Chapter II

Novel murine autoimmune-mediated liver disease model induced by graft-versus-host reaction and concanavalin A.

1. Introduction

Primary biliary cirrhosis (PBC) is still a disease of unknown etiology, with progressively destroyed intra-hepatic bile ducts. In many respects, PBC is analogous to the chronic graft-versus-host reaction (cGVHR) in cases such as bone marrow transplants and in mice sensitized to foreign major histocompatibility complex (MHC) molecules [1,2]. We have analyzed PBC-like hepatic lesions and the appearance of antimitochondrial antibodies (AMA) induced by murine GVHR in B6 T cell- injected MHC class II disparate (bm12 x B6) F1 mice [3-6]. Because bm12 mice have mutations in three amino acids at the *I-A*^{b} regions of MHC, and because the other genetic composition of the mice is identical to the B6, this model could assess MHC class II-different GVHR. In these mice cluster of differentiation (CD)4$^+$ T cells have been considered to be essential for the induction of PBC-like hepatic lesions [7,8]. Histologically, the infiltration of CD4$^+$ T cells around the bile ducts is observed from 5 days after T-cell transfer. To clarify the relationship between the formation of hepatic lesions and the cytokine profile, we have examined the expression level of cytokine mRNA of liver infiltrating CD4$^+$ T cells in this model. Early production of interferon (IFN)-γ and delayed production of interleukin (IL)-10 was observed, and these might play an important role in the formation of our GVHR hepatic lesions [5].

However, this GVHR system did not progress into fibrosis or cirrhosis, that is, it was self- limited [7]. Therefore, this model is insufficient for the analysis of the progression of the autoimmune-related mechanism. We then focused on the T-cell-activate function of concanavalin A (Con A). Concanavalin A is a lectin and a T-lymphocyte mitogen *in vitro* that leads to the production of cytokines and lymphocyte proliferation [9]. Moreover, it has been demonstrated recently that Con A induced T cell-mediated hepatic lesions [10]. Tiegs *et al.* reported that mice developed severe liver injury when an intravenous dose of >1.5 mg/Kg of Con A was administered, as assessed by transaminase release within 8 h [10]. Confluent hepatic necrosis, preferentially in the intermediate zone, occurred at 8h after Con A injection, and continued to develop at 24h [11]. After 24h, a moderate infiltration of mononuclear and polymorphonuclear cells, including a large number of CD4$^+$ T cells, were observed in the portal area within the Glisson's capsules and the central veins [12]. Mice with severe

combined immunodeficiency syndrome as well as athymic nude mice were resistant against Con A. The pretreatment with monoclonal antimouse CD4 antibodies fully protected mice against Con A, whereas monoclonal antimouse CD8 antibodies failed to protect [10]. Taken collectively, $CD4^+$ T cells might be implicated in the pathogenesis of Con A-induced hepatic injury.

In PBC and autoimmune hepatitis, $CD4^+$ T cells are considered to be an important subset for the initiation of autoimmune-mediated hepatic injury [13-15]. Therefore, we are interested in the analysis of the autoimmune mechanism underlying murine GVHR administrated Con A in which $CD4^+$ T cells play an important role in the formation of the disease.

The aim of the present study is to clarify whether Con A deteriorates autoimmune-mediated hepatic lesions induced by GVHR, and to elucidate the participation of the cytokines of liver-infiltrating $CD4^+$ T cells.

2.Materials and Methods

Animals

C57BL/6(B6)[*H-2K*^{b}, *I-A*^{b}, *H-2D*^{b}] mice were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). B6.*C-H-2*^{bm12}(bm12)[*H-2K*^{b}, *I-A*^{bm12}, *H-2D*^{b}] mice with a mutation at *I-A* region, which originated from the Jackson Laboratory (Bar Harbor, ME, USA), were used to obtain (bm12 x B6) F1 hybrid mice. All animals received human care in accordance with the guidelines of the University of Tsukuba for the care of laboratory animals.

Treatment of mice

The female F1 mice between 10 and 15 weeks of age received donor B6 T cells for the induction of GVHR as previously reported [16]. Briefly, spleen cell suspension was treated with 0.83% ammonium chloride-Tris buffer to remove red blood cells, followed by passage through a nylon wool column for purification of T cells. Sex-matched 1-2 x 10^7 B6 T cells were injected into recipients via the tail vein. Concanavalin A (Vector laboratories, Burlingame, CA, USA) was dissolved in pathogen-free saline, and injected intravenously at a dose of 15 mg/Kg. We divided the mice into four groups (Fig. 2-1). Normal control mice (NML) were assigned to group 1. Group 2 (Con A) mice were injected with Con A without the induction of GVHR. Group 3 (GVHR) mice were induced with GVHR, and group 4 (GVHR + Con A) mice were injected with Con A 5 days after the induction of GVHR. At day 14, cell-transferred mice (groups 3 and group 4) were killed as well as group 1 mice at the same age. Group 4 mice were evaluated 9 days after Con A injection. Similarly, group 2 mice were sacrificed 9 days after Con A injection. We performed liver perfusion to eliminate blood contained in liver by the injection of 2 ml of phosphate-buffered saline (PBS) via the portal vein. Immediately after the perfusion, the liver and spleen were removed.

Assessment of the occurrence of graft-versus-host reaction

The spleen was weighed separately to calculate the spleen index by the following formula:

Spleen index = spleen weight (mg)/body weight (g).

The occurrence of GVHR in the cell-transferred group was confirmed by the

elevated spleen indices as compared with that of non-cell-transferred mice [3,4].

Assay of serum transaminase and alkaline phosphatase activity

At the time when the mice were killed, approximately 1mL of blood was drained through the heart, and serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), and serum alkaline phosphatase (ALP) activities were measured by using the JSCC (Japan Society of Clinical Chemistry) consensus method.

Autoantibodies titers

Levels of antipyruvate dehydrogenase (PDH) complex immunoglobulin (Ig)G antibodies, which are members of AMA [17], and antinuclear IgG antibodies (ANA) were measured by using an enzyme-linked immunosorbent assay (ELISA). Samples of serum that were diluted at 1:1000 and 1: 200 were applied to 96-well microplates with MESACUP mitochondria M2 (MBL, Nagoya, Japan) and MESACUP ANA test (MBL, Nagoya, Japan), respectively. After a 1-h incubation, peroxidase conjugated $F(ab')_2$ fragments of goat antimouse IgG specific antibodies (Caltag, San Francisco, CA, USA) at 1:3000 in a dilution buffer was added to each well. After the incubation, *o*-phenylenediamine substrate at a concentration of 0.4 mg/mL sodium phosphate and 0.1 mol/L citrate buffer, pH 5.0, were added. The absorbance was measured at 492 nm for AMA and 450 nm for ANA by using an Emax precision microplate reader (Wako, Osaka, Japan).

Histological analysis

After the mice were killed, small blocks of the liver left lobe were excised from each mouse and fixed in 10% buffered formalin. Paraffin sections were made and stained with hematoxylin-eosin and silver by using standard methods for histological examination.

Preparation of $Thy1.2^+CD4^+$ T cells

The liver was passed through a 200-gauge stainless steel mesh and suspended in a minimal essential medium supplemented with 5% fetal calf serum. Hepatic mononuclear cells were prepared by using 35% Percoll (Pharmacica Biotech, Tokyo, Japan) solution and red blood cells lysis solution

as previously described [18,19]. We then isolated 5 x 10^4 Thy1.2$^+$CD4$^+$ T cells by using a FACS vantage cell sorter (Becton Dickinson, Sunnyvale, CA, USA). In brief, after blocking Fc receptors on hepatic mononuclear cells by Anti-Fc receptor monoclonal antibodies (mAbs; 2.4G2, rat IgG2), the cells were stained with FITC-conjugated anti-Thy1.2 mAbs and R-Phycoerythrin (PE)-conjugated anti-CD4$^+$ mAbs (Caltag laboratories, San Francisco, CA, USA) for 30 min. Liver-infiltrating Thy1.2$^+$CD4$^+$ T cells (5 x 10^4) were sorted from two- to three-pooled cell-transferred mice and also from six-pooled untreated mice. Purity of the sorting was more than 98%.

Semiquantitative analysis of cytokine mRNA using reverse transcription-polymerase chain reaction

Total RNA was prepared from 5 x 10^4 liver-infiltrating Thy1.2$^+$CD4$^+$ T cells by Isogen-LS (Nippon Gene, Osaka, Japan) according to the manufacture's instructions. RNase-free DNase I (Nippon Gene, Osaka, Japan) was used to avoid the contamination of genomic DNA during the total RNA extraction. Total RNA (5 μL) was reverse transcribed to cDNA in a total volume of 20 μL by using a SuperScript preamplification system (Gibco-BRL, Gaithersburg, MD, USA). Commercially available PCR primers specific for IFN-γ, IL-10, β-actin (Clontech laboratories, Palo Alto, CA, USA), transforming growth factor (TGF)-β1 primers (5'-CCT CCC CCA TGC CGC CCT CG-3' as the forward primer, 5'-CCA GGA ATT GTT GCT ATA TTT CTG as the reverse primer)[20], and tumor necrosis factor (TNF)-α primers (5'-GGT GCC TAT GTC TCA GCC TCT-3', 5'-CAT CGG CTG GCA CCA CTA GTT-3')[21] were used. Amplification was carried out in a Program Temp Control System PC-700 (Astec, Fukuoka, Japan) after an initial denaturation at 95°C for 1.5 min. Different conditions were selected empirically for different cytokines by choosing the annealing temperature and cycles at which the maximum amplification could be achieved with minimal non-specific bands. The annealing temperature of IL-10 and IFN-γ was 55°C, whereas that of TNF-α, TGF-β1, and β-actin was 60°C. β-actin was used for the internal standard and validation of the RNA extraction and RT- PCR. The PCR products were visualized by using ethidium bromide-stained 2% agarose gel electrophoresis. Six independent RT-PCR experiments were performed. In the experiments involving a quantitative assessment of IFN-γ (Th1) and IL-10 (Th2) mRNA,

the amounts of fluorescence intensity from each group were measured by using a FluorImager (Molecular Dynamics, Sunnyvale, CA, USA) [22]. The data were expressed relative to the amounts of β- actin mRNA.

Statistical analysis

The results are expressed as Mean ± SE in each group. An ANOVA test was applied to the survival rate, spleen index, the level of the serum transaminase, AMA and ANA titers, and the semiquantitative analysis of cytokine mRNA. A level of $P<0.05$ was judged to be significant.

3. Results

Survival rate

All the mice of group 3 (GVHR) survived 14 days after the cell transfer. The survival rate of group 2 (Con A) and group 4 (GVHR + Con A) mice was 73.6 ± 2.9% and 56.1 ± 6.7% (Mean ± SE), respectively. The average survival rate of group 4 mice was significantly decreased compared to that of group 2 mice ($P<0.05$). The results indicated the average of six independent experiments using four to six mice per experiment. All the dead mice had died within 24 h after the injection of Con A.

Evaluation of changes in the spleen index

We examined the induction of GVHR by using the spleen index. As shown in Table 2-1, the average of the spleen index was 3.3 in group 1, 5.3 in group 2, 12.7 in group 3, and 11.8 in group 4. In GVHR-induced mice (groups 3 and 4), the average spleen index was significantly increased compared with that of group 1 (NML) and group 2 (Con A; $P<0.001$). However, there was no significant difference between groups 3 and 4 mice.

Biochemical analysis of liver injury

In order to substantiate the inflammation of the liver biochemically, we examined the serum level of transaminase. In the previous GVHR model, the increase of the serum transaminase was not recognized. Importantly, at day 14, GVHR + Con A mice (group 4), and at day 9 Con A-only injected mice (group 2), revealed a significant increase of AST ($P<0.001$) and ALT ($P<0.05$) levels compared with control mice (Fig. 2-2). In contrast, there was no significant change in serum alkaline phosphatase level in each group (data not shown).

Autoantibodies titers

To analyze the features of the autoimmune-mediated hepatic injury, we measured the titers (OD) of AMA and ANA. As shown in Table 2-2, GVHR-induced groups (groups 3 and 4) produced a higher level of AMA and ANA than the other groups. However, there was no significant difference between groups 3 and 4 mice.

Morphological changes in graft-versus-host reaction hepatic lesions by concanavalin A

On histological examination, a moderate degree of mononuclear cell infiltration was observed in the portal area and around the central veins in the hepatic lobule in group 3 mice at day 14 as previously described (Fig. 2-3A)[3-7]. Focal intraepithelial lymphocyte infiltration was noticed. In the mice from group 4, mononuclear cell infiltration in the portal area worsened, and the foci of piecemeal necrosis were observed at day 14 (Fig. 2-3B). The bile duct epithelia of group 4 showed a swelling of the nucleus and cytoplasm. Intraepithelial infiltration of mononuclear cells was also observed (Fig. 2-3C). In contrast, in the mice from group 2, quite mild infiltration of mononuclear cells was shown in the portal area 9 days after the Con A injection. Neither piecemeal necrosis nor bile duct lesions were observed (Fig. 2-3D). In the portal areas of group 4, we recognized granulomatous lesions (Fig. 2-3E). Furthermore, focal portal-central and portal-portal bridging necrosis was observed in group 4 (Fig. 2-3F). In the mice from group 4, these hepatic lesions were constantly observed for up to 56 days. Focal parenchymal necrosis localized in the subcapsular area was observed in the mice both of groups 2 and 4, however, these lesions disappeared over time following the observation period.

Cytokine profiles of liver-infiltrating Thy1.2$^+$CD4$^+$T cells

To elucidate the cytokine profiles of liver-infiltrating CD4$^+$ T cells in this model, we performed RT-PCR. We used the expression of β-actin mRNA as an internal standard. The expressions of IFN-γ and TGF-β1 mRNA were detected in all groups, while the bands of IL-10 and TNF-α were visualized only in groups 3 and 4. We measured the amounts of fluorescence intensity from each group for the quantitative assessment of IFN-γ (Th1) and IL-10 (Th2) mRNA. As shown in Fig. 2-4A, relative amounts of IFN-γ mRNA in group 4 mice (0.77 ± 0.07) were significantly higher than that in group 3 mice (0.57 ± 0.09), whereas concerning IL-10 mRNA (Fig. 2-4B), there was no significant difference between groups 3 and 4 (0.59 ± 0.06, 0.51 ± 0.07, respectively).

4. Discussion

We have reported PBC-like hepatic lesions induced by murine GVHR. In GVHR mice, damage to the epithelia of the small intrahepatic bile ducts and mononuclear cell infiltration were observed [4,5,23]. However, they did not progress into fibrosis or cirrhosis. Nevertheless, the suitable animal model that demonstrates the deterioration of liver injury or fibrosis mediated by autoimmune reaction is considered to be important to investigate the mechanism of the progression.

Therefore, we examined whether or not Con A deteriorates murine hepatic lesions induced by GVHR. The reason was that $CD4^+$ T cells have been reported to be crucial in both hepatic lesions [7,8,10,12]. Serum transaminase levels were slightly elevated in both groups of GVHR-added Con A mice and Con A only injected mice. Mice from both groups showed focal parenchymal necrosis localized in the subcapsular area 9 days after Con A injection; however, these lesions disappeared over time. Although bridging necrosis was observed in only GVHR-added Con A mice, there was no significant difference regarding the elevation of serum transaminase levels between these two groups at this point. No change was observed in serum transaminase levels in GVHR mice. Concanavalin A might be involved in the mild elevation of serum transaminase levels. Antimitochondrial antibodies and ANA appeared in both groups of GVHR mice and GVHR-added Con A mice. Namely, the pathogenic mechanism of the autoimmunity of GVHR was preserved in the GVHR-added Con A mice. However, a significant decrease of the survival rate and the worsened portal inflammation, piecemeal necrosis and bridging necrosis were observed only in the GVHR-added Con A mice. These findings cannot be explained by the additive effect of Con A. Concanavalin A might have synergistic destructive effects or blocking effects of the self-limited mechanism on the undergoing GVHR hepatic lesions. Pathological findings of GVHR-added Con A mice are not typical for PBC and autoimmune hepatitis, but show some autoimmune phenomena in the liver as the production of autoantibodies. We could show the animal model of GVHR-added Con A mice which demonstrated the progression of autoimmune-mediated hepatobiliary injury with the elevation of transaminase levels.

We speculated several mechanisms to explain why GVHR-added Con A mice showed the progression of liver injury. They include: (1) The infiltrating $CD4^+$T cells in the liver of GVHR mice might be activated followed by Con A; (2) $CD4^+$T cells were supposed to recognize the Con A-modified MHC of macrophage [10,24], therefore Con A might elicit new a autoreactive reaction undergoing GVHR; and (3) The blood flow disturbance by Con A might succeed to the inflammation induced by GVHR. It was reported that Con A activated T cells directly *in vitro* [9], The cytokines produced from the activated T cells might deteriorate the inflammation in the liver. Moreover, Con A might influence the interaction of macrophage and $CD4^+$ T cells by a mechanism different from that of LPS, because Hayashi *et al.* demonstrated that LPS did not modify the liver injury undergoing murine MHC class II GVHR [25]. On the other hand, Miyazawa *et al.* showed the involvement of intrasinusoidal hemostasis in Con A hepatitis [11]. Therefore, the blood flow disturbance might affect the GVHR-induced hepatic lesion.

In this study we sorted liver-infiltrating $CD4^+$ T cells and performed RT-PCR for cytokines mRNA to analyze the cytokine profile in liver. As IFN-γ (Th1) and IL-10 (Th2) have been suggested to play an important role in the formation of GVHR hepatic lesions [5], we focused on these cytokines for the quantitative assessment in this study. The elevation of IFN-γ mRNA in GVHR-added Con A mice was observed. The expression level of IL-10 in GVHR-added Con A mice was similar to that in GVHR mice, although it was significantly higher than in other groups. We have reported that IFN-γ might play an important role in the pathogenesis of the PBC model using GVHR mice [5]. In Con A hepatitis, IFN-γ [26–30] or TNF-α[12,21,31] is suggested to be a crucial cytokine. Interferon-γ regulates MHC class I and class II protein expression on a variety of immunologically important cell types [32-34], and activates cytotoxic T cells and macrophages [33,35]. In our GVHR-added Con A model, IFN-γ might participate in the progression of the inflammation. Interleukin-10 is suggested to be produced locally in the liver during Con A-[36] or CCl_4-[37,38] induced liver injury. Moreover, we have recently reported the delayed IL-10 mRNA expression of liver infiltrating $CD4^+$ T cells in GVHR mice [5]. Therefore, IL-10 might be produced in the liver by a reactive mechanism during liver injury. The ability of endogenous and exogenous IL-10 to modulate the inflammatory response and to decrease hepatotoxicity was

shown in several models of liver injury [36,39-41]. Moreover, IL-10 was demonstrated to be antifibrogenic in the mouse model of liver injury induced by carbon tetrachloride [37,38]. In contrast, IL-10 was suggested to have an immunostimulating effect on $CD8^+$ T cells [42], and to exacerbate the murine GVHR [43]. Louis *et al.* showed murine liver fibrosis induced by repeated Con A challenge and the implication of IL-10 in liver [44]. Interleukin-10 and TGF-β1, which is one of the fibrogenic factors detected in our GVHR-added Con A model, might play an important role in our model whether or not developing into fibrosis. Therefore, in our model, analyzing the function of IL-10, especially derived from liver-infiltrating $CD4^+$ T cells, might lead us to the elucidation of the immunopathogenesis mechanism and application to autoimmune-mediated liver injury.

5. Summary

We have shown that Con A deteriorates hepatic lesions undergoing self-limited murine GVHR due to MHC class II disparity. An advantage of our model is the exacerbation in comparison with the GVHR model, and the durability of inflammation with Con A hepatitis. Interferon-γ and IL-10 might be involved in the progression of liver injury. Although the pathological findings are not typical of autoimmune hepatitis and PBC, this model might offer a unique aspect for the investigation of the progressive mechanism of T cell-mediated hepatobiliary injury. With this inducible animal model, it might be possible to characterize factors or pharmaceuticals leading to downregulation of liver injury.