Leflunomide attenuates hepatocyte injury by inhibiting Kupffer cells

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INTRODUCTION

Tissue inflammation plays a critical role in liver pathology via induction of cellular injury. In fact, infiltration of mononuclear phagocytes into the liver correlates with the severity of liver injury. Moreover, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) have been shown to be involved in the promotion of liver injury[4,5]. Kupffer cells (KCs) are among the first cells that respond to endotoxins, including lipopolysaccharides (LPS), and are considered to be the primary macrophages involved in the clearance of gut-derived bacteria or bacterial toxins. High portal levels of LPS could lead to a pronounced secretion of proinflammatory mediators by KCs and ultimately to endotoxin-induced liver injury[6,7]. KCs are located in hepatic sinusoids and lie in between or on top of endothelial cells. However, they do have direct cell contacts with parenchymatous hepatocytes (HCs) through their cytoplasmic extensions. Cellular communication between KCs and HCs has been thought to occur mainly by production of cytokines and excretion of proinflammatory mediators such as eicosanoids, nitric oxide (NO), and/or reactive oxygen species (ROS)[8,9]. Proinflammatory cytokines, TNF-α and IL-1, in particular, have been shown to be early and important mediators of HC injury[8,10].

Leflunomide, an isoxazole derivative and a unique immunomodulatory agent, is capable of treating rheumatoid arthritis, allograft and xenograft rejection, systemic lupus erythematosus, Crohn’s disease, and prostate cancer[10-22]. Leflunomide is a produg that can be rapidly converted in the cell to an active metabolite, 771726. Our investigations demonstrated that leflunomide had therapeutic actions on acute and chronic liver diseases[20-22]. In order to study the mechanisms of leflunomide on liver injury, the interaction between KCs and HCs in vitro, and the effect of leflunomide’s active metabolite, 771726, on TNF-α and IL-1 were investigated.

MATERIALS AND METHODS

Animals and reagents

Male Sprague-Dawley rats weighing 200-250 g were purchased from Animal Center of Anhui Medical University. Rats were allowed to take food and tap water ad libitum. Powdered 1640 medium was obtained from GIBCO Co., USA. Leflunomide and its active metabolite, 771726, were kindly donated by Cinkate Co., USA. Leflunomide and its active metabolite, 771726, were kindly donated by Cinkate Co., USA.

Preparation of inflammatory liver injury

Each rat was injected with 15 mg BCG in 0.2 mL saline via tail vein, and 14 d later with 10 µg LPS in 0.2 mL saline. At 16 h post-injection of LPS, rats were anesthetized and KCs were isolated.

Preparation of HCs

The procedure for isolation of rat HCs was based on Seglen’s method[24], with some modifications. The obtained cell suspension was diluted in modified Hanks’ buffered salt solution.
Experimental design

After a recovery period of 24 h at 37 °C and 50 mL/L CO₂, the medium was replaced by a medium containing 0, 1, 5, or 10 µg/mL LPS. Because HCs are known to produce various important serum compounds such as LPS-binding protein, medium of KC cultures still contained 50 mL/L FBS. After 2, 4, 8, and 24 h of incubation, tissue culture supernatants were collected for analysis of cytokines. To study the effect of A_{771726} on excretion of TNF-α and IL-1 in DC coculture supernatants, A_{771726} was dissolved with RPMI 1640 medium and added to DC cocultures from 0 h to 4 h (0-4 h), to 24 h (0-24 h) and from 4 h to 24 h (4-24 h) after incubation with LPS, respectively.

Measurement of TNF-α and IL-1

TNF-α and IL-1 concentrations in cell culture supernatants were measured using commercial ELISA kits with recombinant rat TNF-α and IL-1 as standard. Measurements were performed in duplicate.

Semi-quantitative RT-PCR assay for TNF-α mRNA in KCs of inflammatory liver tissue

After incubation with or without A_{771726} (0.001-10 µmol/L), KCs of inflammatory liver tissue were harvested and kept at -70 °C until RNA extraction. Total cellular RNA was extracted using RNA easy kits (Invitrogen, USA). To test the efficacy of reverse transcriptase, RT-PCR was performed for GPDH mRNA. Briefly, the first strand of cDNA was synthesized by reverse transcriptase and pooled. The resulting cDNA samples were adjusted to PCR buffer conditions and run for PCR simultaneously. The primers for TNF-α were 5’-CGAGTGACAAGCCCGTAGCC and 5’-GAGTAACAGCCGCAATG and the primers for GPDH were 5’-CCACCCTAGCCTATCCGGCA and 5’-TCTAGACGGCAGTCTGGCC. The amplification of TNF-α and GPDH genes was expected to generate 753 bp and 600 bp fragments, respectively. Amplification was performed for 35 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, and extension at 72 °C for 2 min. Ten µL of reaction mixture was loaded to 10 g/L agarose gel containing 0.5 µg/mL ethidium bromide for electrophoresis, the gel was then placed under ultraviolet ray for semi-quantitation detection.

Statistics

Unless stated otherwise, data were expressed as mean±SD and evaluated using two-way ANOVA followed by Student’s t test for comparison between 2 groups. P<0.05 was considered statistically significant.

RESULTS

Proinflammatory cytokine production after stimulation with LPS

The production of TNF-α and IL-1 was measured after incubation of HC and KC cultures, and DC cocultures in medium with or without LPS. A time course study showed that maximal cytokine levels in KC cultures and DC cocultures were observed after 4 h incubation for TNF-α and 24 h incubation for IL-1 respectively (Figure 1). Moreover, TNF-α and IL-1 levels in culture supernatants were positive correlated with the concentration of LPS (Figure 2). TNF-α and IL-1 levels in culture supernatants were significantly higher than those in KC or HC cultures (Figure 1). However, two cytokines levels in HC cultures showed no statistically significant changes throughout the experiments (Figures 1-2).

Effect of A_{771726} on TNF-α and IL-1 production in DC cocultures after stimulation with LPS

Based on the results as above, we selected DC cocultures stimulated with 10 µg/mL LPS as targets, and A_{771726} was added to DC cocultures with different time course. After 4 h incubation (0-4 h), TNF-α and IL-1 production in DC cocultures stimulated with LPS (10 µg/mL) was significantly inhibited in A_{771726} (0.1, 10 µmol/L) and dexamethasone (2×10^(-5) µmol/L) group (Figure 3 A). Likewise, TNF-α and IL-1 production in DC cocultures was also significantly inhibited after 24 h incubation (0-24 h) in A_{771726} (10 µmol/L) group (Figure 3B). Because the concentration of TNF-α reached its maximal level after 4 h incubation, we designed a protocol that A_{771726} was added to DC cocultures from 4 h to 24 h after incubation (4-24 h). The results showed
that the concentration of TNF-α and IL-1 in DC cocultures supernatants was not inhibited in A771726 (10 µmol/L, 0-24 h) group (Figure 3B). Furthermore, TNF-α and IL-1 levels in A771726 (10 µmol/L, 0-24 h) group were significantly lower than those in A771726 (10 µmol/L, 4-24 h) group.

**Effect of A771726 on TNF-α mRNA in KCs of inflammatory liver tissue**

A771726 had significantly inhibitory actions on the production of TNF-α in supernatants of DC cocultures. To gain insights into the potentially inhibitory action of A771726 on TNF-α via transcriptional level, TNF-α mRNA was analyzed by RT-PCR in KCs during liver inflammation induced by injection with BCG and LPS. Gel electrophoresis and semiquantitation analysis showed that A771726 significantly reduced the expression of TNF-α mRNA in KCs of inflammatory liver tissue induced by BCG and LPS (Figure 4, Table 1).

**Figure 1** Changes of TNF-α and IL-1 levels in different type culture supernatants with LPS (10 µg/mL) (n=3, mean±SD). DC, solid line; KC, dot line; HC, dashed line. P <0.05, P <0.01 for IL-1 levels in DC, KC, and HC vs. control, P <0.01 vs KC and HC. A: Changes of TNF-α levels in different type culture supernatants with LPS. B: Changes of IL-1 levels in different type culture supernatants with LPS.

**Figure 2** Changes of TNF-α and IL-1 levels in culture supernatants of HC, DC, KC after 4 h and 24 h incubation with various concentrations of LPS (n=3, mean±SD). P <0.01 for TNF-α and IL-1 levels in DC, KC vs control; P <0.01 for DC coculture vs other cultures after 0, 1, 5, and 10 µg/mL LPS. A: Changes of TNF-α levels in culture supernatants of HC, DC, KC after 4 h incubation with various concentrations of LPS. B: Changes of IL-1 levels in culture supernatants of HC, DC, KC after 24 h incubation with various concentrations of LPS.

**Figure 3** Effect of A771726 on TNF-α and IL-1 levels in culture supernatants of DC stimulated by LPS (10 µg) with different administration time (n=3, mean±SD). P <0.05, P <0.01 vs model; P <0.01, vs 0-24 h group.

**Figure 4** Effect of A771726 on TNF-α mRNA of KCs in immunological liver injury rats. 1: DNA marker; 3: model; 2: Dexamethasone; 4-8: A771726 at concentration of 1x10⁻³, 1x10⁻², 1x10⁻¹, 1x10⁻⁰, 1x10⁰ µmol/L.

| Group                | Dose (µmol/L) | TNF-α (ng/mL) |
|----------------------|---------------|---------------|
| Model                | —             | 2.669±0.252   |
| A771726              | 1x10⁻³        | 2.507±0.051   |
|                      | 1x10⁻²        | 2.213±0.044   |
|                      | 1x10⁻¹        | 1.955±0.080   |
|                      | 1x10⁰         | 1.432±0.067   |
|                      | 1x10¹         | 0.987±0.048   |
| Dexamethasone        | 2x10⁰         | 1.021±0.110   |

P <0.05, P <0.01, vs Model.
DISCUSSION

In the intact liver, HC s are in direct contact with KC s. The present experiments were designed to assess whether direct contact between HC s and KC s was of influence on the LPS-induced inflammatory response. When compared the production of TNF-α and IL-1 in the different culture types, direct cell-to-cell contact between KC s and HC s seemed to be essential, because an 4- or 7-fold increase of TNF-α and IL-1, could be observed in DC cocultures. Although the cytokine levels in KC cultures were markedly lower than those observed in DC cocultures, incubation of KC cultures with LPS still resulted in an abundant cytokine response. LPS-induced cytokine expression in DC cocultures or KC cultures has been extensively studied, but in this study we showed that direct contact between KC s and HC s significantly increased TNF-α and IL-1 production by rat KC s. However, the levels of two cytokines in HC cultures showed no statistically significant changes throughout the experiments. The results indicated proinflammatory mediators such as TNF-α and IL-1 in culture supernatants were mainly produced by KC s, especially 0-4 h incubation, and reduced production of IL-1 by A0-24 h. However, TNF-α stimulated by inflammatory stimuli. We found that TNF-α is a key mediator of inflammation and the activation of nuclear factor kappa B, a potent mediator of inflammation when stimulated by inflammatory stimuli. We found that TNF-α and IL-1 levels in supernatants of DC cocultures were apparently inhibited by A71726 (0.1, 10 µmol/L, 0-4 h) and A71726 (10 µmol/L, 0-24 h). However, TNF-α and IL-1 levels in supernatants of DC cocultures were not affected in A71726 (10 µmol/L, 4-24 h) group, and the levels of the two cytokines in A71726 (10 µmol/L, 4-24 h) group were significantly lower than those in A71726 (10 µmol/L, 4-24 h) group. The results indicated that the inhibitory action of A71726 on TNF-α and IL-1 levels was generated mainly from 0-4 h incubation, and reduced production of IL-1 by A71726 was associated with the inhibitory action of A71726 on TNF-α. Furthermore, RT-PCR analysis showed that A71726 significantly reduced the expression of TNF-α mRNA in KC s of inflammatory liver tissue induced by BCG+LPS. The results showed A71726 had significantly inhibitory effects on production of TNF-α in KC s at transcriptional level.

Although further work is required to demonstrate whether A71726 has other targets in inflammatory liver injury, A71726 can inhibit hepatocyte damage by inhibiting proinflammatory cytokine release from KC s.

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