showed favorable outcome in patients with prothrombin activity of more than 40% or PT-INR of less than 1.5, but the prognosis was not good enough in those with prothrombin activity of 40% or lower and/or PT-INR of 1.5 or higher. The efficacy of pulse steroid treatment for AIH with severe liver disease needs to be clarified in future'.] If anything, pulse steroid treatment increased a risk of death from infectious disease in patients showing prothrombin activity of 40% or lower and/or PT-INR of 1.5 or higher. The physicians should pay attention to the development of infectious disease, especially fungal infection, when pulse steroid treatment is performed.[α]

Human leukocyte antigen DR status is one of the important genetic risk factors for AIH. DR3 and DR4 in Caucasians and DR4 in Japanese are independently susceptible to type 1 AIH.[18] HLA DR status influences the clinical features. In Caucasians, patients with DR3 develop the disease at younger age and show severer form than those with DR4, while patients with DR4 have higher frequencies of extrahepatic concurrent autoimmune diseases, especially autoimmune thyroiditis. Autoimmune thyroiditis has been reported to be associated with DR4.[19] In this study, there was no difference in frequency of DR4 between patients in the acute hepatitis phase and those in the acute exacerbation phase, and this HLA allotype did not influence the outcomes. Furthermore, the frequency of DR4 in this study is similar to the previous national survey reported in 1997 in which most patients showed histological chronic disease.[6] Thus, in Japanese, HLA DR status may not influence the form of clinical onset and the degree of intrahepatic inflammation. On the other hand, in this study, patients with DR4 had a higher frequency of extrahepatic concurrent autoimmune diseases, especially autoimmune thyroiditis. This is consistent with the report in Caucasians.

In conclusion, we evaluated the clinical features of type 1 AIH patients showing acute presentation. The survival of the patients showing acute presentation is generally good, although approximately 10% of them reach fatal outcomes and may require liver transplantation. Prothrombin activity and PT-INR are useful prognostic factors and approximately 50% of patients showing prothrombin activity of 40% or lower and/or PT-INR of 1.5 or higher at presentation reach fatal outcomes.[α] Conventional corticosteroid treatment is effective for the patients showing acute presentation. On the other hand, the efficacy of pulse steroid treatment for AIH with severe liver disease has been uncertain. [Correction made on 30 November 2012, after first online publication: 'the efficacy of pulse steroid treatment is similar to that of conventional corticosteroid treatment' was corrected to 'the efficacy of pulse steroid treatment for AIH with severe liver disease is uncertain'.] If anything, pulse steroid treatment may increase a risk of death from infectious disease. The above information will be useful for clinical physicians. In addition, the results of this study indicate that type 1 AIH patients in the acute hepatitis phase will reach fatal outcomes more frequently than those in the acute exacerbation phase. However, the sample size of this study is small. In order to confirm these findings, further studies with large sample size are required.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinical features of hepatocellular carcinoma in patients with autoimmune hepatitis in Japan

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Abstract
Background The occurrence of hepatocellular carcinoma (HCC) in patients with autoimmune hepatitis (AIH) is rare compared to that in patients with viral hepatitis. To clarify the status of HCC in patients with AIH in Japan, the clinical features of HCC in patients with AIH were analyzed.
Methods A primary survey gathered data from 496 member institutions of the Liver Cancer Study Group of Japan, and a secondary survey collected additional information from 250 HCC patients out of a total 4869 AIH patients who were identified in the primary survey.
Results Of the 250 patients identified through the secondary survey, 127 patients (50.8 %) from throughout Japan were found to have HCC. The mean age at diagnosis of HCC was 69 years, and the male-to-female ratio was 1:5.7. The mean period from diagnosis of AIH to detection of HCC was 8 years, and Child–Pugh status at the time of HCC diagnosis was class A in 61.8 %; of the patients analyzed, 77.9 % also had cirrhosis of the liver. The mean value of maximum tumor diameter was 4.3 cm, and clinical stages were I in 20.1 % of patients, II in 47.6 %, III in 23.4 %, and IV in 8.9 %. The therapeutic modality used was surgery in 30.2 %, percutaneous therapy in 29.5 %, transcatheter arterial chemoembolization in 36.4 %. Cumulative survival rate was 85.4 % at one year.
Conclusion The survey results showed that HCC developed in 5.1 % of patients with AIH in Japan, with cirrhosis of the liver commonly found in elderly individuals; when HCC was diagnosed at an early clinical stage, in many cases, the liver function was relatively preserved. After diagnosis of AIH, observation of its progression with close attention to potential HCC complications is necessary.

Keywords Autoimmune hepatitis · Hepatocellular carcinoma · Cirrhosis · Diabetes mellitus · Liver Cancer Study Group of Japan

Introduction

The risk of hepatocellular carcinoma (HCC) developing in patients with autoimmune hepatitis (AIH) is rare, and its incidence is much lower than that in patients with viral hepatitis [1, 2]. In recent years, however, since the disease concept of AIH and its diagnostic criteria have become better understood, cases of long-term survival have increased, and consequently there have been sporadic reports of complications with HCC [3–5]. In Japan, no studies involving more than 100 cases had yet been examined; typically, only a few cases arise in a single facility, so it is difficult to ascertain the overall situation and understand the pathology. With the convening of the 47th Liver Cancer Study Group of Japan in July 2011, we had an opportunity to survey the member institutions by questionnaire. Based on this survey, we report the clinical features of HCC in Japanese patients with AIH.

Participating investigators other than the authors are listed in the Appendix.

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Methods

A primary survey was first carried out among the 496 member institutions of the Liver Cancer Study Group of Japan, and a secondary survey was then carried out to analyze 250 cases of HCC in patients with AIH, a sample that represented 5.1 % of an initial 4869 cases reviewed. The database search was limited to the period from 1990 to the present. The diagnosis of AIH was based on the International Autoimmune Hepatitis Group (IAHG) criteria. Patients with HCC were diagnosed with imaging studies, clinical data, and/or histopathologic studies. Patients positive for the hepatitis C virus (HCV) antibody and hepatitis B surface (HBs) antigen were excluded. Survey forms were distributed to obtain the following information on the patients: age at the time of HCC diagnosis, sex, body mass index (BMI), history of alcohol consumption, liver cirrhosis status, use of steroids or other immunosuppressants, complication with primary biliary cirrhosis (PBC), comorbidity with diabetes, years after AIH diagnosis, Child–Pugh status, number of tumors, maximum tumor diameter, clinical stage according to the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) [6], therapeutic modality, prognosis, and laboratory findings (ALT, platelet count, antinuclear antibodies (ANA), α-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), HBc antibody). We defined an alcohol consumer as someone with a mean ethanol intake of more than 20 g/day. Liver cirrhosis was comprehensively diagnosed by liver biopsy findings or clinical parameters and imaging findings. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki.

Statistical analysis

Continuous data are presented as means and ranges. Cumulative survival among HCC patients with AIH was calculated using the Kaplan–Meier method. A commercially available computer program, Prism (version 4.0a, GraphPad Software, Inc.), was used for all statistical calculations.

Results

Responses were received from 54 institutions throughout Japan (response rate: 127/250, 50.8 %). Table 1 shows clinical features at the time of HCC diagnosis for 127 cases. Mean age at diagnosis was 69 years, and the male-to-female ratio was 1:5.7. Prevalence of liver cirrhosis was high (77.9 %). There were no cases of obesity (mean BMI of 23.8), and percentage of patients with diabetes mellitus was high (29.2 %). Frequency of steroid use was high (57.2 % of patients), but the percentage of patients using other immunosuppressants was low (7.3 %). The median period until detection of HCC after diagnosis of AIH was 8 years, but the period was as long as 29 years in some cases. The mean and maximum survival periods after diagnosis of HCC were 3.3 years and 17 years, respectively.

Table 1 Clinical features at diagnosis of hepatocellular carcinoma in patients with autoimmune hepatitis

Number	127
Age (years)	69 (36–84)
Male:female	1:5.7
Body mass index (kg/m^2)	23.8 (15.9–37.0)
Alcohol consumption (%)	6.9
Cirrhosis at accession (%)	77.9
Steroid therapy (%)	57.2
Azathioprine therapy (%)	7.3
PBC overlap (%)	5.6
Diabetes mellitus (%)	29.2
Period from AIH diagnosis to HCC development (years)	8.0 (0–29)
Period from HCC development to death (years)	3.3 (0–17)

PBC primary biliary cirrhosis, *AIH* autoimmune hepatitis, *HCC* hepatocellular carcinoma

Table 2 shows laboratory findings at the time of HCC diagnosis. Mean alanine aminotransferase (ALT) was low (40.8 IU/l), and mean platelet count was slightly low (13.6×10^4/μl). The mean AFP was 25599 ng/ml and positivity was 67.2 %, with the level in 34.4 % of the positive patients being 200 ng/ml or higher. The mean DCP was 4132 mAU/ml and positivity was 51.8 %, with the level in 24.5 % of the positive patients being 400 mAU/ml or higher. With respect to the role of the hepatitis virus, HBc antibody positivity was 27.7 %.

Table 3 shows clinical features of HCC. Numbers of tumors were 1 in 63.2 % of the patients, 2 in 18.4 %, and multiple in 18.4 %. The mean value of maximum tumor diameter was 4.3 cm, and maximum diameters were 2 cm or lower in 20.5 % of the patients, 2.1–5 cm in 54.1 %, and 5.1 cm or more in 25.4 %. Rates of Child–Pugh status were comparatively good, with class A in 61.8 %, class B in 30.9 % and class C in 7.3 %. Clinical stages were I in 20.1 % of the patients, II in 47.6 %, III in 23.4 %, and IV in 8.9 %. Therapeutic modality was analyzed for 129 treatments representing 116 cases, some of which included multiple therapies; 11 cases were excluded due to missing data. Treatment was by resection of the tumor in 30.2 % of the patients, by percutaneous therapy, including radiofrequency ablation (RFA) and percutaneous ethanol injection therapy (PEIT), in 29.5 %, by transcatheter arterial

Table 2 Laboratory features at diagnosis of hepatocellular carcinoma in patients with autoimmune hepatitis

ALT (IU/l)	40.8 (5–197)
Platelets ($\times 10^4/\mu l$)	13.6 (2.6–60.5)
ANA median (range)	160 (0–20480)
AFP (ng/ml)	
Mean (range)	25,599 (2–1816620)
<10 (%)	32.8
10–200 (%)	32.8
>200 (%)	34.4
DCP (mAU/ml)	
Mean (range)	4132 (0.05–105000)
<40 (%)	48.2
40–400 (%)	27.3
>400 (%)	24.5
HBc antibody-positive (%)	27.7

ALT alanine aminotransferase (normal 8–42 IU/l), *ANA* antinuclear antibody (<40×), *AFP* α-fetoprotein (<10 ng/ml), *DCP* des-γ-carboxy prothrombin (<40 mAU/ml)

Table 3 Clinical features of and therapy for hepatocellular carcinoma in patients with autoimmune hepatitis

Number of HCC tumors	
Single (%)	63.2
Double (%)	18.4
Multiple (%)	18.4
Maximum tumor size	
Mean (range) (cm)	4.3 (1.0–30.0)
<2 (%)	20.5
2.1–5.0 (%)	54.1
>5.1 (%)	25.4
Child–Pugh score	
A/B/C (%)	61.8/30.9/7.3
Stage	
I/II/III/IV (%)	20.1/47.6/23.4/8.9
Therapy choices for HCC	Total 129
Surgery (%)	39 (30.2)
RFA, PEIT (%)	38 (29.5)
TAE (%)	47 (36.4)
Others (%)	5 (3.9)

HCC hepatocellular carcinoma, *RFA* radiofrequency ablation, *PEIT* percutaneous ethanol injection therapy, *TAE* transcatheter arterial embolization

chemoembolization (TACE) in 36.4 %, and by other means (liver transplantation, proton-beam radiotherapy, sorafenib) in 3.9 %.

Figure 1 shows cumulative survival according to the Kaplan–Meier method based on 117 cases; 10 cases were excluded due to missing data. Cumulative survival rates were 85.4 % at 1 year, 65.8 % at 3 years, 56.4 % at 5 years, and 39.4 % at 10 years.

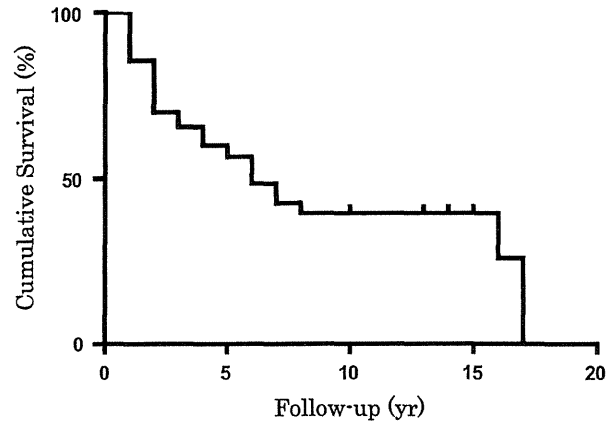

Fig. 1 Kaplan–Meier curve showing survival of autoimmune hepatitis patients with hepatocellular carcinoma. Median survival at 1 year for all patients was 85.4 %

Discussion

As the first analysis of over 100 cases of HCC in patients with AIH, our survey showed the clinical features of these types of cases in Japan. Mean age at the time of HCC diagnosis was relatively high (69 years). The male-to-female ratio was 1:5.7, a higher ratio of males than the ratio of 1:6 or 1:7 in prior reports on AIH [7, 8]; Table 4 demonstrates these changes [9, 10]. Liver cirrhosis was previously reported as a risk factor [9–13], and the rate of liver cirrhosis was also high in our study. Past studies reported that AIH patients with an older age of onset are likely to develop cirrhosis [14], while cases of HCC from liver cirrhosis of non-viral hepatitis has also been reported, but these cases are thought to include AIH or nonalcoholic steatohepatitis (NASH) [15]. Complications with diabetes in our study were also comparatively frequent, but this finding may reflect the influence of therapeutic use of steroid agents in the majority of cases and the inclusion of many liver cirrhosis cases.

In the EU and USA, and even in Japan, steroid resistance in AIH patients using other immunosuppressants (e.g., azathioprine), and attendant carcinoma has also been reported in these cases [16, 17], but in our study, the frequency of use of other immunosuppressants was low (7.3 %). While steroids are used as the first-line of therapy for AIH, about 10 % of AIH patients in Japan have demonstrated resistance to steroid therapy. For steroid-resistant patients, azathioprine, which is not covered by national health insurance, is considered first-line therapy. When the maintenance dose of steroid was higher, the incidence of HCC was significantly higher. However, neither steroid nor azathioprine therapies were significant factors for the development of HCC, andthere was no significant difference between the two therapies [18]. In our survey, the

Table 4 Comparison of clinical features of reported hepatocellular carcinoma in patients with autoimmune hepatitis

Author	Yeoman	Montano-Loza	Watanabe	Our study
Year	2008	2008	2009	2012
Number	15	9	38	127
Mean age (years)	61	49	68	69
Male:Female	1:4	1:1.3	1:4.4	1:5.7
Cirrhosis at accession (%)	73.3	44	58.1	77.9
Period from AIH diagnosis to HCC development (years)	NA	NA	10.2 (0–22)	8.0 (0–29)
Period from HCC development to death (years)	2.3 (0.1–17)	1.3 (0.2–2.8)	1.2 (0.2–3)	3.3 (0–17)
Maximum tumor size				
Mean ± SD (cm)	2.8 ± 1.6	NA	3.7 ± 2.4	4.3 ± 4.4
AFP				
Mean ± SD (ng/ml)	6291 ± 15562	600 ± 100	2340 ± 8823	25599 ± 180028

AIH autoimmune hepatitis, *HCC* hepatocellular carcinoma, *AFP* α-fetoprotein (<10 ng/ml), *NA* not available

proportion of cases positive for HBc antibody was particularly high (approximately 30 %). In Japan, approximately 25 % of cases have a prior history of infection, and the role of prior history of HBV infection in hepatocarcinogenesis is unknown. A recent study shows that 73 % of patients with apparently unidentifiable causes for HCC were HBV-related [19].

As shown in Table 4, the mean period until detection of HCC after diagnosis of AIH was 8 years, which is shorter than the period of approximately 10 years in prior reports [20]. This difference may reflect the fact that age at diagnosis of HCC in our study was older than other reported cases. The rate of HCC onset has also been reported to increase at 10 years or longer after diagnosis of AIH [21]. Positivities for the HCC tumor markers AFP and DCP were 67.2 % and 51.8 %, respectively, on par with follow-up study results for primary liver cancer as a whole [22]. These markers are unrelated and are thought to improve diagnostic capability in complementary fashion through combination assay of both markers. Number of tumors, maximum tumor diameter, and other clinical features of HCC often reflect a single focus, but the mean maximum tumor diameter in our survey was larger than that of general HCC [23]. This finding may be due to the fact that the risk of carcinogenesis in AIH is lower than that in viral hepatitis and image-based screening is thus less frequent.

In our study, Child–Pugh status in many cases was good, at class A, and clinical stage was also I or II in many cases, but treatments such as percutaneous therapy and TACE were more frequent than surgery. We attributed this to the large proportion of elderly patients in our study and the tendency to select less invasive treatment. Mean survival period was 3.3 years, and 10 patients survived for 10 years or longer. Cumulative survival rates were 85.4 % at 1 year, 65.8 % at 3 years, 56.4 % at 5 years, and 39.4 % at 10 years. These rates were better than the cumulative survival rates reported by the Follow-up Study Committee of the Liver Cancer Study Group of Japan: 79.1 % at 1 year, 55.0 % at 3 years, 37.9 % at 5 years, and 16.5 % at 10 years [22]. These differences may reflect the fact that many cases in our study were in early clinical stages of disease at the time of HCC diagnosis and preserved liver function was maintained. Indeed, the mean maximum tumor diameter of 4.3 cm in our survey was larger than that of HCC in viral hepatitis, but there was only one tumor in most of our cases. As of yet, there are no reports on recurrence of HCC in AIH, but one report has shown that the cause of death in cases of HCC in AIH is most frequently liver failure, unlike HCC in viral hepatitis and other such diseases, in which cancer deaths are frequent [20]. AIH patients with liver cirrhosis, who are at high risk for HCC, should be screened by ultrasonography and measurement of AFP and DCP [24]. Since AIH patients with an older age of onset are likely to develop cirrhosis, careful observation is necessary for such patients. Because our study was conducted with a survey at a single point in time, and we have not yet reaffirmed the data, there is a possibility that we do not show the actual features of HCC in patients with AIH. Further study with a view to long-term prognosis is needed.

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Conflict of interest The authors declare that they have no conflict of interest.

Appendix

In addition to the authors, members of the Liver Cancer Study Group of Japan who participated in this study and contributors to this study were as follows: Masahiro Arai

(Toshiba Hospital), Hirotoshi Ebinuma (Keio University School of Medicine), Nobuyuki Enomoto (University of Yamanashi), Shin-ichi Fujioka (Okayama Saiseikai General Hospital), Shigetoshi Fujiyama (NTT West Japan Kyushu Hospital), Yoshihide Fujiyama (Shiga University of Medical Science), Masaru Harada (University of Occupational and Environmental Health), Yoshinobu Hata (Sapporo Social Insurance General Hospital), Hisashi Hidaka (Kitasato University), Keisuke Hino (Kawasaki Medical School), Takaaki Ikeda (Yokosuka Kyosai Hospital), Yoshitaka Inaba (Aichi Cancer Center Hospital), Toru Ishikawa (Saiseikai Niigata Daini Hospital), Akira Jikko (Ako City Hospital), Koji Joko (Matsuyama Red Cross Hospital), Eiji Kajiwara (Nippon Steel Yawata Memorial Hospital), Takashi Kaiho (Kimitsu Chuo Hospital), Akira Kaneko (NTT West Osaka Hospital), Hiroyuki Kirikoshi (Yokohama City University), Masahiko Koda (Tottori University), Kazuhiro Kondo (University of Miyazaki), Kazuyoshi Kon (Juntendo University), Osamu Kurai (Osaka City Juso Hospital), Yasutaka Nagao (Matsushita Memorial Hospital), Masafumi Naitou (Osaka Koseinenkin Hospital), Kazuaki Nakanishi (Hokkaido University Hospital), Hirokazu Nishino (The Jikei University School of Medicine Daisan Hospital), Shunsuke Nojiri (Graduate School of Medical Sciences, Nagoya City University), Norihiro Nomura (Showa University Toyosu Hospital), Makoto Nishiwaki (Hamamatsu Red Cross Hospital), Hisamitsu Miyaaki (Nagasaki University), Yasuhiro Miyake (Okayama University), Hideaki Mizumoto (Funabashi Municipal Medical Center), Satoshi Mochida (Saitama Medical University), Shinji Mukai (Ohta Nishinouchi Hospital), Jun Ohmori (Kameda Medical Center), Takumi Ohmura (Sapporo-Kosei General Hospital), Yoshiaki Okumura (Social Insurance Shiga Hospital), Naoya Sakamoto (Tokyo Medical and Dental University), Kunitoshi Sakurai (Fukuoka University), Masataka Seikke (Oita University), Keiko Shiratori (Tokyo Women's Medical University), Yasuyuki Suzuki (Kagawa University), Hiroko Takami (National Kyushu Medical Center Hospital), Akihiro Tamori (Graduate School of Medicine, Osaka City University), Atsushi Tanaka (Teikyo University), Eiji Tanaka (Shinshu University), Shinji Uemoto (Graduate School of Medicine, Kyoto University), Hiroshi Watanabe (Fukuoka Red Cross Hospital), Sachiro Watanabe (Gifu Prefectural General Medical Center), Junji Yamamoto (National Defense Medical College), Masashi Yoneda (Aichi Medical University) and Hiroshi Yoshida (Nippon Medical School Tama Nagayama Hospital).

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Activated natural killer T cells producing interferon-gamma elicit promoting activity to murine dendritic cell-based autoimmune hepatic inflammation

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Summary

As natural killer (NK) T cells play an important role in the development of autoimmune diseases, they should have significant roles for the pathogenesis of autoimmune liver disease. Implication of the NK T cells in the generation of autoimmune-related hepatic inflammation was investigated using a novel mouse model. Immunization of mice with dendritic cells (DCs) loaded with hepatocyte-mimicking hepatocellular carcinoma cells (DC/Hepa1-6) induces cytotoxic T lymphocytes (CTL) capable of killing hepatocytes. Subsequent administration of interleukin (IL)-12, a potent interferon-gamma (IFN-γ) inducer, to the immunized mice generates autoimmune hepatic inflammation (AHI), as reported previously. Upon onset of the AHI response, the number of intrahepatic $CD3^+NK1{\cdot}1^+$ NK T cells increased markedly, along with a decrease in the number of splenic NK T cells, augmented expression of CXCR6 on intrahepatic NK T cells and CXCL16 in hepatic tissue, suggesting that NK T cells were recruited into the inflamed liver. The NK T cells were strongly positive for CD69 and produced IFN-γ, but not IL-4. AHI activity was attenuated markedly in $CD1d^{-/-}$ NK T cell-deficient mice, indicating that NK T cells play a pivotal role in the development of AHI. Mice treated with DC/Hepa1-6 and alpha-galactosylceramide, a potent NK T cell activator, also exhibited similar hepatic inflammation, in which activated NK T cells producing IFN-γ and $CD8^+$ T cells cytotoxic to hepatocytes were induced in liver-infiltrating mononuclear cells. Activated NK T cells producing IFN-γ potentiate DC-based AHI in the mouse model.

Keywords: α-GalCer, autoimmune hepatic inflammation, cytotoxic T lymphocyte, interferon-gamma, natural killer T cell

Introduction

Autoimmune hepatitis (AIH) is an immune-mediated chronic inflammatory liver disease that predominantly affects women genetically predisposed to its development [1]. Although the pathogenic mechanism of AIH and aetiological agents involved are not known, it has been postulated that autoreactive T cells targeting at hepatocytes play a central role in the development of AIH [2]. Until recently, autoreactive $CD4^+$ T cells were considered to be critical for disease development [3], but increasing evidence has shown that $CD8^+$ T cells also play significant roles [4]. $CD8^+$ T cells are observed mainly in areas of interface hepatitis of AIH, and $CD4^+$ T cells are found in the central part of the portal tract [5]. Peripheral $CD8^+CD25^+$ lymphocytes are more prevalent in patients with acute-onset AIH than those with chronic AIH. After treatment with immunosuppressive therapies, the number of $CD8^+CD25^+$ T lymphocytes in the blood decrease in parallel with the serum aminotransferase level [6]. Although hepatic tissue in AIH exhibits abundant apoptosis of hepatocytes [7], and highly activated infiltrating T lymphocytes [8] may play an important role in the induction of hepatocyte apoptosis, the mechanism of establishment and progression of AIH remains unclear.

Natural killer (NK) T cells are innate immune cells that were described originally as expressing both T cell and NK cell phenotypes [9]. NK T cells are activated in a CD1d-dependent manner in response to glycolipid antigens such as α-galactosylceramide (α-GalCer) [10,11], and rapidly produce large amounts of T helper type 1 (Th1) cytokine,

interferon-gamma (IFN-γ) and Th2 cytokine, interleukin (IL)-4 [12]. The implication of NK T cells in autoimmune diseases such as multiple sclerosis [13–15] and diabetes [16–18] has been shown using well-established animal models. In mice, NK T cells represent up to 30% of T cells in the liver, where they reside within the sinusoids and appear to provide intravascular immune surveillance [19,20], and may also be associated with the development of liver injury in the setting of hepatitis [21,22]. In humans, it has not been clarified whether NK T cells are beneficial or harmful in the setting of liver disease [23]. The importance of NK T cells in the pathogenesis of autoimmune liver disease also remains unknown.

We have reported previously a mouse model of autoimmune hepatic inflammation (AHI) generated by immunization of C57BL/6 mice with dendritic cells (DC) loaded with well-differentiated hepatocellular carcinoma cells (Hepa1-6), followed by IL-12 administration [24,25]. In this model, liver specific inflammation is mediated by hepatocyte-responsive autoreactive T cells. Our findings indicate that two independent steps are necessary for the development of autoimmune-mediated liver damage: one step concerns the induction of autoreactive T cells responsive to hepatocytes. Because of similar phenotypic expression between normal hepatocytes and Hepa1-6 cells, cytotoxic T lymphocytes (CTLs) recognizing shared antigen between them are induced by immunization of mice with DCs loaded with Hepa1-6 [24]. The other step is the modulation of the hepatic microenvironment to promote recruitment of autoreactive T cells into the liver and CTL response to hepatocytes, because the sole induction of autoreactive T cells cannot generate autoimmune hepatic injury *in vivo*. The key cytokine for this response is IFN-γ induced by IL-12, which provides the enhanced expression of major histocompatibility complex (MHC) class I, adhesion molecules and chemokines on hepatocytes [24]. In fact, treatment of the immunized mice with anti-IFN-γ monoclonal antibody or immunization of IFN-γ knock-out mice abolishes AHI activity [24]. Although, unlike human AIH, histological features of AHI are characterized by an acute inflammatory response located mainly in hepatic parenchyma, this model could contribute to analysis of the mechanism of the liver-specific autoimmune response [25].

In this study we show, using our mouse model, that intrahepatic NK T cells play a pivotal role in promoting the CTL-mediated autoimmune hepatic inflammation.

Materials and methods

Animals

Eight-week-old female C57BL/6 wild-type (WT) mice were purchased from Sankyo Labo Service Co., Ltd (Tokyo, Japan). CD1d$^{-/-}$ mice (C57BL/6 genetic background) were kindly provided by Dr Shinsuke Taki (University of Shinshu, Japan). All animals were maintained in our facilities and received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences (NIH publication 86-23, revised 1985). Mice were used at the age of 8–10 weeks and were matched for sex and age.

Cell lines, cytokines and antibodies

Hepa1-6, a well-differentiated murine hepatocellular carcinoma cell line, was obtained from the American Type Culture Collection (Manassas, VA, USA). Recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF) and recombinant IL-2, IL-4 and IL-12 were purchased from PeproTech (Rocky Hill, NJ, USA). Anti-CD8a monoclonal antibody (mAb) (clone 53-6·7) conjugated with fluorescein isothiocyanate (FITC) was purchased from BD Biosciences (San Diego, CA, USA). Anti-CD69 mAb (clone H1·2F3) conjugated with allophycocyanin (APC) and anti-CD62L mAb (clone MEL-14) conjugated with phycoerythrin (PE) were purchased from BioLegend (San Diego, CA, USA). Anti-CXCR6 mAb (clone 221002) conjugated with PE was purchased from R&D Systems, Inc. (Minneaoplis, MN, USA). Anti-IFN-γ (clone XMG1·2), anti-IL-4 (clone 11B11) conjugated with APC for intracellular cytokine staining were purchased from eBioscience (San Diego, CA, USA).

Treatment of mice

AHI was induced in mice as described previously [24,25]. Briefly, bone marrow-derived DCs loaded with Hepa1-6 cells (DC/Hepa1-6) were generated by quick treatment of a mixture of the DCs and Hepa1-6 cells with 50% polyethylene glycol (Peg solution; Sigma-Aldrich, Inc., St Louis, MO, USA). DC/Hepa1-6 cells were injected subcutaneously into 8-week-old female C57BL/6 WT mice or CD1d$^{-/-}$ mice on days 1 and 14. Then, IL-12 (500 ng/mouse) was injected intraperitoneally on days 15, 17 and 19. The mice were sacrificed on day 21. To analyse the role of activated NK T cell in this mouse model, α-GalCer (KRN7000; Funakoshi Co., Ltd, Tokyo, Japan) was dissolved in 0·1 ml phosphate-buffered saline (PBS) and injected intraperitoneally (0·5 μg/mouse) instead of IL-12 to DC/Hepa1-6 pretreated and untreated WT mice on day 15. The mice were sacrificed 48 h after α-GalCer administration.

Assay for serum transaminase levels

Serum alanine aminotransferase (ALT) levels were measured using the DriChem system (L3500V; Fuji Film Medical Co., Ltd, Tokyo, Japan), according to the manufacturer's instructions.

Histology

Liver tissue was fixed in 10% formalin for at least 24 h and paraffin-embedded. Sections of 2 μm thickness were stained with haematoxylin and eosin (H&E) to determine morphological changes. The numbers of inflammatory foci were determined as described previously [24].

Preparation of liver mononuclear cells

Hepatic mononuclear cells (MNCs) were isolated from murine liver, as described previously [24].

Quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

Liver RNA extraction and messenger RNA (mRNA) quantification by real-time qRT–PCR were performed as described previously [25]. The expression levels of CXCL16 were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Flow cytometry and intracellular cytokine staining

For cell-surface staining, after blocking with anti-FcR (clone 93; eBioscience), cells were incubated with various mAbs in darkness at 4°C for 30 min and examined by flow cytometry [fluorescence activated celll sorter (FACS)Calibur, BD Biosciences Immunocytometry Systems, San Jose, CA, USA]. For intracellular cytokine staining, isolated intrahepatic MNCs were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma-Aldrich, Inc.) and ionomycin (1μg/ml; Sigma-Aldrich, Inc.) in the presence of Brefeldin A (10 μg/ml; Sigma-Aldrich, Inc.) for 5 h. After labelling the cell-surface antigens, cells were fixed and permeabilized using a Cytofix/Cytoperm plus kit (BD Biosciences) and then stained with anti-IFN-γ or IL-4 conjugated with APC. The stained cells were analysed by flow cytometry. The data were analysed using CellQuest Pro version 5·2 software (BD Biosciences Immunocytometry Systems, San Jose, CA, USA).

Isolation of hepatocytes and cytotoxicity assay

Hepatocytes were isolated as described previously [24]. A cytotoxicity assay against primary murine cultured hepatocytes was performed as described previously [24]. Briefly, isolated hepatocytes were seeded at 1×10^4 cells/well into 96-well collagen-coated plates (Iwaki, Asahi Techno Glass, Chiba, Japan). After overnight incubation, 4×10^5 effector, intrahepatic whole MNCs or $CD8^+$ T cells or non-$CD8^+$ T cells, which were isolated using a magnetic cell sorting system ($CD8^+$ T Cell Isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany), were co-cultured for 24 h. Aspartate aminotransferase (AST) activity of the culture supernatant was determined and percentage of cytotoxic activity was calculated as [(experimental AST release – spontaneous AST release)/(total AST release – spontaneous AST release) × 100], according to the formula described previously [24].

Statistical analysis

The significance of difference among the groups was analysed with Tukey's test for multiple group comparisons. Unpaired Student's *t*-test was used for comparison of means in two groups. Differences were considered to be significant at a *P*-value less than 0·05.

Results

Accumulation of activated NK T cells producing IFN-γ in the AHI liver

As shown in Fig. 1a,i, the number of NK T cells increased significantly in the AHI liver generated by treatment of mice with DC/Hepa1-6 and IL-12. The number of NK T cells was higher in combined treatment with DC/Hepa1-6 and IL-12 than treatment with DC/Hepa1-6 or IL-12 alone (Supplementary Fig. S1A-a). The absolute number of intrahepatic $CD69^+$ activated NK T cells was high in AHI (Fig. 1a,ii). Conversely, the number of NK T cells in the spleen was lower than in the untreated control at maximum hepatic inflammation (Fig. 1a,iii). Expression levels of CXCL16, a ligand of CXCR6, in hepatic tissue and the population of $CXCR6^+$ intrahepatic NK T cells were elevated in AHI liver (Fig. 1b,i–iii). Although the population of IFN-γ-producing intrahepatic NK T cells increased at maximum hepatic inflammation (Fig. 1c,i,iii), the population of IL-4-producing intrahepatic NK T cells was not affected (Fig. 1c,ii,iii). These results suggest that activated NK T cells which show a Th1 phenotype might have been accumulated into the liver of AHI. Because of a vigorous increase in the number of intrahepatic T cells by generation of AHI, frequency of NK T cells in the intrahepatic MHCs was decreased in spite of an increase in absolute number of NK T cells (Supplementary Fig. S2).

The activity of AHI was suppressed in the livers of $CD1d^{-/-}$ mice

When AHI was generated in NK T cell-deficient $CD1d^{-/-}$ mice, the inflammatory activity was markedly attenuated (Fig. 2a). The level of serum ALT, number of clusters of MHCs (inflammatory foci, IF) in hepatic tissue and absolute number of MNCs in the liver (Fig. 2b–d) were significantly lower in the AHI livers of $CD1d^{-/-}$ mice compared with those in AHI of WT mice. Although $CD8^+$T cells were found in $CD1d^{-/-}$ mice, they were significantly fewer than in

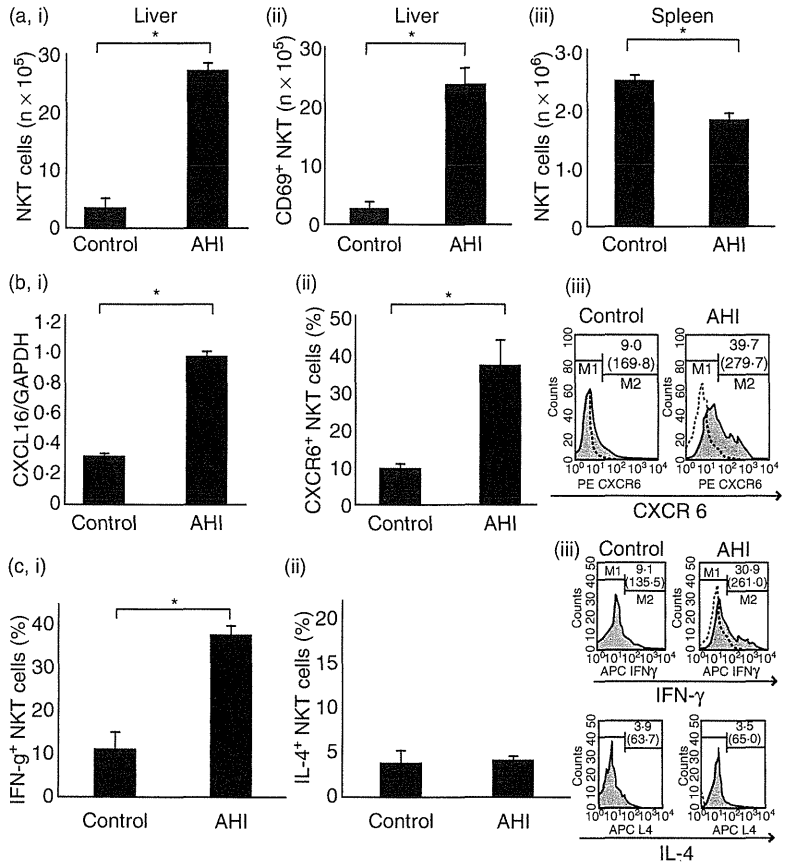

Fig. 1. The dynamic statistics of natural killer (NK) T cells in the autoimmune hepatic inflammation (AHI) liver and spleen. (a) Absolute number of total NK T cells (i) and CD69$^+$NK T cells (ii) in the liver and NK T cells in the spleen (iii) in AHI. The number was determined as [total number of mononuclear cells (MNCs) in the liver or spleen] × [the frequency of CD3$^+$NK1·1$^+$ cells or CD3$^+$NK1·1$^+$ CD69$^+$cells] in each group [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (b) (i) Expression of CXCL16 in hepatic tissue. Levels of CXCL16 mRNA in each group were determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Bars indicate mean ± s.d., $^*P < 0{\cdot}001$. (ii) Population of intrahepatic CXCR6$^+$NK T cells ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (iii) Representative flow cytometry of intrahepatic CXCR6$^+$NK T cells in control and AHI. Grey-filled histograms; CXCR6$^+$cells, dotted histogram; isotype control. The value shows the frequency of CXCR6$^+$ cells and the numbers in parentheses shows mean fluorescence intensity (MFI) of CXCR6. (c) (i) Frequency of interferon (IFN)-γ^+ NK T cells in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (ii) Frequency of interleukin (IL)-4$^+$ NK T cells in control and AHI. (iii) Representative flow cytometry of IFN-γ^+ or IL-4$^+$ intrahepatic NK T cells in each group. Grey-filled histograms; IFN-γ^+ or IL-4$^+$ cells, dotted histogram; isotype control. The value shows the frequency of IFN-γ- or IL-4-producing cells and the numbers in parentheses shows MFI of IFN-γ or IL-4. All experiments were repeated at least three times.

AHI liver of WT mice (Fig. 2e). These results demonstrate that NK T cells play a pivotal role in the establishment of AHI.

α-GalCer treatment of DC/Hepa1-6 immunized mice produced severe hepatic inflammation

Hepatic inflammation was induced when mice immunized with DC/Hepa1-6 were treated with α-GalCer instead of IL-12 (Fig. 3a,i). This was characterized by the emergence of abundant IF in hepatic lobules, similar to the AHI generated by treatment with DC/Hepa1-6 and IL-12. Importantly, the hepatic inflammatory activity generated by treatment with DC/Hepa1-6 and α-GalCer was greater than that induced by treatment with α-GalCer alone (Fig. 3a,i,ii,b,c). The population of IFN-γ-producing NK T cells were enhanced but the population of IL-4-producing NK T cells was not affected in hepatic inflammation induced by treatment with α-GalCer alone or DC/Hepa1-6 and α-GalCer (Fig. 3d,i,ii).

Autoreactive CD8$^+$ CTLs cytocidal to hepatocytes were induced by treatment of mice with DC/Hepa1-6 and α-GalCer

When DC/Hepa1-6 immunized mice were treated with α-GalCer and hepatic inflammation was generated, the population of CD8$^+$ T cells in intrahepatic MNCs was increased (Fig. 4a,i). Among these CD8$^+$ T cells, the percentage of CD62L$^-$ active cells was increased (Fig. 4a,ii,iii).

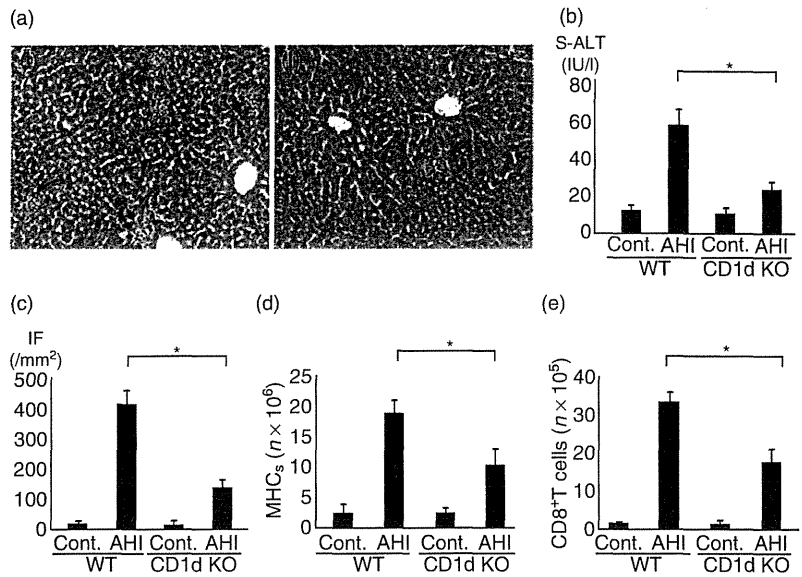

Fig. 2. Autoimmune hepatic inflammation (AHI) activity was attenuated in natural killer (NK) T cell-deficient CD1d$^{-/-}$ mice. AHI was generated in wild-type (WT) or CD1d$^{-/-}$ mice. (a) Histological change of AHI liver (haematoxylin and eosin staining, × 200). Arrows indicate inflammatory foci (IF). (i) Representative hepatic tissue of AHI in WT mice. (ii) Representative hepatic tissue of AHI in CD1d$^{-/-}$ mice. (b) Serum alanine aminotransferase (ALT) levels in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$). (c) Number of IF of AHI in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (d) Absolute number of intrahepatic mononuclear cells (MNCs) in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$) (E) Absolute number of intrahepatic CD8$^+$T cells in WT and CD1d$^{-/-}$ mice ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). All experiments were repeated at least three times.

Notably, CD8$^+$ T cells from the livers of mice treated with DC/Hepa1-6 and α-GalCer showed high cytotoxic activity against hepatocytes, while the cytotoxic activity of intrahepatic CD8$^+$ T cells of mice treated with α-GalCer alone was lower (Fig. 4b). These results indicate that treatment of mice with DC/Hepa1-6 and α-GalCer could induce CTLs cytocidal to autologous hepatocytes, as for the treatment of mice with DC/Hepa1-6 and IL-12.

Discussion

AHI is generated in mice by induction of hepatocyte-responsive CTLs by immunization with DC/Hepa1-6 and accumulation of these autoreactive CTLs to the liver by enhanced expression of adhesion molecules, chemokine ligands and MHC in hepatic tissue by IFN-γ induced by IL-12 [24]. As IFN-γ makes hepatocyte vulnerable to CD8$^+$CTL attack by up-regulation of MHC class I expression [3], IFN-γ is a key cytokine to generate AHI in this mouse model [24]. It is probable that activated NK T cells, accumulated into the liver upon onset of AHI, may contribute to the generation of the AHI as producers of IFN-γ. Because IL-12 is a potent IFN-γ inducer [26], it may stimulate various immune cell types to produce IFN-γ. Thus, the involvement of NK T cells as IFN-γ producers in the generation of AHI has not been clear. When CD1d$^{-/-}$ mice, which show NK T cell deficiency, were immunized with DC/Hepa1-6 and treated with IL-12, the resultant AHI was suppressed significantly, indicating that IFN-γ produced by NK T cells has an important role in development of AHI. Although the number of CD8$^+$ T cells in the AHI liver was decreased significantly in CD1d$^{-/-}$ mice, CD8$^+$ T cells existed in infiltrating cells of the AHI liver of CD1d$^{-/-}$ mice. This result suggests that IFN-γ produced by IL-12-activated immune cells other than NK T cells might have stimulated the CD8$^+$T cell response.

The involvement of NK T cells in the generation of AHI was studied further by treatment of DC/Hepa1-6-immunized mice with α-GalCer. Because treatment with α-GalCer itself causes hepatic injury [27], the minimum dose of α-GalCer that induced hepatic injury (0·5 μg/mouse) was used to analyse the combined effect with DC/Hepa1-6 immunization and α-GalCer on generation of hepatic inflammation. The activity of hepatic inflammation was significantly higher in mice treated with DC/Hepa1-6 and α-GalCer than α-GalCer alone. CD8$^+$ T cells from mice treated with DC/Hepa1-6 plus α-GalCer showed higher percentage of CD62$^-$ cells and significant cytotoxicity against primary cultured hepatocytes, but CD8$^+$ T cells from mice treated with α-GalCer alone showed low cytotoxicity. These results suggest strongly that antigen-specific activated T cells capable of killing hepatocytes, which were induced by immunization with DC/Hepa1-6, had accumulated in the liver following modulation of the hepatic microenvironment by IFN-γ secreted from α-GalCer-activated NK T cells. Although infiltration of CD8$^+$ T cells was seen in the liver of mice treated with DC/Hepa1-6 alone, inflammatory activity of the liver in such mice was somewhat low, as reported previously [24]. Accordingly, because of few numbers of intrahepatic CD8$^+$ T cells of mice treated with DC/Hepa1-6 alone, it was extremely hard to collect them and examine their cytotoxic activity. Cytotoxic activity of CD8$^+$ T cells in the AHI liver of CD1d$^{-/-}$ mice is important for interpretation of the role of NK T cells. According to our previous data, splenic CD8$^+$ T cells from sole DC/Hepa1-6 immunized mice without IL-12 treatment could elicit significant cytotoxic activity to autologous hepatocytes *in vitro*. However, without the effect

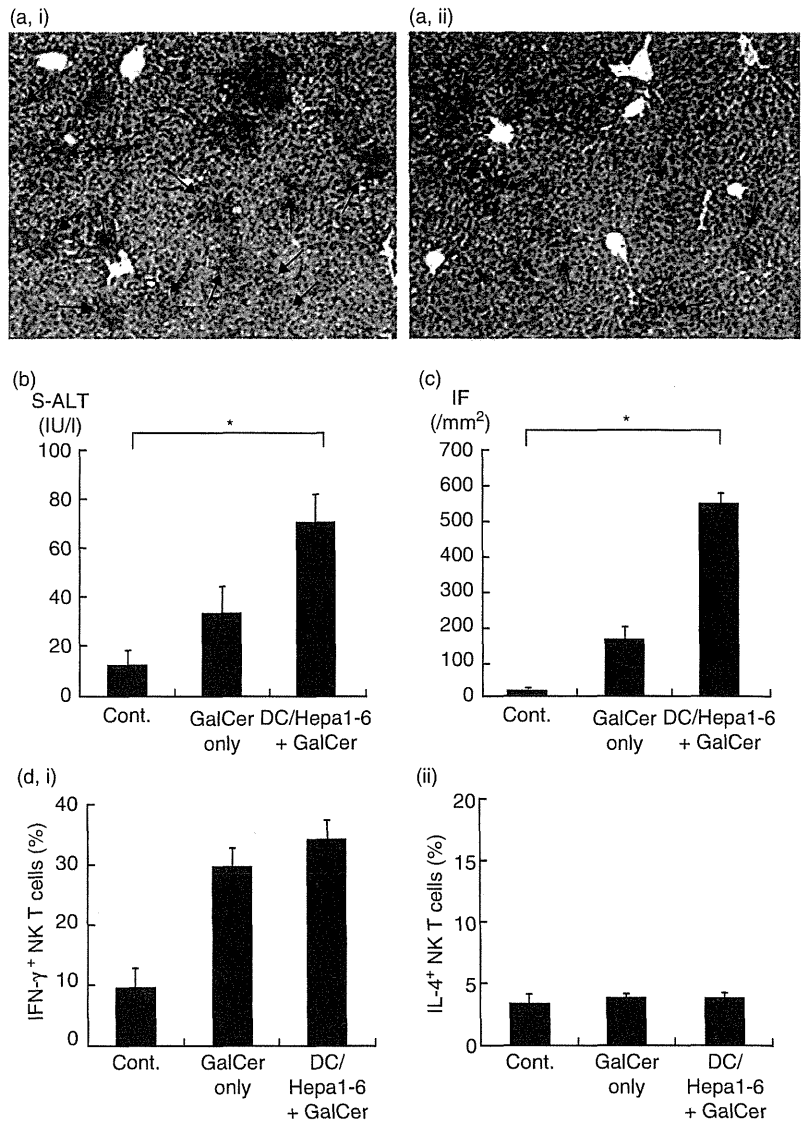

Fig. 3. Treatment of dendritic cell (DC)/Hepa1-6 pre-immunized mice with α-galactosylceramide (α-GalCer) generated marked hepatic inflammation with induction of interferon (IFN)-γ-producing natural killer (NK) T cells. (a) Histological changes in the liver (haematoxylin and eosin staining, × 100). Arrows indicate inflammatory foci (IF). (i) Hepatic tissue of DC/Hepa1-6 pre-immunized mice treated with α-GalCer. (ii) Hepatic tissue of mice treated with α-GalCer alone. (b) Serum alanine aminotransferase (ALT) levels of untreated control mice, mice treated with α-GalCer alone and DC/Hepa1-6 pre-immunized mice treated with α-GalCer [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (c) Numbers of IF in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (d) (i) Frequency of IFN-γ$^+$ intrahepatic NK T cells in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (ii) Frequency of IL-4$^+$ intrahepatic NK T cells in each group. All experiments were repeated at least three times.

of IFN-γ, which was provided by systemic IL-12 treatment or activation of intrahepatic NK T cells, to increase the expression of MHC class I or several adhesion molecules in hepatic tissue, such CD8$^+$ cytotoxic T cells could not recruit into the liver. Thus, it might be probable that CD8$^+$ T cells in the AHI liver of CD1d$^{-/-}$ mice could show cytotoxic activity to hepatocytes, but because of lack of IFN-γ effect provided by activated NK T cells and less infiltration of CD8$^+$CTLs into the liver, hepatic inflammatory activity was reduced in CD1d$^{-/-}$ mice.

Non-CD8$^+$ T cells among the hepatic MHCs showed considerable cytotoxic activity to hepatocytes, although the activity was lower than that of CD8$^+$ T cells. Under Th1 conditions rich in IFN-γ, activated intrahepatic bystander cells such as macrophages and NK or NK T cells might elicit non-specific cytotoxic activity to hepatocytes [28]. As intrahepatic NK T cells express FasL [29], it cannot be excluded that activated NK T cells might elicit direct hepatocyte injury, possibly through Fas-FasL interaction, and this point should be studied further.

Concanavalin A (ConA) hepatitis is used widely as a mouse model of immune-related hepatitis, although the specific autoimmune response to hepatocytes has not been defined. Several studies have shown convincingly the involvement of NK T cells in the pathology of ConA hepatitis. Depletion of hepatic NK T cells in ConA-administered mice, or treatment of NK T cell-deficient mice with ConA, reduced the activity of ConA hepatitis significantly [30]. Moreover, in the interaction between CD8$^+$ T cells responsive to ovalbumin in a MHC class I-restricted manner (OTI T cells) and hepatocytes expressing transferrin-membrane-bound ovalbumin (Tf-mO transferrin-mOVA) in Tf-mOVA mice, specific effector function to antigen was stimulated by co-activation of Vα14 NK T cells using α-GalCer [31]. Also,

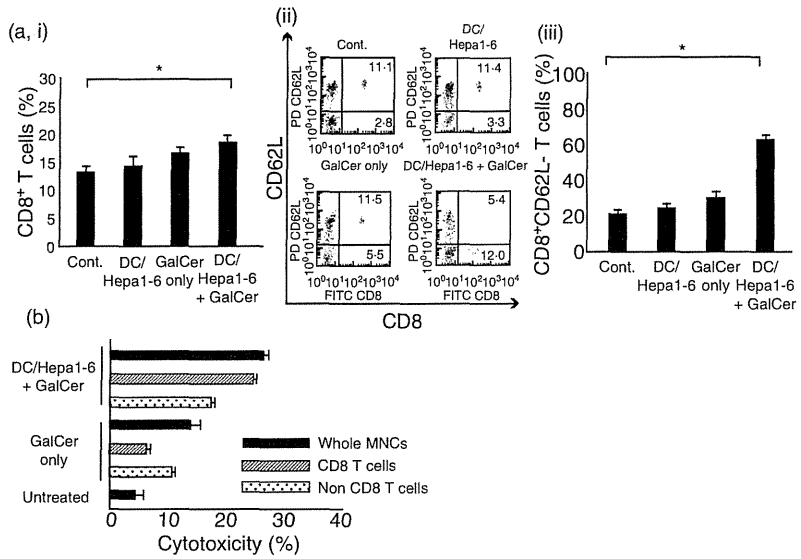

Fig. 4. Treatment of dendritic cell (DC)/Hepa1-6 pre-immunized mice with α-galactosylceramide (α-GalCer) generated active $CD8^+$ T cells which were cytocidal to hepatocytes. (a) (i) Population of intrahepatic $CD8^+$ T cells in each group. The mice were sacrificed 48 h after α-GalCer administration (17 days after the first DC/Hepa1-6 treatment). Intrahepatic mononuclear cells (MNCs) were isolated and the percentage of $CD8^+$ T cells was determined by flow cytometry [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (ii) Representative flow cytometry of each group. Intrahepatic MNCs were isolated and analysed by flow cytometry using anti-CD8 and anti-CD62L monoclonal antibodies (mAbs). Upper left; control, upper right; DC/Hepa1-6, lower left; α-GalCer only, lower right; DC/Hepa1-6 + α-GalCer. Values in the upper and lower right indicate the frequency of cells in each area. (iii) Frequency of $CD8^+$ $CD62L^-$ activated T cells in total intrahepatic $CD8^+$ T cells of each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (b) Cytotoxic activity of intrahepatic whole MNCs, $CD8^+$ T cells and non-$CD8^+$ T cells to autologous hepatocytes. Cytotoxic activity to primary cultured autologous hepatocytes was examined using aspartate aminotransferase (AST) release assay. Effector : target ratio was 40:1. All experiments were repeated at least three times.

in an animal model of primary biliary cirrhosis, in which infection of mice with *Novosphingobium aromaticivorans* induces antibody against mitochondrial component and T cell-mediated autoimmunity, disease induction requires NK T cells [32]. These results suggest that NK T cells play pivotal roles for development of immune-related liver disease.

Our AHI model seems to be an artificial model generated by extreme treatment of mice. However, we demonstrated that in order to generate liver-specific autoimmune response, two independent factors, induction of hepatocyte-responsive $CD8^+$ T cells by immunization with DC/Hepa1-6 and recruitment of such $CD8^+$ T cells into the liver by modulation of hepatic environment with IFN-γ, were required. In humans, the similar mechanism for development of autoimmune hepatitis might be considered. Prior to onset of human autoimmune hepatitis, infection of hepatitis virus or drug-induced liver injury might contribute to induction of hepatocyte-responsive T lymphocytes just as does immunization with DC/Hepa1-6. Some promoting factors such as massive cytokine production from NK T cells for activation of these autoreactive T cells might be associated with onset of autoimmune hepatitis.

Although this study showed an important role for activated NK T cells in the generation of AHI, the implication of NK T cell activation in patients with autoimmune hepatitis remains obscure. Several potential physiological ligands for NK T cells have been reported [33,34], but the specific endogenous ligand for NK T cell activation remains unknown [35]. Recently, several reports suggested the involvement of Toll-like receptors (TLRs) in the pathogenesis of autoimmune diabetes, inflammatory bowel diseases, multiple sclerosis and systemic lupus erythematosus [36,37]. It may be possible that the inflammatory activity in human AIH is affected by activation of IFN-γ-producing NK T cells in the liver through TLR stimulation by intestinal microbial components. If so, the regulation of intrahepatic NK T cell activity might lead to the establishment of a new modality for controlling disease activity in human AIH.

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Disclosure

The authors declare that there are no conflicts of interest.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. The dynamic statistics of natural killer (NK) T cells in the autoimmune hepatic inflammation (AHI) liver and spleen. (A) Absolute number of NK T cells in the liver (a) and spleen (b) in AHI. The number was determined as [total number of mononuclear cells (MNCs) in the liver or spleen] × [the frequency of $CD3^+NK1{\cdot}1^+$ cells] in each group [$n = 5$, mean ± standard deviation (s.d.), $^*P < 0{\cdot}001$]. (B) (a) Population of intrahepatic $CXCR6^+$ NK T cells ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (b) Expression of CXCL16 in hepatic tissue. Levels of CXCL16 mRNA in each group were determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). Bars indicate mean ± s.d., $^*P < 0{\cdot}001$. (C) (a) Frequency of interferon (IFN)-γ^+ NK T cells in each group ($n = 5$, mean ± s.d., $^*P < 0{\cdot}001$). (b) Frequency of interleukin (IL)-4^+ NK T cells in each group. All experiments were repeated at least three times.

Fig. S2. Frequency of natural killer (NK) T cells in the liver and spleen in autoimmune hepatic inflammation (AHI). Frequencies of NK T cells in intrahepatic major histocompatibility complexes (MHCs) and splenocytes were determined by flow cytometry ($n = 5$, mean ± standard deviation, $^*P < 0{\cdot}001$). Experiments were repeated at least three times.

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Review

Diagnostic Criteria for Autoimmune Hepatitis : Historical Review and Present Problems

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ABSTRACT

Autoimmune hepatitis (AIH) is a chronic hepatitis of unexplained etiology. Because no specific clinical marker has been identified, ruling out other liver diseases of known etiology is important when diagnosing AIH. The International Autoimmune Hepatitis Group (IAIHG) has prepared diagnostic criteria aimed at standardizing diagnosis. The IAIHG scoring system has been used extensively for diagnosing AIH. However, because this scoring system covers a variety of elements, using it at the bedside can be difficult. Recently, the IAIHG proposed simplified criteria system composed of only 4 elements which reportedly has excellent diagnostic capabilities. Problems have also been identified in assays for serum autoantibodies. Although the IAIHG recommends the indirect immunofluorescent method with frozen sections of rodent liver, kidney, and stomach to check for autoantibodies involved in AIH, this method is now used at only a few institutions, and a enzyme-linked immunosorbent assay and a method with established cell lines are more widely used. In any event, the method for autoantibody detection must be standardized and quantified. Liver biopsy is important for diagnosis ; however, histological findings are not always specific. In this review we describe the history of the diagnosis of AIH and related problems. (Jikeikai Med J 2011 ; 58 : 89-93)

Key words : autoimmune hepatitis, diagnostic criteria, autoantibody

INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic hepatitis of unexplained etiology. It has been strongly suggested that autoimmune mechanisms are intimately involved in the onset and progression of AIH[1] Clinically, AIH has been characterized by elevated serum levels of gamma-globulin or immunoglobulin (Ig)G ; the presence of autoantibodies, e.g., antinuclear antibodies (ANAs) and anti-smooth muscle antibodies ; histological signs of highly active chronic hepatitis ; and an abundance of plasma cells among infiltrating cells. However, these signs and findings are not always specific to AIH but are also seen in cases of viral hepatitis and drug-induced liver injury. To date, no clinical marker specific to AIH has been identified. For this reason, ruling out other liver diseases of known etiology is important in the diagnosis of AIH, as well as checking for the above-mentioned clinical manifestations. Furthermore, because cases of AIH can be atypical, e.g., complicated by or overlapping with other autoimmune diseases or autoimmune liver diseases[2], the diagnosis of AIH becomes more difficult. Because a delay in the diagnosis of AIH can lead to a delay in the start of treatment and a poor prognosis, prompt diagnosis is essential. Patients with AIH, particu-

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larly Japanese patients with AIH, usually respond well to corticosteroid therapy, and a definite diagnosis of AIH can be made in suspected cases by evaluating the responses to corticosteroid therapy, i.e., therapeutic diagnosis. However, if AIH becomes severe because diagnosis has been delayed, the response to corticosteroid therapy can be unsatisfactory. Therefore, the early, definite diagnosis of AIH is important.

GENETIC FACTORS RELATED TO DIAGNOSIS

Some persons have increased genetic susceptibility to AIH. Genes reported to confer increased susceptibility to AIH include human leucocytes antigen (HLA)-DR4 for Japanese people[3] and HLA-DR3 for people in Europe and the United States[4]. Because HLA-DR3 is seldom found in Japanese people, the clinical features of AIH in Japan differ from those in Western countries. Subsequent studies have demonstrated that in HLA-DR3-free patients with AIH in Western countries HLA-DR4 serves as a second disease susceptibility gene and that the clinical features of AIH in HLA-DR4-positive patients in Western countries are similar to those of AIH in Japanese patients in that the prevalence among middle-aged women is high and responses to treatment are good[5]. Briefly, there are 2 susceptibility genes for AIH, and the clinical features of AIH differ slightly depending on the gene. Interestingly, subsequent studies have revealed that the peptide-binding site is similar for both HLA-DR3 and HLA-DR4[6]. Despite these findings, the target antigen for AIH has not been identified, and the etiology of AIH remains unclear. Nevertheless, the major clinical findings of AIH are similar in patients with HLA-DR3 and patients with HLA-DR4 and have allowed international diagnostic criteria to be established.

DIAGNOSTIC SCORING SYSTEMS

Considering these findings, the International Autoimmune Hepatitis Group (IAIHG) has prepared diagnostic criteria aimed at standardizing the diagnosis of AIH and has proposed a highly convenient scoring system for the diagnosis of AIH[7]. Table 1 shows the brief history of the established criteria, with a focus on the criteria of the IAIHG. The scoring system, proposed in 1998, was aimed at eliminating, as far as possible, factors known to be involved in the onset of hepatopathy. This diagnostic system has enabled the pathophysiological assessment of AIH to be standardized, thereby establishing a firm basis for research on AIH. This scoring system has been extensively used as a means of diagnosing AIH[8]. If this scoring system were applied, most patients with AIH in Japan would receive diagnoses of suspected or definite AIH[9]. When the ratings based on this diagnostic system were reviewed in North America[10], Europe[11], and Japan[12] the sensitivity was 97% to 100% and the overall rate of accurate diagnosis was 89.8%. We may thus say that, by and large, a consensus has been reached regarding the validity of this scoring system.

However, because this scoring system aimed at standardizing the diagnosis of AIH covers a variety of elements, it can be difficult to use at the bedside. In addition, the diagnosis of AIH with this scoring system can be delayed owing to several problems, such as cases diagnosed as AIH despite low scores and the large number of criteria, including items for which data collection is difficult[13].

The IAIHG has recently proposed simplified criteria to facilitate clinical application[14]. The simplified criteria system includes only 4 elements (i.e., seropositivity for anto-antibodies, elevated serum levels of IgG, histological features, and ruling out viral infection responsible for liver damage) and has been reported to have excellent diagnostic capabilities, with a specificity of greater than 99% and a sensitivity of 81%. Because adequate follow-up assessments of the simplified criteria system have not been performed, we can draw no conclusions about it. The diagnostic capability of the simplified criteria system is reportedly low in atypical cases of AIH[15] and is insufficient in cases of acute-onset AIH[16]. However, the simplified criteria system appears to be useful for rapidly identifying typical cases of AIH and starting treatment on the basis on this rapid diagnosis. Katsushima et al. have reviewed 59 cases of AIH in Japanese patients using this new criteria system and found it simple to use and highly useful[17]. According to their report, the percentage of definite cases with the new scoring system was 74.6% and markedly higher than with the original revised scoring system (37.6%). We may, therefore, say that this set of criteria enables an early start to treatment and is of high clinical value for bedside use.

On the basis of the diagnostic criteria reported to date,

Table 1. Brief history of classification of autoimmune hepatitis by International autoimmune hepatitis Study Group (IAIHG)

Year	IAIHG activities	Publications
1967	A classification of chronic hepatitis and advocated	Mackay IR,Whittingham S.
	the term of autoimmune hepatitis	Postgrad Med 1987 ; 41 : 72-83.
1992	The first meeting at IASL Brighton	JohnsonPJ, McFarlane IG, and IAIHG members.
	First IAIHG group chair : I. R. McFarlane 1992-2006	Hepatology 1993 ; 18 : 998-1005.
	followed by D Vergani (2006-)	
1994	IASL Meeting Cancun : Classification of chronic hepatitis	Desmet V, Gerber B, Hoofnagle J, et al.
		Hepatology 1994 ; 19 : 1513-20.
1998	AASLD : IAIHG Report : Review of criteria for diagnosis	Alvarez F, Berg PA, Bianchi L, et al.
	of autoimmunehepatitis	J Hepatol 1999 ; 31 : 929-38.
	Scoring system was firstly proposed involving descriptional criteria	
	Many papers have been published for evaluating this score system	
2004	IAIH serology	Vergani D, Alvarez F, Bianchi FB, et al.
	In this paper, rodent frosen tissue should be uused for detecting ANA	J Hepatpl 2004 ; 41 : 677-83.
2005	AASLD : Simplified scoring system	Abstract only
2008	Simplified Criteria	Hennes EM, Zeniya M, Czaja AJ, et al.
		Hepatology 2008 ; 18 : 169-76.
2009	Pediatric autoimmune hepatitis	Mieli-Vergani G, Heller S, Jara P, et al.
		J Pediatr Gastroenterol Nutr 2009 ; 49 : 158-64.

liver biopsy is indispensable. Histological features of AIH include interface hepatitis with plasma cell infiltration, hepatocyte rosette formation, and emperiporesis. However, none of these features are specific for AIH, and making a definitive diagnosis of AIH is difficult on the basis of liver biopsy findings alone. However, liver biopsy is useful for ruling out other diseases for the differential diagnosis of AIH. Another problem with the simplified criteria system is confusion about how to incorporate these characteristic pathological features into the diagnosis. The criteria fail to describe in detail about when the presence of pathologically typical features may be affirmed (e.g., when all findings presented are typical or when at least 2 of the presented findings are typical). According to our empirical rules, the finding of interface hepatitis accompanied by at least one of the typical pathological features of AIH (hepatocyte rosette formation, plasma cell infiltration, and emperiporesis) will justify affirmation of the presence of pathologically typical features, and all findings need not be typical. However, the validity of this empirical approach is not assured because the criteria do not clearly specify how pathological findings should be selected. Further review of this point for verification is essential. Because a fundamental step in the diagnosis of AIH is to rule out other diseases similar to AIH (diagnosis by exclusion), liver biopsy is useful. However, difficulties can be encountered when attempting to perform liver biopsy in a timely fashion. This difficulty of timely liver biopsy is a significant problem with current diagnostic criteria. We often encounter cases in which treatment is started when a diagnosis of AIH is suspected but not yet proven with biopsy ; the diagnosis of AIH is then established by the marked response to treatment with corticosteroids. Further attempts with a similar approach are important for achieving the goal of establishing a simpler and more rapid means of diagnosing AIH.

The Problems of ANA Assays

Problems have been noted regarding assays for serum autoantibodies, a striking feature of AIH. Although the