

# Nuclear factor kB activity in patients with acute severe cholangitis

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## Abstract

**AIM:** To determine the NF-kB activity in peripheral blood mononuclear cells (PBMC) in patients with acute cholangitis of severe type (ACST) and correlate the degree of NF-kB activation with severity of biliary tract infection and clinical outcome.

**METHODS:** Twenty patients with ACST were divided into survivor group (13 cases) and nonsurvivor group (7 cases). Other ten patients undergoing elective gastrectomy or inguinal hernia repair were selected as control group. Peripheral blood samples were taken 24 hours postoperatively. PBMC were separated by density gradient centrifugation, then nuclear proteins were isolated from PBMC, and Electrophoretic Mobility Shift Assay (EMSA) used determined. The results were quantified by scanning densitometer of a Bio-Image Analysis System and expressed as relative optical density (ROD). The levels of TNF- $\alpha$ , IL-6, and IL-10 in the plasma of patients with ACST and healthy control subjects were determined by using an enzyme-linked immunoassay (ELISA).

**RESULTS:** The NF-kB activity was  $5.02 \pm 1.03$  in nonsurvivor group,  $2.98 \pm 0.51$  in survivor group and  $1.06 \pm 0.34$  in control group. There were statistical differences in three groups ( $P < 0.05$ ). The levels of TNF- $\alpha$  and IL-6 in plasma were  $(498 \pm 53) \text{ ng} \cdot \text{L}^{-1}$  and  $(587 \pm 64) \text{ ng} \cdot \text{L}^{-1}$  in nonsurvivor group,  $(284 \pm 32) \text{ ng} \cdot \text{L}^{-1}$  and  $(318 \pm 49) \text{ ng} \cdot \text{L}^{-1}$  in survivor group and  $(89 \pm 11) \text{ ng} \cdot \text{L}^{-1}$  and  $(102 \pm 13) \text{ ng} \cdot \text{L}^{-1}$  in control group. All patients with ACST had increased levels of TNF- $\alpha$  and IL-6, which were many-fold greater than those of control group, and there was an evidence of significantly higher levels in those of nonsurvivor group than that in survivor group ( $P < 0.05$ ). The levels of IL-10 in plasma were  $(378 \pm 32) \text{ ng} \cdot \text{L}^{-1}$ ,  $(384 \pm 37) \text{ ng} \cdot \text{L}^{-1}$  and  $(68 \pm 11) \text{ ng} \cdot \text{L}^{-1}$  in three groups, respectively. All patients had also increased levels of IL-10 when compared with control group ( $P < 0.05$ ), but the IL-10 levels were not significantly higher in nonsurvivors than in survivors ( $P > 0.05$ ).

**CONCLUSION:** NF-kB activity in PBMC in patients with ACST

**increases markedly and the degree of NF-kB activation is correlated with severity of biliary tract infection and clinical outcome.**

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## INTRODUCTION

Acute cholangitis of severe type (ACST) is a common problem facing today's surgeons<sup>[1-3]</sup>. Despite a multitude of advances in the area of surgical infection and surgical or nonsurgical interventions to treat biliary tract diseases, ACST and biliary sepsis remain a significant cause of morbidity and mortality<sup>[4-9]</sup>. Many reports have focused on aspects of the proinflammatory cytokines which are believed to be central to the pathophysiology of the sepsis syndrome<sup>[10-11]</sup>. Recent investigations have shown that expression of many cytokines is closely linked to the activation of transcriptional factors<sup>[12-13]</sup>. Among several transcriptional regulatory factors involved in immuno-regulatory genes expression, nuclear factor kappa B (NF-kB) acts as a critical step for directing the transcription of many proinflammatory cytokine genes in animal models of sepsis or endotoxemia<sup>[14-16]</sup>. Investigations regarding the role of NF-kB in human inflammatory diseases are scarce<sup>[17-20]</sup>. So far, no study has aimed to examine in patients with ACST the relationship among NF-kB activity in peripheral blood mononuclear cells (PBMC), the concentrations of the pro-inflammatory cytokines in plasma, and clinical outcome. The purpose of this study was to determine the NF-kB DNA binding activity in circulating blood cells and the cytokines tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-6, and IL-10 profile in patients with ACST. Attempts were made also to correlate degree of NF-kB activation with severity of biliary tract infection and clinical outcome.

## MATERIALS AND METHODS

### Patients

The study population was recruited from a series of 20 patients with a clinical diagnosis of ACST. Among them, 13 were male, and 7 female, ranging in age from 27 to 78 yr. All patients had manifestations of fever, chill, jaundice, and right upper quadrant pain. Other manifestations included two or more of the following clinical conditions: Blood cultures were positive; Core body temperature  $>39^\circ\text{C}$  or  $<36^\circ\text{C}$ ; Heart rate  $>120$  beats/min; Hypotension: A systolic blood pressure of  $<12.0$  kPa or a reduction of  $>5.33$  kPa from baseline in the absence of other causes of hypotension; White blood cell count  $>1.5 \times 10^9 \text{ L}^{-1}$ . These patients were divided into nonsurvivor group (7 cases) and survivor group (13 cases). Ten patients undergoing elective gastrectomy or inguinal hernia repair were selected as control group. Peripheral blood samples were taken 24h postoperatively.

### Isolation of PBMC

PBMC were separated by density gradient centrifugation, as previously described<sup>[18]</sup>. In brief, PBMC were isolated from blood

freshly collected on sodium citrate by centrifugation on Ficoll-Hypaque. Before Ficoll, a fraction of the blood was centrifuged 5min at 1500r·min<sup>-1</sup> and 1mL of plasma was collected and put immediately at -20°C for further cytokine measurements.

### Isolation of nuclear proteins

Nuclear proteins were isolated from PBMC extract by placing the sample in 0.8mL of ice-cold hypotonic buffer [10mmol·L<sup>-1</sup> HEPES (pH7.9), 10mL KCL, 0.1mmol·L<sup>-1</sup> EDTA, 0.1mmol·L<sup>-1</sup> ethylene glycol tetraacetic acid, 1mmol·L<sup>-1</sup> DTT; Protease inhibitors (aprotinin, pepstatin, and leupeptin, 10mg·L<sup>-1</sup> each)]. The homogenates were incubated on ice for 20min, vortexed for 20s after adding 50μL 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 were suspended in an ice-cold hypertonic buffer [20mmol·L<sup>-1</sup> HEPES (pH7.9), 0.4mol·L<sup>-1</sup> NaCl, 1mmol·L<sup>-1</sup> EDTA, 1mmol·L<sup>-1</sup> DTT; Protease inhibitors], incubated on ice for 30min at 4°C, mixed frequently, and centrifuged for 15min 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.

### Electrophoretic Mobility Shift Assay (EMSA)

NF-κB binding activity was performed in a 10-μL binding reaction mixture containing 1×binding buffer [50mg·L<sup>-1</sup> of double-stranded poly(dI-dC), 10mmol·L<sup>-1</sup> Tris-HCl (pH 7.5), 50mmol·L<sup>-1</sup> NaCl, 0.5mmol·L<sup>-1</sup> EDTA, 0.5mmol·L<sup>-1</sup> DTT, 1mmol·L<sup>-1</sup> MgCl<sub>2</sub>, and 100mL·L<sup>-1</sup> glycerol], 5μg of nuclear protein, and 35 fmol of double-stranded NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG-3') that was endly labeled with γ-32P(111TBq mmol<sup>-1</sup> at 370GBq L<sup>-1</sup>) using T4 polynucleotide kinase. The binding reaction mixture was incubated at room temperature for 20min and analyzed by electrophoresis on 7 per cent nondenaturing polyacrylamide gels. After electrophoresis the gels were dried by Gel-Drier (Bio-Rad Laboratories, Hercules, CA) and exposed to Kodak X-ray films at -70°C.

### Quantifying with the Phosphor Imager

The binding bands were quantified by scanning densitometer of a Bio-Image Analysis System. The results were expressed as relative optical density (ROD).

### Measurement of cytokines in plasma

TNF-α, IL-6, and IL-10 levels in the plasma of patients with ACST and healthy control subjects were determined with using an enzyme-linked immunoassay (ELISA). The detection limits of the assays were 50ng·L<sup>-1</sup> (TNF-α), 49ng·L<sup>-1</sup> (IL-6), and 49ng·L<sup>-1</sup> (IL-10). All cytokines assays were performed in duplicate and had intra- and interassay variations lower than 8% and 11%, respectively.

### Statistical Analysis

Data were analyzed with using Microsoft Excel with Astute statistical add-in and were expressed as median ± standard error. A value of  $P \leq 0.05$  was considered statistically significant.

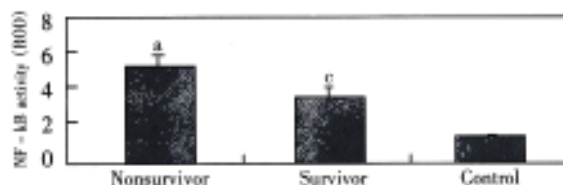
## RESULTS

### Methods

The patients of nonsurvivor group died within 35d, all from complications of SIRS, sepsis or MOF. The patients of survivor group were well discharged from hospital postoperatively in 28 days. The median age in nonsurvivor group was 54 yr, which was not significantly different from that of survivor group (53yr) ( $P > 0.05$ ).

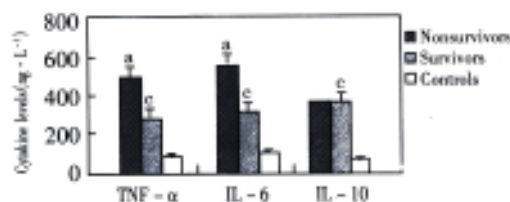
An admission APACHE II score in nonsurvivor group was 25, which was also not different from that of survivor group (24) ( $P > 0.05$ ).

The NF-κB activity was  $5.02 \pm 1.03$  in nonsurvivor group,  $2.98 \pm 0.51$  in survivor group and  $1.06 \pm 0.34$  in control group. There were statistical differences in three groups ( $P < 0.05$ ). The NF-κB activity increased in all patients with ACST, versus the control group ( $P < 0.05$ ), and the patients of nonsurvivor group had higher levels of NF-κB activation than those of survivor group ( $P < 0.05$ , Figure 1).



**Figure 1** The activity of NF-κB in three groups. <sup>a</sup> $P < 0.05$ , vs other groups, <sup>c</sup> $P < 0.05$ , vs control.

The levels of TNF-α and IL-6 in plasma were  $(498 \pm 53)$ ng·L<sup>-1</sup> and  $(587 \pm 64)$ ng·L<sup>-1</sup> in nonsurvivor group,  $(284 \pm 32)$ ng·L<sup>-1</sup> and  $(318 \pm 49)$ ng·L<sup>-1</sup> in survivor group and  $(89 \pm 11)$ ng·L<sup>-1</sup> and  $(102 \pm 13)$ ng·L<sup>-1</sup> in control group. All patients with ACST had increased levels of TNF-α and IL-6, which were many-fold greater than those of control group, and there was an evidence of significantly higher levels in those of nonsurvivor group than that in survivor group ( $P < 0.05$ ). The levels of IL-10 in plasma were  $(378 \pm 32)$ ng·L<sup>-1</sup>,  $(384 \pm 37)$ ng·L<sup>-1</sup> and  $(68 \pm 11)$ ng·L<sup>-1</sup> in three groups, respectively. All patients had also increased levels of IL-10 when compared with control group ( $P < 0.05$ ), but the IL-10 levels were not significantly higher in nonsurvivors than in survivors ( $P > 0.05$ ). (Figure 2).



**Figure 2** Changes of cytokines in plasma. <sup>a</sup> $P < 0.05$ , vs other groups, <sup>c</sup> $P < 0.05$ , vs control

## DISCUSSION

The overwhelming inflammatory response in patients with ACST is a major cause which induces systemic inflammatory response syndrome (SIRS) and MOF<sup>[1-4]</sup>. Mortality in patients with ACST reflects a multifactorial pathology, and neither cytokine concentrations in plasma nor even the APACHE II score can be expected to accurately predict patients' outcomes<sup>[5-9]</sup>. NF-κB is a protein found in inflammatory cells such as lymphocytes, monocytes, and macrophages. NF-κB activation is stimulated by LPS, TNF-α, and IL-1, the very early mediators or factors in the inflammatory cascade<sup>[21,22]</sup>. Once stimulated NF-κB activates various parts of the inflammatory responses: TNF-α, IL-6 and IL-10, and adhesion molecules such as selectins and integrin<sup>[23-25]</sup>. These mediators and factors then promote further activity of the inflammatory cascade and "off" goes the SIRS and MOF<sup>[2,8,9,24]</sup>. Therefore, we chose to focus on NF-κB as an important regulatory factor to regulate the expression of multiple cytokine genes. NF-κB is a ubiquitous transcription factor involved in the signal transduction pathway of many inducers of the inflammatory response and is therefore a potentially attractive target for

immunomodulation to reduce sepsis and organ dysfunction<sup>[26]</sup>, but we are not yet clear about changes of NF- $\kappa$ B and relation between NF- $\kappa$ B and cytokines in patients with ACST. Foulds *et al*<sup>[20]</sup> reported that levels of nuclear-bound NF- $\kappa$ B (activated NF- $\kappa$ B) were greater in patients who developed organ dysfunction after surgery, and patients with lower levels of nuclear NF- $\kappa$ B who recovered from surgery without organ dysfunction. Bohrer *et al* investigated activity of NF- $\kappa$ B in nuclear extracts from PBMC of 15 patients with sepsis, of whom 10 survived. NF- $\kappa$ B activity was measured on days 1, 2, 3, 4, 5, 6, 8, 10, and 14 after admission where available. All patients with NF- $\kappa$ B binding activity exceeding 200% of day 1 died. This small study concluded that NF- $\kappa$ B activation might be an important event in clinical sepsis. But, Adib-Conquy *et al*<sup>[27]</sup> found the expression of NF- $\kappa$ B was significantly reduced for all patients with sepsis and trauma as compared with control subjects. In our study, by comparing the predictive value of measuring NF- $\kappa$ B activity in PBMC and the concentrations of some pro- and anti-inflammatory mediators in plasma in patients with ACST, we found that the NF- $\kappa$ B activity measured in the PBMC was a better overall predictor of mortality than the balance and time course of pro- and anti-inflammatory cytokines released in plasma. On the basis of these results NF- $\kappa$ B would seem to be a more sensitive molecular marker assay when compared with cytokines used as an indicator of sepsis. The expressions of many genes involved in the inflammatory and immune processes are regulated by NF- $\kappa$ B, TNF- $\alpha$ , IL-6, and IL-10, and they possess NF- $\kappa$ B binding sites in the promoter region, enabling messenger ribonucleic acid to express in response to extracellular stimuli<sup>[28-31]</sup>. TNF- $\alpha$ , IL-6, and IL-10 have all been implicated in the pathogenesis of SIRS and MOF that results from trauma, injury, infection, and sepsis<sup>[32-43]</sup>. In the present study, we found that TNF- $\alpha$  and IL-6 were elevated in the patients with ACST and there were higher levels of TNF- $\alpha$  and IL-6 in patients who survived than in patients who died, which is in agreement with previous reports. These previous studies also showed that levels were higher in patients with sepsis when compared with trauma patients. We found significant relationship between NF- $\kappa$ B activation and circulating concentrations of TNF- $\alpha$  and IL-6. IL-10, as an anti-inflammatory mediator, was elevated in the patients with ACST but reduced in patients who died. There might be other transcription factor as AP-1 involved in the signal transduction pathway of the inflammatory response<sup>[16,21,23,44-48]</sup> and its mechanism is going on.

In conclusion, we have shown NF- $\kappa$ B activation in PBMCs in patients with ACST increased markedly before death and was related to plasma TNF- $\alpha$  and IL-6 concentrations. These findings have important implications for the development of future therapeutic interventions in the critically ill and support the need for further study of the role of NF- $\kappa$ B activation in mortality from ACST and MOF.

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