Expression and Importance of Interleukin-17 and Transforming Growth Factor-β in the Spleens of Hypersplenic Rats

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Received date: October 07, 2017; Accepted date: October 24, 2017; Published date: October 31, 2017

Abstract

**Objective:** We analyzed the expression of interleukin-(IL)-17 and transforming growth factor (TGF)-β in the spleens of rats suffering from liver cirrhosis and hypersplenism, and investigated the cause of peripheral cytopenias.

**Methods:** Fifty-five male Sprague–Dawley rats were divided randomly into two groups. The control group (n=15) was gavaged with physiologic (0.9%) saline. The experimental group (n=40) was gavaged with 40% CCL4/peanut oil solution at 0.3 mL/100 g twice a week for 8 weeks, and a 15% alcohol solution was given as drinking water, to establish a model of liver cirrhosis and hypersplenism. After confirmation of liver cirrhosis and hypersplenism, spleen tissue was collected to determine expression of IL-17 and TGF-β by Immuno Histo Chemical (IHC), western blotting, and real-time fluorescence-based quantitative polymerase chain reaction (PCR).

**Results:** IHC staining revealed positive expression of IL-17 and TGF-β in the experimental group to be 36.67% and 73.33% respectively, which was significantly higher than that in the control group (6.67% and 13.33%, respectively; χ²=4.60, P<0.05; χ²=14.46, P<0.01). Western blotting revealed the relative expression of IL-17 and TGF-β in the experimental group (1.09 ± 0.39, 1.51 ± 0.22) to be significantly higher than that in the control group (0.53 ± 0.25, 0.63 ± 0.17) (t= -5.96, P<0.01; t= -7.35, P<0.01). Real-time fluorescence-based qPCR showed relative expression of IL-17 and TGF-β mRNA in the experimental group to be 2.81 ± 0.70 and 2.91 ± 0.63, respectively, which was significantly higher than that in the control group (1.06 ± 0.21 and 0.99 ± 0.052, respectively) (t= -5.96, P<0.01; t= -7.35, P<0.01). Pearson correlation analyses showed the relative mRNA expression of IL-17 to be positively correlated with that of TGF-β (R=0.520, P<0.01).

**Conclusion:** Expression of IL-17 and TGF-β was increased significantly and positively correlated in the spleens of rats suffering from liver cirrhosis and hypersplenism, which may have caused peripheral cytopenias.

Keywords: Hypersplenism; Spleen; IL-17; TGF-β; Immunohistochemical; Western blotting; Real-time fluorescence-based quantitative polymerase chain reaction

Introduction

Often, hypersplenism (i.e., an overactive spleen) is secondary to hepatic cirrhotic portal hypertension. Major manifestations include splenomegaly and peripheral cytopenias [1], which can affect the prognosis [2]. Various theories have been postulated with regard to the cause of peripheral cytopenias [3]. Nevertheless, the spleen is the largest lymphoid organ in humans, so the cytokines secreted by lymphocytes cannot be underestimated [4,5]. Approximately 35–50% of splenic lymphocytes are T lymphocytes, and 50–65% is B lymphocytes. T lymphocytes are involved in cellular immune responses, and play important part in anti-tumor immune responses. B lymphocytes differentiate into plasmocytes, which produce antibodies under antigenic stimulation, and then participate in the humoral immune response. The main role of B lymphocytes is the immune response to infection. T lymphocytes are classified into T helper 1 (Th1), Th2, Th17, and T regulatory (T-reg) cells according to the regulation by different transcription factors. The balance between Th1 and Th2 has an important regulatory role in the immune system [2]. Th17 cells are a newly discovered subset of T helper cells, and they secrete interleukin (IL)-17 specifically. Th17 cells are involved in immune responses against intracellular bacterial and fungal infections, and the autoimmune inflammatory response. T-reg cells mainly secrete immune regulatory factors such as transforming growth factor (TGF)-β and IL-10. T-reg cells control the activation and proliferation of auto reactive T cells and various immune functions. T-reg and Th17 cells have been reported in some autoimmune and inflammatory diseases, such as primary biliary cirrhosis and rheumatoid arthritis [6–8]. The aim of the present study was to investigate the relationship between expression of IL-17 and TGF-β in the spleens and peripheral circulation of hypersplenic rats.

Materials and Methods

Ethical approval of the study protocol

The study protocol was approved by the Ethics Committee of Hainan Provincial People’s Hospital (Haikou, China).
Liver cirrhosis and creation of a model of hypersplenism

Fifty-five male Sprague-Dawley rats were divided randomly into two groups. The control group (n=15) was gavaged with physiologic (0.9%) saline. The experimental group (n=40) was gavaged with 40% carbon tetrachloride (CCL₄)/peanut oil solution at 0.3 mL/100 g body weight twice a week for 8 weeks, and a 15% alcohol solution was administered as drinking water, to establish a rat model of liver cirrhosis and hypersplenism. After the induction of anesthesia (1% pentobarbital sodium, i.v.), orbital blood samples were obtained for testing of liver function and other blood components. One rat was killed randomly, and its liver and spleen tissues harvested. These tissues were fixed in 10% neutral formalin solution, embedded in paraffin, sectioned, and stained with Hematoxylin & Eosin (H&E) and Masson’s trichrome. Liver cirrhosis and hypersplenism were confirmed by a very experienced pathologist based on analyses of sections. Finally, both groups of rats were killed. Freshly harvested spleen tissue was used for immune histo chemical (IHC; fixation in 10% formaldehyde solution), western blotting (preservation at -80°C), and real-time fluorescence-based quantitative polymerase chain reaction (qPCR) analyses.

IHC analyses

Splenic tissue fixed with 10% formaldehyde solution was cut into sections (thickness, 4-μm) dewaxed and hydrated. Next, sections were pretreated by pressure cooking in pH 6.0 citrate buffer, then blocked in 3% hydrogen peroxide for 5 min, and washed with phosphate-buffered saline. Then, sections were incubated with primary antibodies against IL-17 (1:100 dilution) and TGF-β (1:100) for 2 hat 37°C, washed with phosphate-buffered saline to remove excess primary antibodies, and incubated with secondary antibody immunoglobulin-G for 15 min at 37°C. After color development with Diaminobenzidine for 3–5 min, counterstaining with hematoxylin for 1 min, and differentiation with 1% hydrochloric acid in alcohol for 8–9s, sections were rinsed with running water for 5–10 min, dehydrated with 80%, 95%, and 100% anhydrous ethanol for 1 min, cleared with xylene, and mounted with neutral gum. Positive expression of TGF-β and IL-17 was observed under light microscopy. Brown-yellow cytoplasm in the field of view indicated positive expression of TGF-β or IL-17. Five non-overlapping fields of view were selected randomly from each film, and photographed at high power (×400 magnification) (1600 × 1200 pixels). Prevalence of positive expression was calculated using the following equation:

\[
\text{Prevalence of positive expression} = \frac{\text{positive cell count}}{\text{total cell count}} \times 100\%
\]

Western blotting

Each sample was treated with 300 μL of cell lysis buffer and mixed until complete lysis of cells. Next 10 μL of the sample mixture was mixed with 10 μL of 2 × sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) loading buffer. The mixture was heated for 5 min at 100°C, cooled on ice, and centrifuged at 12,000 x g for 5 min to remove sediment.

Then, samples were separated by 10% SDS–PAGE at 20 μL per well. Then, membranes were transferred and blocked, incubated with primary antibodies for 2 hours at 37°C, then with a secondary antibody for 2 hours at 37°C, developed, fixed, and photographed with a gel-documentation system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference protein. Relative expression was based on the ratio of target protein expression to GAPDH expression.

Real-time fluorescence-based qPCR

Total RNA was extracted from spleen samples. Absorbance at 260 nm (A260) and at A280 was determined, and the A260/A280 ratio calculated to determine the purity of total RNA. If the A260/A280 ratio was 1.8–2.0, reverse transcription was carried out. Briefly, the reaction system consisted of 10 μL of 2x reverse transcription buffer, 1 μL of oligo-dT reverse transcription primer (20 μM), 2 μL of total RNA, 0.2 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL) and diethyl pyrocarbonate-treated water to a final volume of 20 μL. Reaction conditions were: 42°C for 30 min; 95°C for 10 min. The fluorescence-based qPCR reaction system consisted of 10 μL of 2 × quantitative PCR Master Mix, 0.08 μL of upstream primer (20 μM), 0.08 μL of downstream primer (20 μM), 2 μL of cDNA template, and double-distilled water to a final volume of 20 μL. Reaction conditions were: 95°C for 3 min; 95°C for 12 s; 62°C for 40 s for 40 cycles.

Statistical analyses

All data had a normal distribution, and are presented as the mean ± standard deviation. Thus, the independent-samples t-test and χ² test were carried out to compare levels of chemokine receptor proteins and the prevalence of positive cells between the two groups. Differences were considered significant at P<0.05. All analyses were processed by SPSSv19.0 (IBM, Armonk, NY, USA).

Results

Physical condition of rats

Rats in the control group had glossy fur and a good appetite, were active and in good spirits. Rats in the experimental group had dull-yellow messy fur/fur loss, were not eating, with no weight increase/loss administration. They were inactive, in poor spirits, insensitive to external stimuli, and had jaundice. Symptoms became increasingly severe. Finally, symptoms of approaching death (weak breathing and inability to stand) were observed. Eventually, 10 rats died (25%) and 30 model rats were produced.

Changes in peripheral blood cell counts

Changes in peripheral blood cell count in the experimental and control groups are shown in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=15)</th>
<th>Experimental group (n=30)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (×10³/L)</td>
<td>6.25 ± 2.22</td>
<td>25.67 ± 8.97</td>
<td>-5.589</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>RBC (×10¹²/L)</td>
<td>7.95 ± 0.57</td>
<td>6.18 ± 1.27</td>
<td>3.485</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PLT (×10³/L)</td>
<td>1115.14 ± 148.09</td>
<td>432.47 ± 127.81</td>
<td>11.112</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Table 1: Comparison of peripheral blood cell counts (±S) between the two groups.
Changes in liver function

Levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin in the experimental group were significantly higher than those in the control group (P<0.01). Levels of total protein and albumin were significantly lower in the experimental group than in the control group (P<0.01) (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=15)</th>
<th>Experimental group (n=30)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>25.14 ± 4.26</td>
<td>267.93 ± 30.65</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>123.86 ± 18.60</td>
<td>714.73 ± 143.56</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TBIL(µmol/L)</td>
<td>1.43 ± 0.38</td>
<td>23.49 ± 6.36</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>64.9 ± 2.71</td>
<td>55.24 ± 5.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>30.23 ± 1.51</td>
<td>24.94 ± 2.94</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 2: Comparison of liver function (±S) between the two groups.

Pathologic changes in the liver

For rats in the control group, the liver was bright red, sharp-edged, and had a tough texture. Liver cells were ordered, with only a small amount of fibrous tissue. For rats in the experimental group, the liver was enlarged, gray-brown or yellow-brown, with unequal-sized diffuse nodules on the surface, a thickened capsule, and a blunt edge. Liver cells were disordered and many collagen fibers, as well as fibrous septa, were observed. The normal lobular structure of the liver was destroyed, and pseudo-lobules were formed (Figure 1).

The spleen index was 3.32 ± 1.02 for the experimental group, which was significantly higher than that for the control group (2.31 ± 1.13) (t = -2.452, P<0.05).

Pathologic changes in the spleen: For rats in the control group, the spleen was dark red, crisp and soft in texture with a sharp upper edge and a relatively blunt lower edge. White pulp was well-developed and clearly demarcated from red pulp. Splenic nodules were round or oval. Germinal centers were apparent and scattered in red pulp, with very few (and relatively thin) reticular fibers. For rats in the experimental group, the spleen was swollen, with a blunt edge. The splenic capsule was thickened. The splenic sinus and red pulp were enlarged, where as white pulp was diffus. The boundary zone had disappeared and fibrous tissue had proliferated. Splenic arteries were thickened, with reduced luminal volume. The splenic trabecula was thickened. Red-stained non-structural material was observed in the inner membrane (Figure 2).

Spleen index and pathologic changes

Spleen index: The spleen index is given by the following equation:

Spleen index = spleen weight (mg)/body weight (g)
Figure 3: Immunohistochemical staining (hematoxylin, ×400) for IL-17 and TGF-β. A: IL-17 expression in the control group; B: IL-17 expression in the experimental group; C: TGF-β expression in the control group; D: TGF-β expression in the experimental group.

Figure 4: Positive expression of IL-17 and TGF-β (%) in splenic tissue.

Western blotting

Relative expression of IL-17 and TGF-β in the experimental group was 1.09 ± 0.39 and 1.51 ± 0.22, respectively. These data were significantly higher than those for the control group (0.53 ± 0.25 and 0.63 ± 0.17, respectively) (t= -3.227, P<0.01; t= -9.264, P<0.01) (Figures 5 and 6).

Real-time fluorescence-based qPCR

Relative mRNA expression of IL-17 and TGF-β in the spleen in the experimental group was 2.81 ± 0.70 and 2.91 ± 0.63, respectively. These values were significantly higher than those in the control group (1.06 ± 0.21 and 0.99 ± 0.052, respectively) (t= -5.96, P<0.01; t= -7.35, P<0.01) (Figure 7).
Pearson correlation analyses

Pearson correlation analyses revealed positive correlation between the relative expression of IL-17 mRNA and TGF-β mRNA (R = 0.520, P<0.01) (Figure 8).

Discussion and Conclusion

Liver cirrhosis can be induced in rats by gavage with 40% CCL4 [9]. Hypersplenism can be induced by gavage for 8 weeks. Splenic enlargement, a significantly increased spleen index, expansion of the splenic sinus, increased amounts of fibrous tissue, and trabecular thickening in the spleen, as well as significantly reduced RBC and platelet counts in peripheral blood, were observed in the present study. Increased WBC counts were due to a systemic inflammatory response after modeling. This method had high mortality (25%, mainly due to improper administration), but it is reliable and conducive to hypersplenism research.

As demonstrated by IHC staining, western blotting, and real-time fluorescence-based qPCR, positive expression, relative protein expression, and relative mRNA expression of IL-17 and TGF-β in rats suffering from cirrhosis and hypersplenism were significantly higher than those in the control group. These observations are in accordance with the reports of other scholars [10–12]. We noted a positive correlation between relative mRNA expression of IL-17 and TGF-β (R=0.520, P<0.01). Nonetheless, the IL-17/TGF-β ratios in the experimental group (0.50, 0.84, 0.97) were not significantly different from those of the control group (0.50, 0.72, 1.07) (P>0.05). The roles of IL-17 and TGF-β in hypersplenism are based mainly on reducing peripheral blood cell counts in different ways.

IL-17 is a potent pro-inflammatory factor involved in immune responses against bacterial and fungal infections, and the autoimmune inflammatory response. A significant increase in IL-17 expression can induce production of large amounts of anti-RBC antibodies, which attack RBCs and lead to their extensive dissolution and destruction [13]. Kim et al. [14] suggested that the effect of IL-17 on changes in blood cells is mainly through regulation of hematopoietic and immune functions as well as stimulation of the development of eosinophils and B lymphocytes. Increased numbers of eosinophils in the spleen enhance the phagocytosis and destruction of blood cells. IL-17 may also induce macrophages, monocytes or dendritic cells to produce factors that activate B lymphocytes, there by stimulating the latter to secrete large amounts of anti-platelet antibodies. The latter make platelets susceptible to destruction by the mononuclear phagocyte system (mainly in the spleen) and reduce platelet formation by inhibiting megakaryocyte maturation. IL-17 is also a key factor affecting bone-marrow stem cells [15–18]. It suppresses bone-marrow hematopoiesis by stimulating macrophages to secrete negative hematopoietic regulators (e.g., IL-6), resulting in reduced hematopoiesis [19].

TGF-β is the most potent inhibitor of blood cells. It inhibits progression of cells from the G1 phase to the S phase by regulation of the expression of cell cycle-related molecules. In this way, it maintains hematopoietic stem cells and progenitor cells in the resting state, thereby inhibiting cell proliferation and promoting differentiation and apoptosis, resulting in reduced hematopoiesis [20]. It has been reported that long-term injection of recombinant human TGF-β into mice leads to severe, progressive inhibition of erythropoiesis. Such mice develop severe anemia, with decreased counts of reticulocytes, bone marrow and spleen erythroid progenitor cells in vivo. This effect could be prevented by injection of erythropoietin. It may be because TGF-β injection in mice induces expression of tumor-necrosis factor-α, which reduces the erythropoietin level in vivo, thereby resulting in inhibition of the effects of erythropoietin [21]. TGF-β can inhibit the proliferation and activation of human hematopoietic progenitor cells directly, and can suppress hematopoiesis indirectly. In addition, splenic fibrosis is a prominent pathologic change in splenomegaly due to portal hypertension. TGF-β is a key mediator of splenic fibrosis [22]. Increases in TGF-β levels can lead to retention of many blood cells in the spleen. This phenomenon is conducive to phagocytosis and destruction by macrophages, there by resulting in peripheral cytopenias.

Funding Source

This work was supported by the Hainan Provincial Key Scientific and Technological Research and Development Projects (ZDYF2016158).

References