Role of NF-kB in multiple organ dysfunction during acute obstructive cholangitis

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MATERIALS AND METHODS

AIM: To elucidate the role of NF-kB activation in the development of multiple organ dysfunction (MOD) during acute obstructive cholangitis (AOC) in rats.

METHODS: Forty-two Wistar rats were divided into three groups: the AOC group, the group of bile duct ligation (BDL group), and the sham operation group (SO group). All the animals in the three groups were killed in the 6th and 48th hour after operation. Morphological changes of vital organs were observed under light and electron microscopy. NF-kB activation was determined with Electrophoretic Mobility Shift Assay (EMSA). Arterial blood gas analyses and the serum levels of LDH, ALT, BUN and creatinine were performed. The concentrations of TNF-α and IL-6 in plasma were also measured.

RESULTS: The significant changes of histology and ultrastructure of vital organs were observed in AOC group. By contrast, in BDL group, all the features of organs damage were greatly reduced. Expression of NF-kB activation in various tissues increased in AOC group when compared to other two groups. At 6 h, the arterial pH in three groups was 7.52±0.01, 7.46±0.02, and 7.45±0.02, and the blood pCO₂ was 33.9±0.95 mmHg, 38.1±0.89 mmHg, 38.9±0.94 mmHg; there was difference in three groups (P<0.05). At 48 h, the blood pH values in three groups was 7.33±0.07, 7.67±0.04, and 7.46±0.03, and blood HCO₃⁻ was 20.1±1.29 mmol·L⁻¹, 26.7±1.45 mmol·L⁻¹ and 27.4±0.35 mmol·L⁻¹; there was also difference in three groups (P<0.05). In AOC group, Levels of LDH, ALT, BUN and creatinine were 16359.9±2278.8 nkat·L⁻¹, 5796.2±941.9 nkat·L⁻¹, 55.7±15.3 mg/dl, and 0.72±0.06 mg/dl, which were higher than in SO group (3739.1±570.1 nkat·L⁻¹, 288.4±71.7 nkat·L⁻¹, 12.5±2.14 mg/dl, and 0.47±0.03 mg/dl) (P<0.05). Levels of plasma TNF-α and IL-6 in AOC at 48 h were 429±56.62 ng·L⁻¹ and 562±57 ng·L⁻¹, which increased greatly when compared to BDL group (139±16 ng·L⁻¹, 227±43 ng·L⁻¹) and SO group (74±10 ng·L⁻¹, 113±19 ng·L⁻¹) (P<0.05).

CONCLUSION: The pathological damages and the NF-kB activation of many vital organs exised during AOC. These findings have an important implication for the role of NF-kB activation in MOD during AOC.

INTRODUCTION

In humans with acute obstructive cholangitis (AOC) or other sepsis, the onset of multiple organ dysfunction (MOD), especially involving the liver, the heart, the lungs, and the kidneys, is a well known complication that is associated with a high mortality rate[1-10]. MOD in either humans or animals appears to emerge as a consequence of progressive development of activation of mononuclear phagocytes system and an unregulated release into the blood of a variety of proinflammatory mediators (interleukins, cytokines, chemokines)[11-14]. NF-kB is highly activated at sites of inflammation in diverse diseases and can induce transcription of proinflammatory cytokines[15-17]. For example, NF-kB is overexpressed in neutrophi, peripheral blood mononuclear cells (PBMC), dendritic cells (DC), and Kupffer cell, and so on. Its activity may enhance recruitment of inflammatory cells and production of proinflammatory mediators like IL-1, IL-6, IL-8, and TNF-α[18-21]. These events may be associated with the development of MOD.

In our original study of patients with AOC, the changes of NF-kB activation were documented[11], but evidence of MOD and the effects of NF-kB were not assessed. In the current studies, we observed the pathological changes of the damages of many vital organs and correlated the role of NF-kB activation with MOD in animal model with AOC.

MATERIALS AND METHODS

Reagents

E. coli and type IV of collagenase were obtained from Sigma Chemical Company (St. Louis, Mo.). The kits of TNF-α and IL-6 were obtained from Beijing Bang Bing Co.. Other reagents were purchased from Ke Hua Co. or Zhong Sheng Co.

Animals and experimental groups

Forty-two male Wistar rats, weighing 250-300 g, were obtained from the Laboratory Animal Center of Chongqing University of Medical Science. The animals were fed with standard rat food and water ad libitum for 1 wk before use and kept in a climate-controlled environment with a 12 h light-dark cycle. The animals were handled in accordance with the guidelines set by the Experimental Animals Society of Chongqing University of Medical Science. These animals were randomized into three groups: the AOC group, the group of bile duct ligation (BDL), and sham operation (SO). All the animals in these groups were killed in the 6th and 48th hour after operation. Seven rats from each group at each time point were examined.
Preparation of animal models
All rats were starved overnight and underwent an upper midline laparotomy with an intraperitoneal injection of 30 mg·kg⁻¹ sodium pentobarbital for anesthesia. In AOC group, a median incision was made on the upper abdomen. The common bile duct was mobilized and doubly ligated. 0.2 ml of the E. coli suspension (5×10⁵cfu·L⁻¹) was injected into the ligated common bile duct. In BDL group, the common bile duct was doubly ligated but no E. coli suspension was injected. In SO group, neither E. coli suspension injection nor common bile duct ligation was done, but only routine operative procedure was performed.

Histology
Morphological changes of liver, heart, lungs, and kidneys were observed by light microscopy and transmission electron microscope (TEM). These samples from different organs were fixed with 10 % buffered formalin or 2.5 % glutaraldehyde immediately. For optical microscopy, the tissue blocks were embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E). For TEM, the tissue blocks were embedded in Epon 618 resin and ultrathin sections were stained with uranyl acetate and lead citrate. A transmission electron microscope (JEM-2000) was used.

Electrophoretic mobility shift assay for NF-kB
Isolation of nuclear proteins
Nuclear proteins were isolated as previously described [2,12]. In brief, the liver, heart, lungs, and kidneys tissues were placed in 0.8 mL of ice-cold hypotonic buffer [10 mmol·L⁻¹ HEPES (pH7.9), 10 mL KCl, 0.1 mmol·L⁻¹ EDTA, 0.1 mmol·L⁻¹ ethylene glycol tetraacetic acid, 1 mmol·L⁻¹ DTT; Protease inhibitors (aprotinin, pepstatin, and leupeptin, 10 mg·L⁻¹ each)]. The homogenates were incubated on ice for 20 min, vortexed for 20 s after adding 50 µL of 10 per cent Nonidet P-40, and then centrifuged for 1 minute at 4 °C in an Eppendorf centrifuge. Supernatants were decanted, the nuclear pellets after a single wash with hypotonic buffer without Nonidet P-40 and then centrifuged for 1 minute at 4 °C in an Eppendorf centrifuge. Supernatants were collected as nuclear extracts and stored at -70 °C. Concentrations of total proteins in the samples were determined according to the method of Bradford.

Electrophoretic mobility shift assay (EMSA)
NF-kB binding activity was performed in a 10-uL binding reaction mixture containing 1 binding buffer [50 mmol·L⁻¹ Tris·HCl (pH 7.5), 50 mmol·L⁻¹ NaCl, 0.5 mmol·L⁻¹ EDTA, 0.5 mmol·L⁻¹ DTT; Protease inhibitors], incubated on ice for 30 min at 4 °C, mixed frequently, and centrifuged for 15 min at 4 °C. The supernatants were collected as nuclear extracts and stored at -70 °C. Concentrations of total proteins in the samples were determined according to the method of Bradford.

Arterial blood gas analyses
In AOC group, arterial blood gases showed early respiratory alkalosis and late metabolic acidosis. At 6 h there was a mild respiratory alkalosis, with the arterial pH rising from 7.45±0.02 to 7.52±0.01, the blood pCO₂ fell from a value of 37.9±0.86 to 34.23±0.89. At 48 h, similar group showed evidence of respiratory alkalosis, with the arterial pH rising from 7.46±0.03 to 7.33±0.007, and blood HCO₃⁻ (mmol·L⁻¹) falling from 27.4±0.35 to 20.1±1.29. In contrast, in BDL group, the blood pH and pCO₂ values were the same as those values obtained in SO group (Table 1).

Biochemical parameters
Biochemical parameters in three groups were showed in Table 2.

Cytokines contents in plasma
In AOC group, Levels of plasma TNF-α and IL-6 increased greatly in 6 h and 48 h. Levels of two cytokines were over 4-fold and 5-fold higher than that in other two groups. In BDL group, the levels of TNF-α and IL-6 in 48 h increased modestly when compared to that in SO group (P<0.05). Low levels of TNF-α and IL-6 was detectable in plasma in SO group (Table 3).
Table 1  Arterial blood gas analyses in three groups animals

| Parameter | Early Phase (6 h) | Late Phase (48 h) |
|-----------|-------------------|-------------------|
|           | AOC | BDL | SO | AOC | BDL | SO |
| pH | 7.52±0.01a | 7.46±0.02 | 7.45±0.02 | 7.33±0.07a | 7.67±0.04 | 7.46±0.03 |
| pCO₂ (mmHg) | 33.9±0.95a | 38.1±0.89 | 38.9±0.94 | 40.4±4.23 | 37.8±3.51 | 37.5±0.72 |
| HCO₃⁻ (mmol/L) | 26.6±0.63 | 28.3±0.86 | 27.8±1.04 | 20.1±1.57a | 26.7±1.45 | 27.82±1.25 |
| pO₂ (mmHg) | 105.1±6.4 | 108.3±4.2 | 106.5±5.8 | 67.3±6.9a | 104.7±5.3 | 110.7±4.6 |

*P <0.05, vs other groups.

Table 2  Biochemical parameters in three groups animals

| Parameter | Early Phase (6 h) | Late Phase (48 h) |
|-----------|-------------------|-------------------|
|           | AOC | BDL | SO | AOC | BDL | SO |
| LDH (nkat·L⁻¹) | 13632.7±891.8a | 4107.4±951.9 | 3655.7±576.8 | 16359.9±2278.8a | 6793±885.1c | 3742.4±570.1 |
| ALT (nkat·L⁻¹) | 2213.8±391.7a | 343.4±103.3 | 311.7±91.7 | 5796.2±941.9a | 955.2±175c | 288.4±75 |
| BUN (mg/dl) | 16.3±13.3 | 12.9±2.8 | 11.8±2.2 | 55.7±15.3a | 16.7±4.6 | 12.5±2.14 |
| Cr (mg/dl) | 0.48±0.03 | 0.42±0.02 | 0.41±0.03 | 0.72±0.06a | 0.51±0.03 | 0.47±0.03 |

*P <0.05, vs other groups.  *P <0.05, vs SO group.

**Figure 1** Fatty degeneration, focal cytoplasmic degeneration and myelin figures in the cytoplasm of hepatocyte in AOC group TEM×12 000.

**Figure 2** Kupffer cells showed proliferation and focal cytoplasmic degeneration and many myelin figures could be seen in the cytoplasm in AOC group TEM×15 000.

**Figure 3** Myocyte mitochondria of heart were swollen or even vacuolated in AOC group TEM×20 000.

**Figure 4** Proximal convoluted tubules was full of electron dense phagosomes, the cells had lost their cell membranes and exhibited mitochondrial swelling together with intracellular edema In AOC group TEM×3 000.
but evidence of MOD and the effects of NF-κB were not assessed. So it would be helpful to further analyze the role of NF-κB in AOC, the changes of NF-κB activation were documented, and published information about a key role of NF-κB in sepsis and shock associated with MOD is limited but shows that NF-κB is increased during inflammation and sepsis[1-5].

The role of NF-κB activation in sepsis, AOC and MOD is linked to the outcome in sepsis. In our original study of patients with severe sepsis, Pennington et al determined NF-κB activation in mononuclear and neutrophils of 34 patients with severe sepsis and serial concentrations of inflammatory cytokines and mediators are therefore a potentially attractive target inducing a rapid normalization of pathological damages of many vital organs, NF-κB activation adds further validity to this assay.

The present study shows a correlation among the pathological damages of many vital organs, NF-κB activation and release of proinflammatory cytokines. This is only one published investigation about the correlation of NF-κB activation and MOD during AOC. The present study demonstrated that NF-κB activation plays a key role during AOC. The finding that prompt surgical inflammatory condition of the inflammatory focus results in a rapid normalization of NF-κB activation adds further validity to this assay.

Figure 5 Activation of NF-κB in AOC group. Lane 1, 2, 3, and 4 represents the Liver, the lungs, the heart, and the kidneys in 4 kw, respectively. Lane 5, 6, 7, and 8 represents the Liver, the lungs, the heart, and the kidneys in 8 kw, respectively.

Table 3 TNF-α and IL-6 levels in plasma (n=7)

| Groups | TNF-α (ng L⁻¹) | IL-6 (ng L⁻¹) |
|--------|----------------|--------------|
|        | 6h             | 48h          | 6h             | 48h            |
| AOC    | 235±39.23      | 429±56.62    | 253±23.41      | 562±57.59      |
| BDL    | 73±11.52       | 139±16.21    | 113±21.39      | 227±43.02      |
| SO     | 72±12.45       | 74±10.53     | 109±18.37      | 113±9.81       |

*p<0.05, vs other two groups. **p<0.05, vs SO group.

DISCUSSION

AOC in humans often leads to progressive MOD, which is still associated with a high mortality rate[1-5]. Recent studies showed NF-κB is rapidly in response to many pathologic signals that may be relevant during surgical trauma, including cytokines, adhesion molecules, endotoxin, hypoxia, and shear[23-27]. Activation of NF-κB results in the transcrition of genes that may be relevant during surgical trauma, including cytokines, adhesion molecules[28-32]. These cytokines and proinflammatory mediators are therefore a potentially attractive target inducing MOD during sepsis and endotoxemia[47,48]. Information on the role of NF-κB in sepsis, AOC and MOD is limited but shows that NF-κB is increased during inflammation and sepsis[1-5].

The role of NF-κB in sepsis is a debatable issue. Pennington et al[29] observed the degree of NF-κB activation with severity of acute appendicitis and found that NF-κB binding activity is elevated in these patients and correlates with symptoms longer than 24 hours. Arnalich et al[30] determined NF-κB activation in peripheral blood mononuclear cells of 34 patients with severe sepsis and serial concentrations of inflammatory cytokines and resulted the prognosis value of early measurement of NF-κB activity in patients with severe sepsis. Gong and Paterson et al[1,29] determined NF-κB activity in mononuclear and neutrophils from critically ill patients and compared NF-κB activity with circulating concentrations of IL-6, IL-8 and soluble intercellular adhesion molecule (sICAM)-1, and found NF-κB activity in patients systemic inflammatory response syndrome, which increased makedly before death. However, Chen et al[30] observed intrahepatic changes in TNF-α and NF-κB activation in Sprague-Dawley rats with cecal ligation and puncture, and their results implied NF-κB activation was not linked to the outcome in sepsis. In our original study of patients with AOC, the changes of NF-κB activation were documented, but evidence of MOD and the effects of NF-κB were not assessed. So it would be helpful to further analyze the expression with the inflamed tissues. There is, however, no published information about a key role of NF-κB in development of multiple organ dysfunction during biliary infection. In this study, we investigated the role NF-κB in the development of MOD in rats with AOC.

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