Decrease of CD56$^+$T Cells and Natural Killer Cells in Cirrhotic Livers With Hepatitis C May Be Involved in Their Susceptibility to Hepatocellular Carcinoma

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CD56$^+$T cells and CD56$^+$natural killer (NK) cells are abundant in the human liver. The aim of this study was the further characterization of these cells in the liver with or without hepatitis C virus (HCV) infection. Liver mononuclear cells (MNC) were isolated from liver specimens obtained from the patients during abdominal surgery. In addition to a flow cytometric analysis, liver MNC and PBMC were cultured with the immobilized anti-CD3 Ab, IL-2, or a combination of IL-2 and IL-12 and their IFN-γ production and the antitumor cytotoxicity were assessed. The liver MNC of HCV (−) patients contained 20% CD56$^+$T cells whereas the same proportions decreased to 11% in chronic hepatitis livers and to 5% in cirrhotic livers. The proportion of NK cells also decreased in the cirrhotic livers. On the other hand, the populations of these cells in PBMC did not significantly differ among patient groups. The IFN-γ production and the cytotoxicity against K562 cells, Raji cells, and a hepatocellular carcinoma, HuH-7 cells, greatly decreased in the cirrhotic liver MNC. In contrast, the cytotoxicity in PBMC did not significantly differ among the patient groups and was lower than that in the liver MNC of HCV (−) patients. CD56$^+$T cells and NK cells but not regular T cells purified from liver MNC cultured with cytokines showed potent cytotoxicities against HuH-7 cells. These results suggest that a decreased number of CD56$^+$T cells and NK cells in cirrhotic livers may be related to their susceptibility to hepatocellular carcinoma. (HEPATOLOGY 2000;32:962-969.)

Chronic viral hepatitis patients, especially hepatitis C patients, often fall victim to liver cirrhosis and subsequent hepatocellular carcinoma (HCC).[1] It is now believed that the HCV infection in hepatocytes itself is not cytopathic whereas the cellular immune response to infected hepatocytes may indeed cause hepatocyte injury.[1] It has been suggested that nonspecific NK cell activation[1,2] and viral antigen-specific activation of either CD4$^+$T cells or cytotoxic CD8$^+$T cells may be responsible for hepatocyte injury.[1,3-6] Consistent with this hypothesis, it has also been reported that liver lymphocytes expressed T helper 1 cytokine, IFN-γ, and IL-2 messenger RNA, and the serum levels of these cytokines were elevated in the patients with HCV.[7,8]

On the other hand, livers from mice and humans have recently been reported to contain not only a large population of NK cells but also of T cells with NK cell markers.[9-13] Namely, mouse NK1.1 antigen$^+$T (NKT) cells and human CD56$^+$T cells are abundant in the livers. NKT cells in mice were activated by IL-12 and inhibited tumor metastases in the liver of mice[10,11,14-16] (for a review, see Seki et al.,[16]). Human peripheral blood CD56$^+$T cells were also activated *in vitro* by IL-12 and thus acquired an antitumor cytotoxicity against NK-resistant tumors.[13] Furthermore, strongly activated mouse liver NKT cells destroyed the syngeneic hepatocytes.[17] Therefore, the possibility has been raised that human CD56$^+$T cells may also play an important role in both hepatocyte injury in chronic viral hepatitis and antitumor immunity in the liver. In the present study, we show that human liver MNC activated by anti-CD3 Ab or a combination of IL-2 and IL-12 (or IL-2 alone) both produced IFN-γ and killed tumors more effectively than did PBMC. Furthermore, human liver CD56$^+$T cells and CD56$^+$NK cells gradually decreased in parallel with the progress of the hepatitis C and diminished in livers with cirrhosis. These liver MNC from cirrhotic livers could not effectively produce IFN-γ and could not effectively kill not only K562 cells and Raji cells but also a human HCC cell line, HuH-7 cells, thus suggesting that the decrease in CD56$^+$T cells and NK cells may be one of the mechanisms explaining why HCC frequently originates from cirrhotic livers.

PATIENTS AND METHODS

Patients and Liver Specimens. Liver specimens were obtained during surgery from the patients listed in Table 1 after obtaining their informed consent. Liver specimens obtained from the anti-HCV Ab– and HBs antigen (Ag)–negative patients with cancers other than HCC and were regarded as the HCV (−) liver specimens. Other liver specimens were from anti-HCV Ab–positive patients with chronic hepatitis C or with liver cirrhosis. Peripheral blood samples were also obtained during surgery. All liver specimens were obtained from areas other than tumor nodules.

Reagents. Anti-CD3 Ab (UCHT1, mouse IgG1) were purchased from PharMingen (San Diego, CA). Recombinant human IL-2 and IL-12 were purchased from PEPRO TECH EC (London, UK). Recombinant human IL-15 was purchased from Genzyme (Cambridge, MA).

Abbreviations: Ag, antigen; ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; HCV, hapatitis C virus; MNC, mononuclear cells; NK, natural killer; NKT cells, NK1.1 Ag$^+$T cells.

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TABLE 1. Patients and Their Backgrounds

	Nonhepatitis (n = 12) HCV (−)	Hepatitis (n = 12) HCV (+)	Cirrhosis (n = 6) HCV (+)
Age (y) mean ± SD	60.2 ± 8.9	67.6 ± 11.1	71.0 ± 5.0
Gender male/female	5/7	5/7	3/3
T-Bil (mg/dL)	0.8 ± 0.3	0.6 ± 0.3	1.1 ± 0.2
AST (IU/L)	22 ± 7	54 ± 15	32 ± 8
ALT (IU/L)	23 ± 7	53 ± 18	27 ± 5
PLT × $10^4/\mu$L	29.9 ± 14.7	27.1 ± 10.3	11.1 ± 2.6
PT (%)	85.1 ± 6.2	86.5 ± 8.16	70.2 ± 4.9
Disease			
Stomach cancer	3	3	1
Colorectal cancer	6	5	1
Hepatocellular carcinoma	0	1	3
Liver metastasis	3	3	1

Isolation of Liver MNC and PBMC. The liver specimens were cut into small pieces with scissors and then treated with collagenase (0.5 mg/mL) and DNAse (100 μg/mL) (Sigma Chemical Co., St Louis, MO) at 37°C for 20 minutes. Treated samples of the liver were pressed through a 200-gauge stainless mesh and then were suspended in RPMI 1640 medium. After washing 3 times with medium, the cells were resuspended in osmolarity- and pH-adjusted 33% Percoll solution containing 100 U/mL heparin and then were centrifuged at 2,000 rpm for 15 minute at room temperature.[14,16] The pellet was resuspended in a red blood cell lysis solution, then washed twice in 5% FBS RPMI. PBMC were obtained from blood samples using a Lymphocyte Separation Medium (ICN Biomedicals Inc., Aurora, OH).

Flow Cytometric Analysis and Cell Culture. Hepatic MNC or PBMC were stained with FITC-conjugated anti-NKR-P1 (CD161, DX12, PharMingen) mAb, phycoerythrin (PE)-conjugated anti-CD56 mAb (NKH-1, Beckman Coulter), phycoerythrin-cyanin 5.1 (PE-Cy5)–conjugated anti-$\alpha\beta$TCR mAb (Beckman Coulter), and gated lymphocytes were analyzed by 3-color flow cytometric analysis using EPICS XL (Beckman Coulter). One hundred microliters (10 μg/mL) of anti-CD3 mAb (UCHT1, mouse IgG1) was incubated overnight at 4°C in flat-bottomed 96-well plates to immobilize Ab, and then the plates were washed 3 times before starting the culture. Liver MNC or PBMC (2×10^5) in 200 μL of RPMI 1640 containing 10% human serum were cultured with immobilized anti-CD3 Ab in 5% CO_2 at 37°C in flat-bottomed 96-well plates. MNC were also incubated with human IL-12 (20 ng/mL) and IL-2 (100 ng/mL) or IL-2 alone in flat-bottomed 96-well plates. After the 48-hour culture, the supernatants were harvested and stocked in −80°C for enzyme-linked immunosorbent assay (ELISA). After the 5 days of culture, the cells were harvested and then subjected to cytotoxic assays.

Assays for IFN-γ Levels. IFN-γ in MNC culture supernatants were evaluated using the cytokine-specific ELISA kit (Endogen, Inc.).

Cytotoxic Assay. NK-sensitive K562 cells, NK-resistant Raji cells and a human HCC cell line, HuH-7 cells were used as targets. HuH-7 cells established from HCC tissue from HBsAg-negative HCC patients[18] were provided by Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University, Japan and were maintained in 10% FBS RPMI 1640. K562 cells or Raji cells were labeled with 100 μCi Na_2 $(^{51}Cr)O_4$ for 60 minutes at 37°C in RPMI 1640 medium containing 10% FBS, washed 3 times with medium, and then were subjected to cytotoxicity assays. The labeled K562 cells or Raji cells (2×10^3 to 1×10^4/well) were incubated in a total volume of 200 μL with effector cells in RPMI 1640 in round-bottomed 96-well microtiter plates (Effector/Target [E/T]) ratio, 10:1). The plates were centrifuged after incubation for 4 hours, after which the supernatants were harvested and counted with a gamma counter. In the case of HuH-7 cells, 10^3 cells were incubated in 10% FBS RPMI 1640 medium in a flat-bottomed 96-well microtiter plate for 4 days before the cytotoxic assay and then were labeled with Na_2 $(^{51}Cr)O_4$ (1 μCi/well) overnight in 5% CO_2 at 37°C in RPMI 1640 medium before undergoing the cytotoxic assays. The plates were washed 3 times with medium, and adherent HuH-7 cells were incubated with effector cells for 4 hours, and thereafter the supernatants were harvested and counted with a gamma counter. The cytotoxicity was calculated as a percentage of releasable counts after the subtraction of spontaneous release. The spontaneous release was less than 15% of the maximum release.

Cell Sorting and Culture. Liver MNC from patients without HCC were cultured with IL-12 (20 ng/mL), IL-2 (100 ng/mL), and IL-15 (5 ng/mL) in a flat-bottomed 96-well plate thereafter in a flat-bottomed 24-well plate for 3 weeks. Cytokines and 10% FBS containing complete medium were changed twice a week. After staining cultured liver MNC with anti-CD56 Ab and anti-$\alpha\beta$TCR Ab, $CD56^+$NK cells, $CD56^+$T cells, and regular $CD56^-$T cells were purified by a cell sorter (EPICS ELITE, Beckman Coulter) and subjected to cytotoxic assays.

Analysis of Class I Expression of Tumor Cells. K562 cells (0.5×10^6), Raji cells, and HuH-7 cells were stained with FITC-anti-human HLA-A, B, C Ab (mouse IgG2a, Beckman Coulter) at 4°C for 20 minutes and analyzed by EPICS XL. FITC-conjugated isotype control Ab (mouse IgG2a, Beckman Coulter) was also used.

Statistical Analysis. The differences between 2 groups were analyzed by the Mann-Whitney U test, and the differences among the 3 groups were analyzed by an ANOVA analysis with the Scheffe' F using the Stat View program on an Apple computer (Cupertino, CA). Differences were considered to be significant when P was $<.05$.

RESULTS

Phenotypic Profile of Liver MNC. Liver MNC from HCV (−) patients contained not only regular $CD56^-$T cells (41%) but also $CD56^+$T cells (20.4%), and approximately 80% of $CD56^+$T cells were $CD161^+$ (Fig. 1). PBMC contained 3.5% $CD56^+$T cells, and approximately half of them expressed CD161 (Fig. 1). CD161 is also referred to as NKR-P1A[19] and is expressed in most of NK cells but not in a majority of regular $CD56^-$T cells. Most of $CD56^+$T cells in the liver and PBMC were $CD8^+V\alpha24^-$ (data not shown). The hepatic MNC also contained a large population of $CD3^-$ $CD56^+$NK cells (31.0%) (Fig. 1). Although we showed that approximately 14% were NK cells in PBMC in our recent report,[13] NK cells in PBMC had a large population of NK cells (22.7%) in this study

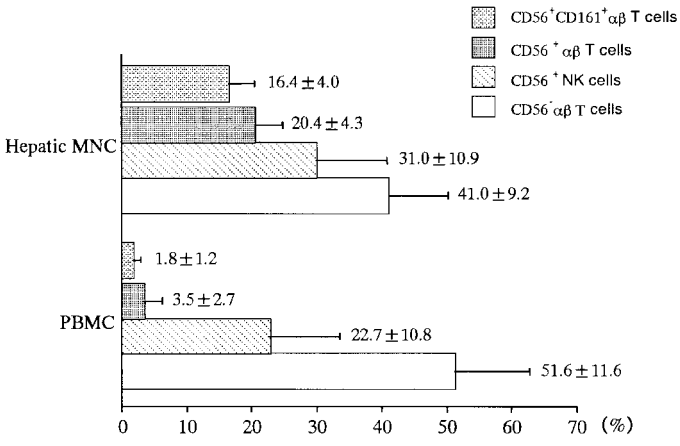

FIG. 1. Human livers contain a large fraction of $CD56^+CD161^+\alpha\beta$T cells. Liver MNC and PBMC were isolated from liver specimens from nonhepatitis patients and were stained with FITC-anti-CD161 Ab, PE-anti-CD56 Ab, PE-Cy5 anti-$\alpha\beta$TCR Ab. Lymphocytes were gated by the forward scatter and side scatter and were analyzed. The numbers represent the means (%) ± SD of the indicated lymphocyte populations in total lymphocytes from 12 patients.

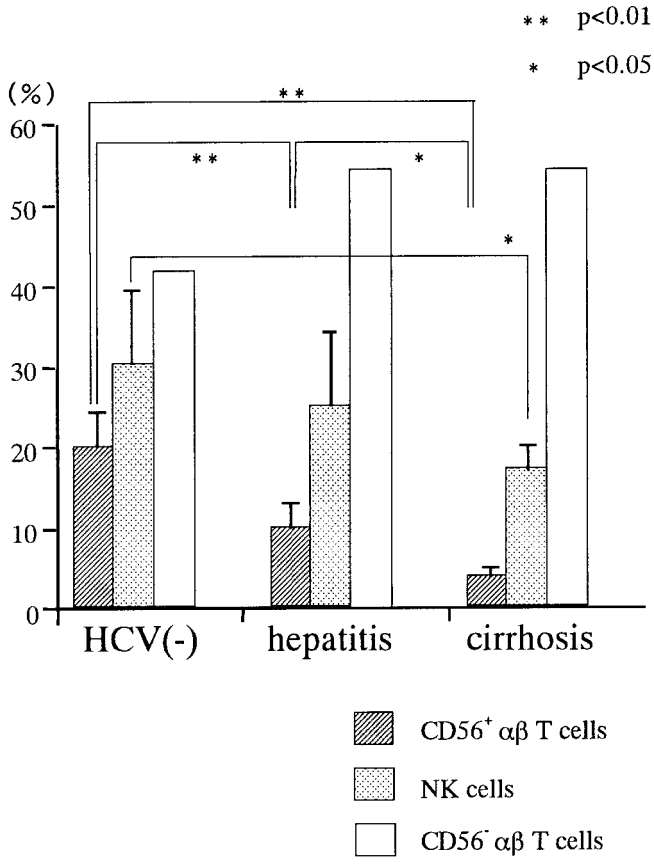

FIG. 2. Decrease of $CD56^+$ $\alpha\beta$T cells and $CD56^+$NK cells in the livers with cirrhosis. Liver MNC of HCV (−) patients (n = 12), chronic hepatitis patients (n = 12), and patients with liver cirrhosis (n = 6) were analyzed by the flow cytometry. The data represent the means (%) ± SD.

(Fig. 1). This discrepancy was the result of the fact that all patients in this study were cancer patients, and some of them had substantially higher populations of NK cells in PBMC. The numbers of liver MNC obtained were approximately 3.0×10^6/g from HCV (−) livers, 3.5×10^6/g from HCV (+) hepatitis livers, and 2.0×10^6/g from cirrhotic livers.

Liver CD56+T Cells and CD56+ NK Cells Decreased in the Livers With Cirrhosis. The proportion of $CD56^+$T cells in the HCV (−) livers were 20.4% although it decreased to 11% ($P < .01$) and 5% ($P < .01$) in the livers with chronic hepatitis and with cirrhosis, respectively (Fig. 2). Although the proportional decrease of NK cells in the livers with chronic hepatitis was not statistically significant, the proportion of NK cells also significantly decreased in the cirrhotic livers (31% vs. 18%, $P < .05$) (Fig. 2). In contrast, the number of regular $CD56^-$T cells did not decrease (Fig. 2). However, proportions of these cells in PBMC did not significantly differ among PBMC from HCV (−) patients (Fig. 1), hepatitis patients, and cirrhosis patients (data not shown).

Liver MNC Produced Larger Amounts of IFN-γ and Acquired More Potent Antitumor Cytotoxicities Than Did PBMC in Response to Either Immobilized Anti-CD3 Ab or IL-2. HCV (−) liver MNC produced approximately 12 ng/mL of IFN-γ by anti-CD3 Ab stimulation for 48 hours whereas PBMC produced 5 ng/mL of IFN-γ (Fig. 3, *left column*). The liver MNC also produced greater amounts of IFN-γ in response to IL-2 than did PBMC (Fig. 3, *left*). However, the IFN-γ amounts produced by the stimulation of IL-2 and IL-12 did not significantly differ between the liver MNC and PBMC (Fig. 3, *left*). In addition, HCV (−) liver MNC stimulated with either anti-CD3 Ab, IL-2, or a combination of IL-2 and IL-12 acquired a more potent cytotoxicity against K562 tumor cells than did PBMC (Fig. 3, *right*).

Decreased IFN-γ Production From the Liver MNC of Cirrhosis Patients in Response to Anti-CD3 Ab or IL-2. The liver MNC from cirrhotic livers produced significantly lower amounts of IFN-γ than did normal liver MNC when they were stimulated with either anti-CD3 Ab or IL-2 whereas cirrhotic liver MNC stimulated with IL-2 and IL-12 produced amounts of IFN-γ comparable with those from HCV (−) liver MNC (Fig. 4). Although anti-CD3-stimulated IFN-γ production from PBMC of cirrhosis patients tended to decrease as compared with that from the PBMC of HCV (−) patients, the differences were not statistically significant, and cytokine-stimulated IFN-γ production did not significantly differ among PBMC from HCV (−) patients, hepatitis patients, and cirrhosis patients (data not shown).

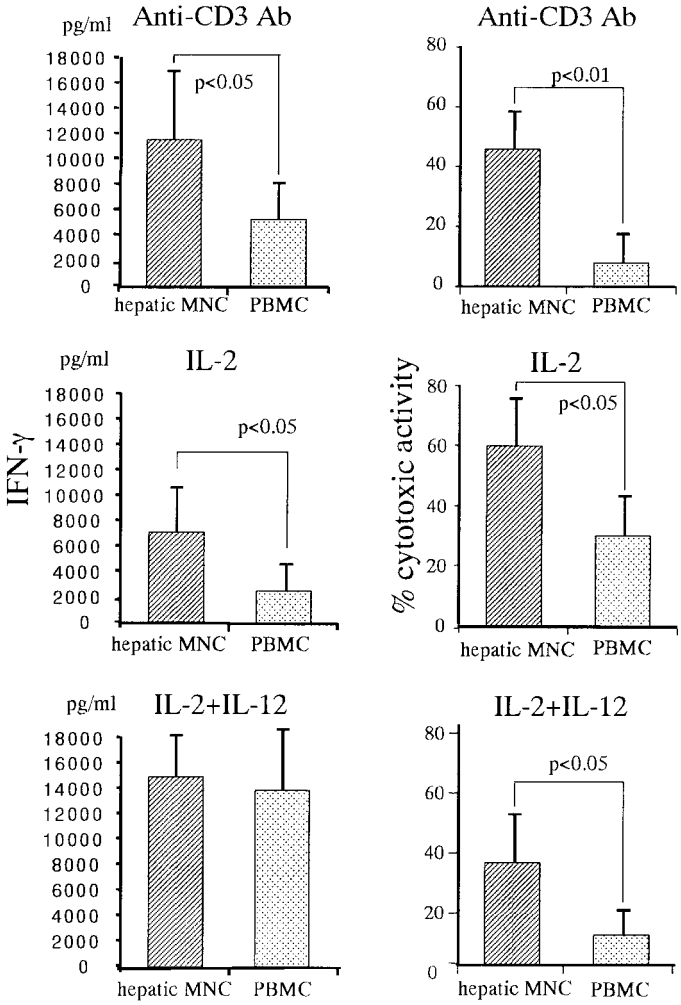

FIG. 3. Comparison of the IFN-γ production and the cytotoxicities between liver MNC and PBMC from nonhepatitis patients. 2×10^5 liver MNC and PBMC from HCV (−) patients were stimulated with anti-CD3 Ab, IL-2, or a combination of IL-2 and IL-12 for 48 hours in a flat-bottomed 96-well plate in 10% human pooled sera containing RPMI 1640, and supernatants were harvested and subjected to an IFN-γ ELISA assay. After 4 days of culture, the MNC were subjected to cytotoxic assays against K562 cells (E/T ratio, 10:1). The data represent the means ± SE from 12 HCV (−) patients.

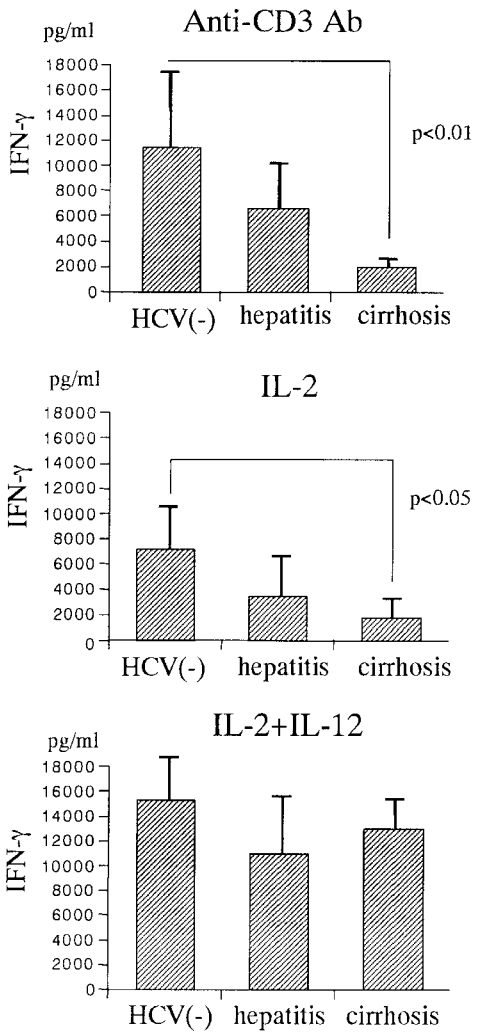

FIG. 4. Comparison of IFN-γ production among liver MNC from nonhepatitis livers, chronic hepatitis livers, and cirrhotic livers. The liver MNC of each group were stimulated for 48 hours with anti-CD3 Ab, IL-2, or IL-2 and IL-12 in a flat-bottomed 96-well plate with 10% human pooled sera containing RPMI 1640, and supernatants were subjected to ELISA. The data represent the means ± SE from 12 nonhepatitis livers, 12 chronic hepatitis livers, and 5 cirrhotic livers.

Decreased Antitumor Cytotoxicities of Cirrhotic Liver MNC Stimulated With Anti-CD3 Ab or Cytokines. Liver MNC from cirrhotic livers acquired a lower cytotoxicity against NK-sensitive K562 cells after either by CD3 or by IL-2 stimulation than MNC from HCV (−) livers or chronic hepatitis livers (Fig. 5, *left*). IL-2– and IL-12–stimulated cirrhotic liver MNC also showed lower cytotoxicities against K562 cells than those from the MNC of chronic hepatitis livers (Fig. 5, *left*). Either CD3 or cytokine-stimulated liver MNC from cirrhotic livers also showed a much lower cytotoxicity against NK-resistant Raji cells (Fig. 5, *right*). On the other hand, anti-CD3 or cytokine-stimulated antitumor cytotoxicities did not significantly differ among PBMC from HCV (−) patients, hepatitis patients, and cirrhosis patients (data not shown).

Decreased Cytotoxicities of Cirrhotic Liver MNC Against HuH-7 Cells. Furthermore, cultured MNC from cirrhotic livers showed a lower cytotoxicity against a human HCC cell line, HuH-7 cells than did those from HCV (−) livers (Fig. 6).

Liver NK Cells and CD56$^+$T Cells but Not CD56$^-$T Cells Were Cytotoxic Against HuH-7 Cells. Because IL-15 reportedly activate NK cells and sustain their survival,[20] liver MNC from livers without HCC were cultured with a combination of IL-2 (100 ng/mL), IL-12 (20 ng/mL), and IL-15 (5 ng/mL) for 3 weeks to obtain more numbers of liver MNC. After the culture, the proportion of CD56$^+$T cells increased to 50% to 60% in liver MNC whereas the proportion of NK cells decreased to approximately 10% and approximately 30% were regular CD56$^-$T cells. CD56$^+$T cells, CD56$^-$T cells, and CD56$^+$NK cells were purified by a cell sorter from cultured liver MNC and cytotoxicities against K562 cells, Raji cells, and HuH-7 cells of these populations were examined. The result showed that CD56$^+$T cells and NK cells but not regular CD56$^-$T cells exerted potent cytotoxicities against tumors (Fig. 7).

These results suggest that NK cells as well as CD56$^+$T cells in the liver MNC were thus the main cytotoxic effectors against HuH-7 cells.

HuH-7 Cells Expressed a Low Level of MHC Class I Ag. The K562 cells lacked any MHC class I (HLA-A, B, C) expression. However, the Raji cells showed a strongly positive MHC class I expression whereas HuH-7 cells showed only a weakly positive MHC class I expression (Fig. 8).

DISCUSSION

The presence of HCV had been expected in the patients with non-A, non-B hepatitis since the 1970s,[21-23] and the cDNA of this RNA virus was identified in 1989.[24,25] Chronic hepatitis C, which is a parenterally transmitted liver disease, is now one of the major causes of liver cirrhosis and HCC.[1] Initially, hepatitis C patients are usually asymptomatic for

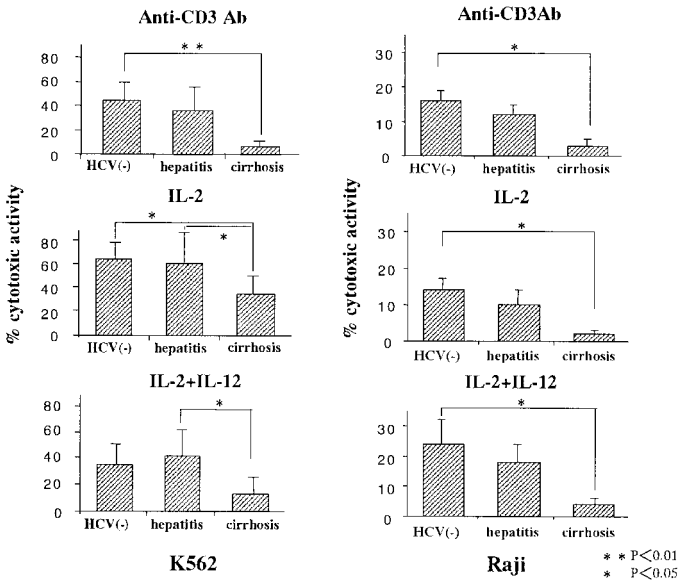

FIG. 5. Comparison of the cytotoxicities against K562 cells and Raji cells among liver MNC from nonhepatitis livers, chronic hepatitis livers, and cirrhotic livers. The cytotoxicities against NK-sensitive Raji cells (*left panels*). The data represent the means ± SE from 12 nonhepatitis livers, 12 chronic hepatitis livers, and 5 cirrhotic livers. The cytotoxicities against NK-resistant Raji cells (*right panels*). The data represent the means ± SE from 4 nonhepatitis livers, 4 chronic hepatitis livers, and 3 cirrhotic livers. The liver MNC of each group were cultured for 5 days with anti-CD3 Ab, IL-2, or IL-2 and IL-12 in a flat-bottomed 96-well plate with 10% human pooled sera containing RPMI 1640 and subjected to cytotoxic assays against K562 cells or Raji cells (E/T ratio, 10:1).

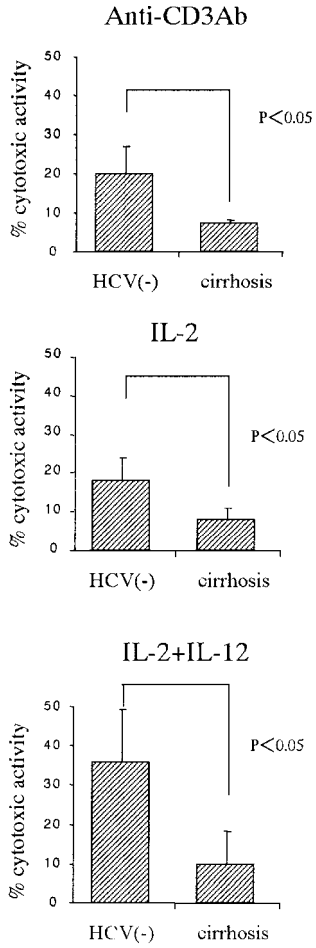

FIG. 6. Comparison of the cytotoxicities against HuH-7 cells between the liver MNC from nonhepatitis livers and from cirrhotic livers. The liver MNC of each group were cultured for 5 days with anti-CD3 Ab, IL-2, or IL-2 and IL-12 in a flat-bottomed 96-well plate with 10% human pooled sera containing RPMI 1640 and subjected to cytotoxic assays against a human HCC cell line, HuH-7 cells. The data represent the means ± SE from 6 HCV (−) livers and 4 cirrhotic livers.

relatively long periods (1 or 2 decades) while thereafter some patients progress into liver cirrhosis and HCC.[1] Cellular immunity by NK cells and T cells has been suggested to play an important role in the hepatocyte injury of hepatitis C.[1-6] However, the conditions and functions of NK cells and T cells with the NK cell marker, CD56, in the liver with or without hepatitis C have not been well defined.

In the present study, we showed that liver MNC displayed the potent capacity to produce IFN-γ and also exerted antitumor cytotoxicity by CD3 stimulation or Th1 cytokine stimulations. The capacity of liver MNC was much larger than PBMC. It is also important to note that $CD56^+$T cells in the liver (most of which were $CD161^+$)[19,26] progressively decreased in parallel with the progress of the hepatitis. Normal liver specimens contained 20.4% $CD56^+$T cells in total liver MNC whereas cirrhotic liver MNC had only 5% of $CD56^+$T cells. In addition, although less dramatically than $CD56^+$T cells, the proportion of $CD56^+$NK cells also significantly decreased in cirrhotic liver MNC. Consistent with these findings, the IFN-γ production and antitumor cytotoxicity of liver MNC, in general, were observed to steadily decrease as the disease progressed. Furthermore, MNC of cirrhotic livers were poorly cytotoxic against not only K562 cells and Raji cells but also against HuH-7 cells, and this was the result of the decrease of $CD56^+$T cells and NK cells in the liver as evidenced by the cytotoxic assays of purified each population from liver MNC cultured with cytokines.

Mouse liver NKT cells have been reported to decrease in number in CCl_4-induced experimental liver cirrhosis in mice, and the cytotoxicity of liver MNC against a mouse HCC cell line, MH134, was also found to be markedly disturbed.[27] However, the depletion of Kupffer cells did not affect the number of NKT cells.[27] Because some liver MNC firmly adhered to parenchymal hepatocytes, it was suggested that hepatocytes play a role as stromal-like cells for NKT cells.[27] In fact, hepatocytes were found to express IL-7 messenger RNA, which was reported to be an important cytokine for NKT cell development.[28] The present results in human liver MNC were consistent with these findings in mice. In addition, we and others reported that the adult mouse liver contains pluripotent hematopoietic stem cells that can produce all lineage leukocytes.[29,30] O'Farrelly et al., also reported that human livers contain pluripotent stem cells and RAG 1–positive T cell precursors.[31,32] These findings suggest that liver T cells with NK cell markers in the livers of mice and humans may

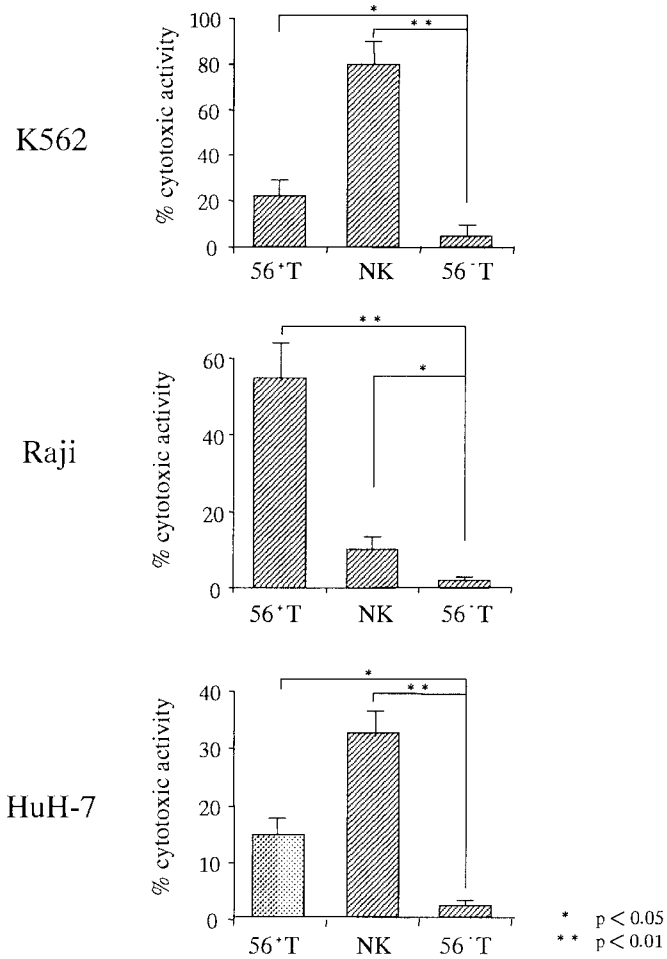

FIG. 7. NK cells and $CD56^+$T cells but not $CD56^-$T cells cultured with cytokines were cytotoxic against tumors. Liver MNC from patients without HCC were cultured with IL-2, IL-12, and IL-15 for 3 weeks and each cell population was purified by a cell sorter and was subjected to a cytotoxic assay. The data represent the means ± SE from 3 independent experiments.

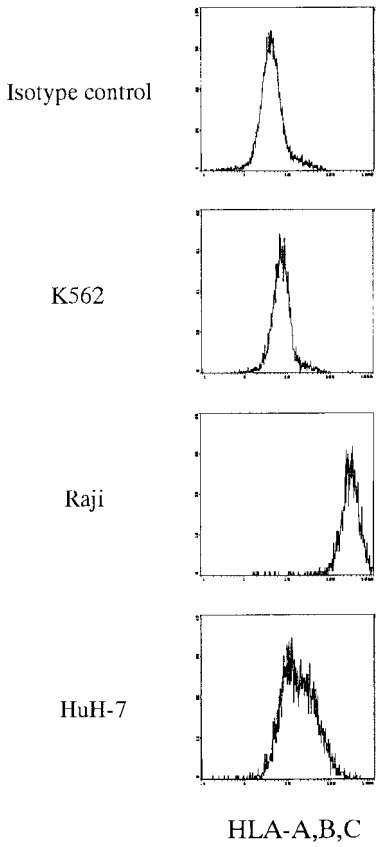

FIG. 8. Surface MHC class-I (HLA-A, B, C) expressions of the tumors. K562 cells, Raji cells, and HuH-7 cells were stained with anti-HLA-A, B, C Ab. K562 cells were also stained with isotype control Ab (*top panel*).

thus develop *in situ* from their liver or bone marrow precursors whereas liver cirrhosis may inhibit the development or proliferation of these cells in the liver.

We and others recently reported that mouse NKT cells in the liver were specifically activated *in vivo* by IL-12 to produce IFN-γ and acquired a potent antitumor cytotoxicity and inhibited tumor growth and metastases in the livers.[10,11,14-17] $CD56^+$T cells in the PBMC of humans were also strongly activated by IL-2 and IL-12 *in vitro* and acquired a potent antitumor cytotoxicity against NK-resistant tumors.[13] In the present study, we extended our study into these cells in human livers, especially in HCV-infected livers. Normal liver MNC activated by various stimuli produced a large amount of IFN-γ and acquired a potent cytotoxicity against tumor cells including HuH-7 cells. Because the present study showed that the liver NK cells as well as $CD56^+$T cells were mainly responsible for the antitumor cytotoxicity *in vitro*, these cells in the livers are therefore considered to play a crucial role in the antitumor immunity of the liver.

$CD4^+$T cells and $CD8^+$T cells, either from the liver or peripheral blood of HCV-infected patients have been reported to recognize HCV Ags[1-6] and were also activated to produce Th1 cytokines, such as IFN-γ and IL-2.[7,8] NK cells in the livers of chronic viral hepatitis patients were also suggested to be activated.[1,2] In addition, mouse NKT cells were reported to be activated in livers with viral hepatitis.[33] We also showed that human liver NK cells and $CD56^+$T cells exerted potent cytotoxicities against tumors including HuH-7 cells in an MHC-unrestricted manner. However, it should be noted that liver MNC from cirrhotic livers or PBMC of nonhepatitis patients, when stimulated with IL-2 and IL-12, produced IFN-γ comparable with those from HCV (−) liver MNC. Therefore, regular T cells also have the capacity to produce IFN-γ under certain conditions. Taken together, it is suggested that Ag specific regular T cells, NK cells, and $CD56^+$T cells are all activated to produce Th1 cytokines and eradicate virus-infected hepatocytes so as to inhibit the replication of hepatitis virus. In fact, IFN-γ receptor mutant mice have been reported to be susceptible to coronavirus-induced hepatitis.[34] However, it should be noted that because HCV replicates at a rapid rate but lacks proofreading ability, it thus has a large genetic diversity.[1] It may therefore avoid effective surveillance of the host cellular immunity unless it is eradicated in the acute phase of hepatitis C.

On the other hand, 80% of $CD56^+$T cells in the human liver were NKRP-1 $(CD161)^+$ whereas most of them were Vα24$^-$. NKT cells in mice mainly use Vα14 gene products for their T cell receptors[35,36] and Vα24$^+$T cells in humans have been suggested to be the counterparts of mouse NKT cells because the Vα14 gene of mice and the Vα24 gene of humans have a sequence homology,[36] and both responded CD1 dependently to α-galactosylceramide to produce IFN-γ and acquire an antitumor cytotoxicity.[37-39] However, Vα24$^+$T cells were rarely found in both human PBMC and the liver MNC in the present study, and liver MNC and PBMC stimulated with α-galactosylceramide did not produce IFN-γ and did not acquire antitumor cytotoxicity (our unpublished observation, March, 2000). It is therefore suggested that although mouse Vα14$^+$T cells and human Vα24$^+$T cells are indeed a counterpart of each other, the role in host immune responses of human Vα24$^+$T cells might be more limited than that of mouse Vα14$^+$T cells. Because NK1.1 Ag of mice and CD161 in humans are both belong to the NKR-P1 family,[26] we proposed that $CD56^+$T cells (more precisely, $CD161^+CD56^+$T cells) are a functional counterpart of NKT cells in mice as evidenced by their common properties of the tissue localization, IFN-γ production, and also their antitumor function.[16] In other words, human $CD56^+$T cells constitute more heterogeneous populations than mouse NKT cells as was also pointed out by Doherty et al.[40] Because Kupffer cells, which were activated with various bacterial stimuli produced IL-12 and activated NKT cells as well as NK cells[41,42] and the *in vivo* depletion of Kupffer cells in mice suppressed the antitumor activity of liver MNC, [42] Kupffer cells may also play an important role in the antitumor immunity of the liver.

Liver MNC of chronic hepatitis patients activated with IL-2 and IL-12 exerted an even stronger cytotoxicity than did HCV (−) liver MNC, thus suggesting that they are activated by an HCV infection even though NK cells and $CD56^+$T cells slightly decreased in comparison with those of HCV (−) livers. It is also noteworthy that IL-2–activated liver MNC exerted even a stronger cytotoxicity against K562 cells than did IL-2 and IL-12–activated liver MNC. This is probably a result of the fact that IL-12 inhibits the moderate or high dose of IL-2–induced proliferation of NK cells.[43] In fact, the proportion of NK cells decreased when PBMC were cultured with IL-2 and IL-12[13] and also when liver MNC were cultured with a combination of IL-2, IL-12, and IL-15 as shown in the present study.

MHC class I expression of tumors has been reported to be inversely correlated with susceptibility to NK cell–mediated

lysis.[44-46] The enhancement of MHC class I expression of tumor cells by IFN-γ induced the resistance of tumors to NK cell–mediated cytolysis[44] while a decrease of surface MHC class I expression of NK-resistant tumors (including Raji cells) by citric acid treatment makes them susceptible to NK cell–mediated lysis.[46] Because K562 cells lacked an MHC class I expression[46] while HuH-7 cells were MHC class I weakly positive and Raji cells were MHC class I strongly positive, it is reasonable to assume that cytokine-activated NK cells effectively killed K562 cells and HuH-7 cells whereas CD56$^+$T cells effectively killed Raji cells. It is therefore suggested that the degree of MHC class I expression of HCC may determine its susceptibility to either NK cells or CD56$^+$T cells.

We recently found by sorting and culture experiments of PBMC that the IFN-γ production from CD3-stimulated CD56$^+$T cells was much greater than that from regular T cells and IFN-γ production from either NK cells or CD56$^+$T cells stimulated with IL-2, IL-12, and IL-15 was much greater than that from regular T cells (T. Ohkawa et al., submitted), thus indicating that the abundance of CD56$^+$T cells and NK cells and the decrease of these cells in the liver are responsible for the potent IFN-γ production in the MNC from livers without cirrhosis and the decreased IFN-γ production in the MNC from cirrhotic livers, respectively.

In conclusion, NK cells and CD56$^+$T cells in humans are therefore considered to play an important role not only in the innate antitumor immunity of the liver but possibly also in the hepatocyte injury in hepatitis C patients, and the decrease of CD56$^+$T cells and NK cells in cirrhotic livers may therefore be one of the important mechanisms explaining why HCC frequently originates in cirrhotic livers.

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