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Original Article

Kinetics of cytokine expression in cirrhotic rats

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Abstract

Background: Cytokines are involved in liver injury and cirrhosis and systemic and hepatic cytokine levels may help predict cirrhosis evolution. However, the relevant survey has not been performed.
Methods: Male Sprague-Dawley rats (240–270 g) received either common bile duct ligation (BDL, animal model of cholestatic liver injury) or sham operation (control). Five rats were sacrificed and liver and serum were collected from each in weeks 1, 2, 4, 6, 8 and 10 after surgery. Hepatic expression of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) were analyzed by immunohistochemial staining. The corresponding serum levels were measured by ELISA.
Results: Compared to the corresponding sham groups, hepatic expression of these cytokines in BDL rats was significantly and progressively enhanced during cirrhosis development. However, serum IFN-γ levels of BDL rats did not change significantly. Serum TNF-α of BDL rats increased gradually and reached a peak in week 6. Serum TGF-β level was elevated up to week 8, whereas IL-10 level decreased progressively until week 6.
Conclusion: Cirrhosis development in BDL rats is associated with progressively enhanced expression of hepatic pro-inflammatory and anti-inflammatory cytokines, which is not in accord with the corresponding serum concentration. The circulating cytokine concentration may not totally reflect the hepatic expression level throughout the development of cirrhosis.

Keywords: cytokines; experimental cholestasis; experimental portal hypertension

1. Introduction

Cytokines are soluble, hormone-like mediators synthesized by a variety of cells in response to various stimuli, including inflammation and tissue injury.[1] The liver is a major organ in the production as well as the elimination of cytokines and all cell types in the liver are capable of producing cytokines.[1] Cytokines in the liver and the association with liver diseases has been investigated.[1–4] Upon liver injury, the activated Kupffer cells and T cells secret cytokines and soluble factors that activate hepatic stellate cells (HSC). One of the key cytokines involved in the activation of HSC is transforming growth factor-β (TGF-β).[5] Furthermore, tumor necrosis factor-α (TNF-α) participates in HSC extracellular matrix protein synthesis.[6] Other pro-inflammatory cytokines e.g., interferon-γ (IFN-γ) and anti-inflammatory cytokines e.g., interleukin-10 (IL-10) have also been implicated in the process of fibrosis and cirrhosis.[7,8]

Changes of serum cytokine level and the progression of hepatic damage in rats with common bile duct ligation

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(BDL)-induced cholestatic liver injury have been reported.[9–11] However, there has been no systematic approach to evaluate the whole picture of hepatic and circulating cytokine changes throughout the formation of cirrhosis: cytokine levels in acute liver damage induced by BDL when cirrhosis had not been established has been reported.[12,13] Some surveyed the serum and hepatic cytokine changes in late cirrhosis.[10] Since the serial changes of cytokine production may contribute to a better understanding and prediction of cirrhosis progression, we investigated the kinetics of hepatic and serum cytokine expression during the course of cirrhosis development induced by BDL in rats.

2. Methods

2.1. Animal model

Male Sprague-Dawley rats weighing 240–270 g at the time of surgery were used in this study. Rats were housed in plastic cages with free access to food and water. All rats were fasted for 12 h before the operation. Secondary biliary cirrhosis was induced by BDL.[14] Under ketamine anesthesia (100 mg/kg, intramuscularly), the common bile duct was exposed through a midline abdominal incision. The common bile duct was dissected and doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second was made above the entrance of the pancreatic duct. The incision was then closed and the animal allowed to recover. A high yield of secondary biliary cirrhosis was noted four weeks after the ligation.[14,15] To avoid coagulation defects, BDL rats received a weekly injection of vitamin K (50 μg/kg intramuscularly). The control group (sham group) was prepared by a technique similar to that described above for the BDL group, except the common bile duct was not ligated. This study was approved by the Taipei Veterans General Hospital Animal Committee. The principles of laboratory animal care were followed as described in the Guide for the Care and Use of Laboratory Animals (DHEW publication no. [NIH] 85-23, rev. 985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, USA).

2.2. Immunohistochemical (IHC) staining

Frozen sections (5 μm thick) were fixed in acetone for 10 min and then treated with H_2O_2 to block endogenous peroxidase activity. After normal serum blocking, the sections were incubated with primary antibodies, then secondary antibodies and then peroxidase anti-peroxidase complex with intensive washing with phosphate-buffered saline between steps. A positive stain was seen as the deposition of a stain color by the diaminobenzidine substrate solution in the presence of the peroxidase. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted. The final results were observed under a visible light microscope.

The degree of color development in the stained section treated with a negative control primary antibody, such as NS-1, normal goat serum or normal rabbit serum was defined as grade 0. The degree of color development after treatment with the positive control β-actin antibody was defined as grade 5. The intensity and percentage of staining of IFN-γ, TNF-α, TGF-β, and IL-10 were graded (with some modification) as follows: significantly strong positive (5+), dark brown staining in more than 75% of cells completely obscuring cytoplasm and nucleus; strong positive (4+), lower degree of brown staining in $>$ 75% of cells; intermediate strong positive (3+), brown staining in 50–75% of cells; intermediate weak positive (2+), brown staining in 25–50% of cells; weak positive (1+), brown staining in $<$ 25% of cells; absent (0), no appreciable staining.[16]

2.3. Serum cytokines

The concentration of cytokines in serum samples was determined by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions for using kits selective for rat IFN-γ and TNF-α (American Research Product Inc., Belmont, MA 02478 USA) as well as TGF-β and IL-10 (R&D Systems Co., Minneapolis, MN, USA). Briefly, serum samples from rats were incubated in immunoassay plates coated with anti rat IFN-γ, anti rat TNF-α, anti rat IL-10 or anti rat TGF-β. After washing, the plates were interacted with polyclonal antibodies against rat IFN-γ, anti TNF-α, anti IL-10 or anti TGF-β, respectively, followed by addition of secondary antibody–alkaline phosphatase conjugates and the chromagen *p*-nitrophenyl phosphate. The concentration of each cytokine in each serum sample was calculated from a calibration curve constructed with cytokine standards.

2.4. Determination of liver biochemistry parameters

Blood was collected from the inferior vena cava into a pyrogen-free syringe containing 75 U of heparin sodium. Plasma was separated by centrifugation (3000 rpm for 10 min) at 4 °C with and then stored at −70 °C in Eppendorf tubes. The serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (Alk-P), albumin and total bilirubin was evaluated with a Vitro DT chemistry system (Johnson & Johnson Inc., New York, NY, USA).

2.5. Statistical analysis

The data were assessed using the Friedman test for six related samples and post hoc analysis with Wilcoxon signed-rank tests if needed for comparison among BDL or sham-operated rats at different time intervals after surgery. The Mann–Whitney U test for two independent samples was used to analyze the differences between BDL and the corresponding sham-operated groups. Linear trends were determined by polynomial contrasts. The statistically significant difference was set at $p \leq 0.05$ and the results are shown as mean ± SD.

3. Results

3.1. Mortality rates and histological changes in the liver of BDL rats

The mortality rates of BDL rats at weeks 6, 8 and 10 were 100%, 94.12% and 100%, respectively.

Fig. 1 shows the histological findings for BDL rats at the 6 weeks post surgery. In the liver from BDL rats at 6 and 8 weeks post surgery, areas of remaining liver cells were surrounded by bile duct proliferation and fibrotic tissue. The intrahepatic architecture was interrupted by fibrotic bands connecting the portal tracts and the original lobules were replaced by pseudo-lobules of unusual shapes and variable size.

In BDL rats 10 weeks post surgery, bile-duct proliferation was extensive and some bile ducts appeared to be distorted and distended. Most of the necrotic tissue had been replaced by proliferating bile ducts. It was thus difficult to identify portal tracts and many hepatocytes had disappeared.

3.2. Serum cytokine concentrations

The circulating cytokine levels in BDL rats and time-matched, sham-operated groups are shown in Table 1.

3.3. Serum cytokine concentrations and hepatic expression of IFN-γ

There was no significant change of serum IFN-γ concentration in either sham-operated or BDL groups throughout the experimental course (Fig. 2A).

The circulating IFN-γ levels were significantly higher in BDL rats in week 6 ($p < 0.01$) as compared with the time-matched, sham-operated rats. Immunohistochemical staining of liver tissue showed that IFN-γ production was hardly detectable in the sham-operated group whereas it was up-regulated gradually in the BDL group from weeks 1 to 8 and was declined slightly lowered in week 10 (Fig. 2B). As compared to the sham-operated group, the degree of IFN-γ IHC staining was elevated significantly from weeks 1 to 10 (sham-operated vs. BDL, $p < 0.05$ in week 1, <0.01 in week 2, <0.01 in week 4, <0.01 in week 6, <0.05 in week 8, and <0.01 in week 10, respectively). The representative IHC staining pattern (magnification 100×) of livers from BDL and sham-operated rats are shown below.

3.4. Serum cytokine concentrations and hepatic expression of TNF-α

The serum level of TNF-α in the BDL group increased gradually, reached a peak in week 6 and then decreased to the basal level in the later stages, but was not significantly different from those of weeks 1, 2, 4, 8 or 10 (Fig. 3A). Moreover, they were not significantly higher than those of the corresponding sham-operated groups.

Hepatic production of TNF-α, as reflected by the degree of IHC staining in BDL rats, increased gradually and the peak level was noted in week 8 (Fig. 3B). Compared to the sham-operated rats, the degree of IFN-γ IHC staining was elevated significantly in weeks 1 to 10 (BDL versus sham-operated: $p < 0.05$ in week 1, <0.01 in week 2, <0.01 in week 4, <0.05 in week 6, <0.01 in week 8, and <0.01 in week 10). The representative IHC staining pattern (magnification 100×) of livers from BDL and sham-operated rats are shown below.

3.5. Serum cytokine concentrations and hepatic expression of TGF-β

Serum TGF-β in the BDL group increased gradually, reached a peak in week 8 and then decreased slightly in week 10. Fig. 4A shows that compared to the sham-operated group, a significant difference was reached in weeks 6 ($p < 0.01$) and 8 ($p < 0.01$).

Hepatic expression of TGF-β in the BDL group was up-regulated gradually, reached a peak in week 6 then declined slightly in weeks 8 to 10, whereas it was almost undetectable in the liver in the sham-operated group (Fig. 4). The degree of IHC staining was significantly higher in the BDL group compared to the sham-operated group in week 1 ($p < 0.05$), week 2 ($p < 0.01$), week 4 ($p < 0.01$), week 6 ($p < 0.05$), week 8 ($p < 0.01$), and week 10 ($p < 0.01$), respectively. The representative IHC staining pattern (magnification 100×) of livers from BDL and sham-operated rats is shown below.

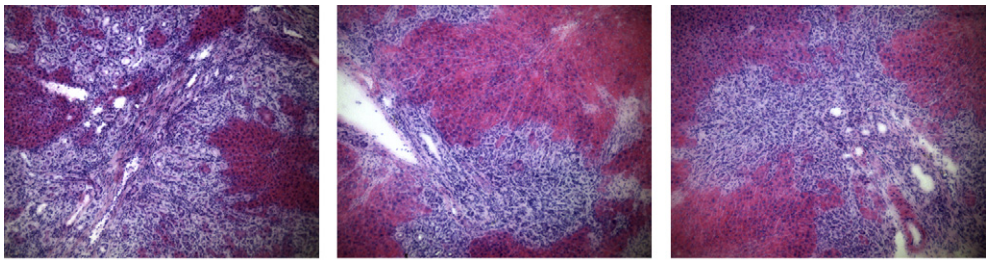

Fig. 1. The histological change of livers from rats after BDL for 6, 8 and 10 weeks, respectively (H & E staining; magnification 100×). Most of the hepatocytes have been replaced by regenerating bile ducts and fibrotic bands.

Table 1
Serum concentration of cytokines in BDL and time-matched, sham-operated rats

	IFN-γ (ng/ml)	TNF-α (pg/ml)	TGF-β (ng/ml)	IL-10 (pg/ml)
Sham−operated				
Week 1	17.2 ± 7.8 (18.7)	24.5 ± 13.8 (19.0)	42.1 ± 9.2 (37.0)	25.3 ± 1.0 (25.4)
Week 2	19.0 ± 14.2 (11.7)	21.7 ± 10.2 (17.7)	51.2 ± 26.3 (57.1)	25.9 ± 1.8 (26.3)
Week 4	20.0 ± 12.1 (19.9)	47.4 ± 20.5 (46.6)	42.3 ± 22.9 (36.3)	24.2 ± 2.0 (24.1)
Week 6	8.2 ± 4.0 (8.4)	44.2 ± 23.9 (43.7)	24.1 ± 5.3 (25.9)	22.9 ± 2.2 (23.1)
Week 8	8.9 ± 2.0 (8.8)	18.4 ± 5.3 (19.6)	38.4 ± 12.0 (33.1)	26.1 ± 2.3 (26.2)
Week 10	7.6 ± 1.9 (8.4)	15.7 ± 2.6 (15.9)	33.9 ± 8.4 (29.6)	25.1 ± 1.3 (24.8)
BDL				
Week 1	58.1 ± 74.1 (29.4)	20.9 ± 5.1 (19.2)	49.6 ± 12.2 (50.3)	25.4 ± 2.4 (24.4)
Week 2	28.8 ± 17.6 (29.5)	16.4 ± 3.8 (15.1)	60.0 ± 16.8 (59.2)	24.9 ± 2.1 (25.0)
Week 4	52.9 ± 30.2 (52.7)	39.5 ± 17.4 (28.9)	59.5 ± 26.4 (54.2)	22.1 ± 2.1 (22.1)
Week 6	33.3 ± 32.6 (19.6)*	79.4 ± 16.3 (83.6)	84.2 ± 47.2 (58.5)*	19.6 ± 3.3 (18.3)
Week 8	34.1 ± 40.9 (18.2)*	20.2 ± 6.3 (20.1)	168.2 ± 60.5 (161.1)*	25.0 ± 1.5 (24.4)
Week 10	18.5 ± 13.6 (24.0)	29.1 ± 16.9 (28.0)	149.9 ± 123.3 (118.0)*	27.3 ± 3.0 (27.5)

The data are expressed as mean ± SD (median). Statistically significant difference (*$p < 0.05$) between BDL and time-matched, sham-operated rats.

3.6. Serum cytokine concentrations and hepatic expression of IL-10

Serum level of IL-10 in BDL rats decreased gradually from weeks 1 to 6 and increased gradually thereafter (Fig. 5A). There was no significant difference between the BDL and sham-operated groups.

In BDL rats, the hepatic IHC staining degree of IL-10 increased gradually from weeks 1 to 4 then declined and was significantly higher than that of the corresponding

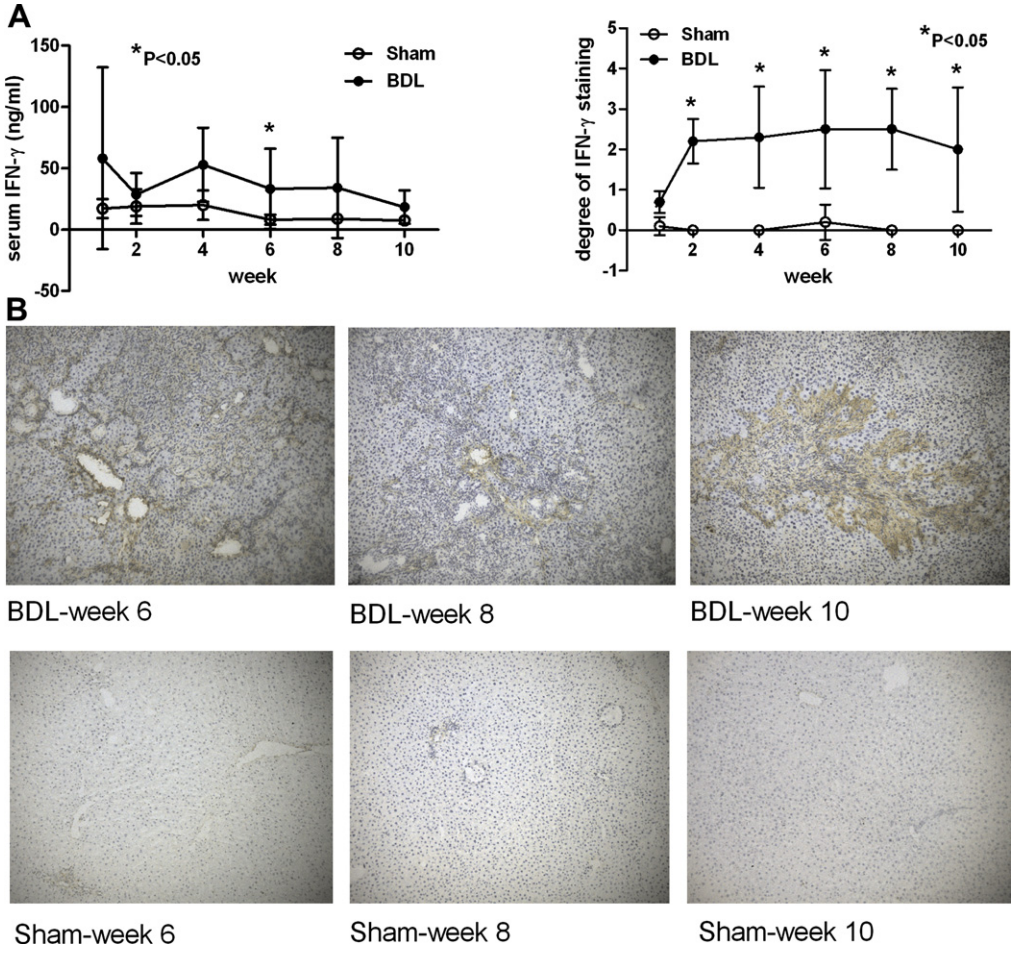

Fig. 2. Kinetics of IFN-γ expression in serum and liver tissue at 1, 2, 4, 6, 8 and 10 weeks after sham and BDL surgery. The data are expressed as mean ± SE ($n = 5$ for each group). (B) Representative IHC staining patterns at different time points are shown. Magnification 100×.

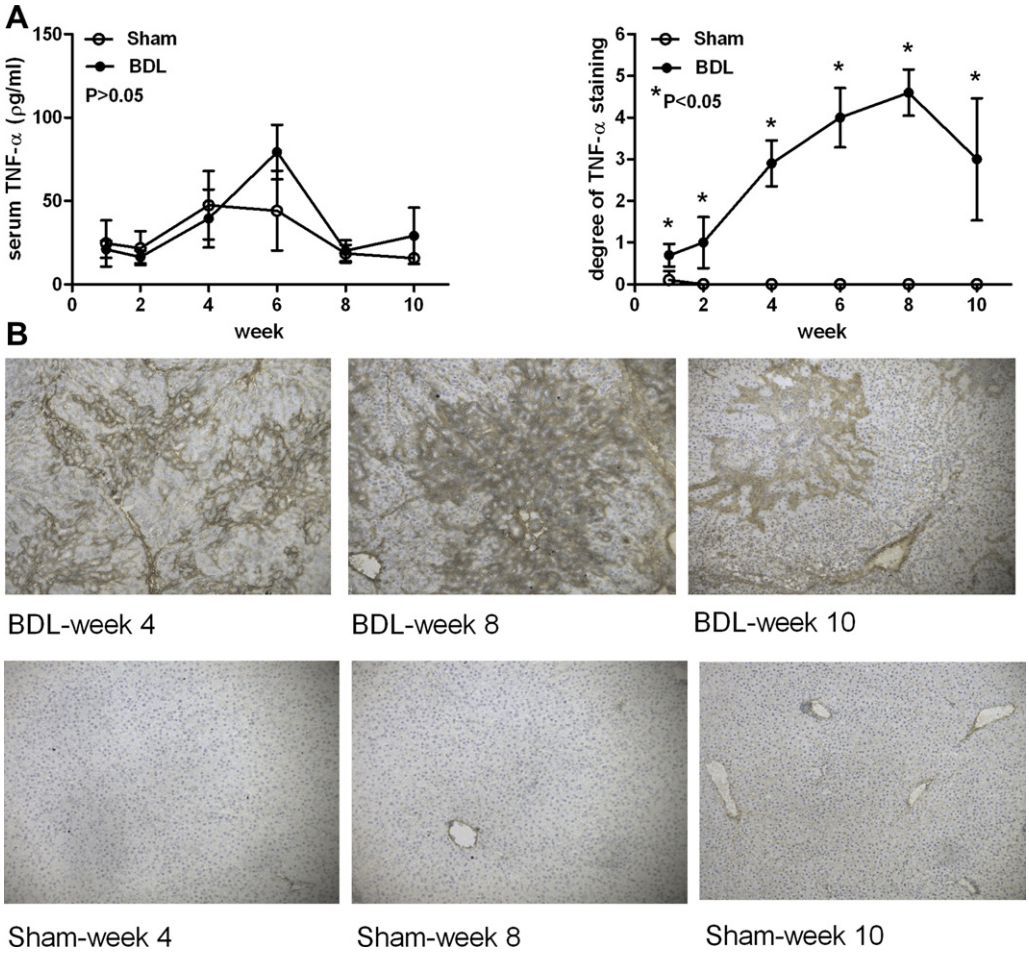

Fig. 3. (A) Kinetics of TNF-α expression in serum and liver tissue at 1, 2, 4, 6, 8 and 10 weeks after sham and BDL surgery. The data are expressed as mean ± SE ($n = 5$ for each group). (B) Representative IHC staining patterns at different time points are shown. Magnification 100×.

sham-operated group in week 1 ($p < 0.01$), week 2 ($p < 0.05$), week 4 ($p < 0.01$), week 6 ($p < 0.01$) and week 8 ($p < 0.01$), while it was barely detected in the sham-operated group (Fig. 5B). The representative IHC staining pattern (magnification 100×) of livers from BDL and sham rats is shown below.

3.7. β-Actin

The expression of β-actin was constant and stable in liver tissue of both the BDL and the sham-operated groups from weeks 1 to 10 (data not shown).

3.8. Correlation between hepatic expression and serum level of cytokines

When combining the sham-operated rats and BDL rats, the hepatic IHC staining degree of TGF-β was positively correlated with the serum level of TGF-β ($r = 0.416$, $p < 0.05$), whereas the hepatic IL-10 expression level was negatively correlated with its corresponding serum concentration ($r = -0.485$, $p < 0.05$). There was no significant relationship between hepatic and serum levels of IFN-γ and TNF-α (Fig. 6).

3.9. Biochemical tests

As compared to the sham-operated group, plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALK-P) and total bilirubin of the BDL group were significantly higher throughout the experiment and the albumin concentration was significantly lower after week 4 (Fig. 7).

4. Discussion

Common bile duct ligation (BDL) is a well established animal model used to induce cholestatic liver injury with inflammation, fibrosis and cirrhosis.[15] Histological examination has shown that hepatic lobular areas were gradually replaced by proliferated bile ducts and fibrous tissue, which led to typical biliary cirrhosis in four to eight weeks after

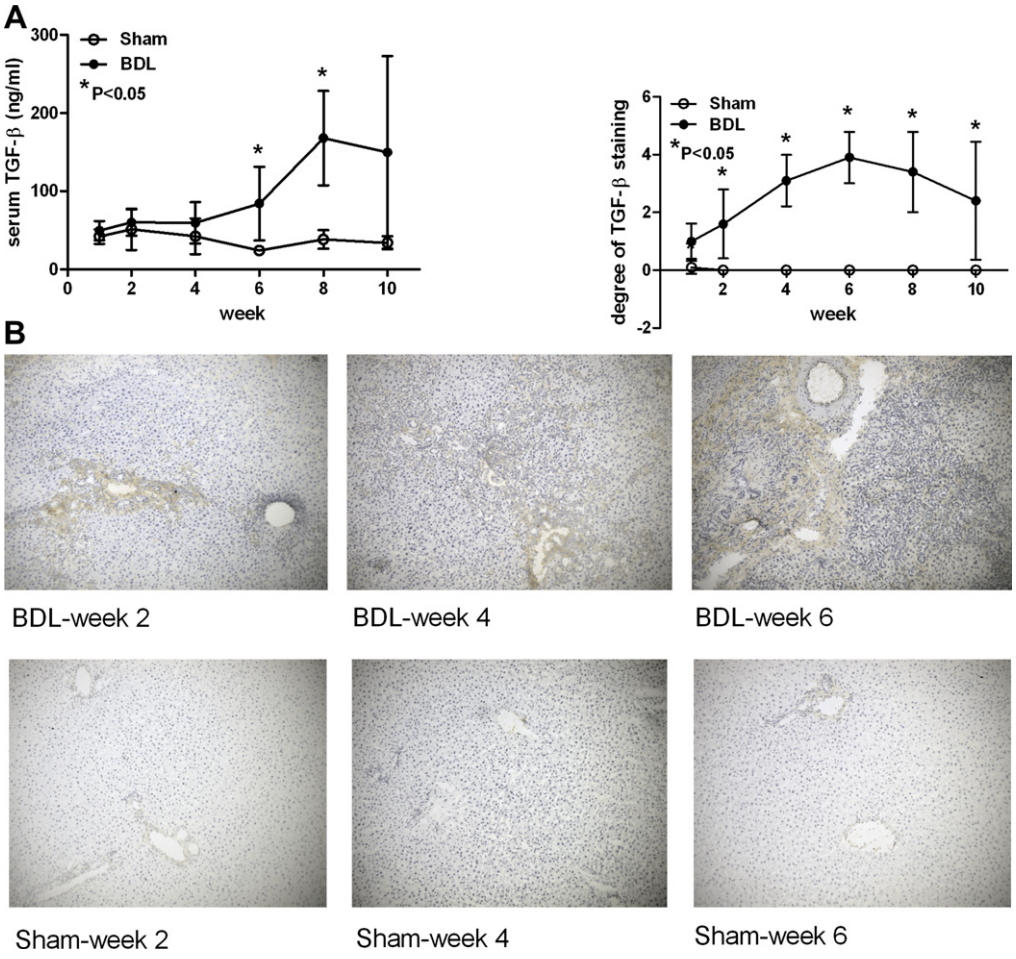

Fig. 4. (A) Kinetics of TGF-β expression in serum and liver tissue at 1, 2, 4, 6, 8 and 10 weeks after sham and BDL surgery. The data are expressed as mean ± SE ($n = 5$ for each group). (B) Representative IHC staining patterns at different time points are shown. Magnification 100×.

BDL.[11] In the current study, compared to the sham-operated rats, BDL rats showed significantly elevated serum concentrations of ALT, AST, ALK-P and total bilirubin, which is consistent with hepatic inflammation and injury elicited by BDL. The albumin level decreased from week 4 after BDL, which also reflects the development of liver cirrhosis with impaired albumin production.

With this model, we systematically revealed the whole profile of hepatic and circulating cytokine changes, including IFN-γ, TNF-α, IL-10 and TGF-β during cirrhosis development. The roles of cytokines facing hepatic damage are complicated: they may be responsible for the establishment and progression of hepatic injury, fibrosis and cirrhosis or participate in liver regeneration.[1] For instance, TNF-α, IL-6 and IL-1β are responsible for the activation of stellate cells, which play pivotal roles in liver inflammation, fibrosis and cirrhosis.[1] In the current study, to our surprise, some of the patterns of serum cytokine concentration changes were not consistent with those of hepatic expression levels. This suggests that the dynamic changes of hepatic cytokine expression may not be merely reflected by the corresponding serum levels.

After BDL, the hepatic IFN-γ expression level elevated progressively and reached a peak value in week 4. However, the serum concentration of IFN-γdid not change significantly and was not correlated with its hepatic expression level. Shindo et al had revealed that hepatic IFN-γ mRNA correlated with the severity of liver inflammation.[17] Furthermore, hepatic IFN-γ expression was higher in patients with primary biliary cirrhosis compared to patients with chronic hepatitis and normal controls.[7] Some authors describe a low but significant increment in hepatic IFN-γ mRNA, due to a fibrotic process that is correlated with the degree of liver damage.[18,19] The earlier study noted that the plasma concentration of IFN-γ was increased in a patient with chronic liver disease, but such an elevation is not different between cirrhotic and non-cirrhotic patients.[20] It seems that the serum concentration of IFN-γ is not a good indicator of liver cirrhosis.

The hepatic TNF-α expression was elevated and was significantly higher in BDL rats than that in the corresponding sham-operated rats. Although the serum TNF-α level increased progressively, it did not reach a statistically significant difference as compared to the sham-operated rats. Besides, the hepatic and circulatory TNF-α levels were not significantly correlated. Tumor necrosis factor-α has been associated with hepatic necrosis, because an elevated local or systemic TNF-α induced focal hepatic necrosis[1] and increased serum levels of transaminases.[21] Furthermore, hepatic necrosis

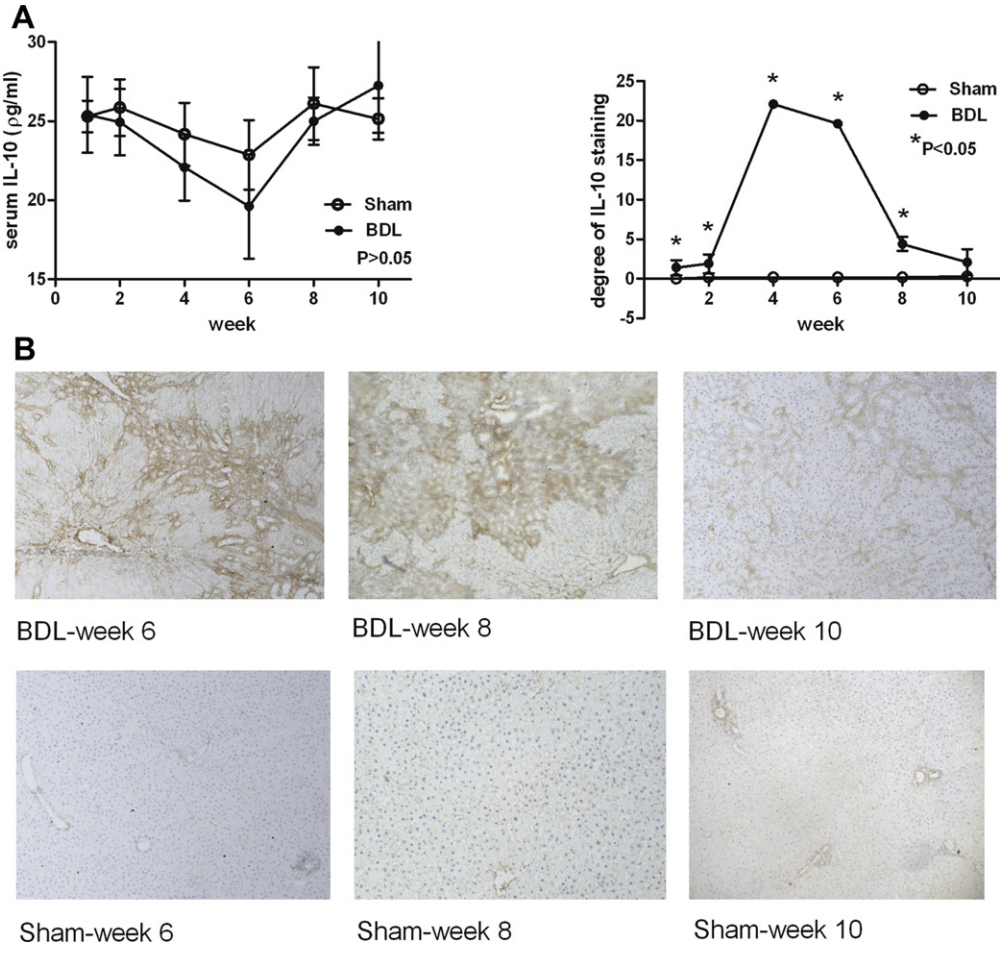

Fig. 5. (A) Kinetics of IL-10 expression in serum and liver tissue at 1, 2, 4, 6, 8 and 10 weeks after sham and BDL surgery. The data are expressed as mean ± SE ($n = 5$ for each group). (B) Representative IHC staining patterns at different time points are shown. Magnification 100×.

could be prevented by TNF-α inhibition.[1,22] In human studies, hepatic TNF-α mRNA expression was increased in cirrhotic patients.[18] Regarding the circulating TNF-α, an elevated plasma concentration of TNF-α reported in patients with chronic liver disease which was significantly higher in cirrhotics than in noncirrhotics.[20] However, in Sprague-Dawley rats, Plebani et al[9] found a stable hepatic TNF-α level throughout the 28 days following BDL. They argued that synthesis and release of TNF-α in biliary cirrhosis appears to be a continuous process that is caused by cholestasis, which partially interferes with the normal metabolism of this cytokine regulated by immunostimulation. Altogether, our data suggest that in biliary cirrhosis, although synthesis of hepatic TNF-α is augmented, the same statistical significance is not found in the concentration of circulating TNF-α due to the change of its release and metabolism.

Compared to the sham-operated group, the hepatic TGF-β expression was enhanced significantly until 10 weeks after BDL. Meanwhile, the serum TGF-β concentration showed a positive correlation with the hepatic expression level and was significantly higher at 6 and 8 weeks after BDL. Clinically, the serum level of TGF-β reflects the severity of liver damage in patients with primary biliary cirrhosis.[23] Studies with animals indicated that TGF-β1 is correlated with hepatic fibrosis and an enhanced hepatic expression of TGF-β1 has been found in BDL rats.[9] Consistent with the results reported by Lee BS et al,[11] the serum TGF-β level reached a peak in the 6th to 8th week in BDL rats. Our finding of hepatic TGF-β expression is also partially in accord with that noted by Plebani et al,[9] showing that the hepatic TGF-β1 expression was augmented fom the 14th to 28th day after BDL in rats.

The liver expression of IL-10 increased progressively after BDL, reached a peak in week 4, and was significantly higher than that of the corresponding sham-operated groups throughout the time course. IL-10 can be synthesized by several cell types in the liver in response to various stimuli.[24] It is known as a cytokine synthesis inhibitory factor for T lymphocytes[25] with potent anti-inflammatory and anti-fibrotic properties.[26] In the study led by Fernandez-Martinez E et al,[10] the amount of hepatic IL-10 was enhanced significantly after BDL. Wang et al[27] reported that in either *in vitro* or *in vivo* condition, the hepatic stellate cells from Wistar rats underwent BDL and showed high increments of IL-10 mRNA and protein on the 7th day after BDL. However, this cytokine induction disappeared in the advanced fibrotic stage (19th day after BDL), indicating that the activation of hepatic stellate cells induced an increased autocrine expression of IL-10 that possessed negative regulatory effects on the production of

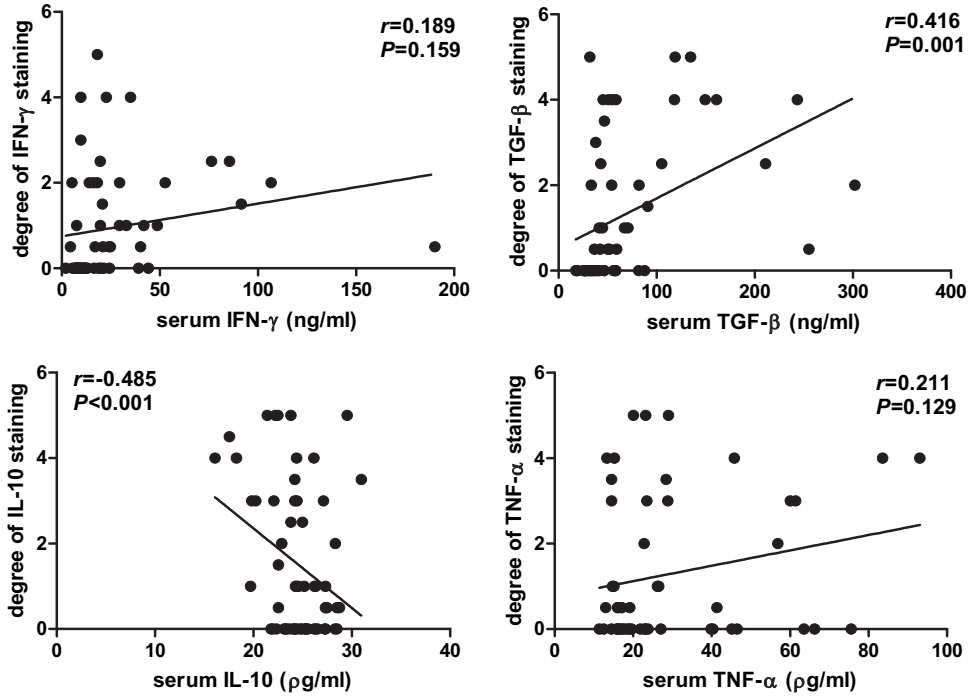

Fig. 6. The correlation between corresponding hepatic expression and serum levels of IFN-γ, TNF-α, TGF-β and IL-10 in all rats.

collagen and promoted collagenase activity. Although we found that the hepatic IL-10 expression remained high throughout the 10 weeks following BDL, this may not be contradictory because we evaluated the whole liver rather than stellate cell IL-10 expression.

To our surprise, the serum IL-10 concentration was negatively correlated with the hepatic IL-10 expression. Furthermore, although not statistically significant, the serum IL-10 concentration of BDL rats tended to be lower than that of the sham-operated rats. The trend is compatible with the finding that in patients with alcoholic cirrhosis, the serum level of IL-10 was lower than that of healthy control subjects.[28] Furthermore, compared with the healthy controls, patients with compensated alcoholic cirrhosis were characterized by

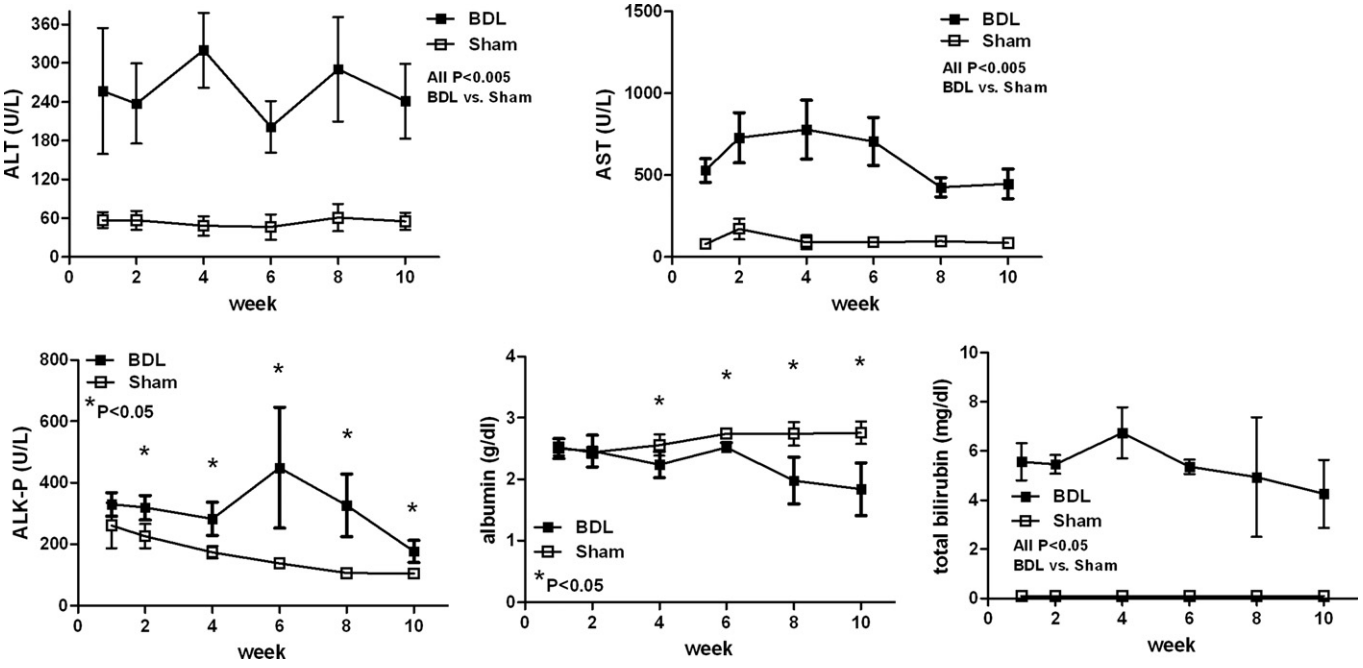

Fig. 7. Plasma levels of ALT, AST, ALP-P, albumin and total bilirubin at 1, 2, 4, 6, 8 and 10 weeks after sham and BDL surgery. The data are expressed as mean ± SE ($n = 5$ for each group).

a decreased serum IL-10 level, whereas the IL-10 level in patients with decompensated alcoholic liver cirrhosis was similar to that in controls.[29] In another human study, most cases of fibrosis in children were associated with low ($< 47^{th}$ percentile) IL-10 factor scores.[30] The underlying mechanism remains obscure, but a recent study noted that 3 weeks after BDL in rats, the liver contributed to the rapid plasma disappearance of IL-10, probably due to an increase in hepatic IL-10 receptor expression.[31] An intriguing possibility is that in cholestatic injury and fibrosis induced by BDL, the counter-regulatory hepatic production of anti-inflammatory and anti-fibrotic cytokine IL-10 was induced. Meanwhile, the intrahepatic IL-10 receptor was up-regulated and the serum IL-10 concentration decreased accordingly.

In conclusion, cirrhosis development in BDL rats is associated with progressive enhancement of both pro-inflammatory and anti-inflammatory cytokine expression in liver. However, only parts of the changes of serum cytokine concentration are consistent with those of hepatic expression, implying a complex interplay of cytokine production, utilization and metabolism in liver injury.

Acknowledgments

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