulated inflammasome activation and increased IL-1β production, generating uncontrolled inflammation in the intestine system.

Therefore, inflammasomes are assumed to mediate host defense against microbial pathogens and gut homeostasis, so that their disregulation might contribute to IBD pathogenesis. Improving knowledge of the inflammasome could provide insights into potential therapeutic targets for patients with IBD.

Th17 cells develop from naïve T lymphocytes through distinct pathways from classical Th1 and Th2 cells. Th17 cells secrete IL-17, which promotes the recruitment of inflammatory cells into the intestinal mucosa via its ability to enhance the synthesis of chemoattractants and adhesion molecules on epithelial, endothelial and mesenchymal cells. A growing evidences in human and mouse model studies have shown that Th17 effector cytokines promote chronic intestinal inflammation through the induction of multiple proinflammatory mediators. Our evaluation mRNA expression of IL-17 revealed significant differences in expression levels between the ileum of rats with ileitis compared to uninflamed control rats. These agree with previous observations, and suggest the involvement of IL-17 in the pathophysiology of IBD.

Weidlich S. et al. showed that mRNA expression of IL-8, IL-17, and IL-10 were upregulated in patients with CD and UC. Sugihara T. et al. showed the increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease.

In inflammatory conditions, HMGB1 is actively secreted from immune cells in the extracellular matrix, where it behaves as a proinflammatory cytokine. Vitali R. et al. showed for the first time in their study that HMGB1 is secreted by human inflamed intestinal tissues and abundantly found in the stools of IBD patients, and it can be considered as a novel marker for intestinal inflammation. Ample evidence indicates that HMGB1 is an important mediator in the pathogenesis of colitis and colitis associated carcinoma. Increased circulating levels of HMGB1 have been observed in animal models of IBD and are positively associated with disease severity. HMGB1 is secreted by inflamed human intestinal tissues, and abundantly found in stools of IBD patients. It is established that high levels of HMGB1 increase epithelial cell permeability and impair intestinal barrier function. All together, these studies suggest that HMGB1 is an important target in IBD, and sequestration and or neutralization of HMGB1 would be valuable for controlling the disease severity in ileitis.

We propose that the identification of abnormal mRNA expression of IL-1β, IL-17A, NLRP3 may be of relevance in the elucidation of the pathogenesis of Crohn's and may also facilitate the definition of future potential targets for therapeutic interventions.

REFERENCES

5. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato