Elevated Serum IL-18 and Interferon-Gamma in Medium-Term Survivors of Biliary Atresia

P. Vejchapipat[1] S. Poomsawat[2] V. Chongsrisawat[3] S. Honsawek[4] Y. Poovorawan[3]

[1] Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[2] Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand
[3] Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[4] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Address for correspondence and reprint requests Paisarn Vejchapipat, M.D., Ph.D., Department of Surgery, Faculty of Medicine, Chulalongkorn University, Rama IV Road, Patumwan, Bangkok 10330, Thailand (e-mail: paisarnv@gmail.com).

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Abstract

Introduction Biliary atresia (BA) is a fatal disease in children. Its main pathological feature is progressive immune-mediated cholangiopathy. Interleukin (IL)-12, IL-18, and interferon-gamma (IFN-gamma) play important roles in various immunological diseases. The objective was to investigate whether these serum markers were associated with clinical outcome in BA.

Methods Serum levels of IL-12, IL-18, and IFN-gamma were determined using enzyme-linked immunosorbent assay from 46 BA patients (median age of 9 years) and 19 normal controls. The BA patients were then categorized into three groups according to their outcome: jaundice-free (29 cases), mild to moderate jaundice (10 cases), and marked jaundice (7 cases). The comparisons of serum IL-12, IL-18, and IFN-gamma levels among groups of the patients were performed using one-way analysis of variance with post-hoc tests. Data are expressed as mean + standard deviation.

Results Serum IL-18 and IFN-gamma in BA patients were higher than the normal controls (IL-18: 113.3 + 82.6 vs. 80.5 + 9.9 pg/mL, $p = 0.011$ and IFN-gamma: 41.7 + 5.1 vs. 38.0 + 1.9 pg/mL, $p < 0.001$). There was no difference in serum IL-12 between BA and controls. Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for trend, $p = 0.004$).

Conclusions Serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression.

Keywords
- biliary atresia
- interleukin-12
- interleukin-18
- interferon-gamma

Introduction

Biliary atresia (BA) remains one of the most intractable liver diseases in pediatric population. It is a disease characterized by an obliterative, inflammatory, and immune-mediated cholangiopathy leading to the obstruction of bile flow in neonates and infants.[1] The cause and pathophysiology of this disease are still unsolved. Without proper treatment,

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the consequence is the development of progressive liver fibrosis, indicated by direct hyperbilirubinemia and acholic stools, leading to biliary cirrhosis within a few months and mortality within 2 to 3 years.[2,3] When patients with BA are left without any surgical correction, the majority will die from hepatic decompensation, esophageal variceal bleeding, or infection.[4,5]

Although there were several studies on serum and tissue markers regarding the pathophysiology of progressive liver fibrosis in BA including various cytokines,[6–9] growth factors,[10,11] nitric oxide,[12,13] and the apoptosis of bile duct cells,[14–16] the exact mechanism is still unclear. Our previous studies demonstrated an evidence of the association between serum inflammatory markers, including intercellular adhesion molecule 1 (ICAM-1), interleukin (IL)-8, nitric oxide, and selectin,[7,9,12,13,17] and clinical outcome in BA. However, the information regarding the association between serum IL-12, IL-18, and interferon-gamma (IFN-gamma) with clinical outcome in BA has been lacking.

IFN-gamma is a soluble inflammatory cytokine. This interferon was originally called macrophage-activating factor. It has been shown that IFN-gamma can prevent liver fibrosis by inhibiting the activation and proliferation of stellate cells, resulting in reduced expression of procollagen without affecting transforming growth factor-β1 expression in pig serum-induced rat liver fibrosis in vivo.[18] In addition, IL-12 and IL-18 are both critical to the induction of IFN-gamma. Various roles for IL-12 and IL-18 in control of inflammation in an IFN-gamma-dependent manner have been demonstrated.[19] Both IL-12 and IL-18 have been characterized as effective IFN-gamma-inducing cytokines. Concomitant treatment with IL-12 and IL-18 has been shown to synergistically induce IFN-gamma synthesis. This was postulated to be an effective therapy for treating immune-mediated diseases and infectious diseases.[20] It has also been suggested that IL-18 contributes to infection through the induction of IFN-gamma production by NK cells, but not through the development of T helper 1 (Th1) cells, under the condition in which IL-12 synthesis is deficient.[21] Hence, evidences as mentioned above indicate that IL-12, IL-18, and IFN-gamma are closely related to each other.

The information of serum levels of IL-12, IL-18, and IFN-gamma in BA patients may improve our understanding of its pathophysiology regarding the inflammatory nature of this disease. Therefore, the objective of this study was to explore possible roles of serum IL-12, IL-18, and IFN-gamma levels in medium-term survivors of BA patients. We hypothesized that serum IFN-gamma, IL-12, and IL-18 can be used as prognostic markers for postoperative BA patients.

Materials and Methods

Patients

The study was approved by the ethical committee. BA patients and their parents were informed and recruited for the study during the annual follow-up. The age- and gender-comparable control group comprised healthy children participating in the vaccination program during the same period. Briefly, peripheral venous whole blood was drawn with a sterile syringe, transferred to a centrifuge tube, allowed to clot, and then centrifuged at 4°C. The sera were stored at −70°C until they could be assayed.

Measurement of Serum IL-12, IL-18, and IFN-Gamma Levels

Serum levels of IL-12, IL-18, and IFN-gamma were measured using available commercial enzyme-linked immunosorbent assay (ELISA) kits according to company's protocols (catalog number D1200 for IL-12, R&D Systems, Minneapolis, MN, USA; code number 7620 for IL-18, MBL International, Chicago, IL, USA; and catalog number DIF50 for IFN-gamma, R&D Systems). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibodies specific for IL-12, IL-18, or IFN-gamma were precoated onto a microplate. Standards and samples were pipetted into the wells and any IL-12, IL-18, or IFN-gamma present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibodies specific for IL-12, IL-18, or IFN-gamma were added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of IL-12, IL-18, or IFN-gamma bound in the initial step. The color development was then stopped and the intensity of the color was measured.

In addition, liver function tests, including serum albumin, total bilirubin (TB), direct bilirubin, and alanine aminotransferase, were performed using an automated chemical analyser (Hitachi 911, Hitachi, Tokyo, Japan) at the central laboratory of the hospital.

Sample Size Calculation

Comparisons of serum IL-18 between BA and normal controls were selected as the primary outcome. From our pilot data, standard deviation (SD) of serum IL-18 from normal controls was 20 pg/mL. The true difference of means between two groups was proposed at 15 pg/mL. By using StatMate 2.00 (GraphPad Software Inc., San Diego, CA, USA), it revealed that sample size of 25 in each group has a 80% power to detect a difference between means of 15 with a significance level (α) of 0.05 (two-tailed). Further adjustment calculation of sample size in ratio of 1:2.5, with the same power, suggested that sample size of BA group is 44 and sample size of control group is 18 (http://www.stattools.net/SSizunequal_Pgm.php).

Categorization of the BA Patients

To compare different clinical outcome among BA patients, they were divided into three groups according to the status of jaundice:

Group A; patients without jaundice (serum TB <2.0 mg%).
Group B; patients with mild to moderate jaundice (serum TB total = 2 to 10 mg%).
Group C; patients with marked jaundice (serum TB > 10 mg%).

Table 1 Demographic and Clinical Data of BA Patients Based on the Status of Jaundice. Data are Shown as Mean + SD and One-Way Analysis of Variance was Used

	Group A	Group B	Group C	p Value
Number of cases	29	10	7	–
Age (years)	10.3 + 5.2	8.3 + 6.5	5.7 + 3.1	0.37
Serum albumin (g/dL)	4.4 + 0.4	3.8 + 0.5	3.4 + 0.4	<0.001
Serum TB (mg%)	0.9 + 0.5	4.5 + 2.6	24.8 + 13.9	<0.001
ALT (IU/L)	125.8 + 174.3	191.9 + 120.8	168.3 + 95.6	0.48
Serum IL-12 (pg/mL)	12.8 + 2.1	12.7 + 1.9	13.1 + 1.5	0.94
Serum IL-18 (pg/mL)	93.1 + 30.4	118.6 + 33.6	189.8 + 190.8	0.017
Serum IFN-gamma (pg/mL)	41.9 + 5.7	40.7 + 4.4	42.1 + 4.0	0.81

BA, biliary atresia; SD, standard deviation; TB, total bilirubin; ALT, alanine aminotransferase; IL, interleukin; IFN-gamma, interferon-gamma.

Further analysis between BA patients based on the status of jaundice and the levels of serum IL-12, IL-18, and IFN-gamma were performed.

Statistical Analyses

Demographic and clinical data between groups were compared by chi-square tests and unpaired *t* tests. The mean and SD were calculated for each variable. The comparisons of these variables between BA patients and controls were performed using parametric tests. The comparisons of serum IL-12, IL-18, and IFN-gamma levels among groups of the patients were performed using one-way analysis of variance with post-hoc tests for linear trend.

All data are expressed as mean + SD. All statistical analyses were performed using SPSS Statistics 17.0 (SPS, Inc., Chicago, IL, USA) and Prism version 5.0 (Graphpad software, Inc., San Diego, CA, USA).

Results

Serum levels of IL-12, IL-18, and IFN-gamma were determined using ELISA from 46 BA patients (median age of 9 years) and 19 normal controls. Serum IL-18 and IFN-gamma in BA patients were higher than normal controls (IL-18: 113.3 + 82.6 vs. 80.5 + 9.9 pg/mL, $p = 0.011$ and IFN-gamma: 41.7 + 5.1 vs. 38.0 + 1.9 pg/mL, $p < 0.001$). However, there was no difference in serum IL-12 between BA and controls (12.8 + 1.9 vs. 12.9 + 1.8 pg/mL, $p = 0.92$).

The BA patients were then categorized into three groups according to their outcome: Group A—jaundice-free (29 cases), Group B—mild to moderate jaundice (10 cases), and Group C—marked jaundice (7 cases). The demographic and clinical data of BA patients are shown in ►Table 1.

Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for linear trend, $p = 0.004$), as shown in ►Fig. 1. In addition, there was no correlation between serum IL-18 and serum IFN-gamma in BA patients.

Discussion

At present, the knowledge of BA has been better understood and its pathophysiology has been widely investigated.[1] It has been suggested that the role of inflammatory signaling pathways in progressive liver fibrosis is critical to the liver pathology.[22] Although there were several reports regarding the association between inflammatory markers and BA, the study of possible roles of serum levels of IL-12, IL-18, and IFN-gamma in BA receives not much attention.

Based on the role of IL-12 as a key proinflammatory cytokine, it is likely that IL-12 is involved in the pathophysiology of BA. Recent studies showed that the loss of IL-12

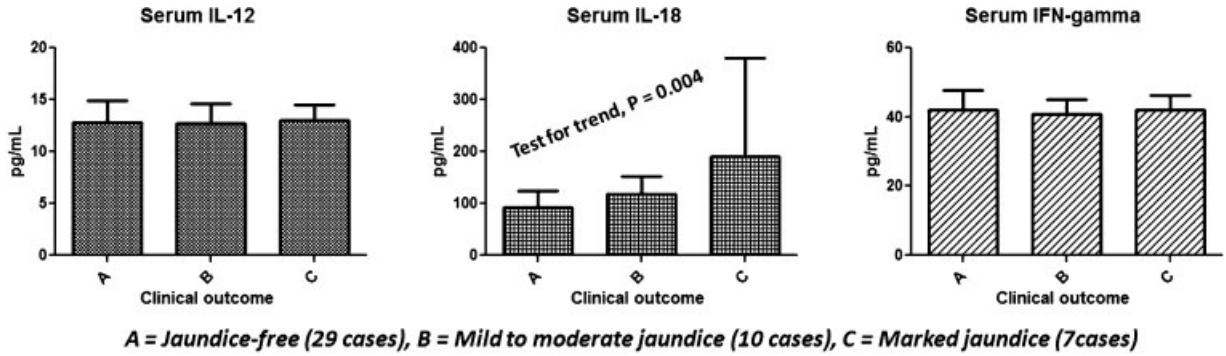

Figure 1 The comparisons of serum IL-12, serum IL-18, and serum IFN-gamma among the three groups of BA patients.

modified the proinflammatory response but did not prevent duct obstruction in experimental BA in animals.[23] Furthermore, IL-18 levels were demonstrated to correlate with IL-12 and IFN-gamma levels through Th1 cytokine production.[24] Mack et al[25] revealed that there were increases in T cells and Kupffer cells in the portal tracts of BA. Analysis of liver tissue from BA patients showed a Th1-type cytokine profile with significant expression of IFN-gamma and IL-12.

The present study clearly showed that serum levels of IL-18 and IFN-gamma were significantly elevated in medium-term survivors of BA compared with healthy children. The results are consistent with previous studies conducted in adult patients demonstrating that serum IL-18 levels were higher in cirrhotic patients including primary biliary cirrhosis and chronic hepatitis C than those of healthy adults.[26,27] Our findings on the correlation of elevated serum IL-18 levels and the severity of jaundice in BA are also agreeable with Urushihara et al.[28] Since IL-18 is a potent proinflammatory cytokine-inducing IFN-gamma production from activated T cells, elevated serum IL-18 levels found in BA patients may be responsible for the elevation of serum IFN-gamma in BA found in this study. Nonetheless, we found no correlation between serum IL-18 and serum IFN-gamma in BA patients. This may be due to the small sample size or the complex pathways involved in the interaction between IL-18 and IFN-gamma. Investigation of these cytokine levels in the liver tissue will elucidate possible roles of IL-18 and IFN-gamma in BA more precisely.

Interestingly, we demonstrated that there was no difference in serum IL-12 levels between BA patients and normal controls. An important biologic activity associated with IL-12 is to promote the differentiation of naive T cells into T-helper cells capable of producing IFN-gamma. Stimulation of IFN-gamma production by IL-12 can be synergistically enhanced by the presence of other proinflammatory cytokines such as tumor necrosis factor, IL-1, and IL-2.[19] Therefore, it is likely that IL-12 is not directly involved in the pathophysiology of BA and the elevation of serum IFN-gamma found in BA patients is not passed through IL-12 signaling pathway but rather via IL-18 pathway in some extent. Additionally, since there is no difference in serum IL-12 among BA patients with different jaundice status, it is likely that the association between serum IL-18 and jaundice status found in this study is not due to poor liver function.

As always, this kind of study regarding serum markers does inevitably have some limitations. First, the serum levels of cytokines did not necessarily reflect the action of their signaling pathways within the disease organs. However, with the supported evidence from other studies on the real association between their serum levels and the process of liver fibrosis, it is likely that the elevated serum levels of IL-18 and IFN-gamma found in BA play roles in its pathophysiology. Second, there are inflammatory cytokines or markers other than IL-12, IL-18, and IFN-gamma that are involved in the pathophysiology of progressive inflammatory liver injury, for example, IL-8,[7] ICAM-1,[17] selectin,[9] and nitric oxide.[13] Third, we would like to emphasize that the results are weak due to very small patient numbers and more data are needed to confirm our findings. Therefore, there are other influential factors outside the boundaries of this study needed to be taken into account. What we attempted to do here is to assemble some small pieces from the big picture of complex pathways.

In conclusions, serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression. Serum IL-18 may be used as a prognostic marker in BA.

Conflict of Interest

None

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