Figure 6 – NK cell activation and anti-metastatic effects in GKO mice. (a) Anti-metastatic effects. GKO mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. After 14 days, the mice were sacrificed to examine tumor development in the liver. The liver weight was compared between the groups ($n = 8$/group). Experiments were performed 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (b) Yac1 lytic ability. GKO mice were hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Four days later, splenocytes isolated from the mice were examined for the lytic ability for Yac1 cells. All experiments were performed at least 3 times and representative data are shown.

effects in SCID mice. Research using a variety of murine models has revealed the direct effects of tumor cells[11] and the CD8 T cell response[12-14] involved in the antitumor effects of IFNα. A recent study[29] using STAT1-deficient animals and STAT1-deficient tumor cells revealed that IFNα activation of host cells, but not tumor cells, is required for antitumor effects in a peritoneal model of melanoma. They also showed the involvement of NK cells in their model. Our data demonstrated that NK cells are critically required and sufficient for IFNα-mediated protection from liver metastasis. However, NK cells are not effective for controlling tumor growth at extrahepatic sites, because IFNα activated splenic (systemic) NK activity but did not elicit antitumor effects against subcutaneously injected CT-26 cells. Subcutaneous tumor growth appeared to be controlled by adaptive immunity rather than innate pathway.

The reason that IFNα-mediated activation of NK cells leads to such a strong antitumor effect in the liver but not under the skin is not known. In the present study, we applied hydrodynamic injection of the IFNα gene to obtain efficient and stable expression of IFNα. Since the hydrodynamic procedure leads to predominant expression of foreign genes in the liver, the concentration of IFNα may be greater in the liver than in circulation. This may be related to the observed strong antitumor effects in the liver. Another possibility is that NK cells are more numerically abundant and functionally potent in the liver than in other organs.[30] In any case, the hydrodynamic injection of the IFNα gene led to higher activation of the NK lytic ability of hepatic mononuclear cells than that of systemic mononuclear cells. This may be related to the stronger antitumor activity in the liver.

An earlier study on STAT1 knockout mice revealed that STAT1 is a critical signaling molecule for IFNα in macrophage and T cells.[9] STAT1-deficient mice showed impairment of NK activity.[31] STAT1-deficient splenocytes did not show increase in NK lytic activity upon IFNα stimulation.[29] Therefore, STAT1 should also play an important role in IFNα-mediated NK cell activation. However, the significance of STAT1 in NK cells on IFNα action had not been fully proven, because splenocytes consist of a variety of lymphocyte subsets. In the present study, we found that NK cells express lower levels of STAT1 than T cells, which is associ-

ated with lower levels of STAT1 expression in SCID splenocytes than those in wild-type splenocytes. Importantly, IFNα phosphorylated STAT1 in SCID splenocytes with similar kinetics to that in wild-type splenocytes even if the signal intensities in the former were lower than those in the latter. In agreement with this, IFNα was capable of activating a variety of genes in SCID mononuclear cells. Thus, IFNα does not require other lymphocyte subsets to activate NK cells and to induce NK cell expression of IFN-regulated genes.

IFNγ was shown to be produced in lymphocytes upon IFNα administration, which is dependent on STAT4 signaling.[32] In the present study, IFNγ was produced in serum after pCMV-IFNa1 injection. Furthermore, the IFNγ gene was activated in SCID NK cells upon IFNα stimulation. However, IFNγ is not necessary for NK cell activation in terms of killing ability as well as an IFNα-mediated antimetastatic effect. NK cells, upon IFNα stimulation, expressed well-established IFN-regulated genes[33] as well as killer cell-specific molecules granzyme B or TRAIL. Although our data showed that hepatic mononuclear cells from mice receiving IFNα can kill CT-26 cells *in vitro*, it remains unclear whether NK cells serve as direct effector cells for ablating CT-26 cells *in vivo*. Further study is needed to find whether killer cell-specific molecules are actually involved in the antimetastatic effects of IFNα.

IFNα has achieved a long record of clinical use in the treatment of hematological malignancy and solid tumors such as melanoma, renal cell carcinoma and Kaposi's sarcoma.[34,35] In therapy for colon carcinoma, special attention has been paid to the use of IFNα in the combination with 5-FU, since IFNα has been shown to modulate 5-FU metabolism and to enhance its cytotoxic activity.[36] Although several clinical trials have evaluated the 5-FU plus IFNα combination for adjuvant therapy of colon carcinomas with encouraging results,[37,38] recent randomized trials revealed that addition of IFNα to 5-FU + levamisole marginally increased the recurrence-free survival time compared to 5-FU + levamisole alone, but did not alter the over-all survival.[39] Therefore, use of IFNα as a modulator of 5-FU activity may have some limitations in future clinical use. In the present study, we demonstrated that

IFNα activates both innate and adaptive immunity and ablates microdisseminated colon carcinoma cells in the liver. There may be a variety of reasons which can explain the difference between the present study and the clinical use in the therapy of metastasizing colon cancer. We found CT-26 far less sensitive to NK cells than Yac1 cells but human colon carcinoma cells might be more resistant to NK cells activated by IFNα in a clinical setting. Systemic administration of recombinant IFNα may be less effective than enforced expression of IFNα gene in the liver. In any way, we used CT-26 cells just as a murine model of hepatic metastasis and observed similar therapeutic effect of the IFNα gene when using another cell line such as BL6 melanoma cells in a C57/BL6 background (our unpublished data). Our study raised the possibility that IFNα therapy may be a promising approach for developing future adjuvant therapy for metastatic liver tumors arising from various organs. Immunological aspect of IFNα is important when considering antimetastatic effect of this cytokine.

In conclusion, IFNα-mediated protection of CT-26 hepatic metastasis critically requires NK cells. NK cells, upon IFNα stimulation, do not require other immune cells such as T cells, B cells and

NKT cells for their activation and protection against hepatic metastasis. NK cell production of IFNγ is not involved in the increase in NK activity and antitumor effect. Our study has shown NK cells to be important mediators in ablating microdisseminating tumors in the liver in IFNα therapy. Eradication of microdisseminated tumor cells by IFNα led to long-lasting adaptive immune responses which may be important for suppressing tumor growth in extrahepatic sites and overall antitumor effects.

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Original Article

Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis

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Aim: Regulatory T cells (Tregs) maintain immunological tolerance and suppress autoreactive immune responses. We evaluated the intrahepatic status of Tregs in patients with autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), or chronic hepatitis B (CH-B).

Methods: We analyzed 85 patients (20 AIH, 22 PBC, 27 CH-C, and 16 CH-B) and 14 controls. Using liver tissue samples obtained by needle biopsy or from marginal parts of resected metastatic liver tumors in the controls, immunohistochemical analyses of forkhead box P3^{+}, which is a specific marker for Tregs, CD4^{+}, and CD8^{+} cells were performed.

Results: Intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls. There were significantly fewer intrahepatic Tregs in the AIH patients than in the PBC patients ($P = 0.037$). Patients with a low frequency of intrahepatic Tregs were detected significantly more in the AIH and CH-B groups than in the PBC and CH-C groups ($P < 0.05$). In addition, the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced. We found significantly less infiltration of CD4^{+} T cells in AIH than in other diseases ($P < 0.05$). Liver-infiltrating CD8^{+} T cells were detected more frequently in the CH-B group than in other groups ($P < 0.003$).

Conclusion: Intrahepatic Tregs were increased in both patients with autoimmune liver diseases and those with viral hepatitis. In autoimmune liver diseases, intrahepatic Tregs were fewer in the AIH patients than in the PBC patients.

Key words: autoimmune hepatitis, chronic hepatitis, forkhead box P3, primary biliary cirrhosis, regulatory T cells

INTRODUCTION

T-CELL RESPONSES are implicated in host immune defense against microbes as well as immunopathogenesis of certain diseases, such as viral hepatitis. An appropriate T-cell response leads to the eradication of microbes, while a weak response may result in persistent infection. If the T-cell activation is too potent, however, severe inflammation or autoimmune disease may develop. The detailed mechanisms that lead to the breakdown of self-tolerance and the subsequent development of autoimmune disease are still unknown; however, the mechanisms are likely to involve the failure of homeostatic processes that keep the response against self-antigens under control.[1]

T-cell populations regulate and control the balance of immune responses. The CD4^{+} and CD25^{+} regulatory T cells (Tregs) are crucial for maintaining immunologic self-tolerance and negative control of various immune responses. The majority of Tregs are produced by the thymus as a functionally distinct T-cell subpopulation and are responsible for maintaining peripheral tolerance. Genetic abnormalities in the development and function of this Treg population can cause autoimmune disease, immunopathology, and allergy in humans.[2] In addition, there are different T-cell subpopulations with regulatory functions, such as natural killer T cells, T helper 3, T regulatory 1, CD8^{+} and CD28^{−}, and γδ T cells. These types of T cells may also prevent the activation of autoreactive T cells and be involved in the failure of homeostasis.[1]

Although several cell-surface molecules, such as CD25, glucocorticoid-induced tumor necrosis factor

receptor family-related gene/protein, and cytotoxic T lymphocyte-associated molecule-4, have been reported as Treg markers, these molecules are also expressed on activated T cells derived from CD4+ and CD25- naïve T cells.[3] Transcription factor forkhead box P3 (FOXP3) is expressed in CD4+ and CD25+ Tregs as a master control molecule for their development and function in mice and humans, thus, FOXP3 is thought to be a specific marker of Tregs.

Autoimmune mechanisms are involved in autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC). AIH is an inflammatory liver disease characterized by high levels of transaminases, circulating autoantibodies, hyper-γ-globulinemia, histological evidence of interface hepatitis, and response to immunosuppressive treatment.[4,5] PBC is an enigmatic liver disease characterized by the chronic non-suppurative destruction of small intrahepatic bile ducts, portal inflammation, and the presence of antimitochondrial antibodies (AMA).[6,7] The presence of AMA and autoreactive T and B cells, in conjunction with the co-occurrence of other autoimmune diseases, characterizes PBC as a typical autoimmune disease.[8] Although the etiology of PBC remains obscure, recent data suggest that autoreactive T-cell responses play a major role in its pathophysiology.[9–12]

Hepatitis C virus (HCV) infection is often asymptomatic, and approximately 80% of infected patients progress to chronic hepatitis.[13] After HCV infection, interaction between the innate and adaptive immune responses plays a pivotal role in perpetuation or clearance of HCV infection. T helper 1-type (Th1) cytokines, such as interferon (IFN)-γ and interleukin (IL)-2, are involved in cell-mediated immunity and play a crucial role in protection against intracellular pathogens.[14] A weak cellular immune response is thought to be one of the mechanisms of HCV persistence.

In hepatitis B virus (HBV) infection, a multispecific CD4+ and CD8+ T cell with a Th1 cytokine profile is also important for control of the infection.[15] These multispecific T-cell responses are maintained for decades after clinical recovery. However, these responses are lacking in patients with chronic HBV infection, and the mechanism of T-cell hyporesponsiveness or tolerance is still unknown.

The frequency of Tregs in the peripheral blood was decreased in patients with AIH and PBC and increased in patients with chronic hepatitis C (CH-C) and chronic hepatitis B (CH-B) compared with the healthy controls.[16] However, there are few reports investigating the intrahepatic status of Tregs. In the present study, we analyzed and compared the intrahepatic status of Tregs

in patients with AIH, PBC, CH-C, and CH-B because liver-infiltrating immune cells should reflect the status of disease and pathogenesis more directly than peripheral cells.

METHODS

Patients and liver tissue

NEEDLE BIOPSIES WERE performed to obtain liver tissue from 85 patients, consisting of 20 AIH patients, 22 PBC patients, 27 CH-C patients, and 16 CH-B patients. All patients had a persistently increased level of serum alanine aminotransferase (ALT; >30 IU/L). The diagnosis of each case was based on reliable clinical and laboratory data and was independently confirmed histologically by two pathologists who specialize in liver diseases. All AIH patients were antinuclear antibody positive or antismooth muscle antibody positive, and all had histological features of interface hepatitis. Patients with morbid changes in bile duct were excluded individually by retrograde radiological cholangiography or magnetic resonance cholangiopancreatography. Patients with overlap syndrome were also excluded from this study. All PBC patients were AMA positive and fulfilled the diagnostic criteria of PBC based on internationally accepted standards. Livers from PBC patients were staged histologically by Scheuer's classification. Seventeen and five patients were of stage 1 and of stages 2/3/4, respectively. We included 14 patients with metastatic liver tumors as the controls. The control patients were not infected with HBV (negative for hepatitis B surface antigen) or HCV (negative for anti-HCV antibody), and they had no history of autoimmune diseases and were negative for autoimmune antibodies. Liver tissue from control patients was obtained from a marginal part of the resected liver in which the histological examination was normal. Table 1 shows the patients' characteristics. All patients gave written informed consent according to a protocol approved by the Ethical Committee of Showa University.

Immunohistochemical staining

Liver needle biopsies and resected tissues were obtained from the 99 patients, as described earlier. All tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 3 μm-thick serial sections were cut from each paraffin block. Each specimen contained at least three portal tracts encompassing interlobular bile ducts, and a total of 297 portal tracts were counted. Antigen retrieval for CD4 and FOXP3 staining

Table 1 Characteristics of the patients and controls analyzed in this study

Group (number)	AIH (20)	PBC (22)	CH-C (27)	CH-B (16)	Control (14)
Female (%)	95.0**,†,††	90.9†,††,‡	40.7	18.8	28.6
Age (years)	55.8 ± 13.8†	55.5 ± 11.2††	49.8 ± 11.9§,§§	38.1 ± 11.6‖	59.1 ± 14.2
ALT (IU/L)	393 ± 462*,**,††	113 ± 139††,‡	85 ± 57§,§§	295 ± 351	17 ± 9
AST (IU/L)	306 ± 393*,**,††	88 ± 78‡	60 ± 40§,§§	148 ± 141	22 ± 9
ALP (IU/L)	495 ± 300*,**,†	842 ± 547†,††,‡	268 ± 85	320 ± 96	316 ± 171
IgG (mg/dL)	2639 ± 1163*,**	1837 ± 584	1794 ± 290	n.d.	n.d.
IgM (mg/dL)	334 ± 355**	466 ± 231†	129 ± 67	n.d.	n.d.

Significance was assessed with Fisher's exact probability test. *P* < 0.05 (*AIH versus PBC, **AIH versus CH-C, †AIH versus CH-B, ††AIH versus Control, ‡PBC versus CH-C, ††PBC versus CH-B, ‡PBC versus Control, §CH-C versus CH-B, §§CH-C versus Control, ‖CH-B versus Control). Values are mean ± standard deviation. AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; IgG, immunoglobulin G; IgM, immunoglobulin M; n.a., not determined; PBC, primary biliary cirrhosis.

was achieved by pressure cooking for 5 min in citrate buffer (pH 7.0), while antigen retrieval for CD8 staining was achieved by microwaving for 15 min in citrate buffer (pH 7.0). For CD4 or CD8 immunohistochemical staining, anti-CD4 monoclonal antibody (mAb; Nichirei Biosciences, Tokyo, Japan) or anti-CD8 mAb (Dako Cytomation, Tokyo, Japan) and biotinylated goat antimouse immunoglobulin G (IgG; Dako ChemMate Envision kit/HRP[DAB], Dako, Japan) were used. FOXP3 expression was analyzed by immunostaining with a goat antihuman FOXP3 polyclonal antibody (ab22510; Abcam, Cambridge, UK) and biotinylated rabbit antigoat IgG (Dako ChemMate Envision kit/ HRP[DAB]). The slides were stained with hematoxylin following immunohistochemical staining.

Evaluation of frequency of FOXP3-, CD4-, and CD8-positive cells

To evaluate and compare the distribution and frequency of cells positive for FOXP3, CD4, and CD8, three small- to medium-sized portal tract areas were selected for investigation with an optical microscope. The same visual fields were chosen and examined using serial sections. The numbers of FOXP3-, CD4-, or CD8-positive cells contained within the three portal tract areas from each specimen were counted at a magnification of ×400 by two independent observers in a blinded fashion. To correct for differences in the sizes of the portal tracts, the proportion of FOXP3⁺ Tregs was determined as follows: %FOXP3 = (counts of FOXP3⁺ Tregs/counts of total mononuclear cells) × 100, which is a total mononuclear cell-corrected value for FOXP3⁺. CD4⁻ and CD8⁻ T cells in total mononuclear cells were also calculated.

Statistical analyses

Significance was assessed with the Mann–Whitney *U*-test or Fisher's exact probability test. Differences between groups were considered statistically significant when the *P*-value was less than 0.05.

RESULTS

Intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls

TO COMPARE THE frequencies of intrahepatic FOXP3⁺ Tregs between the liver diseases, we determined the percentage of FOXP3, as described in Methods. As shown in Figure 1, the frequency of FOXP3⁺ T cells in patients with AIH, PBC, CH-C, or CH-B was significantly much higher than that in the control patients. Interestingly, there were significantly fewer FOXP3⁺ T cells in the liver tissues of AIH patients than in those of PBC patients (*P* = 0.037). The frequency of intrahepatic FOXP3⁺ Tregs in the AIH patients was not different from that in the patients with CH-C or CH-B.

Patients with a low frequency of intrahepatic Tregs were detected significantly more in the AIH and CH-B groups than in the PBC and CH-C groups

Since the patients with numerous intrahepatic FOXP3⁺ Tregs were observed in the PBC and CH-C groups, we separated the patients into two groups according to the frequency of intrahepatic Tregs. The patients were divided into those with FOXP3⁻ cells of less than 9% and those with FOXP3⁻ cells of 9% or more: this level
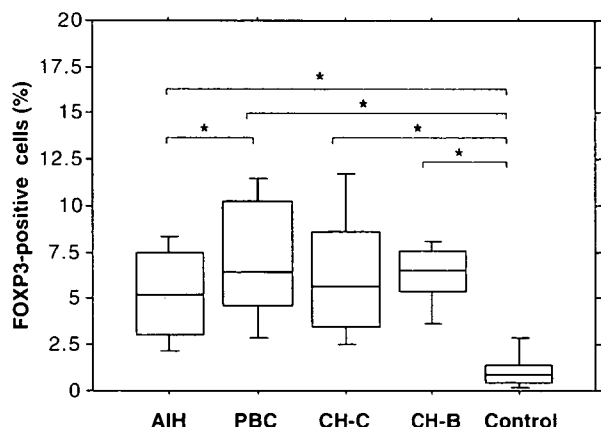
Figure 1 Intrahepatic forkhead box P3⁺ (FOXP3⁺) T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic FOXP3⁺ cell status, intrahepatic FOXP3⁺ cells in patients with AIH, PBC, CH-C, and CH-B were stained. For the enumeration of positive mononuclear cells, mononuclear cells were counted in three high-powered fields (×400) by two independent observers in a blinded fashion. For each sample, the mean percentage of positive cells was chosen. Results are expressed as the median and range of all tested patents in each group. $P < 0.05$.

Table 3 Comparison of the intrahepatic Tregs frequency with histological stages in PBC patients

	<9%	≥9%	Total
Early stage	8	9	17
Advanced stage	5	0	5
Total	13	9	22

$P = 0.034$. PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4). Number of patients with a high frequency of intrahepatic regulatory T cells (Tregs; %FOXP3 ≥9%) and that with a low frequency (%FOXP3 <9%) were compared for each stage. PBC, primary biliary cirrhosis.

was decided arbitrarily as follows: (mean percentage of FOXP3 of controls + 3 standard deviation) × 2. When the number of patients with a high frequency of intrahepatic Tregs (%FOXP3 ≥9%) and that with a low frequency (%FOXP3 <9%) were compared for each liver disease, patients who had a low frequency of intrahepatic Tregs were detected significantly more in the AIH group than in PBC and CH-C groups as shown in Table 2. In addition, more patients with low frequency Treg infiltration were found in the CH-B group than in the PBC and CH-C groups. Thus, PBC is characterized by higher frequency of FOXP3⁺ cells compared

to AIH, whereas the number and profiles of liver-infiltrating T cells are comparable. In viral hepatitis, a higher frequency of FOXP3⁺ cells is observed in CH-C, while higher frequency of CD4⁺ or CD8⁺ cells is characteristic for HBV-infected liver.

When the PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4), the frequency of Tregs was higher than 9% in nine of 17 (53%) PBC patients with early histological stage, while that of Tregs was below 9% in all patients with advanced stage ($P = 0.034$), as shown in Table 3. Furthermore, as shown in Figure 2, more FOXP3⁺ T-cell infiltration was seen in the early stage than in the advanced stage (8.03 ± 3.50 vs 4.47 ± 1.40, $P = 0.041$). Therefore, it was thought that the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced.

Frequency of intrahepatic CD4⁺ T cells was lower in AIH patients, while the frequency of intrahepatic CD8⁺ T cells was higher in CH-B patients

We evaluated the intrahepatic frequencies of CD4⁺ cells as well as CD8⁺ cells to investigate whether these

Table 2 Comparison of the number of patients with high frequency of intrahepatic Tregs with those with low frequency

	<9%	≥9%	Versus PBC*	Versus CH-C**
AIH (n = 20)	20	0	$P = 0.001$	$P = 0.014$
PBC (n = 22)	13	9	–	$P = 0.266$
CH-C (n = 27)	20	7	$P = 0.266$	–
CH-B (n = 16)	16	0	$P = 0.003$	$P = 0.026$
Control (n = 14)	14	0	$P = 0.006$	$P = 0.036$

Significance was assessed with Fisher's exact probability test. P-values are shown as VS PBC group(*) and VS CH-C(**) group.
AIH, autoimmune hepatitis; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; PBC, primary biliary cirrhosis; Tregs, regulatory T cells
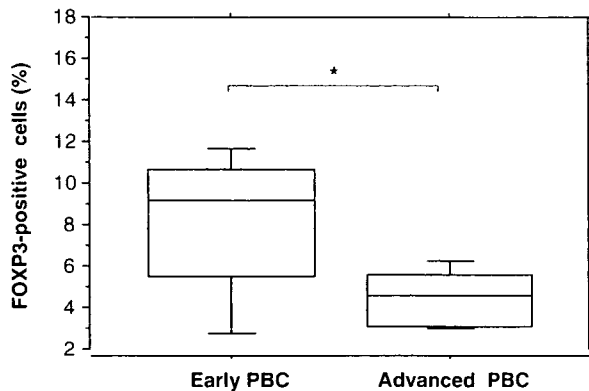
Figure 2 Frequency of regulatory cells (Tregs) in the liver of primary biliary cirrhosis (PBC) patients in terms of pathological stage of the disease advances. Intrahepatic forkhead box P3$^+$ (FOXP3$^+$) T cells in the PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4). Results are shown as the mean percentage of Tregs frequency ± standard deviation in each stage. $P < 0.05$.

Figure 4 Intrahepatic CD8$^+$ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD8$^+$ cell frequency, intrahepatic CD8$^+$ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD8$^+$ cells were counted with the same procedure used for forkhead box P3$^+$ cells. Results are expressed as the median and range of all tested patents in each group. $P < 0.05$.

immune cells were involved in the immunopathogenesis of each liver disease. As shown in Figure 3, the frequency of CD4$^+$ T cells infiltrating the liver tissue was significantly higher in CH-B patients than in the controls. We found significantly less infiltrating CD4$^+$ T
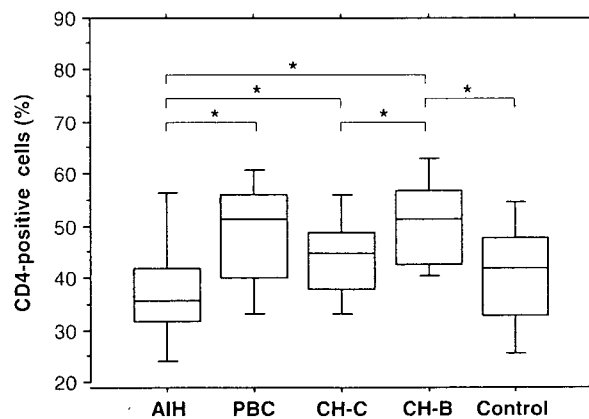

Figure 3 Intrahepatic CD4$^+$ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD4$^+$ cell frequency, intrahepatic CD4$^+$ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD4$^-$ cells were counted with the same procedure used for forkhead box P3$^-$ cells. Results are expressed as the median and range of all tested patients in each group. $P < 0.05$.
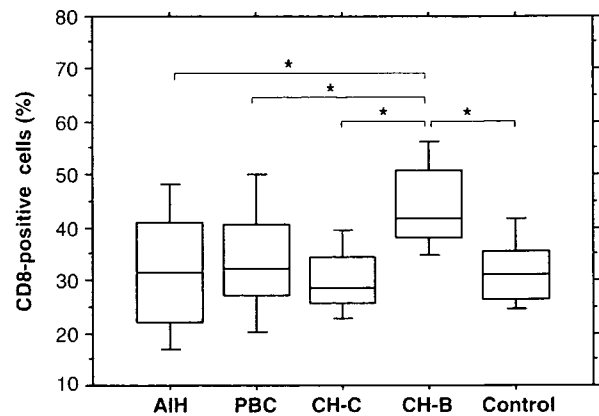
cells in the AIH patients than in the PBC patients ($P = 0.007$), CH-C patients ($P = 0.045$), and CH-B patients ($P < 0.001$). As shown in Figure 4, the frequency of CD8$^+$ T cells was significantly higher in the CH-B patients than in the controls. There were also significantly higher CD8$^+$ T cells in the liver tissues of the CH-B patients than in those of the AIH patients ($P = 0.003$), PBC patients ($P = 0.002$), and CH-C patients ($P < 0.001$).

Furthermore, the CD4$^+$/CD8$^+$ ratio was lower in the CH-B patients than in the PBC patients (1.18 ± 0.26 vs 1.56 ± 0.66, $P = 0.037$) and CH-C patients (1.18 ± 0.26 vs 1.49 ± 0.39, $P = 0.007$). There was no difference in the total infiltration of mononuclear cells between patients with AIH, PBC, CH-C, and CH-B. Intrahepatic CD4$^+$ T cells and CD8$^+$ T cells in the control patients were significantly less than in the CH-B patients ($P = 0.013$ and $P < 0.001$, respectively), although we did not detect any differences between the control group and the other liver disease groups. There was no relationship between the biochemical data or histological activities and infiltration of the immune cells.

Since intrahepatic immune cells may directly affect inflammation in the liver, we compared the biochemical data, such as the serum ALT level, and histological activities with the intrahepatic frequencies of FOXP3$^-$, CD4$^-$, and CD8$^+$ cells. There was no relationship between the ALT, alkaline phosphatase, IgG, immuno-

globulin M level, or histological activities and the frequency of infiltrating immune cells other than described above (data not shown).

DISCUSSION

TREGS ARE THOUGHT to play roles in immune regulation, such as the suppression of severe inflammation and autoimmune diseases. The removal or reduction of Tregs can also enhance immune responses against infectious microbes, thus, Tregs affect the elimination of infectious microbes.[17–24] A higher proportion of CD4+ and CD25+ T cells in peripheral blood was found in patients with chronic HCV infection as compared to recovered patients and normal controls.[25] Tregs secrete transforming growth factor-β_1 and IL-10, and these cytokines may attenuate the function of macrophages. IL-10 also inhibits HCV-specific immunity when administered exogenously in patients with chronic HCV infection.[26] Thus, Tregs may disturb the eradication of HCV and lead to chronic infection. Chronic HBV patients harbor an increased frequency of Tregs in peripheral blood as compared to control patients, and Tregs have an immunosuppressive effect on HBV-specific T helper cells.[27] This may be one of the mechanisms that leads to chronic infection.

Several recent studies have focused on Tregs in patients with autoimmune liver diseases, such as AIH and PBC. Since Tregs prevent the proliferation and effector function of autoreactive T cells[16] and downregulate the production of IFN-γ by CD8+ T cells in a murine model and in humans,[28,29] Tregs may be implicated in the pathogenesis of AIH and PBC. In fact, the relative frequencies of Tregs are decreased in peripheral blood samples of patients with PBC,[30] and Tregs are few in patients with AIH.[16] However, there are only a few reports regarding the status of intrahepatic Tregs.

Tregs maintain the ability to suppress IFN-γ production by CD4+ and CD25− T cells in AIH, and circulating Tregs are significantly less in AIH patients than in controls.[16] However, few details regarding the roles of Tregs in the pathogenesis of AIH have been revealed.

In the present study, we demonstrated that intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls. Indeed there are significantly fewer intrahepatic Tregs in AIH patients and CH-B patients than in PBC patients and CH-C patients, but as a whole, there is more infiltration of FOXP3+ Tregs than in the controls, and there is not a great difference. In addition, we found significantly

fewer infiltrating CD4+ T cells in AIH patients than in the patients with other diseases, whereas CD8+ T cells infiltrating liver tissue were detected with a significantly greater frequency in CH-B patients than in the other patients.

Although both AIH and PBC are representative autoimmune liver diseases, we identified differences in immune cell infiltration between these two autoimmune diseases in the present study. The results indicate that different mechanisms are involved in the pathogenesis of AIH and PBC. However, there are significantly more ratios of Tregs than control, and it seems that only a ratio of Tregs does not relate to the pathogenesis of these diseases.

We found that the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced. A previous report demonstrated that there were few liver-infiltrating Tregs in PBC patients,[30] although it has not been confirmed by other researches. Sasaki et al. recently reported findings similar to ours. They found that the extent of FOXP3+ Tregs in inflamed portal tracts with chronic non-suppurative destructive cholangitis in early stage (Scheuer's classification 1 and 2) of PBC was higher than that in late stage (Scheuer's classification 3, 4) of PBC.[31]

It is not clear whether this decrease of Tregs is a cause or a result of disease progression. Although we cannot explain the reason for these differences in Tregs' infiltration, the race of the study patients may be one of the factors. Functional investigations of intrahepatic Tregs in these autoimmune liver diseases may clarify this issue.

Since the frequency of intrahepatic Tregs in CH-C groups is diverse widely, we could not detect a significant difference in Tregs' accumulation between the CH-C and AIH groups. However, several CH-C patients had a large number of intrahepatic Tregs. When we divided the patients in each group into those with FOXP3+ cells of less than 9% and those with FOXP3+ cells of 9% or more, a significant difference was confirmed. In addition, patients who had a high frequency of intrahepatic Tregs were detected significantly more often in the CH-C group than in the CH-B group. In HCV infection, it has been suggested that HCV itself, especially in the NS3 region, induces Tregs in patients with HCV infection as well as in healthy donors,[32] and these Tregs are involved in the development of viral persistence, which occurs usually in acute HCV infection and rarely in acute HBV infection in adults. Thus, in chronic hepatitis, the pathogenesis of HCV should be different from that of HBV.

There were only a few Tregs in the pathologically normal tissue that surrounded metastatic liver tumors. The same phenomenon has been described in other reports.[30,33] The decreased frequency of Tregs was not likely to be the effect of metastatic tumors, because it has been reported that malignant tumors often induce Tregs.[34–39] In normal liver tissue, Treg infiltration may be suppressed because it is necessary to induce immunity against many pathogens flowing into the liver, rather than prevent inflammation or induction of autoimmunity.

Intrahepatic Tregs may be involved with immunopathogenesis and play a crucial direct role in the development of each liver disease. However, since immune systems in liver diseases are complicated, further investigations are needed to clarify the detailed relationship between Tregs and immunopathogenesis.

ACKNOWLEDGMENT

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Patterns in the prevalence of hepatitis C virus infection at the start of hemodialysis in Japan

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Abstract

Background Although hepatitis C virus (HCV) infection is a persistent public health concern in hemodialysis patients, there seem to have been only a few reports on the prevalence of HCV at the start of hemodialysis. In this study we investigated whether patients starting on hemodialysis therapy are positive for anti-HCV antibody or not.

Methods The 400 patients who began regular hemodialysis between February 2003 and June 2007 were enrolled in this study. Clinical data such as age, anti-HCV antibody and primary cause of end-stage kidney disease (ESKD) were examined. As healthy controls we used 70,717 healthy blood donors in 2005 whose data were obtained from Tokyo Metropolitan Red Cross Blood Center. Anti-HCV antibody was used as an indicator of HCV infection. Since the prevalence of HCV infection is affected by age in Japan, we classified the patients by age group.

Results The anti-HCV antibody prevalence rate among the patients who were new to hemodialysis was 7.3%, as opposed to 0.15% in the healthy volunteers. The prevalence of HCV in the 31–45-, 46–60-, and 61-year-old groups was significantly higher among the hemodialysis patients than among the healthy volunteers ($P = 0.0209$, <0.0001, and <0.0001, respectively). The prevalence rate of anti-HCV antibody was higher among men (10.0%) than among women (1.5%, $P < 0.0001$) in the hemodialysis patients. The anti-HCV-antibody-positive patients were significantly older than the anti-HCV-antibody-negative patients (66.4 ± 14.3 years versus 58.6±16.6 years; $P = 0.0152$). Diabetic nephropathy was a more frequent cause of ESKD among the anti-HCV-antibody-positive patients (30.4%) than among the anti-HCV-antibody-negative patients (19.9%, $P = 0.0122$). Among the anti-HCV-antibody-positive patients, 55.2% had received a blood transfusion. The rate was significantly higher than that among the anti-HCV-antibody-negative patients (19.4%, $P < 0.0001$).

Conclusion The results showed a much higher rate of anti-HCV antibody positivity in patients new to hemodialysis than in healthy volunteers. Older age, blood transfusion, male gender, and diabetic nephropathy seemed to be risk factors for anti-HCV antibody positivity in Japan.

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Introduction

Hepatitis C virus (HCV) infection is a persistent public health concern in hemodialysis patients. Unlike hepatitis B virus (HBV), no vaccine is available for HCV [1]. Patients infected with HCV often have minimal clinical evidence of disease [1, 2], but HCV infection has been associated with greater morbidity and mortality in ESKD patients [2–4].

The number of patients on hemodialysis infected with HCV is rather high [5], mostly as a result of nosocomial infection. Recent dialysis outcomes and practice patterns study (DOPPS) have revealed a mean facility prevalence in France, Germany, Italy, Japan, Spain, the UK, and the US of 13.5% and the mean prevalence according to country ranged from 2.6 to 22.9% [6]. The main causes of nosocomial infection by HCV in hemodialysis patients are filter reuse, use of contamined hemodialysis machines, and contamination of medical staff's hands [7].

Nevertheless, there seem to have been only a few reports on the prevalence of HCV at the start of hemodialysis. Some ESKD patients may be at risk of exposure to HCV associated with medical treatment, including blood transfusion, and ESKD patients are thought to be susceptible to HCV infection because of the decline in immune response. Hepatitis C is both a cause and a complication of chronic kidney disease. Chronic infection with HCV can lead to the immune complex syndromes of cryoglobulinemia and membranoproliferative glomerulonephritis (MPGN). Management of HCV-related cryoglobulinemia and MPGN is difficult: antiviral therapy is effective in clearing HCV infection in a proportion of patients, but these conditions can be severe and resistant to antiviral therapy [8]. Glomerular abnormalities in liver cirrhosis patients are also known, even though their renal insufficiency is generally mild.

An overview of regular dialysis treatment in Japan revealed that the proportion of patients who had been on hemodialysis therapy for less than 2 years who were positive for anti-HCV antibody was 7.6% [9], a higher rate than in the general population in Japan.

We therefore hypothesized that HCV infection is already relatively widespread at the start of hemodialysis therapy, and in the present study, we investigated whether patients who start hemodialysis therapy are already anti-HCV-antibody-positive.

Materials and methods

The 400 patients who started on regular hemodialysis in our kidney center at Tokyo Women's Medical University Hospital between February 2003 and June 2007 were enrolled in this study.

Age, gender, HBs antigen (Ag), HBs antibody (Ab), treponema pallidum latex immuno assay (TPLA), and primary cause of ESKD were examined. The proportions of patients starting on hemodialysis after having been on continous ambulatory peritoneal dialysis (CAPD) or having received a renal transplant were also examined. The blood chemistry, peripheral blood count and whether they had signs of liver fibrosis or hepatocellular carcinoma on

Table 1 Prevalence of HCV in patients new to hemodialysis therapy

	Ant-HCV Ab positive	Ant-HCV Ab negative	P value
Number	29	371	–
Age[a] (years)	66.4 ± 14.3	58.6 ± 16.6	0.0152
Gender (M/F)	27/2	242/129	<0.0001
CAPD[b] (%)	0	3.2	n.s.
Transplantation[b] (%)	10.3	7.8	n.s.
Positive for HBs Ag (%)	0	1.08	n.s.
Positive for HBs Ab (%)	34.8	19.1	n.s.
Positive for TPLA (%)	7.1	1.64	n.s.

CAPD continuous ambulatory peritoneal dialysis, *Ag* antigen, *Ab* antibody

TPLA treponema pallidum latex immuno assay mean ± SD

[a] Age at the start of hemodialysis therapy

[b] Rate of patients switching to hemodialysis from CAPD or transplantation

abdominal echo examinations or had ever received a blood transfusion were examined.

As healthy controls for the prevalence of HCV we used 70,717 healthy first-time blood donors in 2005 whose data were obtained from Tokyo Metropolitan Red Cross Blood Center [10]. Since the prevalence of HCV infection is affected by age, we classified the patients into the following age groups (years): under 31, 31–45, 46–60, and 61–70.

Data are reported as means ± SD. The chi-square (χ^2) test was used for comparisons between categorical variables. Fisher's exact test was used when the criteria for the χ^2 test could not be applied. Student's t-test was used for comparisons between continuous variables. All statistical calculations were performed with Stat View J 5.0 software. A P value of less than 0.05 was considered statistically significant.

Results

The overall anti-HCV antibody prevalence rate among patients new to hemodialysis was 7.3%. Table 1 compares the anti-HCV-antibody-positive and anti-HCV-antibody-negative patients. The anti-HCV-antibody-positive patients were significantly older than the anti-HCV-antibody-negative patients (66.4 ± 14.3 years versus 58.6 ± 16.6 years; $P = 0.0152$). The prevalence rate of anti-HCV antibody was higher among men (10.0%) than among women (1.5%, $P < 0.0001$) in the hemodialysis patients. The proportions of patients starting on hemodialysis after having been on CAPD or having received a transplant were similar in both patients, and the prevalence of HBs Ag or HBs Ab was also similar in both patients. The proportion
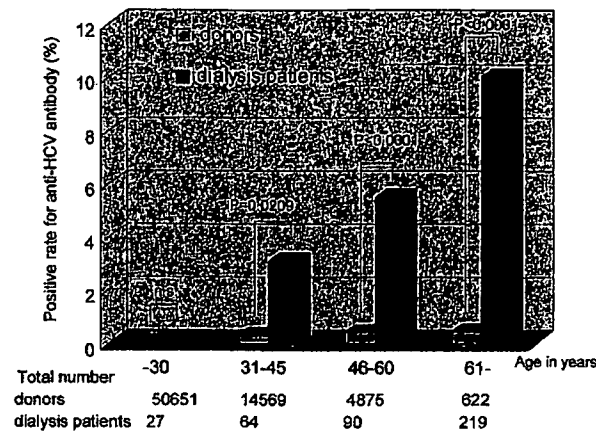
Fig. 1 Comparison between the prevalence of HCV in blood donors in Tokyo in 2005 and a new hemodialysis population in whole gender. Data on the prevalence in blood donors in Tokyo were obtained from the Tokyo Metropolitan Red Cross Blood Center
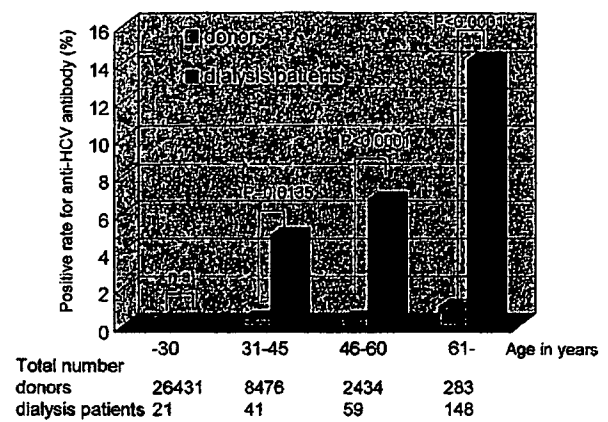

Fig. 2 Comparison between the prevalence of HCV in blood donors in Tokyo in 2005 and a new hemodialysis population in men. Data on the prevalence in blood donors in Tokyo were obtained from the Tokyo Metropolitan Red Cross Blood Center

positive for TPLA tended to be higher in anti-HCV-antibody-positive patients than in the anti-HCV-antibody-negative patients, but the difference was not statistically significant.

The anti-HCV antibody prevalence rate among the 70,717 blood donors in Tokyo in 2005 was 0.15%. Figure 1 compares the prevalence of HCV in the blood donors and the patients new to hemodialysis therapy. None of the patients new to hemodialysis in the under 31-year-old group were positive for anti-HCV antibody, whereas in the 31–45-, 46–60-, and 61-year-old groups the prevalence of HCV was significantly higher in the hemodialysis patients than in the healthy volunteers ($P = 0.0209$, <0.0001, <0.0001, respectively).

Among the 37,624 healthy male volunteers, 72 (0.19%) were positive for anti-HCV antibody, while among the 33,093 healthy female volunteers, 36 (0.11%) were positive. Similar to the trend among hemodialysis patients, the prevalence rate of anti-HCV antibody was also significantly higher among healthy male volunteers than among healthy female volunteers ($P = 0.005$). As only two women were positive for anti-HCV antibody among the hemodialysis patients, we could not compare the difference in HCV prevalence between female hemodialysis patients and female healthy volunteers. Figure 2 compares the prevalence of HCV among the blood donors and patients new to hemodialysis therapy among men. The result was similar to the overall trend for both genders.

Table 2 shows the primary causes of ESKD. Diabetic nephropathy was a more frequent cause of ESKD in the anti-HCV-antibody-positive patients (37.9%) than in the anti-HCV-antibody-negative patients (18.6%, $P = 0.0122$).

Table 3 shows the blood chemistry results, peripheral blood count, results of abdominal echography, and the

history of blood transfusion for the anti-HCV-antibody-positive and anti-HCV-antibody-negative patients new to hemodialysis therapy. The total bilirubin and asparate aminotransferase levels were statistically higher among anti-HCV-antibody-positive patients (0.4 ± 0.4 mg/dl and 28.9 ± 23.3 IU/l, respectively) than among anti-HCV-antibody-negative patients (0.3 ± 0.2 mg/dl; $P = 0.0012$,

Table 2 Primary cause of end-stage kidney disease

Cause of ESKD (%)	Ant-HCV antibody positive	Ant-HCV antibody negative	P value
Chronic glomerulonephritis	6 (20.7%)	150 (40.4%)	n.s.
Chronic pyelonephritis	0 (0%)	1 (0.3%)	n.s.
RPGN	1 (3.4%)	9 (2.4%)	n.s.
Nephropathy of toxemia of pregnancy	0 (0%)	3 (0.8%)	n.s.
Other unclassified nephritis	1 (3.4%)	6 (1.6%)	n.s.
Polycystic kidney disease	1 (3.4%)	14 (3.8%)	n.s.
Nephrosclerosis	1 (3.4%)	51 (13.7%)	n.s.
Diabetic nephropathy	11 (18.6%)	(37.9%)	69 0.0122
Lupus nephritis	2 (6.9%)	4 (1.1%)	n.s.
Urate nephropathy	0 (0%)	2 (0.5%)	n.s.
Urolithiasis	0 (0%)	3 (0.8%)	n.s.
Tumor of kidney or urinary tract	1 (3.4%)	3 (0.8%)	n.s.
Obstructive uropathy	0 (0%)	1 (0.3%)	n.s.
Myeloma kidney	0 (0%)	2 (0.5%)	n.s.
Renal dysplasia	0 (0%)	2 (0.5%)	n.s.
Unknown	0 (0%)	8 (2.2%)	n.s.
After renal transplantation	3 (10.3%)	29 (7.8%)	n.s.
Others	2 (6.9%)	14 (3.8%)	n.s.

RPGN rapidly progressive glomerulonephritis

Table 3 Characteristics of patients new to hemodialysis therapy

Category	Ant-HCV antibody positive	Ant-HCV antibody negative	P value
Total bilirubin (mg/dl)	0.4 ± 0.4	0.3 ± 0.2	0.0012
Asparate aminotransferase (IU/l)	28.9 ± 23.2	18.6 ± 16.7	0.0022
Alanine aminotransferase (IU/l)	25.2 ± 17.4	17.3 ± 25.5	n.s.
Fe (µg/dl)	58.4 ± 60.8	59.4 ± 35.3	n.s.
Total iron binding capacity (µg/dl)	222 ± 58	227 ± 51	n.s.
Ferritin (ng/ml)	354 ± 273	305 ± 472	n.s.
White blood cell count (/µl)	6,400 ± 3,240	6,750 ± 2,720	n.s.
Red blood cell count ($\times 10^6$/µl)	2.78 ± 0.58	2.85 ± 0.54	n.s.
Hemoglobin (g/dl)	8.4 ± 1.4	8.6 ± 1.6	n.s.
Hematocrit (%)	25.5 ± 4.6	26.2 ± 4.9	n.s.
Platelet count ($\times 10^4$/µl)	17.0 ± 7.1	19.8 ± 8.2	n.s.
Liver fibrosis (%)	25.0	4.9	0.0002
Hepatocellular carcinoma (%)	17.9	1.4	<0.0001
Blood transfusion (%)	55.2	19.4	<0.0001

Mean ± SD

18.6 ± 16.7 IU/l; $P = 0.0022$). The alanine aminotransferase level tended to be higher among the anti-HCV-antibody-positive patients (25.2 ± 17.4 IU/l) than among the anti-HCV-antibody-negative patients (17.3 ± 25.5 IU/l). Iron-related markers like the Fe level, the total iron-binding capacity, and the ferritin level were almost the same among anti-HCV-antibody-positive and anti-HCV-antibody-negative patients. Similarly, the white blood cell count, hemoglobin and hematocrit levels were almost the same, but the platelet count tended to be lower among the ant-HCV-antibody-positive patients (17.0 ± 7.1 × 10⁴/µl) than among the anti-HCV-antibody-negative patients (19.8 ± 8.2 × 10⁴/µl). The rates of liver fibrosis and hepatocellular carcinoma were statistically higher among anti-HCV-antibody-positive patients (25.0% and 17.9%) than among anti-HCV-antibody-negative patients (4.9%; $P = 0.0002$, 1.4%; $P < 0.0001$). Among the anti-HCV-antibody-positive patients, 55.2% had received a blood transfusion. This rate was significantly higher than that among the anti-HCV-antibody-negative patients (19.4%, $P < 0.0001$).

Discussion

The prevalence of HCV infection at the start of hemodialysis therapy has never been clearly described in Japan. A study in Italy reported an anti-HCV-antibody-positive rate at the start of hemodialysis therapy of 13% [11]. An anti-HCV prevalence rate of 14.4% at the start of hemodialysis therapy was reported by a study in the US, and age, race, gender, and drug abuse were all independent predictors of anti-HCV antibody positivity in that study population [12]. The US study reported that age (50>) was a significant predictor, that younger patients were more likely to be infected with HCV, and that black men and former or current drug abusers were more likely to test positive for anti-HCV antibody. Presumably, such high-risk behaviors as drug abuse are more common among younger patients, men, and blacks, thereby contributing to the high frequency of HCV infection in incident dialysis patients belonging to these patient groups [12]. The prevalence of anti-HCV antibody among patients new to hemodialysis in our study was 7.3%. As Fig. 1 shows, the prevalence of anti-HCV antibody was significantly higher among patients new to hemodialysis than among the healthy controls in subjects over the age of 31 years. Similar to the results of a study conducted in the US, male gender was a risk factor for anti-HCV-antibody positivity in this study. On the other hand, the anti-HCV-antibody-positive patients were older than the anti-HCV-antibody-negative patients at the start of hemodialysis, and the rate of patients who had received a blood transfusion was higher among the anti-HCV-antibody-positive patients than among the anti-HCV-antibody-negative patients. In contrast to the US, older age and blood transfusion may be risk factors in Japanese patients. Donor blood was not routinely screened for HCV infection in Japan until 1989. Older patients may have received unscreened blood that transmitted HCV infections.

HCV infection may cause ESKD, but none of the patients had ever undergone a renal biopsy and been diagnosed with HCV-related glomerulonephritis. The frequent presence of glomerular abnormalities in patients with liver cirrhosis was first noted during the 1940s. The renal insufficiency is generally mild in such patients. Twenty-five percent of patients positive for anti-HCV antibody already had liver fibrosis. The total bilirubin and asparate aminotransferase levels were statistically higher among the anti-HCV-antibody-positive patients than among the anti-HCV-antibody-negative patients. The alanine aminotransferase level tended to be higher, and platelet count tended to be lower among the anti-HCV-antibody-positive patients than among the anti-HCV-antibody-negative patients. Renal dysfunction secondary to liver fibrosis may have affected their renal survival even though the primary cause of the ESKD was something else, for example diabetic nephropathy.

Our hospital has two hemodialysis rooms, one in the kidney center and the other in the diabetes center. The subjects of this study were patients who started

hemodialysis in the kidney center, so the percentage of patients who started on hemodialysis for ESKD secondary to diabetic nephropathy was relatively low.

A high prevalence of HCV has been reported in patients with type-two diabetes mellitus (DM) [13]. The high prevalence may be related to increased vulnerability to HCV infection because of impaired immune defence mechanisms in DM. Also, some patients with various forms of liver disease are predisposed to impaired glucose tolerance because of corticosteroid and hydrochlorothiazide therapy or the presence of hemochromatosis [14]. In addition to these known risk factors, there are emerging epidemiological data suggesting that HCV infection may also contribute to the development of diabetes [15]. Two reports have mentioned the high prevalence of HCV infection among hemodialysis patients with diabetes mellitus [13, 16]. Our study revealed that diabetic nephropathy was a more frequent cause of ESKD among Japanese patients who were anti-HCV-antibody positive at the start of hemodialysis (37.9%) than among those who were anti-HCV-antibody negative (18.6%, $P = 0.0122$).

The prevalence of HCV was not very high among the patients starting on hemodialysis after having been on CAPD or who had received a transplant. Previous CAPD or transplantation was not a risk factor for HCV infection in this study.

Low levels of iron and ferritin are advantageous for the activity of hepatitis because of the reduced reactive oxidative stress. However, no differences in the iron and ferritin levels were observed between the anti-HCV-antibody-positive and the anti-antibody-negative patients.

One of the limitations of this study is that Tokyo Metropolitan Red Cross Blood Center accepts volunteers who do not have history of blood transfusion, history of viral hepatitis, or other risk factors as blood donors. So the volunteer blood donors, even first time ones, have been documented to have lower infection rates than the general population.

An overview of regular dialysis treatment in Japan reported a 7.6% anti-HCV antibody-positive rate among patients who had been on hemodialysis therapy for less than 2 years [9], and there were no statistical differences between the patients with less than 2 years hemodialysis in their study and the patients new to hemodialysis in our study. Acquisition of hepatitis C from nosocomial sources after starting on dialysis therapy appears to be much less of a factor now.

Conclusion

The results of this study showed a much higher rate of anti-HCV antibody positivity in patients new to hemodialysis than in healthy volunteers. Older age, blood transfusion, male gender, and diabetic nephropathy seemed to be risk factors for anti-HCV antibody positivity in Japan.

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Laboratory and Epidemiology Communications

Positivity Rate of Hepatitis B Surface Antigen in 16-Year-Old First-Time Blood Donors: Effectiveness of Immunoprophylaxis with Hepatitis B Vaccine and Immunoglobulin in Newborn Infants with Mothers Positive for Hepatitis B e Antigen

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In 1986, a program to prevent mother-to-infant transmission of hepatitis B virus (HBV) was initiated in Japan. Since that time, expecting mothers have been routinely tested for hepatitis B surface antigen (HBsAg). When a mother is found to be HBsAg-positive, she is also tested for hepatitis B e antigen (HBeAg). If a mother tests positive for both HBsAg and HBeAg, the newborn infant is administered the hepatitis B vaccine and immunoglobulin at the public's expense. The rate of HBsAg testing of expecting mothers was calculated to be 92-96%, as determined by comparison of the number of expecting mothers tested for HBsAg and the number of births during the following year (1). Moreover, 97-98% of newborn infants with HBeAg-positive mothers were administered the hepatitis B vaccine together with immunoglobulin (1). This mother-to-infant HBV transmission prevention program was expanded in March of 1995 to include not only newborn infants with HBeAg-positive mothers, but also infants with HBeAg-negative mothers.

Since 1995, HBsAg-positivity rate of first-time blood donors in Tokyo, Ibaraki, Tochigi, Kanagawa, and Fukuoka Prefectures has been investigated. Because first-time blood donors are unaffected by prior notification of previous screening test results, the positivity rate of first-time blood donors is thought to reflect the positivity rate of the community in general. To evaluate the effectiveness of the mother-to-infant HBV transmission prevention program, we compared the HBsAg positivity rate of 16-year-old first-time blood donors before and after 2003, because all 16-year-old blood donors in 2003 in these areas were born in the same year, 1986.

The HBsAg positivity rate of 16-year-old first-time blood donors was found to decline yearly starting in 1995, and finally reached zero in 2003 (Fig. 1). In 2004, the positivity rate increased, but it was confirmed that all HBsAg-positive persons identified in that year had been infected via a horizontal transmission route. Because the HBsAg-positivity rate in 2005 returned to zero, no HBV carrier was identified among 16-year-old first-time blood donors for the 3 years from 2003 to 2005. However, one HBV carrier was confirmed in 2006. Investigation revealed that this case was due to a failure of immunoprophylaxis.

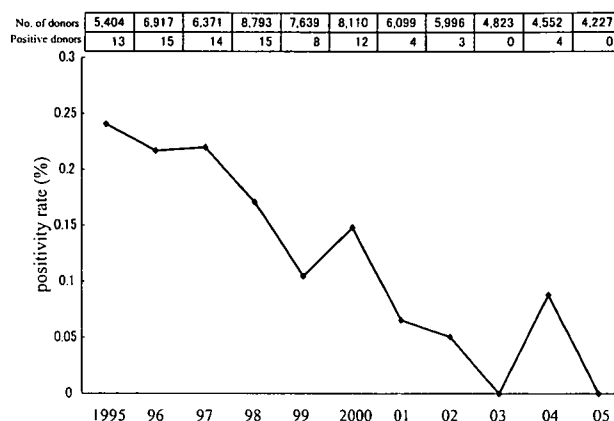| No. of donors	5,404	6,917	6,371	8,793	7,639	8,110	6,099	5,996	4,823	4,552	4,227
Positive donors	13	15	14	15	8	12	4	3	0	4	0

Fig. 1. Positivity rate of HBsAg in 16-year-old first-time blood donors. The investigation was performed in Tokyo, Ibaraki, Tochigi, Kanagawa, and Fukuoka Prefectures.

The effectiveness of this program to prevent mother-to-infant transmission of HBV was confirmed by investigation of the HBsAg-positivity rate of 16-year-old first-time blood donors. It is expected that the continuity of this prevention program will substantially reduce the number of HBV carriers in Japan.

The HBsAg-positivity rate of 16-year-old first-time blood donors was found to decline yearly after the onset of this study in 1995. This decline may be attributed to the following factors: (i) clinical immunoprophylaxis trials using hepatitis B vaccine and immunoglobulin had been carried out prior to the initiation of the national mother-to-infant HBV transmission prevention program, and (ii) the number of infants with HBeAg-positive mothers decreased due to late marriage.

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