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分冊2

厚生労働科学研究費補助金

肝炎等克服緊急対策研究事業（肝炎分野）

C型肝炎における新規治療法に関する研究

平成21～23年度　総合研究報告書

研究代表者　　鈴木　文孝

平成24（2012）年３月

Ⅳ．研究成果の刊行物・別刷

Hepatology Research

Hepatology Research 2011; 41: 160–169 doi: 10.1111/j.1872-034X.2010.00743.x

Original Article

α-Galactosylceramide activates antitumor immunity against liver tumor

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Aim: α-Galactosylceramide (α-GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. We investigated the detailed process of activating liver dendritic cells (DC) and immune cells after α-GalCer treatment in the mouse liver tumor model.

Methods: BALB/c mice bearing CMS4 liver tumor (p53 peptide-expressing tumor) were treated by α-GalCer. We evaluated the activation of liver DC and immune cells after α-GalCer treatment. Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay was performed to detect p53 peptide-specific cytotoxic T lymphocytes (CTL). To assess the impact of systemic acquired immunity by α-GalCer treatment, 28 days after liver tumor treatment, CMS4 cells or Colon26 cells were re-challenged s.c.

Results: The liver weights of α-GalCer-treated mice were significantly lighter than those of vehicle-treated mice. Depletion experiments revealed that natural killer (NK) cells were essential for the antitumor effect of α-GalCer. α-GalCer treatment significantly increased the population of DC and NK cells in the liver. The expressions of co-stimulatory molecules on liver DC significantly increased with the peak at 1 day after α-GalCer administration. IFN-γ ELISPOT assay demonstrated that p53 peptide-specific CTL was generated efficiently in α-GalCer-treated mice. ^{51}Cr-release assay revealed that $CD8^+$, not $CD4^+$, CTL against CMS4 cells were generated in α-GalCer-treated mice. The mice that had been protected from CMS4 liver tumor by α-GalCer injection became resistant against s.c. CMS4 re-challenge, but not against Colon26 re-challenge.

Conclusion: These results demonstrated the therapeutic potential of α-GalCer against liver cancer through activating liver DC and immune cells in the liver.

Key words: α-galactosylceramide, cytotoxic T lymphocytes, dendritic cells, liver, natural killer cells.

INTRODUCTION

THE GLYCOLIPID ANTIGEN α-galactosylceramide (α-GalCer) induced the activation of natural killer (NK) T cells in a CD1d-dependent manner.[1,2] Recently, α-GalCer has been attracting attention as a novel antitumor therapy. In *in vivo* animal studies, systemic administration of α-GalCer showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma and lymphoma) in hepatic and lung metastasis models.[3,4] Based on the promising results of preclinical studies demonstrating the antitumor potential of α-GalCer, several phase 1 clinical studies on cancer immunotherapy by the i.v. administration of α-GalCer has been carried out, but clinical responses of α-GalCer has been limited.[5] No clinical trial against liver cancer has been reported to date. In view of future α-GalCer treatment of liver cancer, the precise mechanism of activation of innate and acquired immunity in the liver by α-GalCer should be examined. However, these are still not fully understood.

The liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells).[6,7] Dendritic cells (DC) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) cells.[8,9] Recent research of DC biology revealed that DC also contribute

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Received 23 July 2010; revision 8 October 2010; accepted 18 October 2010.

to innate immune responses by activating NK cells[10–14] and NKT cells[1,15,16] through interleukin (IL)-12 secretion and direct cellular interaction. Thus, DC can be expected to play critical roles in activating abundant T cells, NK cells and NKT cells in the liver after α-GalCer administration. We previously reported that administration of α-GalCer stimulated hepatic NKT cells and led to activation of hepatic NK cells,[4] and that α-GalCer activated liver DC have higher ability to generate acquired immunity.[17] However, the detailed process of activating liver DC and immune cells after α-GalCer treatment should be elucidated.

In this study, we evaluated the liver DC activation and antitumor effect mediated by both innate and acquired immunity against mouse liver tumor after administration of α-GalCer. Administration of α-GalCer induced early activation of liver DC with upregulation of antigen presenting-related molecules and resulted in complete rejection of local liver tumor by NK cells. Followed by early rejection of liver tumor, tumor antigen-specific CTL were generated and complete rejection in s.c. re-challenge of tumor cells was observed. Sequential activation of liver DC, innate and acquired immune cells in the liver may be an attractive strategy for treatment of local and distant tumor of liver cancer.

METHODS

Mice

SIX-TO-EIGHT-WEEK-OLD female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). All mice were maintained in micro-isolator cages. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines and culture

CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type $p53_{232-240}$ epitope recognized by H-$2K^d$-restricted CTL.[18] Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained as previously described.[19,20]

α-GalCer

α-Galactosylceramide was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*[1]

Animal experiments

BALB/c mice were injected in the liver with 5×10^5 CMS4 cells on day 0. On day 1, BALB/c mice were injected i.p. with α-GalCer (2 μg/100 μL) or 100 μL of vehicle. Two weeks after the tumor injection, the livers of treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.p. injection of α-GalCer, mice were injected intrahepatically with 5×10^5 CMS4 cells on day 0 and i.p. treated with α-GalCer on day 1. On day 28 after α-GalCer treatment, 1×10^6 CMS4 cells or Colon26 cells were injected in the right flank of treated mice. To confirm the involvement of $CD8^+$ T cells in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice. On day 1 and day 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. as previously described.[19] Tumor size was assessed every 3 or 4 days and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± standard deviation (SD).

NK cell depletion experiments

For NK cell depletion, mice were injected with anti-asialo GM-1 (ASGM1) antibody (Ab) (Wako, Osaka, Japan) on day 1, 5, 10, 15 and 20 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using phycoerythrin (PE)-conjugated anti-DX5 monoclonal antibody (BD-Pharmingen, San Diego, CA, USA). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Preparation of hepatic mononuclear cells and liver DC

Hepatic mononuclear cells (MNC) were prepared as previously described.[4] $CD11c^+$ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

Flow cytometry

For phenotypic analysis of liver DC, NK cells, NKT cells and $CD4^+$ and $CD8^+$ T cells, PE- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80, CD86, T-cell receptor [TCR]-β, CD49b [DX5], CD4, CD8, CD69 [all from BD-Pharmingen], major his-

tocompatibility complex [MHC] class II [Miltenyi Biotec]), and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results of flow cytometric analysis are reported in positive cell rates (%) determined by using isotype controls. DC were identified as $CD11c^+$/MHC class II^+ cells. NK cells were identified as $DX5^+$/TCR-β^- cells, NKT cells as $DX5^+$/TCR-β^+ cells, as previously described.[20]

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays for $p53_{232-240}$ peptide-reactive CD8+ T-cell responses

Bone marrow derived DC (BMDC) were generated from normal mice as previously described[21] and were used as peptide-presenting cells. On day 14 after treatment of α-GalCer or vehicle, $CD8^+$ T cells were isolated from the spleen cells of treated mice by using magnetic beads (Miltenyi Biotec). We used a mouse IFN-γ ELISPOT kit (R&D systems, Minneapolis, MN, USA) to detect the $p53_{232-240}$ peptide-specific $CD8^+$ T-cell responses. To evaluate the $p53_{232-240}$ peptide, strongly expressing on CMS4 cells,[18] specific CTL induction, isolated $CD8^+$ T cells (1×10^5 cells/well) were co-cultured with syngeneic BMDC (2×10^4 cells/well) pulsed with $p53_{232-240}$ peptide in an ELISPOT culture plate. BMDC cells without $p53_{232-240}$ peptide served as the negative control, and this value was subtracted from all experimental determinations to establish $p53_{232-240}$ peptide-specific spot numbers. The data are represented as mean IFN-γ spots ± SD per 100 000 $CD8^+$ T cells analyzed.

Cytolytic assays

Splenocytes from α-GalCer or vehicle-treated mice were harvested 14 days after tumor inoculation. After 5 days of *in vitro* stimulation with mitomycin-C (Kyowa Hakko, Tokyo, Japan)-treated CMS4 cells, lymphocytes were analyzed for their ability to kill CMS4 cells in 4-h ^{51}Cr-release assays (effector cells/target cells ratio, 60:1), as previously described.[21] $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively.

Statistical analyses

All experiments with three or more groups in which treatment was applied with a completely random design were first analyzed by a one-way factorial ANOVA. If the resulting *P*-value was less than 0.05, specific pairwise

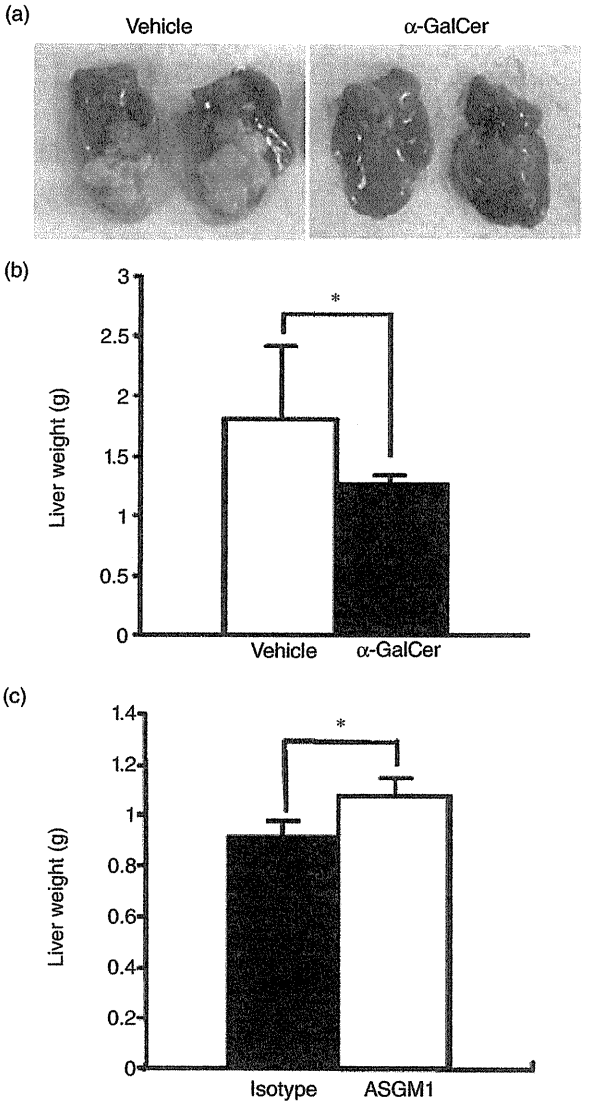


Figure 1 Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intro-hepatically with 5×10^5 CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, $n = 7$). Two weeks after the CMS4 tumor injection, the livers of treated mice were removed. (a) Representative liver macroscopic view of each group. (b) Comparison of liver weight of each group. $^*P < 0.05$. (c) To prove that the therapeutic benefit of α-GalCer treatment in the CMS4 liver tumor model is natural killer (NK)-cell dependent, *in vivo* depletion of NK cells was performed (as described in Methods, ASGM1). In control mice, isotype antibody (Ab) was injected i.p. (isotype). Both mice were treated by α-GalCer. Ab-mediated *in situ* depletion of NK cells markedly reduces the therapeutic efficacy of α-GalCer treatment (all treatment groups $n = 5$). $^*P < 0.05$.

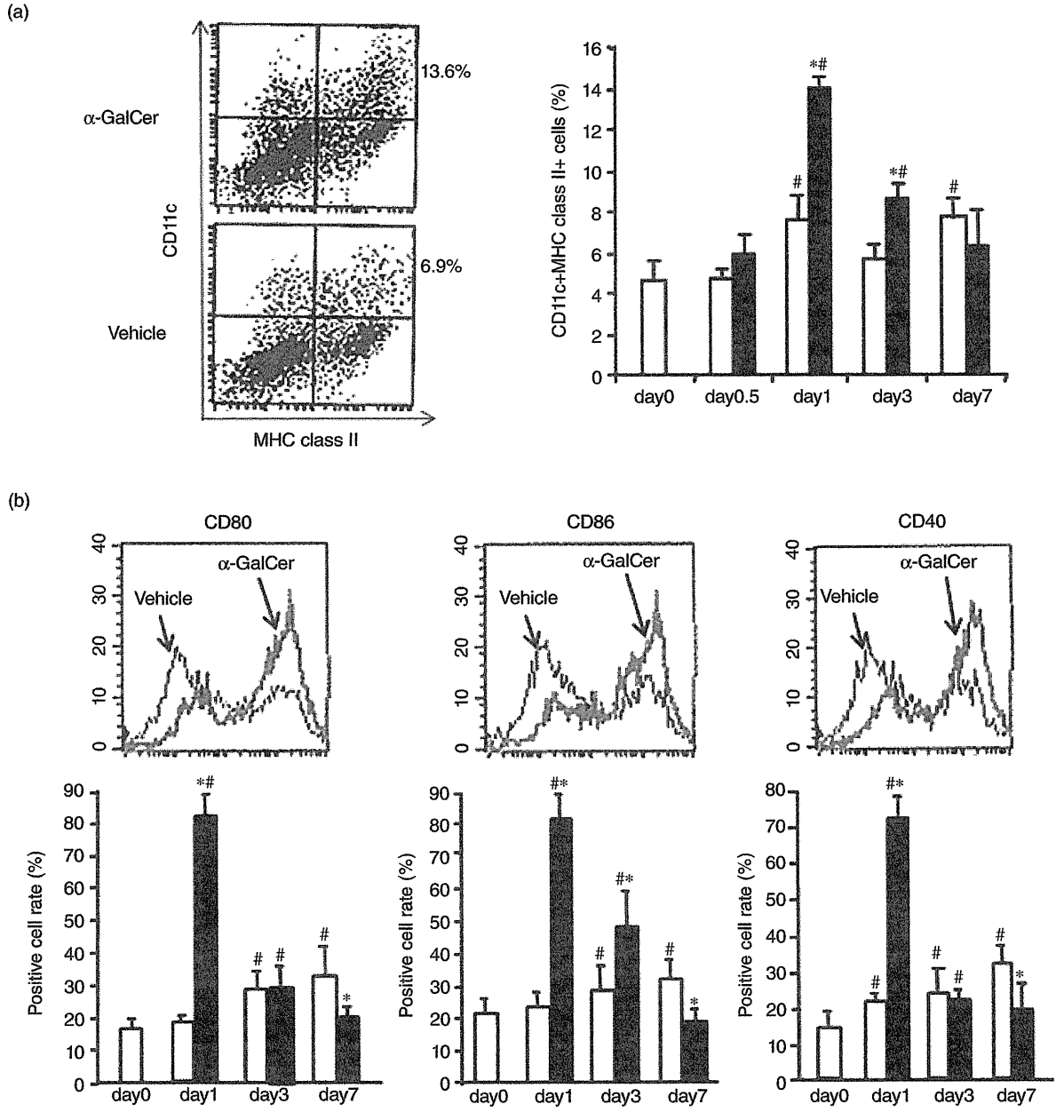


Figure 2 α-Galactosylceramide (α-GalCer) treatment increased dendritic cell (DC) population in the liver mononuclear cells (MNC) and activated DC functions. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 0.5, 1, 3 and 7. DC ($CD11c^+$ major histocompatibility complex [MHC] class II^+ cells) population was evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. Representative dot plots of liver DC ($CD11c^+$ MHC class II^+ cells) at day 1 after α-GalCer or vehicle administration are shown in the left panels. The calculated percentages of liver DC are shown in the right. (b) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 and DC were isolated from liver MNC by a magnetic cell sorting system. For phenotypic analysis, liver DC were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86), and the expressions of these molecules were analyzed by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $^*P < 0.05$ vs vehicle-treated mice, $\#P < 0.05$ vs non-treated mice. Representative histograms of the expressions of CD80, CD86 and CD40 on liver DC at day 1 after α-GalCer or vehicle administration are shown in the upper panels.

contrasts were tested with a Student's *t*-test with Welch's correction for unequal variance as needed.

RESULTS

α-GalCer administration inhibited CMS4 liver tumor mediated by NK cells

WE INITIALLY EXAMINED whether α-GalCer administration could induce antitumor effect against CMS4 liver tumor. As shown in Figure 1(a), no tumors were observed in the livers of α-GalCer-treated mice whereas large tumors were observed in the livers of vehicle-treated mice. The liver weight of the α-GalCer treatment group was significantly lighter than that of the vehicle treatment group (Fig. 1b). Depletion of NK cells significantly inhibited the antitumor efficacy of α-GalCer treatment (Fig. 1c), whereas depletion of neither $CD4^+$ nor $CD8^+$ T cells was inhibited (data not shown). These results suggested that administration of α-GalCer was therapeutic against CMS4 liver tumor and NK cells were the main effector cells in this antitumor immunity.

Administration of α-GalCer increased DC population in the liver MNC and activated DC functions

We investigated the population changes of DC in the liver MNC after α-GalCer or vehicle treatment. On day 1 after α-GalCer administration, liver DC proportion in α-GalCer-treated mice was higher than that in vehicle-treated mice (Fig. 2a). Liver DC proportion increased with the peak at 1 day after α-GalCer administration and the liver DC proportion at 7 days decreased to the same level with that from non-treated mice (Fig. 2a). In contrast, liver DC proportion in vehicle-treated mice exhibited weaker change than those in α-GalCer-treated mice (Fig. 2a). The liver DC number also exhibited increase at the peak of 1 day after α-GalCer administration whereas that from vehicle-treated mice exhibited no change (data not shown). We examined the CD40, CD80 and CD86 expressions of liver DC after administration of α-GalCer, which is an indicator of DC activation. On day 1 after α-GalCer administration, CD40, CD80 and CD86 on liver DC from α-GalCer-treated mice expressed more strongly than those from vehicle-treated mice (Fig. 2b). The expressions of all these molecules on liver DC increased with the peak at 1 day after α-GalCer administration and the expression levels of these molecules at 7 days decreased to the same levels on liver DC from non-treated mice (Fig. 2b). In contrast, the expressions of these molecules on liver DC exhibited weaker change in vehicle-treated mice.

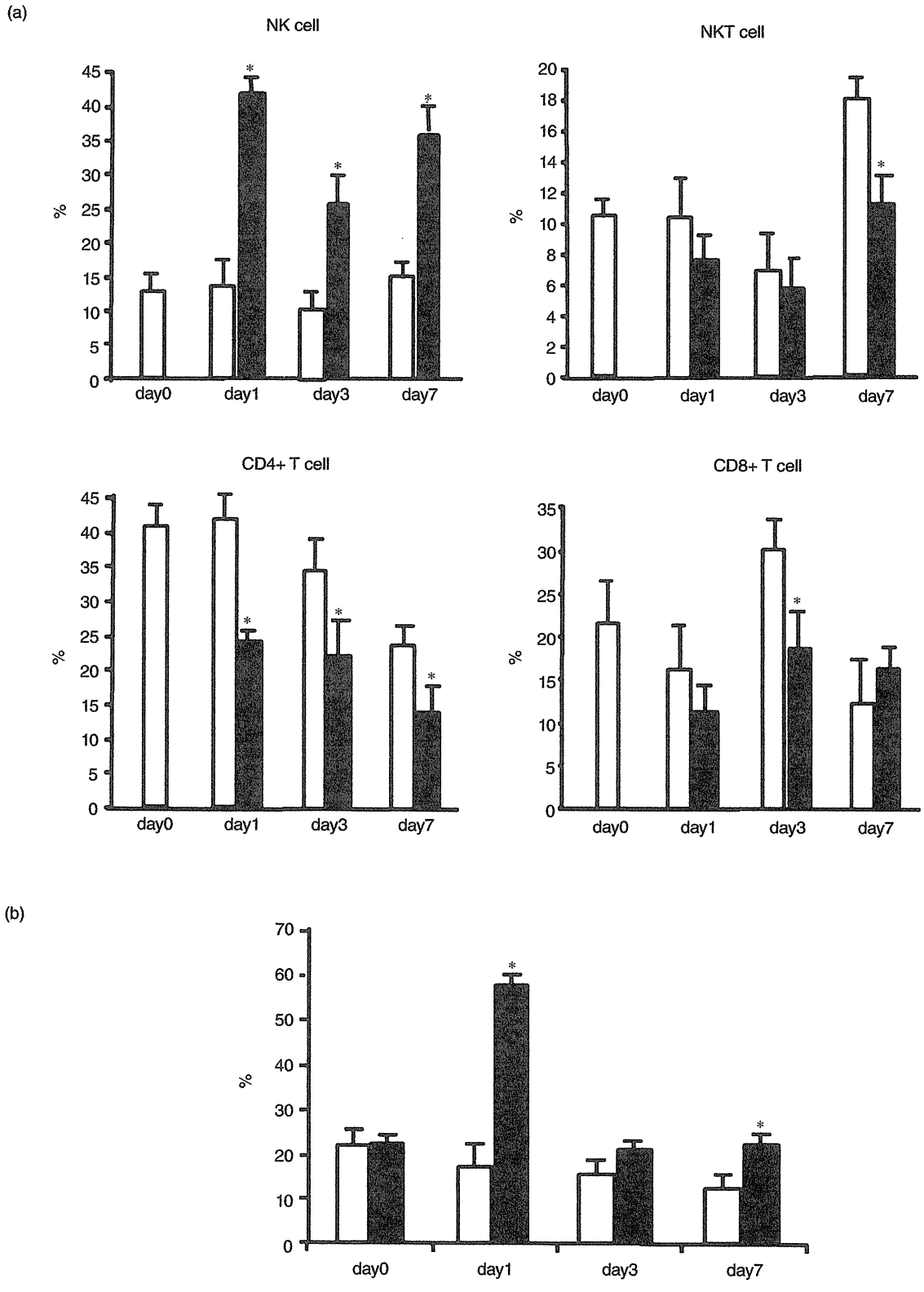
Activated NK cells composed the major subpopulation of hepatic MNC that increased after α-GalCer treatment

We examined the population change of MNC from the livers after α-GalCer or vehicle administration. It is notable that NK cells strikingly increased in proportion after α-GalCer administration, but not after vehicle administration (Fig. 3a). In contrast, the NKT cells decreased at 1 and 3 days after α-GalCer administration and recovered at day 7 after α-GalCer administration. Both $CD4^+$ and $CD8^+$ T cells decreased in proportion after α-GalCer administration, but not after vehicle administration. We also examined the CD69 expressions of NK cells, which is an indicator of lymphocyte activation. The CD69 expressions on liver NK cells increased with the peak at 1 day and gradually decreased at 7 days after α-GalCer administration (Fig. 3b). In contrast, those did not change after vehicle administration. These results demonstrated that the activated NK cells were the major subpopulation of MNC that increased in the liver after α-GalCer administration.

$p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor

We evaluated whether $p53_{232-240}$ peptide-specific CTL were generated after α-GalCer treatment of liver tumor. $CD8^+$ T cells were isolated from the spleen cells of treated mice and then co-cultured with syngeneic DC pulsed with $p53_{232-240}$ peptide strongly expressed on CMS4 cells. As shown in Figure 4(a), the number of

Figure 3 Activated natural killer (NK) cells composed the major subpopulation of hepatic mononuclear cells (MNC) that increased after α-galactosylceramide (α-GalCer) treatment. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 1, 3 and 7. NK cells, NKT cells, $CD4^+$ T cells and $CD8^+$ T cells in liver MNC were evaluated by flow cytometry. (b) The expressions of CD69 on liver NK cells were also evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α-GalCer-treated mice. $^*P < 0.05$ vs vehicle-treated mice.



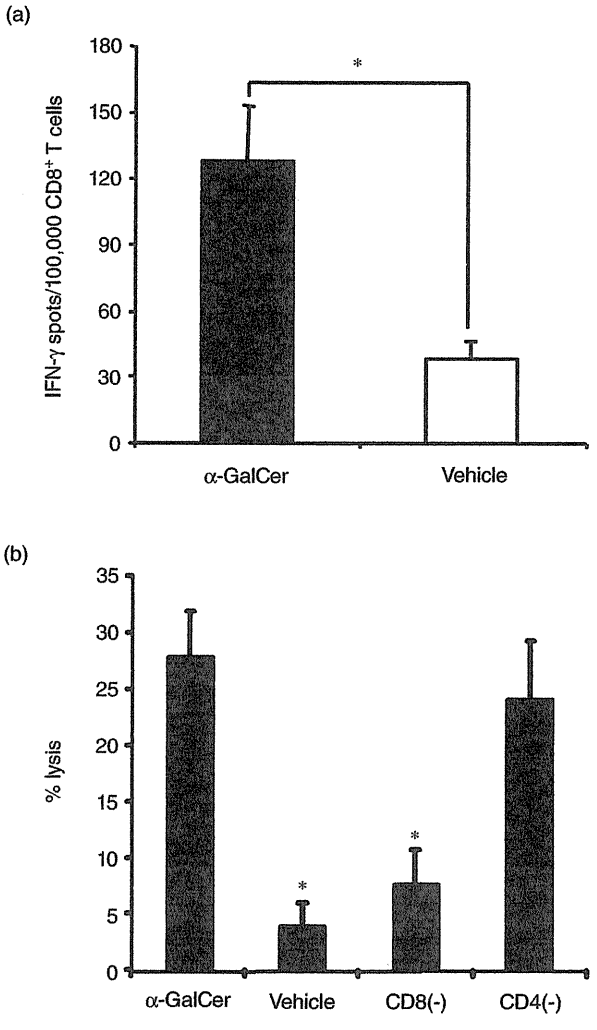


Figure 4 Evaluation of $p53_{232-240}$ peptide-specific $CD8^+$ cytotoxic T lymphocytes (CTL) in α-galactosylceramide (α-GalCer)-treated mice. (a) $CD8^+$ T cells were isolated from the spleen cells of treated mice 14 days after α-GalCer or vehicle treatment. The frequency of $p53_{232-240}$ peptide-specific $CD8^+$ CTL was evaluated by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay. The results are shown in spots/100 000 $CD8^+$ T cells; mean ± standard deviation of triplicate samples. $^*P < 0.05$. (b) Splenocytes from α-GalCer- or vehicle-treated mice were harvested 14 days after tumor inoculation and were analyzed for their ability to kill CMS4 cells in 4 h ^{51}Cr-release assays (effector cells/target cells ratio, 60:1). $CD4^+$ or $CD8^+$ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively. $CD8^-$, $CD8^+$ T-cell-depleted splenocytes. $CD4^-$, $CD4^+$ T-cell-depleted splenocytes. $^*P < 0.05$ vs the cytolytic activity of splenocytes from α-GalCer-treated mice.

IFN-γ spots (per 100 000 $CD8^+$ T cells) observed for T-cell responses against $p53_{232-240}$ peptide in α-GalCer-treated mice were significantly higher than that in vehicle-treated mice. These results suggested that strong $p53_{232-240}$ peptide-specific CTL were generated by α-GalCer treatment of liver tumor. Splenocytes from α-GalCer-treated mice displayed strong cytolytic activity against CMS4 cells, while those from vehicle-treated mice did not (Fig. 4b). $CD8^+$ T-cell-depleted splenocytes from α-GalCer-treated mice displayed significant weak cytolytic activity against CMS4 cells, but $CD4^+$ T-cell-depleted splenocytes did not. These results demonstrated that $CD8^+$ T cells (i.e. CTL) played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice.

Systemic therapeutic antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

Because strong $p53_{232-240}$ peptide-specific CTL were generated in α-GalCer-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in the liver would impact the progression of subcutaneous untreated CMS4 tumors. BALB/c mice were intrahepatically injected with CMS4 tumors and treated by administration of α-GalCer. Twenty-eight days later, 1×10^6 CMS4 cells or Colon26 cells were injected s.c. in the right flank. As shown in Figure 5(a), the non-treated CMS4 tumors in mice receiving α-GalCer treatment were completely rejected in all mice. The growth of non-treated Colon26 tumor in α-GalCer-treated mice was not inhibited (Fig. 5b). These results suggested that systemic CMS4-specific antitumor immunity could be induced by α-GalCer treatment. To confirm the involvement of CTL in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells (s.c. injection of 1×10^6 CMS4 cells) in α-GalCer-treated mice bearing CMS4 liver tumor. On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. As shown in Figure 5(c), antitumor effect against re-challenged CMS4 subcutaneous tumor was diminished in $CD8^+$ T-cell-depleted mice. These results supported that $CD8^+$ T cells (i.e. CTL) play essential roles in the antitumor effect against re-challenge of CMS4 cells in α-GalCer-treated mice.

DISCUSSION

WE PREVIOUSLY DEMONSTRATED that administration of α-GalCer activated both NKT cells and NK cells in the liver, and that liver NK cells were the

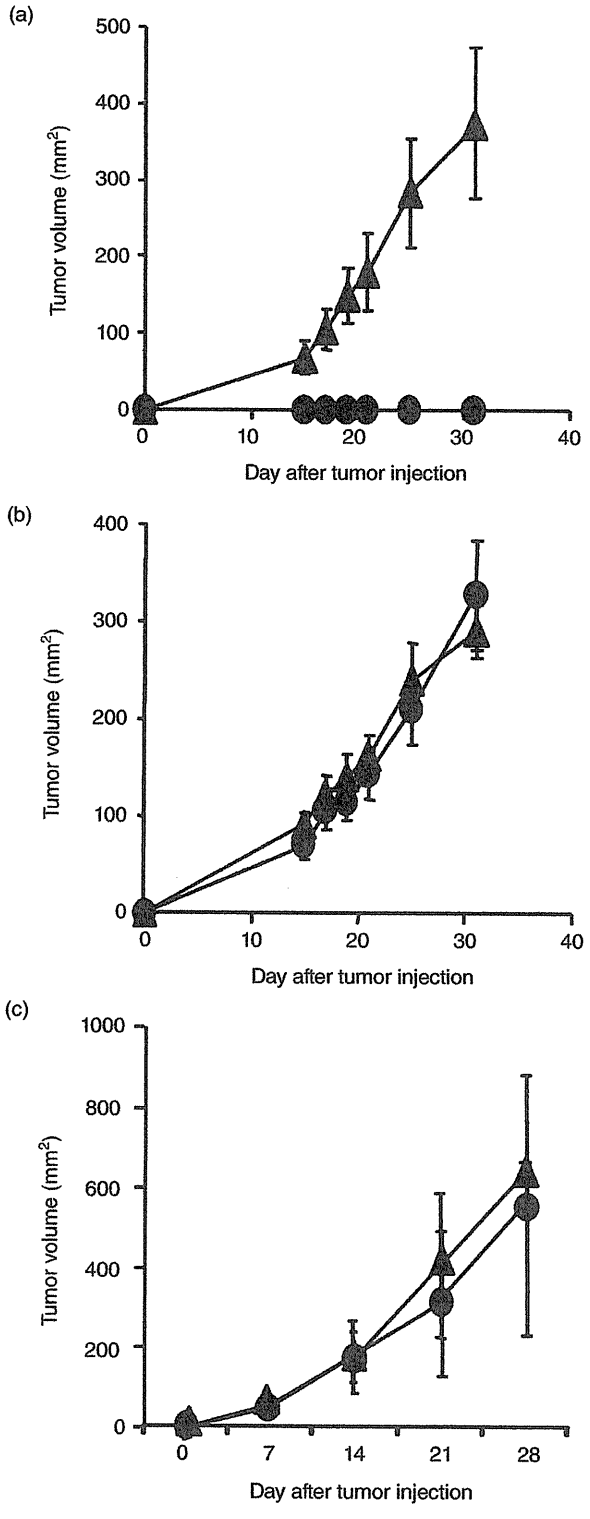


Figure 5 α-Galactosylceramide (α-GalCer) therapy results in the development of systemic antitumor immunity that protects distal tumor re-challenge. BALB/c mice were injected intrahepatically with CMS4 tumors. Twenty-four hours later, the mice were treated with α-GalCer. Twenty-eight days after treatment, α-GalCer-treated mice were re-challenged s.c. with 1×10^6 CMS4 cells (a) or Colon26 cells (b) in the right flank (all treatment groups, $n = 8$). To confirm the involvement of cytotoxic T lymphocytes (CTL) in this antitumor effect, we depleted $CD8^+$ T cells before re-challenge of CMS4 cells in α-GalCer-treated mice bearing CMS4 liver tumor (c). On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody was injected i.p. Tumor size was assessed every 3 or 4 days after s.c. injection of tumor cells (on day 0). As control mice, naïve mice were injected s.c. with 1×10^6 CMS4 cells (a, $n = 8$; c, $n = 6$) or Colon26 cells (b) ($n = 8$) on day 0. (●) α-GalCer-treated mice, (▲) control mice. Each data point represents the mean tumor size ± standard deviations.

main effector cells to kill disseminated hepatoma cells injected from spleen in α-GalCer treatment.[4] In this study, we evaluated α-GalCer treatment in local injected liver tumor, and the α-GalCer treatment resulted in complete rejection of local liver tumor, which had a similar antitumor effect as α-GalCer in a previous metastatic liver tumor model. These findings suggested the ability of α-GalCer treatment to activate the liver NK cells efficiently, which may mainly contribute to eradication of local liver tumor cells. A normal liver contains lymphocytes that are usually enriched with NK and NKT cells; namely, 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells.[6,7] Thus, activation of innate immune cells, NK cells and NKT cells must be important to develop more effective immunotherapy against liver cancer. We believe that α-GalCer treatment must be a good candidate for human liver cancer treatment.

Recently, activated DC have been implicated in the activation of NKT and NK cells in both mice and humans,[1,5,8–12,22] suggesting that DC play crucial roles in the activation of abundant immune cells in the liver. To establish more efficient α-GalCer treatment in liver cancer, the precise mechanism of liver DC activation is needed. Our results demonstrated that the proportion of liver DC in liver MNC increased immediately and reached the peak 1 day after α-GalCer treatment. The infiltration of tumors by mature DC has been reported to correlate with a better prognosis in cancer patients.[23,24] Thus, the increase of liver DC by α-GalCer might contribute to generation of antitumor effect against liver cancer. The expressions of co-stimulatory

molecules on liver DC also increased early after administration of α-GalCer. IL-12 production from DC is key Th1-cytokine to enhance NK and CTL functionality,[25,26] IL-12 production from liver DC after α-GalCer treatment was significantly higher than that after vehicle treatment.[17] These results suggested that α-GalCer treatment resulted in rapid activation of liver DC, which might play important roles in activating liver NK cells and might contribute to the subsequent establishment of acquired immunity against liver cancer. Pillarisetty *et al.* identified new DC subsets, NK–DC, which presented in the liver of mice,[27] which may affect the interpretation of the activation of liver NK cells by α-GalCer. However, we previously demonstrated that α-GalCer had no direct effect on liver NK cells in mice.[4] These results supported the idea that α-GalCer activated liver DC, which activated the liver NK cells secondary.

Interferon-γ ELISPOT assay revealed that the frequency of $CD8^+$ T cells isolated from α-GalCer-treated mice in liver tumors in response to $p53_{232-240}$ peptide were much higher than that from vehicle-treated mice. Mayordomo *et al.* reported that immunization of $p53_{232-240}$ peptide-pulsed DC induced peptide-specific CTL in immunized mice that showed cytolytic activity against CMS4, p53 overexpressing cells.[18] In this study, ^{51}Cr-release assay demonstrated that $CD8^+$ T cell, not $CD4^+$ T cells, played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice, which is consistent with the IFN-γ ELISPOT results. The detection of $p53_{232-240}$ peptide-specific CTL means the generation of CMS4 tumor-specific CTL after eradication of liver tumor by α-GalCer treatment. The activation of NKT cells was associated with an expansion of antigen-specific CTL, as might be expected if the DC that matured *in vivo* in response to NKT cells were capturing antigens.[28–31] Our results suggested that the activation of hepatic DC might be associated with the efficiency of generation of tumor antigen-specific CTL.

Additional experiments using an s.c. re-challenge with tumor demonstrated that α-GalCer treatment of liver tumors not only blocked treated CMS4 liver tumor progression but completely protected against consequent "recurrence" of that same tumor at a distant site. In contrast, Colon26 re-challenge tumor was not inhibited in treated mice, suggesting that CMS4-specific immunity was generated after liver tumor treatment. These results were consistent with the activation of acquired immunity evaluated by IFN-γ ELISPOT assay with increase of the frequency of $p53_{232-240}$ peptide-specific CTL. Taken together, we believe that α-GalCer treatment of liver tumors resulted in rejection of both local liver tumor and distant metastatic tumor.

In summary, we have shown that α-GalCer treatment activated both innate and acquired immune cells in the liver. These findings suggested that the use of α-GalCer might represent a particularly promising approach to suppress tumor growth and to promote regression of metastatic lesions in liver cancer patients.

ACKNOWLEDGMENTS

THIS WORK WAS supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.

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Hepatology Research

Hepatology Research 2011; 41: 30–38 doi: 10.1111/j.1872-034X.2010.00734.x

Original Article

Hepatitis C virus-specific CD8+ T cell frequencies are associated with the responses of pegylated interferon-α and ribavirin combination therapy in patients with chronic hepatitis C virus infection

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Aim: Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon-α (peg-IFNα) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

Methods: CD8+ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN-γ enzyme-linked immunospot (ELISPOT) assay.

Results: The numbers of IFN-γ spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

Conclusions: These results demonstrated that significant increase of HCV-specific CD8+ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFNα and ribavirin.

Key words: chronic hepatitis C, HCV-specific CTL, IFN-γ ELISPOT, peg-IFNα, ribavirin

INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.[1,2] Combination therapy of pegylated interferon-α (Peg-IFNα) plus ribavirin is standard treatment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,[1,2] which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,[3] and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.[4–6] The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.[7] However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

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Received 28 June 2010; revised 20 August 2010; accepted 24 August 2010.

property of Peg-IFNα and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN-α enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.[8] Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports[7,8] , even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN-γ enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the in vivo situation.[9–11] In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN-γ ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFNα plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

MATERIALS AND METHODS

Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFNα plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFNα-2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after 24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLICORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.[12]The characteristics of patients with chronic HCV infection were summarized in Table 1.

CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

IFN-γ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN-γ ELISPOT assay were performed as previously described.[11] Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μg/mL of anti-human IFN-γmAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), 1×10^5 CD8+ T cells were co-cultured with 2×10^4 T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μg/mL). HLA-A2-restricted HCV Core protein derived peptides ($Core_{35-44}$, YLLPRPGPRL, $Core_{131-140}$, ADLMGYIPLV) or NS3 protein derived peptides ($NS3_{1073-1081}$, CINGVCWTV, $NS3_{1406-1415}$, KLVALGINAV) were synthesized as previously described.[13] Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-$nef_{190-198}$ peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Subject	Age	Sex	HCV-RNA (KIU)	ALT (U/l)	Treatment duration	SVR
1	43	F	440	17	48 week	SVR
2	56	M	2000	146	48 week	non
3	49	F	1200	31	72 week	SVR
4	49	M	340	106	48 week	SVR
5	65	F	3800	24	72 week	SVR
6	58	M	320	25	48 week	SVR
7	56	M	2551	24	48 week	non
8	55	M	939	43	48 week	SVR
9	46	M	1200	64	48 week	SVR
10	46	M	1059	42	48 week	SVR
11	43	M	407	91	24 week	non
12	63	F	1621	61	48 week	non
13	63	F	1841	63	48 week	non
14	47	M	458	41	48 week	SVR
15	36	M	1024	79	48 week	non
16	61	F	677	148	48 week	non

ALT, alanine aminotransferase; F, female; M, male; non, non-SVR; SVR, sustained virologic response.

by incubation for 2 h with biotinylated mAb anti-human IFN-γ (7-6B-1, Mabtech) at 2 μg/mL. Plates were washed six times and avidin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) were added for 1 h. Unbound complex was removed by washing and 3-Amino-9-ethylcarbazole substrate (Sigma, St Louis, MO) was added for 5 min. The data are represented as mean IFN-γ spots per 100 000 T cells analyzed.

Statistics

All values were expressed as the mean and standard deviation (SD). The statistical significance of differences between the groups was determined by applying Mann–Whitney *U*-test. We defined statistical significance as $P < 0.05$.

RESULTS

Analysis of HCV derived peptide-specific IFN-γ release of peripheral blood CD8+ T cells in ELISPOT assay

WE ASSESSED PERIPHERAL blood CD8+ T cell responses against HCV derived peptides ($Core_{35-44}$, $Core_{131-140}$, $NS3_{1073-1081}$, $NS3_{1406-1415}$) in 16 HLA-A2+ HCV patients and 6 healthy donors. As shown in Figure 1, the numbers of IFN-γ spots (per 100 000 CD8+ T cells) observed for T cell responses against HCV peptides in pre-IFN patients were as low as those observed in healthy HLA-A2+ donors. In contrast, significant elevations of ELISPOT reactivity to three peptides ($Core_{131-140}$, $NS3_{1073-1081}$, $NS3_{1406-1415}$) were observed in IFN-4week patients compared with healthy donors. The number of IFN-γ spots against $Core_{35-44}$ peptides in IFN-4week patients also tended to be higher than those in healthy donors. In treated HCV patients, the numbers of IFN-γ spots against all four HCV derived peptides in IFN-4week patients were significantly higher than those in pre-IFN patients (Fig. 1). We also examined whether the frequencies of HCV-specific CD8+ T cell responses were associated with sex difference. The frequencies of CTLs against all four peptides were similar between males and females before and 4 weeks after starting treatment (data not shown).

HCV-specific CD8+ T cell responses in pre-IFN patients were not associated with the antiviral activity of the combination therapy of Peg-IFNα-2b plus ribavirin

We examined the association between HCV-specific CD8+ T cell responses in pre-IFN patients and ALT levels or HCV viral load before treatment. No association was observed between the frequencies of HCV-specific CD8+ T cells in pre-IFN patients and ALT levels or HCV viral load of pre-treated patients (Fig. 2).

We next examined whether HCV-specific CD8+ T cell responses in pre-IFN patients were associated with the antiviral activity of this combination therapy. As shown in Figure 3, the frequencies of CD8+ T cell responses against all four HCV proteins-derived peptides in

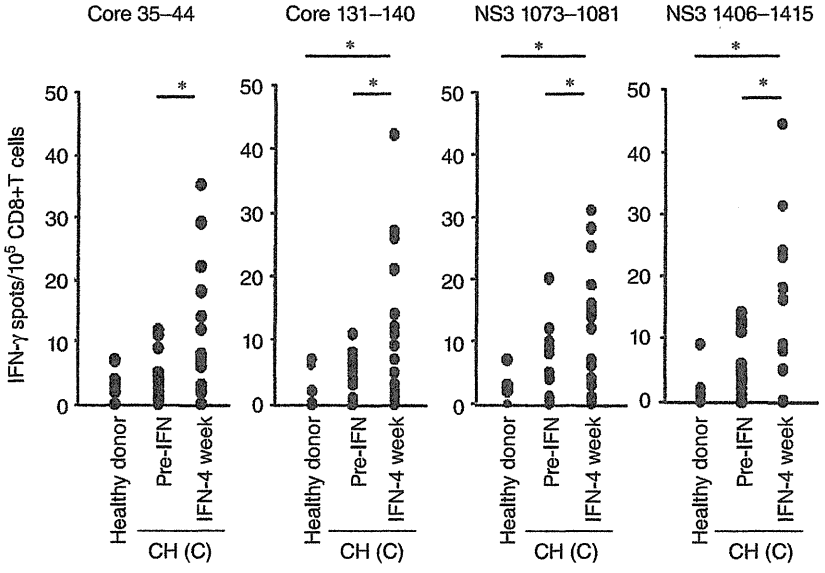

Figure 1 Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis of hepatitis C virus (HCV)-specific CD8+ T cell responses in HCV patients treated with the combination therapy of peg-IFNα plus ribavirin. Peripheral blood CD8+ T cells were isolated from HLA-A2+ healthy donors and chronic hepatitis C (CH-C) patients. The CH-C patients were treated with the combination therapy of peg-IFNα plus ribavirin and PBMC were isolated from pre-treated patients (Pre-IFN) and treated patients 4 weeks after starting treatment (IFN-4week). HCV-specific CD8+ T cell responses were evaluated by IFN-γ ELISPOT as outlined in "Materials and Methods". Data are reported as IFN-γ spots/ 100 000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with HLA-A2-presenting HIV-nef$_{190-196}$ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine HCV specific spots numbers. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core- or NS3-peptides. *$P < 0.05$.

pre-IFN patients were not significantly different between SVR, the group of the patients who were observed SVR, and non-SVR, the group of the patients who were not observed SVR. These results suggested that the baseline HCV-specific CD8+ T cell responses in HCV patients were not associated with the antiviral activity of this combination therapy.

Significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of the combination therapy of Peg-IFNα plus ribavirin

We examined the association between early elevation of HCV-specific CD8+ T cell responses and the antiviral activity of this combination therapy. We evaluated the frequencies of CD8+ T cell responses against HCV proteins-derived peptides before and 4 weeks after starting treatment. As shown in Figure 4, in SVR patients, the frequencies of CD8+ T cell responses against all four HCV peptides (Core$_{35-44}$, Core$_{131-140}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$) increased significantly 4 weeks after starting treatment. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-SVR patients. These results demonstrated that significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of this combination therapy.

DISCUSSION

HCV-SPECIFIC CD8+ CTLs have been reported to play a significant role in the elimination of HCV in acute hepatitis of HCV.[4,9] In contrast, in chronic infection of HCV, HCV-specific CD8+ T cell responses were weak and were directed against a limited series of epitopes compared with acute hepatitis.[9] These might cause persistent infection of HCV in the HCV infected host. However, conflicting results have been reported with respect to HCV-specific CD8+ T cell responses on the antiviral activity of IFN therapy. IFNα monotherapy may promote viral clearance by enhancing the host CTL responses.[14,15] But Rehermann et al. reported that CTL

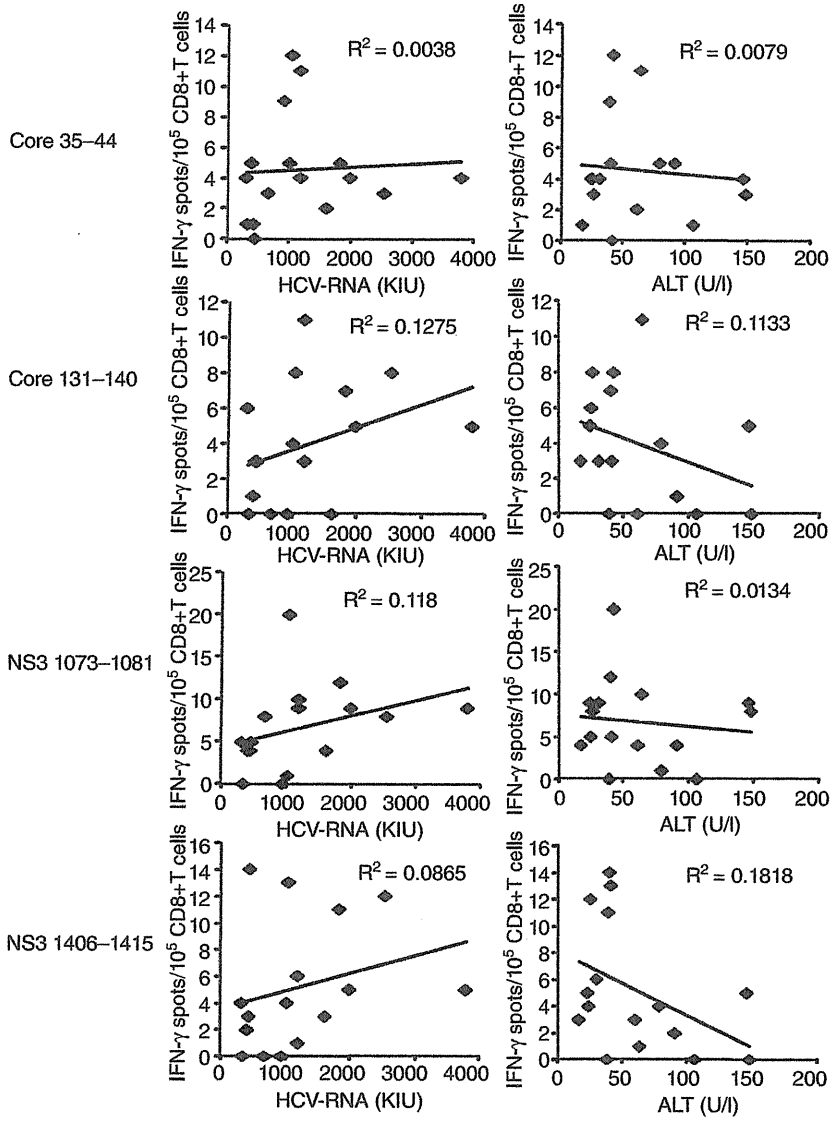

Figure 2 The association between the hepatitis C virus (HCV)-specific CD8+ T cell responses of pre-IFN patients and the serum alanine aminotransferase (ALT) levels or the HCV viral load of patients before treatment. The frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We examined the association between the frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patient and the serum ALT levels or HCV viral loads of patients before treatment.

precursor frequencies against a range of HCV epitopes did not change during or after the course of IFNα monotherapy.[13] Recently, the combination therapy of PegIFNα plus ribavirin is standard treatment in the treatment of HCV infected patients with the better results of viral clearance compared with IFNα monotherapy. This suggested that this combination therapy might modify the HCV specific CD8+ T cell responses. We evaluated HCV-specific CD8+ T cell responses by IFN-γ ELISPOT assay, a functional assay of T cells. Significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. This is consistent with the previous report of evaluating the frequencies of HCV-specific CTLs by direct ex vivo staining with HCV-specific pentamers.[16] Thus the evaluation of reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

It has been reported that complete early virologic response (cEVR), which means HCV RNA negativity at week 12, is strongly related to SVR in the combination therapy of Peg-IFNα plus ribavirin.[12,17] cEVR itself has been reported to be an independent predictive factor of

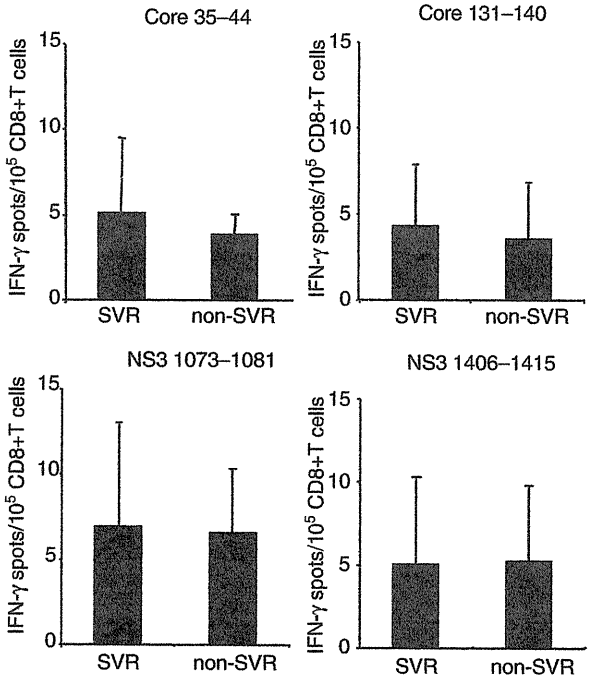

Figure 3 Comparison of the frequencies of hepatitis C virus (HCV)-specific CD8+ T cells in pre-treated HCV patients between sustained virologic response (SVR) and non-SVR. HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-treated HCV patients were evaluated by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). We analyzed the association between the HCV-specific CD8+ T cell responses and the achieving of SVR. SVR: patients who were observed SVR, non-SVR: patients who were not observed SVR.

SVR.[1,12] We also examined the association between cEVR and early elevation of HCV-specific CD8+ T cell responses. The frequencies of CD8+ T cell responses against all four HCV derived peptides in pre-IFN patients were not significantly different between cEVR and non-cEVR (Tatsumi T, unpublished data). In cEVR patients, the frequencies of CD8+ T cell responses against three HCV peptides $Core_{35-44}$, $Core_{131-140}$, $NS3_{1406-1415}$) increased significantly 4 weeks after the starting treatment and those against $NS3_{1073-1081}$ peptide tended to increase although these were not significant. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-cEVR patients (Tatsumi T, unpublished data). The cEVR results were almost similar to those of the SVR results. Although we could not evaluate the HCV RNA levels at 4 week after starting treatment, the cEVR results suggested that early elevation of the frequencies of HCV-specific CD8+ T cell responses might reflect the decrease of viral load of HCV.

CD8+ CTL activities in pre-treated HCV patients have been reported to be very low.[7,18,19] Consistent with the previous observations, the frequencies of HCV specific CD8+ T cell in pre-treated patients were also low in our study. The frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the HCV viral load and the serum ALT levels of patients before treatment. Several reports demonstrated that the baseline presence of HCV-specific CTLs prior to treatment was associated with viral clearance.[7,18] However, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were not associated with the achievement of SVR in our study. In previous other reports, whole PBMC isolated from treated patients were used to evaluate the antiviral activity of HCV-specific CD8+ T cells. In our study, enriched CD8+ T cells obtained by magnetic sorting methods were used to enhance the sensitivity for the detection of HCV-specific CD8+ T cells. Both ELISPOT and staining with tetramers/pentamers could be applied for immunological monitoring for peptide-specific CTLs.[20] ELISPOT can detect activated functional CTLs, and tetramers/pentamers staining can detect peptide-specific CTLs.[20] In our study, we assessed the HCV-specific CD8+ T cell responses by IFN-γ ELISPOT, which is the most well-established methods and has already applied for immunological monitoring in cancer patients.[11] Recently perforin- or granzyme B-ELISPOT assays have also been reported. However, due to limitations in cell numbers of PBMC isolated from HCV patients, we were unable to apply another system of immunological monitoring and test other functional molecules. If we can apply these ELISPOT assays, we could directly evaluate the cytotoxic activity of HCV-specific CTLs.

In our study, the frequencies of HCV-specific CD8+ T cells in pre-treated patients were similar between SVR and non-SVR patients. In contrast, significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be observed in SVR patients, but not in non SVR patients. Caetano et al. evaluated the HCV-specific CD8+ T cells by HLA class I pentamers specific for the one HCV-Core epitope and one NS3 epitope which were same as we used.[16] They demonstrated that the increase of the frequencies of HCV-specific CTLs at 1 month after starting treatment was mainly due to terminally differentiated cells as well as, to a lesser extent, central memory cells in SVR patients and, in contrast, the increase of HCV-specific