pathway in hepatocytes, placing the mitochondrial pathway of apoptosis as a potent loop for amplifying activation of the caspase cascade to execute complete and rapid cell death in hepatocytes.

Acknowledgment: We thank Xiao-Ming Yin (Department of Pathology and Laboratory Medicine, Indiana University School of Medicine) for providing the anti-mouse Bid antibody.

References

1. Guicciardi M, Gores G. Life and death by death receptors. FASEB J 2009;23:1625-1637.
2. Malhi H, Guicciardi M, Gores G. Hepatocyte death: a clear and present danger. Physiol Rev 2010;90:1165-1194.
3. Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, et al. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. HEPATOLOGY 1994;19: 1354-1359.
4. Hayashi N, Mita E. Involvement of Fas system-mediated apoptosis in pathogenesis of viral hepatitis. J Viral Hepat 1999;6:357-365.
5. Feldstein A, Canbay A, Angulo P, Taniai M, Burgart L, Lindor K, et al. Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. Gastroenterology 2003;125: 437-443.
6. Ryo K, Kamogawa Y, Ikeda I, Yamauchi K, Yonehara S, Nagata S, et al. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. Am J Gastroenterol 2000;95:2047-2055.
7. Feldmann G, Lamboley C, Moreau A, Bringuier A. Fas-mediated apoptosis of hepatic cells. Biomed Pharmacother 1998;52:378-385.
8. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 2003;195:158-167.
9. Tait S, Green D. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 2010;11:621-632.
10. Yin X, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, et al. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999;400:886-891.
11. Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK, et al. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. Cell 2007;129: 423-433.
12. Lindsten T, Ross A, King A, Zong W, Rathmell J, Shiels H, et al. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 2000;6:1389-1399.
13. Takehara T, Tatsumi T, Suzuki T, Rucker EB, 3rd, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189-1197.
14. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005; 434:652-658.
15. Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. HEPATOLOGY 2009;50:1217-1226.
16. Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. HEPATOLOGY 2009;50:1972-1980.
17. Wang K, Yin X, Chao D, Milliman C, Korsmeyer S. BID: a novel BH3 domain-only death agonist. Genes Dev 1996;10:2859-2869.
18. Yamagata H, Shimizu S, Nishida Y, Watanabe Y, Craigen WJ, Tsujimoto Y. Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax. Oncogene 2009;28:3563-3572.
19. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, et al. Lethal effect of the anti-Fas antibody in mice. Nature 1993;364:806-809.
20. Wieder T, Essmann F, Prokop A, Schmelz K, Schulze-Osthoff K, Beyaert R, et al. Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. Blood 2001;97:1378-1387.
21. Antonsson B, Montessuit S, Sanchez B, Martinou J. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem 2001;276:11615-11623.
22. Kim T, Zhao Y, Barber M, Kuharsky D, Yin X. Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. J Biol Chem 2000;275: 39474-39481.
23. Schüngel S, Buitrago-Molina L, Nalapareddy P, Lebofsky M, Manns M, Jaeschke H, et al. The strength of the Fas ligand signal determines whether hepatocytes act as type 1 or type 2 cells in murine livers. HEPATOLOGY 2009;50:1558-1566.
24. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, et al. Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x(L). Proc Natl Acad Sci U S A 1999;96:14871-14876.
25. Fujita E, Egashira J, Urase K, Kuida K, Momoi T. Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. Cell Death Differ 2001;8:335-344.
26. Deveraux Q, Reed J. IAP family proteins—suppressors of apoptosis. Genes Dev 1999;13:239-252.
27. Jost P, Grabow S, Gray D, McKenzie M, Nachbur U, Huang D, et al. XIAP discriminates between type I and type II FAS-induced apoptosis. Nature 2009;460:1035-1039.
28. Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1: 489-495.
29. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 2005;1:112-119.
30. Baines C, Kaiser R, Purcell N, Blair N, Osinska H, Hambleton M, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 2005;434:658-662.
31. Caserta T, Smith A, Gultice A, Reedy M, Brown T. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis 2003;8:345-352.
32. Wei M, Zong W, Cheng E, Lindsten T, Panoutsakopoulou V, Ross A, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727-730.
33. Walter D, Schmich K, Vogel S, Pick R, Kaufmann T, Hochmuth F, et al. Switch from type II to I Fas/CD95 death signaling on in vitro culturing of primary hepatocytes. HEPATOLOGY 2008;48:1942-1953.
34. Schmich K, Schlatter R, Corazza N, Ferreira KS, Ederer M, Brunner T, et al. Tumor necrosis factor α sensitizes primary murine hepatocytes to Fas/CD95-induced apoptosis in a Bim- and Bid-dependent manner. HEPATOLOGY 2011;53:282-292.
35. Cardier JE, Schulte T, Kammer H, Kwak J, Cardier M. Fas (CD95, APO-1) antigen expression and function in murine liver endothelial cells: implications for the regulation of apoptosis in liver endothelial cells. FASEB J 1999;13:1950-1960.
36. Baskin-Bey E, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. Am J Transplant 2007;7:218-225.
37. Pockros P, Schiff E, Shiffman M, McHutchison J, Gish R, Afdhal N, et al. Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. HEPATOLOGY 2007;46:324-329.

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

Tomohide Tatsumi,[1] Tetsuo Takehara,[1] Takuya Miyagi,[1] Tsukasa Sugiyama,[1] Hiroshi Aketa,[1] Akira Sasakawa,[1] Tatsuya Kanto,[1] Naoki Hiramatsu[1] and Norio Hayashi[1,2]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka and* [2]*Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan*

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

Correspondence: Dr Tomohide Tatsumi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Email: tatsumit@gh.med.osaka-u.ac.jp

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

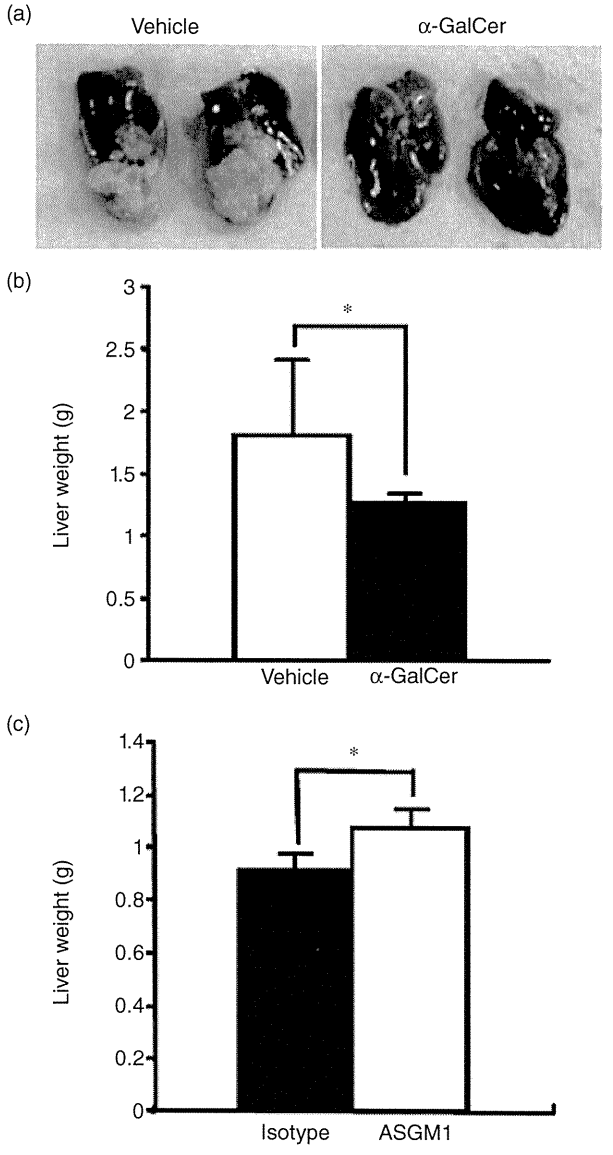


Figure 1 Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intro-hepatically with 5×10^5 CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, $n = 7$). Two 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 $n = 5$). $^*P < 0.05$.

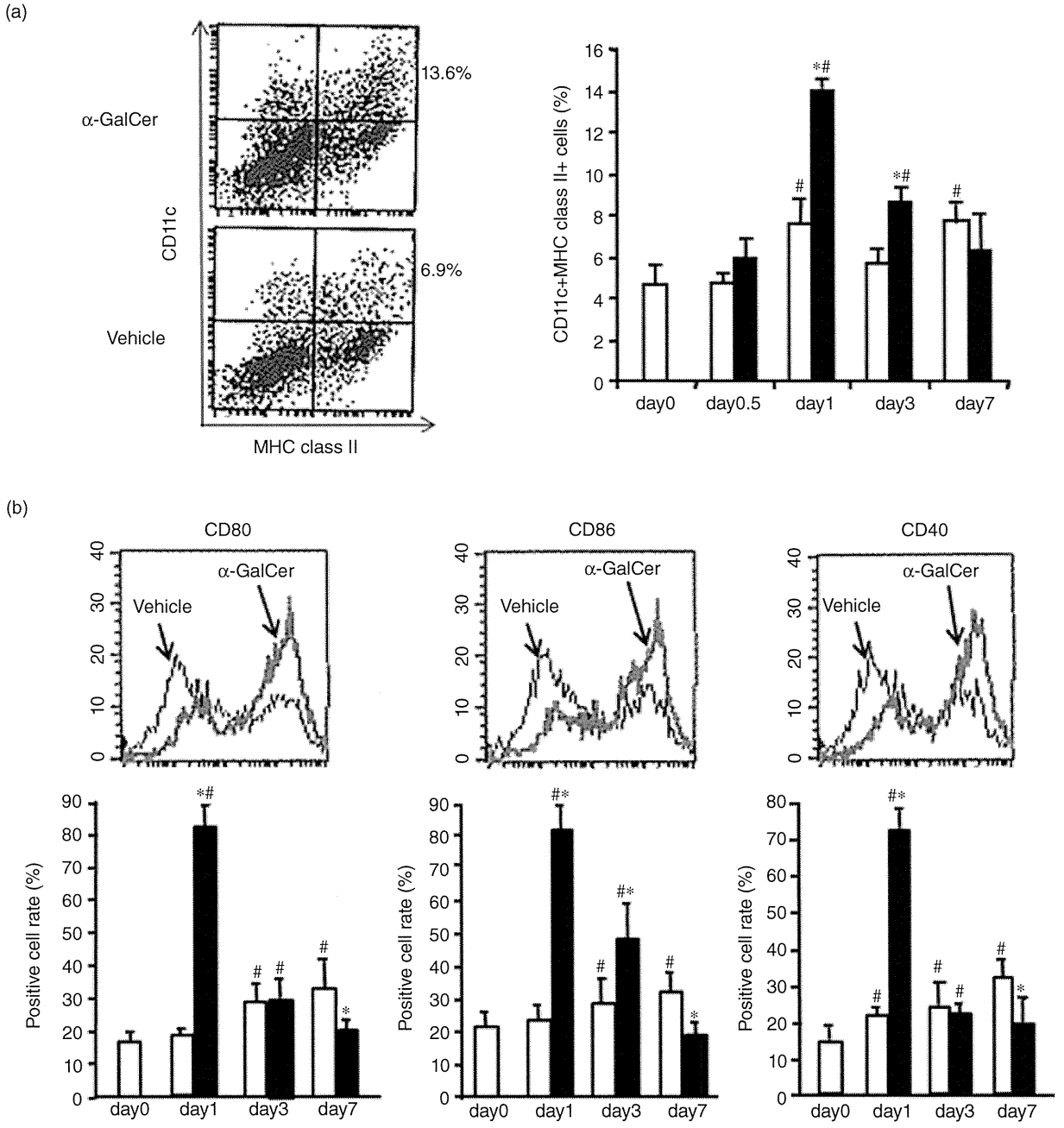


Figure 2 α-Galactosylceramide (α-GalCer) treatment increased dendritic cell (DC) population in the liver mononuclear cells (MNC) and activated DC functions. (a) BALB/c mice were treated with α-GalCer or vehicle. Hepatic MNC were prepared on days 0, 0.5, 1, 3 and 7. DC ($CD11c^+$ major histocompatibility 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.

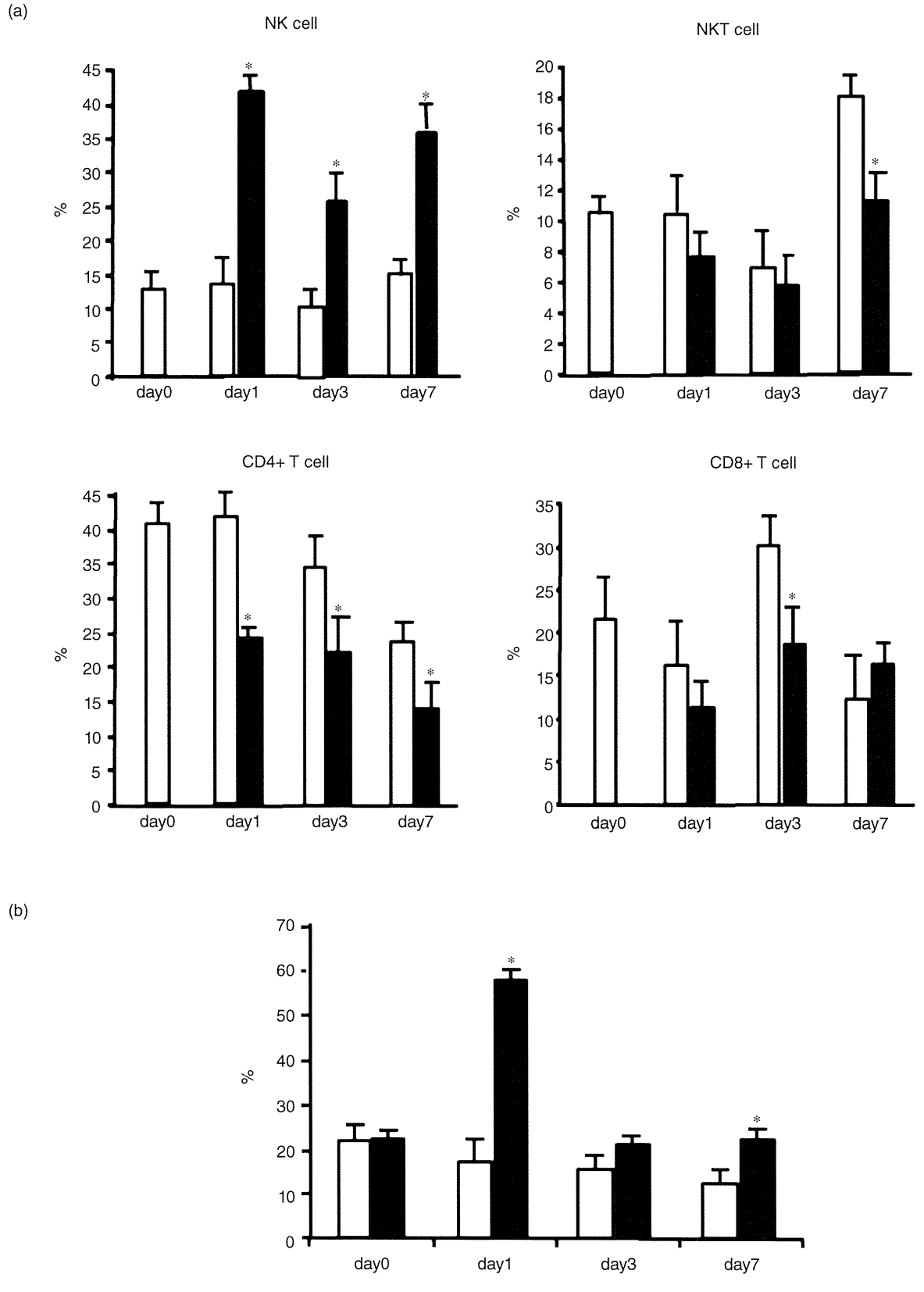
Activated NK cells composed the major subpopulation of hepatic MNC that increased after α-GalCer treatment

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.



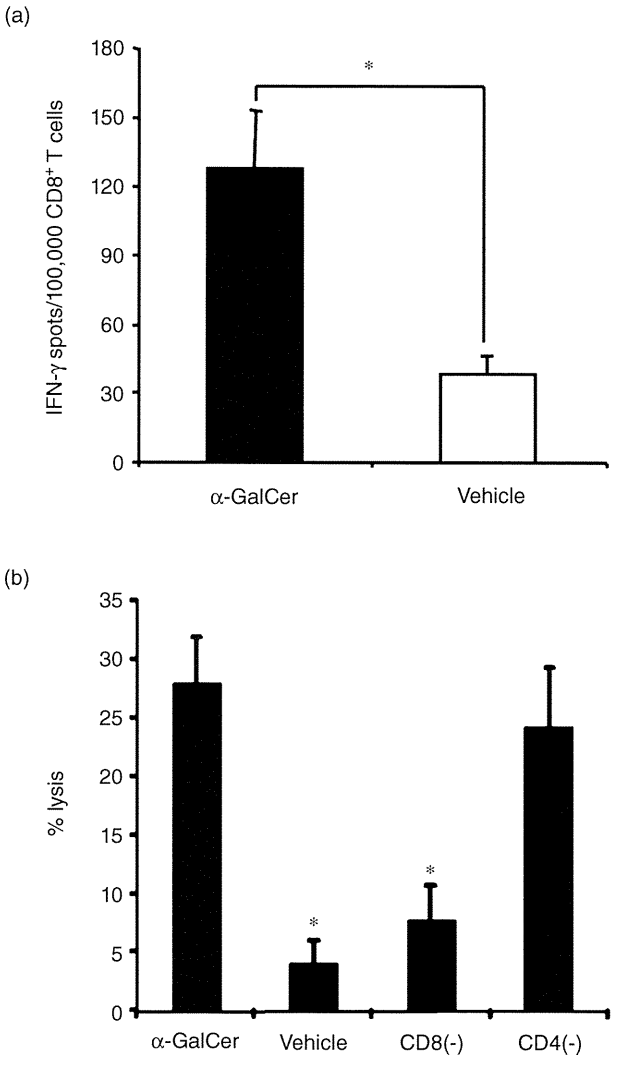


Figure 4 Evaluation of $p53_{232-240}$ peptide-specific $CD8^+$ cytotoxic T lymphocytes (CTL) in α-galactosylceramide (α-GalCer)-treated mice. (a) $CD8^+$ T cells were isolated from the spleen cells of treated mice 14 days after α-GalCer or vehicle treatment. The frequency of $p53_{232-240}$ peptide-specific $CD8^+$ 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

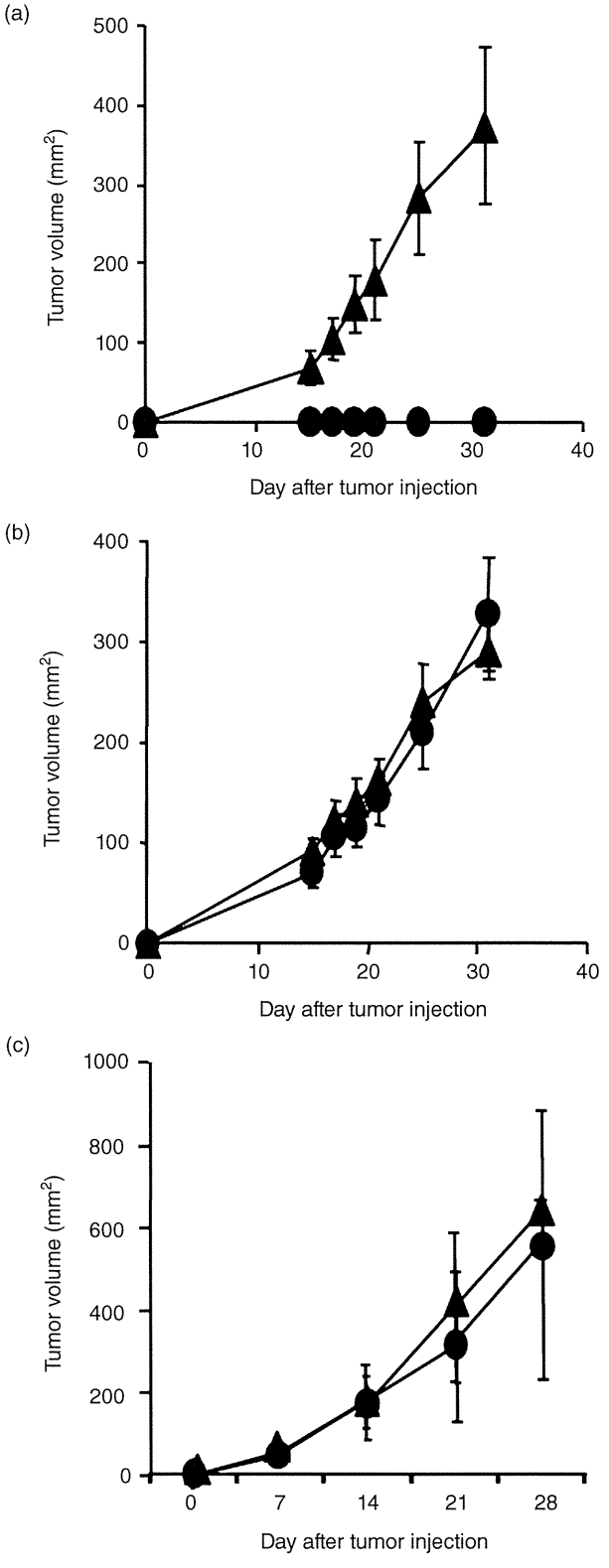


Figure 5 α-Galactosylceramide (α-GalCer) therapy results in the development of systemic antitumor immunity that protects distal tumor re-challenge. BALB/c mice were injected intrahepatically with CMS4 tumors. Twenty-four hours later, the mice were treated with α-GalCer. Twenty-eight days after treatment, 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.

REFERENCES

1 Kawano T, Cui J, Koezuka Y *et al.* CD1d-restricted and TCR-mediated activation of Vα14NKT cells by glycosylceramides. *Science* 1997; **278**: 1626–9.
2 Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ- producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 2002; **3**: 867–74.
3 Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M *et al.* α-Galactosylceramide-activated Vα 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci USA* 2000; **97**: 8461–6.
4 Miyagi T, Takehara T, Tatsumi T *et al.* CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. *Int J Cancer* 2003; **106**: 81–9.
5 Giaccone G, Punt CJ, Ando Y *et al.* A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002; **8**: 3702–9.
6 Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in human liver. *Immunol Rev* 2000; **174**: 5–20.
7 Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gatsroenterology* 2001; **120**: 250–60.
8 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991; **9**: 271–96.
9 Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* 1997; **90**: 3245–87.
10 Fernandez NC, Lozier A, Flament C *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* 1999; **5**: 405–11.

11 Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* 2002; **195**: 327–33.
12 Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting NK cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* 2002; **195**: 343–51.
13 Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. *J Exp Med* 2002; **195**: 335–41.
14 Miller G, Lahrs S, Dematteo RP. Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity. *FASEB J* 2003; **17**: 728–30.
15 Kawano T, Cui J, Koezuka Y *et al.* Natural killer-like non-specific tumor cell lysis mediated by specific ligand-activated Vα14NKT cells. *Proc Natl Acad Sci USA* 1998; **95**: 5690–3.
16 Kitamura H, Iwakabe K, Yahata T *et al.* The natural killer T (NKT) cell ligand α-galactosylceramide demonstrates its immunopoteniating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 1999; **189**: 1121–8.
17 Sasakawa A, Tatsumi T, Takehara T *et al.* Activated liver dendritic cells generates strong acquired immunity in α-galctosylceramide treatment. *J Hepatol* 2009; **50**: 1155–62.
18 Mayordomo JI, Loftus DJ, Sakamoto H *et al.* Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. *J Exp Med* 1996; **183**: 1357–65.
19 Tatsumi T, Huang J, Gooding WE *et al.* Intratumoral delivery of dendritic cells engineered to secrete both interleukin(IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res* 2003; **63**: 6378–86.
20 Uemura A, Takehara T, Miyagi T *et al.* Natural killer cell is a major producer of IFN-γ that is critical for the IL-12-induced anti-tumor effect in mice. *Cancer Immunol Immunother* 2010; **59**: 453–63.
21 Yamaguchi S, Tatsumi T, Takehara T *et al.* Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide pulsed dendritic cell vaccines. *Cancer* 2007; **110**: 1469–77.
22 Ferlazzo G, Munz C. NK cell compartments and their activation by dendritic cells. *J Immunol* 2004; **172**: 1333–9.
23 Takagi S, Miyagawa S, Ichikawa E *et al.* Dendritic cells, T-cell infiltration, and Grp94 expression in cholangiocellular carcinoma. *Hum Pathol* 2004; **35**: 881–6.
24 Iwamoto M, Shinohara H, Miyamoto A *et al.* Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int J Cancer* 2003; **104**: 92–7.
25 Kobayashi M, Fitz L, Ryan M *et al.* Identification and purification of natural killer cell stimulatory factors (NKSF), a cytokine with multiple biological effects on human lymphocytes. *J Exp Med* 1989; **170**: 827–45.
26 Gately MK, Wolitzky AG, Quinn PM, Chizzonite R. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell Immunol* 1992; **143**: 127–42.
27 Pillarisetty VG, Katz SC, Bleier JI, Shah AB, DeMatteo RP. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. *J Immunol* 2005; **174**: 2612–18.
28 Fujii S, Shimizu K, Smith C, Bonifaz L, Steinman RM. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 2003; **198**: 267–79.
29 Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 2004; **199**: 1607–18.
30 Hermans IF, Silk JD, Gileadi U, Salio M *et al.* NKT cells enhance CD4+ and CD8+ T cells responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 2003; **171**: 5140–7.
31 Nishimura T, Kitamura H, Iwakabe K *et al.* The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* 2000; **12**: 987–94.

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

The *let-7* family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma

Satoshi Shimizu[1,†], Tetsuo Takehara[1,†], Hayato Hikita[1], Takahiro Kodama[1], Takuya Miyagi[1], Atsushi Hosui[1], Tomohide Tatsumi[1], Hisashi Ishida[1], Takehiro Noda[2], Hiroaki Nagano[2], Yuichiro Doki[2], Masaki Mori[2], Norio Hayashi[1,*]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan;* [2]*Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan*

Background & Aims: Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is over-expressed in human hepatocellular carcinoma, conferring a survival advantage to tumour cells. The mechanisms underlying its dysregulation have not been clarified. In the present study, we explored the involvement of microRNAs that act as endogenous sequence-specific suppressors of gene expression.

Methods: The expression profiles of microRNAs in Huh7 hepatoma cells and primary human hepatocytes were compared by microarray analysis. The effect of *let-7* on Bcl-xL expression was examined by Western blot and a reporter assay. The involvement of *let-7* microRNAs in human tissues was analysed by western blot and reverse transcription-PCR.

Results: Microarray analysis, followed by *in silico* target prediction, identified *let-7* microRNAs as being downregulated in Huh7 hepatoma cells in comparison with primary human hepatocytes, as well as possessing a putative target site in the *bcl-xl* mRNA. Over-expression of *let-7c* or *let-7g* led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines. Reporter assays revealed direct post-transcriptional regulation involving *let-7c* or *let-7g* and the 3′-untranslated region of *bcl-xl* mRNA. Human hepatocellular carcinoma tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* microRNA expression contributes to Bcl-xL over-expression. Finally, expression of *let-7c* enhanced apoptosis of hepatoma cells upon exposure to sorafenib, which downregulates expression of another anti-apoptotic Bcl-2 protein, Mcl-1.

Conclusions: *let-7* microRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1.

Keywords: Liver; Mcl-1; Bcl-2; Tumour; Epigenetic.
Received 2 July 2009; received in revised form 17 November 2009; accepted 2 December 2009; available online 4 March 2010
*Corresponding author. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail address: hayashin@gh.med.osaka-u.ac.jp (N. Hayashi).
†These authors contributed equally to this work and share first authorship.
Abbreviations: miRNA, microRNA; 3′UTR, 3′-untranslated region; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle medium; RT, reverse transcription; PCR, polymerase chain reaction; 7-AAD, 7-amino-actinomycin D; DMSO, dimethyl sulfoxide.

Introduction

MicroRNAs (miRNAs), a novel class of non-coding, small RNAs, repress gene expression by binding to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) [1]. More than 500 miRNAs have been identified in humans. Each miRNA is capable of modulating the expression of many mRNAs to which it binds by imperfect sequence complementarity, although only a limited number of targeted genes has been identified. Through its activity of gene silencing, miRNA functions in a variety of cellular processes, such as development, organ homeostasis, and cancer development and progression [2]. In the context of cancer development and progression, miRNAs targeting oncogenes function as tumour suppressors, whereas those targeting tumour suppressor genes serve as oncogenes [3]. Accumulating evidence has revealed the aberrant expression of miRNAs in human hepatocellular carcinoma (HCC) [4–6]. *miR-122a* has been reported to be downregulated in HCC, in turn, leading to upregulation of cyclin G1 [7]. On the other hand, recent reports have demonstrated that *miR-21* [8], *miR-221* [9], and *miR-224* [10] are upregulated in HCC, leading to downregulation of PTEN, CDK inhibitors, and API-5, respectively. Furthermore, the miRNA expression signature was reported to be related to the clinical outcome of patients with HCC [11,12]. Thus, miRNAs may play an important role in HCC development and progression by modulating a variety of gene expression and cellular processes.

Apoptosis resistance is an important characteristic of tumour cells, in addition to dysregulated proliferation and aberrant differentiation. Apoptosis is regulated by a fine balance of Bcl-2 family proteins, such as anti-apoptotic Bcl-xL and Mcl-1 and pro-apoptotic Bak and Bax. We previously demonstrated that Bcl-xL

is over-expressed in one-third of human HCC and confers resistance to hepatoma cells toward a variety of apoptotic insults generated by serum starvation and *p53* activation [13]. Patients with Bcl-xL-overexpressing HCC were shown to have significantly shorter disease-free survival after surgery [14]. Recently, it was proposed that autophagy defect is another mechanism of the malignant phenotype of Bcl-xL-overexpressing HCC through interaction between Bcl-xL and Beclin1 [15]. The underlying mechanisms of Bcl-xL over-expression in HCC are not clearly understood. Several reports show that transcription factors such as NF-κB [16] and STAT3 [17] could upregulate Bcl-xL expression in hepatoma cells. In addition, hepatitis C virus-related proteins, such as core [18] and NS5A [19], could upregulate Bcl-xL at a transcriptional level. However, we noticed that Bcl-xL-overexpressing hepatocarcinoma tissues do not always display upregulation of *bcl-xl* mRNA [13]. This observation led us to examine the possibility that post-transcriptional regulation by miRNAs may be involved in Bcl-xL expression in human HCC.

In the present study, we demonstrate that *let-7* family miRNAs, a prototype of human miRNAs [20], negatively regulate Bcl-xL expression in human HCC. *let-7* miRNAs are downregulated in human hepatoma cells and tissues in association with enhanced expression of Bcl-xL. Over-expression of *let-7* miRNAs in hepatoma cells downregulates Bcl-xL in a *bcl-xl* 3′UTR sequence-specific manner and enhances apoptosis induced by sorafenib, a recently approved anti-cancer drug for HCC [21]. The present study demonstrates for the first time that *let-7* miRNAs directly target Bcl-xL and induce apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in HCC.

Materials and methods

miRNA target predictions

The algorithms miRanda (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) were used to predict miRNAs that could potentially bind to *bcl-xl* mRNA.

Cell lines and tissues

Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines, Huh7 and HepG2, were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, St. Louis, MO). HCCs and adjacent non-tumour counterparts were obtained at the time of surgical resection. Written informed consent was obtained from each patient. All tissues were stored at −80 °C until the time of use.

RNA extractions

Total RNA including the miRNA fraction was isolated from cell lines and tissue samples using the miRNeasy Mini Kit (QIAGEN, Valencia, CA). After extraction, the quality of each RNA sample was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

miRNA microarray analysis

RNA labelling and hybridisation were performed using a human miRNA microarray kit and a miRNA complete labelling and hybridisation kit (Agilent Technologies). After washing with Gene Expression Wash Buffer, the slides were scanned with an Agilent Microarray Scanner and analysed by GeneSpring GX software.

Western blot

Cells or tissues were lysed and Western blotted as previously described [22]. For immunodetection, the following antibodies were used: anti-Bcl-xL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 polyclonal antibody (Santa Cruz Biotechnology), anti-Bak polyclonal antibody (Millipore, Billerica, MA), anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA). Optical densities of bands in each blot were analysed using ImageJ 1.40 g (NIH, Bethesda, MD).

Real time reverse transcription (RT)-PCR assays for mature miRNAs

To quantify the expression of mature miRNA, we synthesised cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) specific for *let-7c* (P/N 4373167) and *let-7g* (P/N 4395393). To normalise the expression levels of miRNAs, we used TaqMan MicroRNA Assays specific for RNU6B (P/N 4373381) as the endogenous control.

Real time RT-PCR assays for bcl-xl mRNA

We reverse-transcribed RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and *bcl-xl* mRNA expression was measured using TaqMan Gene Expression Assays (Applied Biosystems, Assay ID: Hs99999146_m1). We also quantified *β-actin* mRNA as an endogenous control (Assay ID: Hs99999903_m1).

Transfections with miRNAs

Huh7 and HepG2 cells were transfected with 50 nM Pre-miR miRNA precursor molecules (Ambion, Austin, TX) of either *let-7c* or *let-7g* using RNAiMAX (Invitrogen, Carlsbad, CA) in six-well plates according to the manufacturer's instructions. Pre-miR negative control (Ambion) was also used as a control.

Luciferase assay

To generate the pMIR-Bcl-xL-3′UTR construct that contains the putative binding site of *bcl-xl* 3′UTR downstream of the firefly luciferase gene, we synthesised oligonucleotides to mimic the target sequence and inserted them into the SpeI–HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Bcl-xL-3′UTR mutant that has a point mutation in the putative binding site, using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Each of these constructs was transfected into Huh7 cells together with 50 nM Pre-miR miRNA precursor molecules and pMIR-REPORT β-Gal vector (Ambion), which contains the β-galactosidase gene for normalisation of transfection efficiency. Transfection was performed using Lipofectamine 2000 (Invitrogen). We measured firefly luciferase activity 24 h after transfection using the Luciferase Assay System (Promega, Madison, WI) and normalised it to the β-galactosidase expression level.

In vitro staurosporine or sorafenib treatment

Huh7 cells were transfected with 50 nM Pre-miR miRNA precursor molecules as described above, and 48 h after transfection, the medium was changed to DMEM containing staurosporine (Calbiochem, Gibbstown, NJ) or sorafenib. Sorafenib was kindly provided by Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ). Cells were additionally cultured and assayed for apoptosis by monitoring the activity of caspase-3/7 using a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega, Madison, WI), or by flow cytometry using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA). We defined apoptotic cells as Annexin V-PE-positive and 7-amino-actinomycin D (7-AAD)-negative cells. Cell viability was determined by the WST assay using cell count reagent SF (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Data are presented as mean ± SD. Comparisons between two groups were performed by the unpaired *t*-test. Multiple comparisons were performed by ANOVA with the Scheffe post hoc test. $p < 0.05$ was considered statistically significant.

Results

let-7 miRNAs were downregulated in hepatoma cells with upregulated expression of Bcl-xL

As observed in human HCC tissues, Bcl-xL was over-expressed, according to Western blot analysis, in Huh7 and HepG2 human hepatoma cell lines compared to normal hepatocytes (Fig. 1A). Previous research established that 30 and 32 kDa species are original and post-translationally modified Bcl-xL, respectively [23]. Mcl-1 was also over-expressed in human hepatoma cells, but the levels of expression of Bak and Bax did not differ between hepatoma cells and normal hepatocytes. We reasoned that miRNA regulating Bcl-xL expression would be downregulated in those hepatoma cell lines. To search for the candidate miRNA, microarray analysis was performed. More specifically, miRNA expression in Huh7 cells and normal hepatocytes was compared. When levels of expression less than 50% were considered significant, 26 miRNAs were identified as being downregulated in Huh7 cells: *let-7b*, *let-7g*, *let-7i*, *miR-127-3p*, *miR-214*, *miR-376a*, *miR-381*, *miR-409-3p*, *miR-376c*, *miR-493**, *miR-432*, *miR-487b*, *let-7d*, *let-7a*, *let-7f*, *let-7c*, *miR-200a*, *let-7e*, *miR-134*, *miR-503*, *miR-34a*, *miR-638*, *miR-150**, *miR-1225-5p*, *miR-21**, and *miR-223*. Among them, *in silico* analysis revealed that only the *let-7* family is capable of potentially targeting the 3′UTR of the *bcl-xl* mRNA. To confirm the results of the microarray analysis, quantitative real time RT-PCR was performed to evaluate the expression of *let-7c* and *let-7g* (Fig. 1B). After normalisation to endogenous RNU6B expression levels, the expression levels of both miRNAs were substantially lower in Huh7 cells than in normal hepatocytes. These results were consistent with the results of microarray analysis. Furthermore, the expression levels of both miRNAs were found to be downregulated in another human hepatoma cell line, HepG2, compared to normal hepatocytes.

let-7c and let-7g downregulate Bcl-xL expression by directly targeting the 3′UTR of bcl-xl mRNA

To examine whether *let-7* miRNAs are capable of suppressing translation of Bcl-xL, hepatoma cell lines were transfected with *let-7c* or *let-7g* or the negative control. Three days after transfection, Huh7 cells showed a decrease in Bcl-xL protein levels in both the *let-7c*-transfected group and the *let-7g*-transfected group in comparison with the negative control group (Fig. 2A). The transfection of *let-7c* and *let-7g* showed suppression of Bcl-xL protein levels in HepG2 cells as well (Fig. 2B). It did not affect expression of Bak and Bax, but increased Mcl-1 expression, which may be a secondary phenomenon of suppression of Bcl-xL. Normal hepatocytes were also transfected with *let-7c* or *let-7g* (Suppl. Fig. 1). The transfection led to a decrease in Bcl-xL expression in normal hepatocytes, but the decline was lesser than that observed in hepatoma cells. This finding may be explained by the observation that endogenous expression of *let-7c* and *let-7g* was extremely high in normal hepatocytes.

To examine whether the downregulation of Bcl-xL by *let-7c* or *let-7g* is caused by direct binding to a putative targeting site in the *bcl-xl* mRNA, we constructed the luciferase reporter plasmid pMIR-Bcl-xL-3′UTR containing the putative *let-7* binding site of *bcl-xl* 3′UTR downstream of the luciferase open reading frame (Fig 3A). The pMIR-Bcl-xL-3′UTR construct was cotransfected with the control pMIR-REPORT β-gal vector into Huh7 cells together with *let-7c* or *let-7g* or the negative control. When *let-7c* or *let-7g* Pre-miR was cotransfected with pMIR-Bcl-xL-3′UTR, the expression of firefly luciferase was significantly reduced compared to the negative control cotransfected group. There was no difference in firefly luciferase expression levels when pMIR-REPORT, which does not contain the putative *let-7* binding site, was cotransfected with *let-7c*, *let-7g* or the negative control (Fig. 3B). We also generated a pMIR-Bcl-xL-3′UTR mutant with a single base mutation in the seed region of the putative binding sequence to investigate whether the downregulation of firefly luciferase can be attributed to the insert (Fig. 3A). A single base mutation prevented the downregulation of firefly luciferase

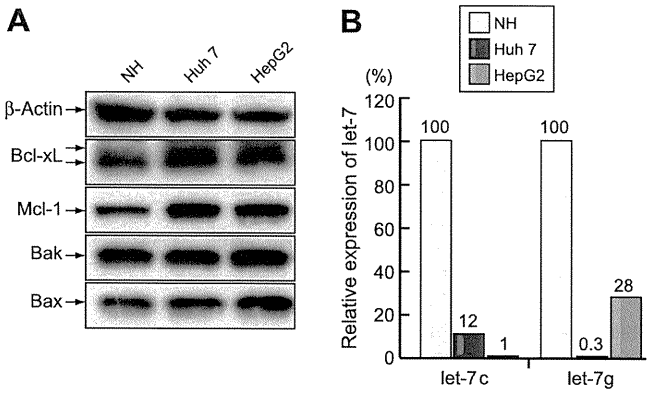


Fig. 1. Expression of Bcl-xL and *let-7* miRNAs in cultured human hepatocytes and hepatoma cells. Human hepatoma cell lines, Huh7 and HepG2, and normal hepatocytes (NH) were cultured and then lysed. (A) Western blot analysis for Bcl-2 family proteins. Bcl-xL migrates as a doublet band (see text). (B) Real time RT-PCR analysis for *let-7c* and *let-7g* expression. After normalisation to endogenous RNU6B expression, the expression of each miRNA in hepatoma cells was expressed in comparison to the levels observed in normal hepatocytes.

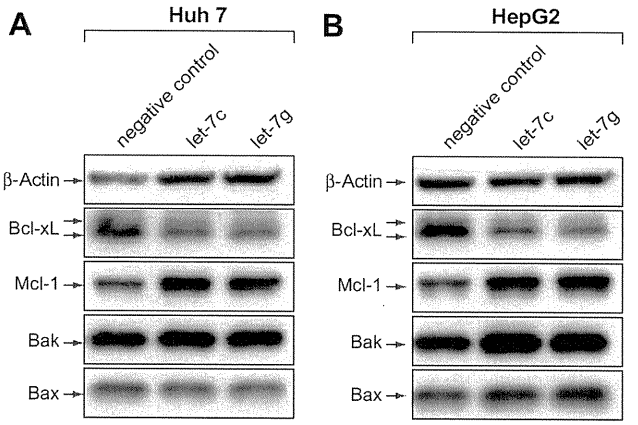


Fig. 2. Over-expression of *let-7* miRNAs downregulates Bcl-xL expression in hepatoma cells. Hepatoma cell lines Huh7 (A) and HepG2 (B) were transfected with *let-7c*, *let-7g*, or negative control miRNA at 50 nM and cultured for 3 days. Expression levels of Bcl-2 family proteins were determined by Western blot analysis.

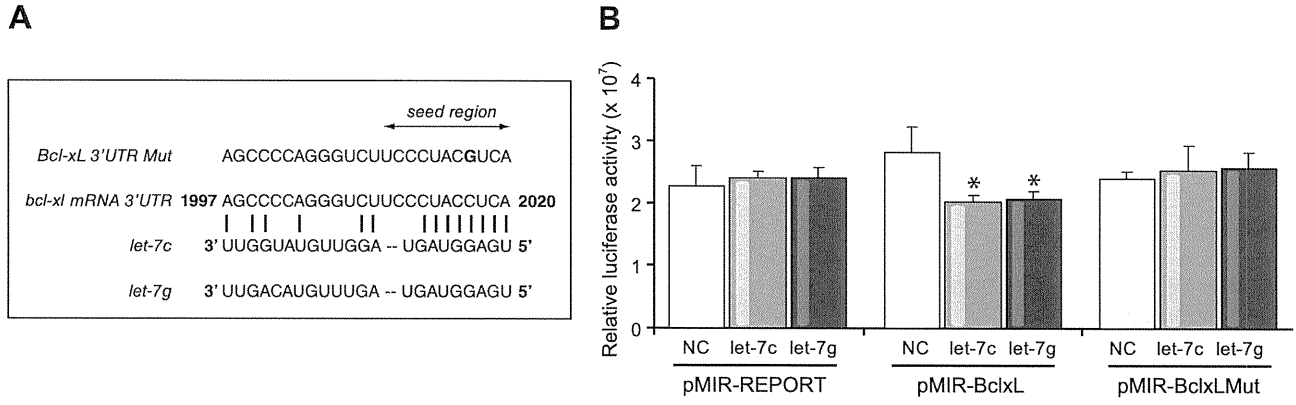


Fig. 3. Sequence-specific suppression of *bcl-xl* gene expression by *let-7c* or *let-7g* miRNAs. (A) The putative target site of *bcl-xl* mRNA 3′UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xL-3′UTR). pMIR-Bcl-xL-3′UTR mutant was also generated with a single mutation (indicated by a bold character) in the target site. (B) Each of these constructs was transfected into Huh7 cells together with *let-7c*, *let-7g* or negative control miRNA (NC). At 24 h after transfection, the activity of firefly luciferase was measured and normalised to β-galactosidase expression levels ($n = 3$). *$p < 0.05$.

induced by *let-7c* or *let-7g*, which strongly suggests a direct inhibitory effect of *let-7* on Bcl-xL expression (Fig. 3B).

Downregulation of let-7c miRNA in human HCC tissues overexpressing Bcl-xL but not bcl-xl mRNA

To investigate the relationship between *let-7* expression levels and Bcl-xL protein levels in human HCC samples, we used 22 pairs of surgically resected human HCC tissue samples and adjacent non-tumour tissue samples with highly preserved RNA. Compared to the non-tumour counterparts, *bcl-xl* mRNA was found to be over-expressed in HCC tissue samples in only two cases; Bcl-xL was also over-expressed at the protein level in these cases. To assess the significance of *let-7* in post-transcriptional regulation of Bcl-xL *in vivo*, we selected 20 pairs of HCC tissue samples that did not over-express *bcl-xl* mRNA. When these samples were divided into two groups according to relative *let-7c* expression levels, the relative expression of Bcl-xL protein was significantly higher in the *let-7c* low expression group than in the *let-7c* high expression group (Fig. 4). By contrast, there was no significant difference in *bcl-xl* mRNA expression between the two groups. We also examined the relationship between relative *let-7g* expression and Bcl-xL expression. The *let-7g* low expression group tended to over-express Bcl-xL protein compared to the *let-7g* high expression group, although the difference did not reach statistical significance (data not shown). These results are consistent with the hypothesis that *let-7* miRNAs negatively regulate Bcl-xL expression independent of transcriptional regulation.

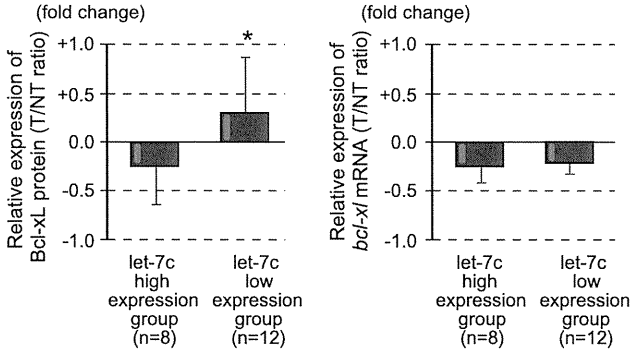


Fig. 4. Expression of Bcl-xL, *bcl-xl* mRNA and *let-7* miRNAs in human HCC tissue. Relationship between *let-7* and Bcl-xL expression in human HCC tissue samples. HCC tissue samples that did not show transcriptional upregulation of *bcl-xl* mRNA were divided into two groups according to relative *let-7c* expression levels with the threshold set at a 0.4-fold change in the tumour to non-tumour (T/NT) ratio. Relative expression of Bcl-xL protein and *bcl-xl* mRNA was calculated as the optical densities of the Bcl-xL blots normalised with the β-actin blots and those of real time RT-PCR assays, respectively, and are shown as the ratio of expression in the tumour to non-tumour expression in $\log_{10}$ scale. *$p < 0.05$.

let-7c miRNA sensitises human Huh7 cells to sorafenib, which downregulates Mcl-1 expression

To investigate the effect of *let-7* in the resistance of hepatoma cells to apoptosis, we transfected Huh7 hepatoma cells with *let-7c* miRNAs and then subjected them to apoptosis analysis and a cell viability assay. There was no significant difference in caspase-3/7 activation or cell viability between *let-7c* miRNA-transfected Huh7 cells and control miRNA-transfected Huh7 cells (represented by the DMSO-treated group of Fig. 5A and B). These results are in agreement with our previous finding that anti-sense oligonucleotide-mediated knockdown of Bcl-xL sensitised hepatoma cells to apoptotic stimuli, such as serum starvation and *p53* activation, but did not induce apoptosis by itself [13]. Next, we exposed miRNA-transfected Huh7 cells to staurosporine, which is a well-established apoptosis inducer. Staurosporine treatment induced apoptosis, as determined by caspase-3/7 activation and decreased the viability of Huh7 cells by itself, but *let-7c* miRNA-transfected Huh7 cells were more susceptible to staurosporine treatment than control miRNA-transfected cells. *let-7c* miRNA-transfected Huh7 cells showed a significant decrease in cell viability, even upon exposure to low-dose of staurosporine at which control miRNA-transfected Huh7 did not show a significant difference in cell viability (Fig. 5B). In addition, the activation of caspase-3/7 was more intense in *let-7c* miRNA-transfected Huh7 cells than in control miRNA-transfected Huh7 cells (Fig. 5A). Thus, suppression of *let-7* expression leading to over-expression of Bcl-xL, may be a mechanism by which hepatoma cells resist apoptotic stimuli. While normal hepatocytes were more sensitive to staurosporine than hepatoma cells, transfection of *let-7* miRNA did not affect sensitivity to staurosporine

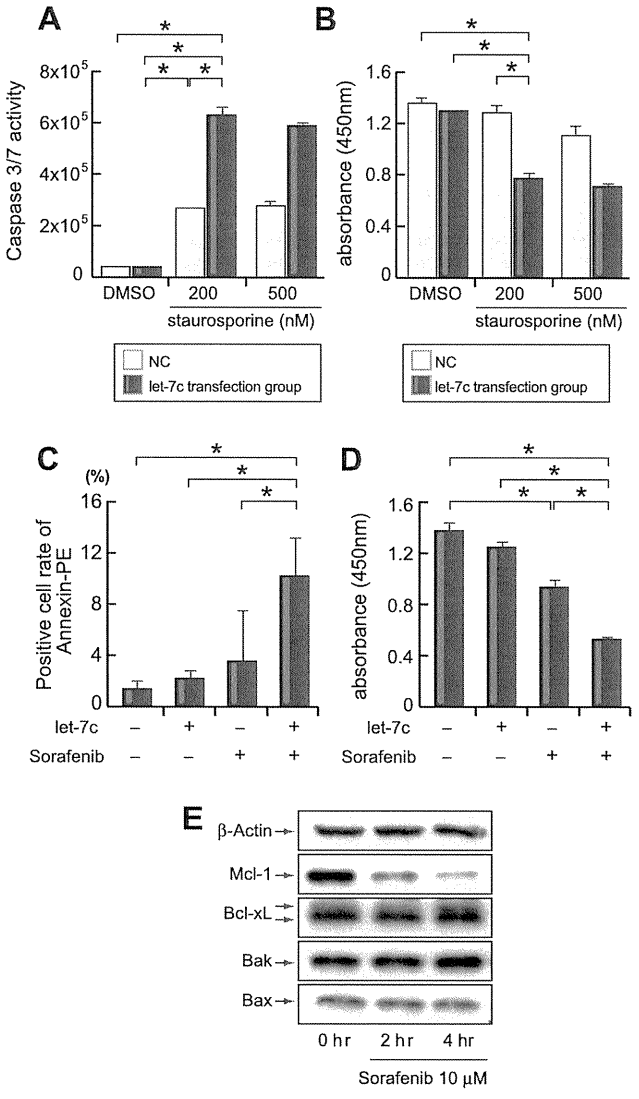


Fig. 5. Introduction of *let-7* miRNAs sensitises hepatoma cells to apoptotic stimuli. (A and B) Response to staurosporine treatment. Huh7 cells were transfected with *let-7c* (grey bars) or control miRNA (white bars) for 48 h and then further treated with staurosporine or DMSO alone for 12 h. The activities of caspase-3 and -7 were determined by luminescent substrate assays for caspase-3 and -7 ($n = 4$) (A). Cell viability was determined by the WST assay ($n = 4$) (B). *$p < 0.05$. (C and D) Response to sorafenib treatment. Huh7 cells were transfected with *let-7c* or control miRNA for 48 h and then further treated with sorafenib (5 μM) or DMSO alone for 48 h (C) or 72 h (D) 7-AAD negative cells were gated and the positive cell rate for annexin V-PE was determined ($n = 4$) (C). Cell viability was determined by the WST assay ($n = 4$) (D). *$p < 0.05$. E. Western blot analysis for Bcl-2 family proteins in lysates of Huh7 cells treated with sorafenib.

in normal hepatocytes (Suppl. Fig. 2), which is in agreement with the modest decline of Bcl-xL expression described earlier.

To examine the impact of *let-7* family miRNAs as a therapeutic tool, we investigated the effect of *let-7* miRNAs on apoptosis resistance to sorafenib, a recently approved anti-cancer drug for HCC. It has been reported that sorafenib was capable of downregulating Mcl-1 expression in tumour cells [24], and HCC has been reported to over-express Mcl-1, which is another anti-apoptotic Bcl-2 protein capable of conferring resistance to apoptosis [24–27]. In agreement with these findings, sorafenib treatment clearly downregulated Mcl-1 expression in hepatoma cells, but did not affect Bcl-xL expression (Fig. 5E). In contrast, sorafenib treatment did not affect Mcl-1 expression in normal hepatocytes (Suppl. Fig. 3). We hypothesised that *let-7* miRNA targeting Bcl-xL may induce apoptosis of hepatoma cells in cooperation with sorafenib. Apoptosis determined by Annexin V staining did not increase in *let-7c* miRNA-treated Huh7 cells compared to control miRNA-treated cells (represented by the DMSO-treated group in Fig. 5C). Sorafenib treatment of Huh7 cells led to a slight increase in the annexin V-positive cell rate, although the difference did not reach statistical significance levels under our experimental conditions (Fig. 5C). Of importance is the finding that sorafenib-induced apoptosis was markedly enhanced in *let-7c* miRNA-transfected cells. In addition, sorafenib treatment significantly reduced the viability of Huh7 cells and this decrease was markedly enhanced in cells transfected with *let-7c* miRNA (Fig. 5D). This finding implies that *let-7* miRNA transfection potentiates sorafenib-induced apoptosis and toxicity in hepatoma cells.

Discussion

Anti-apoptotic members of the Bcl-2 family, which consists of five members, Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1, are critically involved in the mitochondrial pathway of apoptosis [28]. Cancer cells frequently over-express one or more members of this family to acquire a survival advantage [29]. These proteins are over-expressed in a variety of ways, including genetic translocation, particularly in the case of Bcl-2, and transcriptional regulation. Unlike the case of the *bcl-2* gene, mutations or amplification of the *bcl-x* gene have not been demonstrated in tumour cells. With regard to miRNA regulation, previous research clearly demonstrated that Bcl-2 is a direct target of *miR-15* and *miR-16*. The expression levels of *miR-15* and *miR-16* inversely correlate with Bcl-2 expression in chronic lymphocytic leukaemia [30]. More recently, Mcl-1 was reported to be suppressed by *miR-29* [31]. Our present study is the first demonstration of miRNA-mediated regulation of Bcl-xL expression. Since Bcl-xL is over-expressed not only in HCC but also in other tumours, the present findings may shed light on the mechanisms of Bcl-xL over-expression in other malignancies.

While more than 500 human miRNAs have been identified, *let-7* is a prototype of human miRNA and was first identified in 2001 [32]. *let-7* miRNAs are downregulated in several malignancies. A highly characterised example is non-small cell lung cancer in which downregulation of *let-7* miRNAs is well correlated with poor prognosis in patients [33]. In HCC, some reports showed downregulation of *let-7*, while others did not [7]. In the present study, *let-7c* miRNA was under-expressed at less than 40% of the normal level in approximately half of the HCC tissues. Further study is needed to determine the clinical significance of *let-7* miRNA in HCC. Several target genes have been identified for *let-7* miRNA, including Ras [34], Myc [35], HMGA2 [36], CDC25A, and CDK6 [37]. The major function of this miRNA is to promote cell proliferation. Since these proteins could act as oncogenes in tumour cells, *let-7* miRNA is believed to serve as a tumour suppressor [38]. In the present study, we have demonstrated that *bcl-xl* is a direct target for *let-7* miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy.

Sorafenib is a recent FDA-approved anti-cancer drug for HCC [21]. It functions as a multi-kinase inhibitor and can induce

apoptosis at least in part by downregulating Mcl-1 in tumour cells [24]. Like Bcl-xL, several reports have identified Mcl-1 as being over-expressed in some HCCs [25–27]. Since Bcl-xL and Mcl-1 share a similar structure and functions, we reasoned that downregulation of both proteins would efficiently kill hepatoma cells. To verify this hypothesis, we treated hepatoma cells with sorafenib and *let-7* miRNA. As expected, sorafenib treatment downregulated Mcl-1 expression as early as 2 h post-treatment; however, it did not efficiently induce apoptosis. Transfection of *let-7* miRNA itself was not capable of inducing apoptosis of hepatoma cells despite a clear reduction in Bcl-xL expression. Importantly, *let-7* miRNA substantially increased sensitivity to sorafenib. Since both *let-7* miRNA and sorafenib may have pleiotropic effects on gene expression and cellular processes, downregulation of Bcl-xL and Mcl-1 may not be a single mechanism for killing hepatoma cells. However, our study revealed that Bcl-xL-targeting miRNA, *let-7*, controls resistance of hepatoma cells to this novel class of anti-HCC drug.

In conclusion, we have demonstrated that *let-7* miRNA negatively regulates Bcl-xL expression in HCCs. Reconstitution of *let-7* miRNA may reduce apoptosis resistance to anti-cancer drugs targeting Mcl-1 in HCC. Further study is needed to examine the significance of *let-7* miRNA expression for predicting responses to sorafenib therapy in patients with HCC.

Financial support

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to T. Tak).

Disclosures

All authors have nothing to disclose.

Conflicts of interest

All authors have no conflicts of interest.

Acknowledgements

We thank Bayer HealthCare Pharmaceuticals Inc. (Wayne, NJ) for providing sorafenib.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.12.024.

References

[1] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–114.
[2] Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–269.
[3] Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302:1–12.
[4] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25:2537–2545.
[5] Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP, et al. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology 2008;47:1223–1232.
[6] Wong QW, Lung RW, Law PT, Lai PB, Chan KY, To KF, et al. MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1. Gastroenterology 2008;135:257–269.
[7] Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently downregulated in human hepatocellular carcinoma. Cancer Res 2007;67:6092–6099.
[8] Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133:647–658.
[9] Fornari F, Gramantieri L, Ferracin M, Veronese A, Sabbioni S, Calin GA, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene 2008;27:5651–5661.
[10] Wang Y, Lee AT, Ma JZ, Wang J, Ren J, Yang Y, et al. Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem 2008;283:13205–13215.
[11] Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008;47:897–907.
[12] Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008;47:1955–1963.
[13] Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. Hepatology 2001;34:55–61.
[14] Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604–612.
[15] Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, et al. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008;68:9167–9175.
[16] Chiao PJ, Na R, Niu J, Sclabas GM, Dong Q, Curley SA. Role of Rel/NF-kappaB transcription factors in apoptosis of human hepatocellular carcinoma cells. Cancer 2002;95:1696–1705.
[17] Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T, et al. Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. Cancer Res 2006;66:4826–4834.
[18] Otsuka M, Kato N, Taniguchi H, Yoshida H, Goto T, Shiratori Y, et al. Hepatitis C virus core protein inhibits apoptosis via enhanced Bcl-xL expression. Virology 2002;296:84–93.
[19] Sarcar B, Ghosh AK, Steele R, Ray R, Ray RB. Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. Virology 2004;322:51–60.
[20] Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000;408:86–89.
[21] Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology 2008;48:1312–1327.
[22] Takehara T, Tatsumi T, Suzuki T, Rucker 3rd EB, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189–1197.
[23] Takehara T, Takahashi H. Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. Cancer Res 2003;63:3054–3057.
[24] Rahmani M, Davis EM, Bauer C, Dent P, Grant S. Apoptosis induced by the kinase inhibitor BAY 43-9006 in human leukemia cells involves down-regulation of Mcl-1 through inhibition of translation. J Biol Chem 2005;280:35217–35227.
[25] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151–157.
[26] Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25–32.
[27] Schulze-Bergkamen H, Fleischer B, Schuchmann M, Weber A, Weinmann A, Krammer PH, et al. Suppression of Mcl-1 via RNA interference sensitizes human hepatocellular carcinoma cells towards apoptosis induction. BMC Cancer 2006;6:232.
[28] Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47–59.

[29] Lessene G, Czabotar PE, Colman PM. BCL-2 family antagonists for cancer therapy. Nat Rev Drug Discov 2008;7:989–1000.
[30] Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. MiR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 2005;102:13944–13949.
[31] Mott JL, Kobayashi S, Bronk SF, Gores GJ. Mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene 2007;26:6133–6140.
[32] Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001;294:853–858.
[33] Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64: 3753–3756.
[34] Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by let-7 microRNA family. Cell 2005;120:635–647.
[35] Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res 2007;67:9762–9770.
[36] Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogenes. Genes Dev 2007;21:1025–1030.
[37] Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res 2007;67:7713–7722.
[38] Büssing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. Trend Mol Med 2008;14:400–409.

The Bcl-xL Inhibitor, ABT-737, Efficiently Induces Apoptosis and Suppresses Growth of Hepatoma Cells in Combination with Sorafenib

Hayato Hikita,[1]* Tetsuo Takehara,[1]* Satoshi Shimizu,[1] Takahiro Kodama,[1] Minoru Shigekawa,[1] Kyoko Iwase,[1] Atsushi Hosui,[1] Takuya Miyagi,[1] Tomohide Tatsumi,[1] Hisashi Ishida,[1] Wei Li,[1] Tatsuya Kanto,[1] Naoki Hiramatsu,[1] and Norio Hayashi[2]

Tumor cells are characterized by uncontrolled proliferation, often driven by activation of oncogenes, and apoptosis resistance. The oncogenic kinase inhibitor sorafenib can significantly prolong median survival of patients with advanced hepatocellular carcinoma (HCC), although the response is disease-stabilizing and cytostatic rather than one of tumor regression. Bcl-xL (B cell lymphoma extra large), an antiapoptotic member of the B cell lymphoma-2 (Bcl-2) family, is frequently overexpressed in HCC. Here, we present *in vivo* evidence that Bcl-xL overexpression is directly linked to the rapid growth of solid tumors. We also examined whether ABT-737, a small molecule that specifically inhibits Bcl-xL but not myeloid cell leukemia-1 (Mcl-1), could control HCC progression, especially when used with sorafenib. Administration of ABT-737, even at an *in vivo* effective dose, failed to suppress Huh7 xenograft tumors in mice. ABT-737 caused the levels of Mcl-1 expression to rapidly increase by protein stabilization. This appeared to be related to resistance to ABT-737, because decreasing Mcl-1 expression levels to the baseline by a small interfering RNA–mediated strategy made hepatoma cells sensitive to this agent. Importantly, administration of ABT-737 to Mcl-1 knockout mice induced severe liver apoptosis, suggesting that tumor-specific inhibition of Mcl-1 is required for therapeutic purposes. Sorafenib transcriptionally down-regulated Mcl-1 expression specifically in tumor cells and abolished Mcl-1 up-regulation induced by ABT-737. Sorafenib, not alone but in combination with ABT-737, efficiently induced apoptosis in hepatoma cells. This combination also led to stronger suppression of xenograft tumors than sorafenib alone. *Conclusion:* Bcl-xL inactivation by ABT-737 in combination with sorafenib was found to be safe and effective for anti-HCC therapy in preclinical models. Direct activation of the apoptosis machinery seems to unlock the antitumor potential of oncogenic kinase inhibitors and may produce durable clinical responses against HCC. (HEPATOLOGY 2010;52:1310-1321)

The B cell lymphoma-2 (Bcl-2) family proteins regulate the mitochondrial pathway of apoptosis, a major form of cell death.[1] They include five antiapoptotic proteins, Bcl-2, B cell lymphoma extra large (Bcl-xL), myeloid cell leukemia-1 (Mcl-1), Bcl-2–related protien A1 (Bfl-1), and Bcl-2-like 2 (Bcl-w), and two structurally related proapoptotic proteins, Bcl-2–antagonist/killer (Bak) and Bcl-2–

Abbreviations: ALT, alanine aminotransferase; Bad, Bcl-2–associated agonist of cell death; Bak, Bcl-2–antagonist/killer; Bax, Bcl-2–associated X protein; Bcl-2, B cell lymphoma-2; BH3, Bcl-2 homology domain-3; Bid, BH3-interacting domain death agonist; cDNA, complementary DNA; HA, hemagglutinin; HCC, hepatocellular carcinoma; Mcl-1, myeloid cell leukemia-1; mRNA, messenger RNA; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; USP9X, ubiquitin-specific peptidase 9 X-linked; WST, water-soluble tetrazolium.

From the [1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; and [2]Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan.

Received May 26, 2010; accepted June 30, 2010.

**These authors contributed equally to this work This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to Tetsuo Takehara) and Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.*

Address reprint requests to: Tetsuo Takehara, M.D., Ph.D., Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: takehara@gh.med.osaka-u.ac.jp; fax: +81-6-6879-3629.

associated X protein (Bax), as well as several structurally diverse proapoptotic Bcl-2 homology domain-3 (BH3)-only proteins like Bcl-2–associated agonist of cell death (Bad), BH3-interacting domain death agonist (Bid), and Bcl-2-like 11 (Bim). Bak and Bax, effector molecules in this family, homo-oligomerize into proteolipid pores within the mitochondrial outer membrane, leading to release of cytochrome c followed by activation of downstream caspases, such as caspase-3/7, which dismantle a variety of cellular substrates, leading to cell death. Antiapoptotic Bcl-2 proteins function as regulators of apoptosis by directly or indirectly antagonizing Bak and Bax activity to maintain cellular integrity. BH3-only proteins, sensors of apoptosis, are activated by a variety of cellular stresses and either directly activate Bak and Bax or neutralize antiapoptotic Bcl-2 proteins, inducing cell death. Because tumor cells encounter a variety of cellular stresses, such as genotoxic and environmental factors, overexpression of antiapoptotic Bcl-2 family proteins is commonly observed and leads to survival of tumor cells.[2] We and others have reported that Bcl-xL is frequently overexpressed in human hepatocellular carcinomas (HCCs).[3–6] These reports point to the resistance of hepatoma cells to a wide variety of stress-inducing conditions. For example, Bcl-xL blocks p53-induced apoptosis in hepatoma cells, implying that Bcl-xL overexpression may be one of the mechanisms by which HCC survives under genotoxic conditions.[3] In addition, Bcl-xL overexpression was found to be associated with poor overall survival and disease-free survival after surgical resection for HCC.[7] These findings suggest that Bcl-xL may be a therapeutic target for HCC, although this possibility has not yet been addressed. Bcl-xL is also expressed in normal hepatocytes and plays a critical role in maintaining their integrity.[8] Thus, special caution is necessary when Bcl-xL inactivation is applied to therapeutic purposes.

Despite advances in understanding the mechanisms of cell death and the biology of Bcl-2 family proteins, therapeutic strategies for HCC targeting apoptotic molecules have been hampered due to a lack of specific inhibitors. ABT-737 is one of the first small-molecule inhibitors of Bcl-2 family proteins and opens the field for cancer treatment targeting the apoptosis machinery. ABT-737, designed as a Bad mimetic, binds and neutralizes Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or Bfl-1.[9-11] It has single-agent activity in a number of hematopoietic cancers and some solid tumors.[12,13] Its orally available derivative, ABT-263, is in early clinical trials against lymphoid malignancies, small-cell lung cancer, and chronic lymphocytic leukemia, with some promising results.[14] In this study, we investigated the impact of ABT-737 in treating human hepatoma in culture and using a xenograft model. We found that hepatoma cells are relatively resistant to ABT-737, presumably due to reciprocal up-regulation of Mcl-1 upon ABT-737 exposure. Although concomitant Mcl-1 inhibition appears to be effective for inducing apoptosis by ABT-737, it should be done in a tumor-specific manner, because administration of ABT-737 leads to liver deterioration in Mcl-1 knockout mice. Finally, sorafenib, an anti-HCC agent recently approved by the U.S. Food and Drug Administration, down-regulates Mcl-1 expression in a tumor-specific manner and induces apoptosis and tumor growth suppression in cooperation with ABT-737. Combination therapy with sorafenib and a Bcl-xL inhibitor seems to be an attractive strategy for controlling tumor progression in HCC.

Materials and Methods

Cell Lines and Reagents. Primary human hepatocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with the provided medium. Human hepatoma cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO). Cycloheximide was purchased from Nacalai Tesque (Kyoto, Japan), sorafenib tablets were purchased from Bayer HealthCare (Osaka, Japan), and ABT-737 was kindly provided by Abbott Laboratories (Abbott Park, IL). They were dissolved with dimethyl sulfoxide for *in vitro* use.

Hela Cells Expressed Bcl-xL with the Tet-on System. pcDNA3HABcl-xL, an expression vector coding human Bcl-xL tagged with hemagglutinin (HA), was provided by Dr. G. Nunez (University of Michigan Medical School, Ann Arbor, MI). The pcDNA4/TOHABcl-xL was constructed by inserting the complementary DNA (cDNA) for Bcl-xL gene with HA-tag from pcDNA3HABcl-xL into the EcoRI site of pcDNA4/TO (Invitrogen, Carlsbad, CA). TREx-Hela cells (Invitrogen) were transfected with pcDNA4/

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.23836
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.