potential to induce antitumour responses *in vivo*. However, in patients with malignancies,[16,17] NKT cells are reduced in number and activity, and *in vivo* activation by α-GalCer leads to transient activation and long-term unresponsiveness of NKT cells.[18,19] For that reason, adaptive transfer of *in vitro* expanded and/or activated NKT cells is expected to induce effective antitumour responses.

To date, several combinations of cytokines with α-GalCer have been reported to expand NKT cells isolated from peripheral mononuclear cells. However, NKT cells present a diverse range of expansion ratios even among healthy individuals.[20,21] Although a previous study suggested that differences in NKT cell proliferation are associated with the age of the donor,[22] there is still much that remains to be determined concerning additional factors that influence NKT cell proliferation.

In this study, we used inbred mouse strains as an experimental system in which to reveal factors that affect variation in proliferation rates among individuals. Previously, we found that *in vitro* expanded NKT cells from C57BL/6 mice retained an effector-memory-like phenotype and retained the ability to produce cytokines.[23] In addition, we found that there was a marked difference in the NKT cell expansion ratio among various mouse strains and that the differences were closely related to the bias in production of Th1 or Th2 cytokines by NKT cells. Finally, we report that a relatively low rate of proliferation can be enhanced by the addition of IL-4, which creates Th2-biased culture conditions.

## Materials and methods

### *Mice*

Female C57BL/6N, BALB/cA, C3H/HeN, DBA/2N (C57BL/6 × DBA/2)$F_1$ (BDF1), (C57BL/6 × C3H/HeN)$F_1$ (B6C3F1), and SJL/J mice were purchased from Charles River Japan (Kanagawa, Japan). All mice, which were maintained in our animal facilities, were 8–11 weeks of age at the time of the experiment. All animal protocols for this study were reviewed and approved by the committee for ethics of animal experimentation at the National Cancer Center of Japan prior to the beginning of the study.

### *Monoclonal antibodies and reagents*

Anti-IL-4 (clone 11B11) and anti-IFN-γ (clone R4-6A2) monoclonal antigen-neutralizing antibodies (mAbs) were obtained from the supernatant of a hybridoma culture maintained in serum-free medium in a CELLine CL-1000 flask (BD Biosciences, San Jose, CA) and purified by Protein G Sepharose (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden) affinity column chromatography. Anti-CD16/32 (clone 2·4G2) was obtained from a hybridoma supernatant. Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), allophycocyanin (APC)-conjugated anti-IL-4 (11B11), anti-IFN-γ (XMG1·2), and a rat immunoglobulin G1 (IgG1) isotype control (clone R3-34) and Golgi Stop™ were obtained from BD Biosciences. α-Galactosylceramide (α-GalCer) was kindly provided by the Pharmaceutical Research Laboratory, KIRIN Brewery Co., Ltd (Gunma, Japan). The phycoerythrin (PE)-conjugated CD1d/α-GalCer tetramer was prepared using a baculovirus expression system as previously described.[24] Human recombinant IL-2 (rIL-2) was kindly provided by Takeda Chemical Industries Ltd (Osaka, Japan). Mouse rIL-4 was obtained from PeproTech EC Ltd (London, UK).

### *Flow cytometry*

NKT cells were detected by multicolour flow cytometry as previously described.[23] Briefly, cells were preincubated with anti-CD16/32 mAb to block non-specific FcRγ binding and then stained with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Dead cells were excluded by propidium iodide staining and electronic gating. For detection of intracellular cytokines, cells were stimulated for 3 hr with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml), with the last 1 hr of stimulation in the presence of Golgi block, in a 37°, 5% $CO_2$ incubator, and then washed and incubated with anti-CD16/32 mAb, followed by incubation with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Cells were then permeabilized using Cytofix/Cytoperm (BD Biosciences) and IL-4 or IFN-γ was detected using APC-conjugated mAbs. Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences).

### *NKT cell proliferation assay*

Preparation of splenic mononuclear cells and *in vitro* expansion of NKT cells were performed as previously described.[23] Briefly, spleens of each mouse strain were macerated aseptically and pushed through a nylon mesh to obtain single-cell suspensions, and erythrocytes were lysed in ammonium chloride buffer. Mononuclear cells ($1 \times 10^6$ cells/ml) were cultured with α-GalCer (50 ng/ml) and rIL-2 (100 IU/ml) in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), 2-mercaptoethanol ($5 \times 10^{-5}$ M) 100 U/ml penicillin and 100 μg/ml streptomycin for 4 days in a 37°, 5% $CO_2$ incubator. After 4 days in culture, the absolute number of living cells was counted using a microscope after staining of cells with 0·2% trypan blue, and the relative percentages of NKT cells were determined by flow cytometry.

### *Cytokine production*

The cell culture supernatant was collected after 24 hr or 4 days in culture and stored at −20°. The concentrations

 

of IL-4 and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) (OptEIA ELISA set; BD Biosciences).

## Results

### α-GalCer-induced expansion of NKT cells from various mouse strains

Mouse NKT cells show a similar variation in expansion ratios to that observed for human NKT cells. We found that the expansion ratios were different for different mouse strains (Fig. 1). Before culture, spleen cell suspensions contained a small percentage (0·8–1·5%) and a small number ($7–18 \times 10^3$ cells/ml) of NKT cells in each mouse strain. As shown in Fig. 1, culture of spleen cells with α-GalCer and IL-2 induced expansion of NKT cells, except for C3H/HeN mice. After 4 days of culture, NKT cells constituted 6·4–40·7% of cells in the culture and had expanded 7–25-fold in BALB/c, C57BL/6, DBA/2, B6C3F1 and BDF1 mice. The CD1d-restricted TCR α-chain Vα14 dominantly associates with the high-affinity TCR β-chain Vβ8·2, or the lower affinity chain Vβ8·3, Vβ7 or Vβ2, and a genetic defect in Vβ8 is reportedly the cause of the low responsiveness of NKT cells. We next asked if the TCR-β status of NKT cells had an effect on expansion. However, we found no significant differences among the six strains that were tested, and selective proliferation did not occur (data not shown).

### NKT cell proliferation ratio correlates with amount of IL-4 in supernatant from a 4-day culture

Previously, a high concentration of IL-4 and IFN-γ in supernatant from a 4-day culture was observed.[23] Firstly, we measure amounts of IL-4 and IFN-γ in the culture supernatant.

An increase in the number of NKT cells was positively correlated with the production of IL-4 in the 4-day culture (Fig. 2a). However, high levels of IFN-γ were observed in all of the mouse strains, independent of an increase in either NKT cell number or IL-4 production. Almost all CD8 T cells acquired the ability to produce IFN-γ when activated indirectly via NKT cells by α-GalCer (data not shown), so it appears that, in C3H/HeN mice, NKT cells do not proliferate. Instead, it seems reasonable that a large amount of IFN-γ might be produced by the activated NK cells and CD8 T cells.[25,26]

A previous study reported cytokine secretion of NKT cells prior to their proliferation.[2,27] Thus, we harvested culture supernatants at 24 hr, before NKT cell expansion,[27] to determine the status of cytokine production at this early stage, which is the stage at which NKT cells initially respond to culture and initiate production of IL-4. This initial response positively correlated with NKT cell expansion to some degree, although the response was weaker than that observed for cells in culture for 4 days. It is notable that IL-4 production by C3H/HeN was more robust than that observed for C57BL/6, and IFN-γ

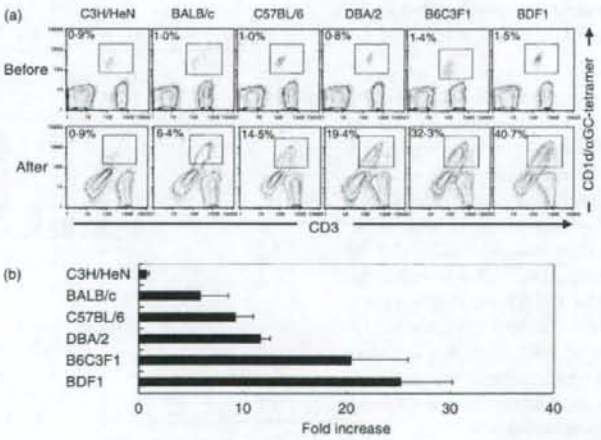


Figure 1. Expansion of natural killer T (NKT) cells *in vitro*. (a) Mouse spleen cells ($1 \times 10^6$ cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days. Cells were stained with anti-CD3 monoclonal antibody (mAb) and CD1d/α-GalCer tetramer and analysed by flow cytometry. The percentage of NKT cells was determined for both fresh (upper row) and cultured (lower row) cells. Representative results from replicate experiments are shown. (b) The fold increase in NKT cells after culture was calculated based on living cell counts and the percentage of NKT cells in the total cell population. Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).


 

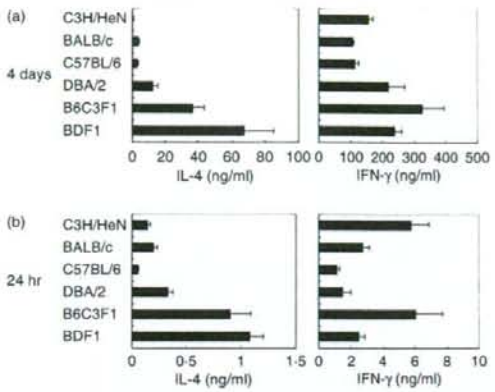


Figure 2. Production of interleukin (IL)-4 and interferon (IFN)-γ in expansion cell culture supernatants. Mouse spleen cells ($1 \times 10^6$ cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days. Supernatants were collected after 24 hr (b) or 4 days (a). The levels of IFN-γ and IL-4 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).

production of C3H/HeN mice was much higher than that of other strains (Fig. 2b). These observations lead us to speculate that IL-4 and IFN-γ produced by NKT cells work as promoting and suppressing factors, respectively, during NKT cell proliferation.

## NKT cell proliferation partially depends on IL-4 and is enhanced by Th2 cytokines

We next examined the influence of IL-4 on NKT cell proliferation *in vitro*. Proliferation of these cells was accelerated by addition of IL-4 at the start of the culture period, an effect that could be partially suppressed by neutralization of IL-4 (Fig. 3). In the C3H/HeN strain, where proliferation of NKT cells was not robust, a more significant induction of proliferation by IL-4 was observed (Fig. 4). In addition, neutralization of IFN-γ using antibodies did not significantly change the proportion of NKT cells in the total cell population. However, this did appear to up-regulate the total number of living cells and lead to a concomitant increase in the total number of NKT cells (Fig. 4b). Only NKT cells can produce IL-4 when cultured with α-GalCer and IL-2,[23] so IL-4 must act as an autocrine growth factor in the expansion of NKT cells in this context.

## The proportion of intracellular IFN-γ high positive NKT cells is reduced by addition of IL-4

Exogenous IL-4 promoted NKT cell expansion in C3H/HeN mice, as shown in Figs 3 and 4. We next examined

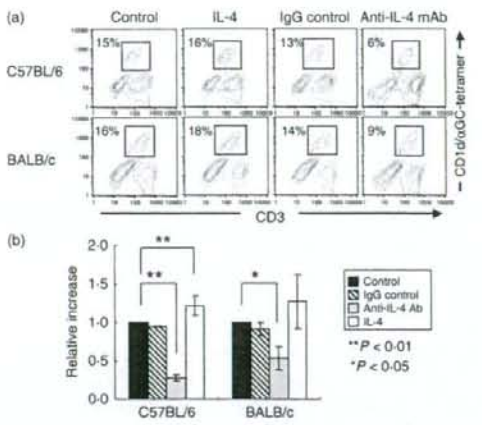


Figure 3. Expansion of natural killer T (NKT) cells in the presence or absence of interleukin (IL)-4. (a) Spleen cells ($1 \times 10^6$ cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days with IL-4 (10 ng/ml) or anti-IL-4 monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with control expansion culture. Data are shown as mean ± standard deviation for five independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·05$; **$P < 0·01$).

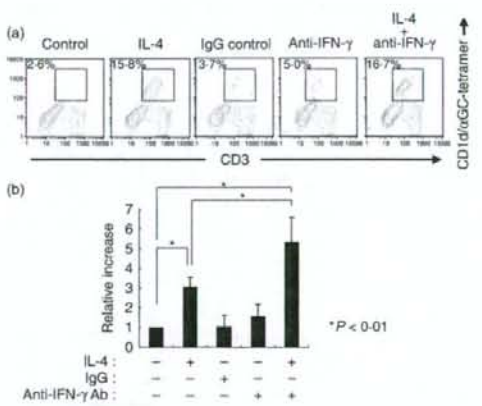


Figure 4. Expansion of natural killer T (NKT) cells from C3H/HeN strain mice in conditions that favour production of T helper type 2 (Th2)-biased cytokines. (a) Spleen cells ($1 \times 10^6$ cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 and with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml) for 4 days. The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as mean ± standard deviation for seven independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·01$).

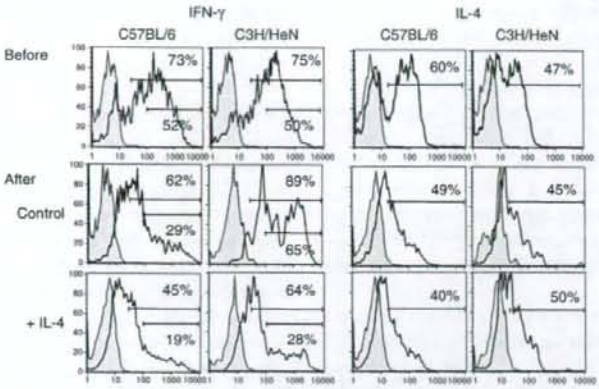


**Figure 5.** Cytokine production profile of natural killer T (NKT) cells treated with interleukin (IL)-4. Intracellular cytokine staining for interferon (IFN)-γ and IL-4 in NKT cells that were fresh (upper), cultured (middle), or cultured with additional IL-4 (lower) is shown. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 3 hr, stained with anti-CD3 monoclonal antibody (mAb), CD1d/α-galactosylceramide (α-GalCer) tetramer and anti-IFN-γ, anti-IL-4, or an isotype control mAb, and then detected and sorted via flow cytometry. Histogram panels for CD1d/α-GalCer-tetramer$^+$ CD3$^+$ cells are shown. Closed histograms indicate isotype controls. The percentage of total positive and high positive cells are indicated in the histograms. Data are representative of replicate experiments.

whether NKT cells cultured in Th2 conditions produced IFN-γ and IL-4. After 4 days of culture with α-GalCer and IL-2, intracellular IFN-γ- and IL-4-positive NKT cells were observed in both strains of mice. However, the proportion of intracellular IFN-γ high positive NKT cells was reduced when the cells were cultured with additional IL-4 (Fig. 5). In contrast to IFN-γ, the proportion of IL-4-positive NKT cells did not differ between cultures with and without IL-4. Therefore, NKT cells expanding as a result of induction with additional IL-4 displayed a polarized Th2 phenotype.

### NKT cell expansion is accelerated by Th2-biased cytokine conditions

The SJL/J mouse strain is defective in cytokine production by NKT cells, as a consequence of a loss of high-affinity TCR to CD1d, which results from a deletion of the TCR Vβ8 subfamily genomic loci.[28,29] The proportion of NKT cells in the spleens of these mice was lower than that observed for other strains (Fig. 6a), and IFN-γ and IL-4 production after α-GalCer stimulation was also lower than that observed for other strains tested in this study (data not shown). NKT cells from SJL/J mice proliferated even in the absence of additional IL-4, as was observed for NKT cells from C57BL/6 mice. Moreover, similar to findings for NKT cells from C3H/HeN mice, the NKT cell proliferation effect could be enhanced by addition of IL-4 and further enhanced by addition of IL-4 combined with neutralization of IFN-γ (Fig. 6b).

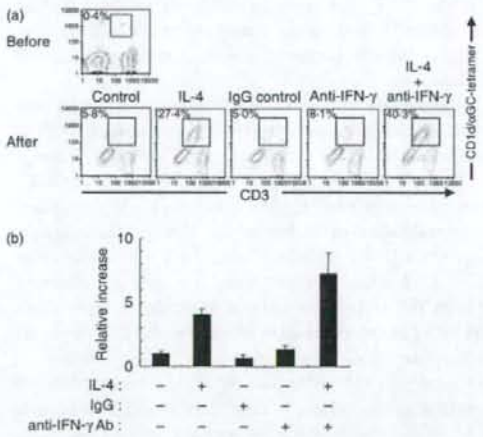


**Figure 6.** Expansion of natural killer T (NKT) cells from SJL/J mice *in vitro*. (a) Spleen cells ($1 \times 10^6$ cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as the mean of three wells ± standard deviation. Similar results were obtained in two independent experiments.

## Discussion

In a previous study in which we induced expansion of NKT cells collected from human peripheral blood, we


 

observed wide variation in the efficiency of NKT cell expansion.[21] Similarly, when mouse NKT cells were induced to proliferate using similar methods in the present study, the ratios of expanding cell types were distinctly different in cells obtained from different mouse strains (Fig. 1). This suggests that genetic background influences or controls the difference in proliferation efficiency observed in humans and mice. However, we could not rule out the alternative possibility that the effect was a result of bipolar expansion of the cells, rather than originating from genetic variation in one or a few loci.

In this study, we have shown that the amount of IL-4 in the culture supernatant was related to the efficiency of NKT cell expansion induced by α-GalCer and IL-2. Previous studies revealed that addition of exogenous IL-2, IL-7 and IL-15 was able to augment NKT cell expansion by α-GalCer.[30–34] Similarly, in the present study we found that exogenous IL-2 augmented α-GalCer-induced NKT cell expansion in various mouse strains, with the exception of C3H/HeN mice. Moreover, addition of exogenous IL-4 promoted α-GalCer-induced NKT cell expansion in spleen cells from C3H/HeN mice. It has been shown that only NKT cells have the ability to produce IL-4 in this culture.[23] IL-4 might therefore be an autocrine or paracrine growth factor in α-GalCer-induced NKT cell expansion.

NKT cells, NK cells and some T cells when cultured with α-GalCer and IL-2 produce IFN-γ.[23] In contrast to IL-4, the amount of IFN-γ did not correlate with the efficiency of NKT cell expansion. Furthermore, we found that NKT cell proliferation in C3H/HeN mice was slightly increased by neutralization of IFN-γ in the culture. These results suggest that IFN-γ partially inhibits NKT cell expansion by α-GalCer. Interestingly, we found an inverse correlation between the IFN-γ:IL-4 ratio in the culture supernatant after 24 hr of culture and the efficiency of NKT cell proliferation (data not shown). Although higher amounts of IL-4 were detected in the culture of cells from C3H/HeN mice than in the culture of cells from C57BL/6 mice after 24 hr of culture, α-GalCer stimulated spleen cells from C3H/Ne mice produced higher amounts of IFN-γ and exhibited the highest IFN-γ:IL-4 ratio of all mouse strains tested. These results may explain the failure of NKT cell expansion in spleen cells from C3H/HeN mice.

The balance between the production of IFN-γ and the production of IL-4 by NKT cells is influenced by microenvironmental factors such as cytokines and antigen-presenting cells.[20,35–38] IL-7 and IL-12 selectively enhance IL-4 production by NKT cells.[35,36] Antigen-presenting cells such as α-GalCer-pulsed B cells selectively elicit weak IL-4 but not IFN-γ production from NKT cells.[37] There is a high IFN-γ:IL-4 ratio in cultures of spleen cells from C3H/HeN mice, which is caused by splenic NKT cells (A. Iizuka *et al.*, unpublished data) Moreover, it has been reported that the balance of IFN-γ:IL-4 production by NKT cells is developmentally controlled.[39,40] At immature stages, NKT cells predominantly produce IL-4, whereas IFN-γ secretion increases during the course of development.[39] Moreover, immature NKT cells have the ability to proliferate as compared with mature NKT cells.[39] Therefore, NKT cells in the spleen of C3H/HeN mice may be more mature than those of C57BL/6 mice, or contain only a few immature NKT cells. We assume that the failure of proliferation and the high IFN-γ:IL-4 cytokine production ratio of NKT cells in the spleen of C3H/HeN mice were attributable to their maturation stage.

Although IL-4 has opposite effects to IFN-γ and suppresses the Th1 immune response, IL-4 induces proliferation of human IL-13$^+$ NK cells[41] and CD8$^+$ T cells.[42] We found that Th2 culture conditions (in the presence of IL-4 and anti-IFN-γ mAb) facilitated NKT cell expansion induced by α-GalCer and IL-2 even in C3H/HeN and SJL/J mice. IL-4 also induces IFN-γ production by NK and NKT cells *in vivo*.[43] However, the proportion of IFN-γ-positive, but not IL-4-positive, NKT cells decreased when cells were cultured in the presence of IL-4. As in human immature IL-13$^+$ NK cells,[41] IL-4 may induce expansion of developmentally immature NKT cells which have a Th2-biased phenotype.

NKT cell maturation is controlled by the transcription factor T-bet.[44,45] Terminally differentiated NKT cells acquire a strong ability to produce IFN-γ and elicit cytotoxicity.[44] Assuming that expanded Th2-biased NKT cells after culture with α-GalCer, IL-2 and IL-4 are immature cells, it will be possible to induce terminally differentiated Th1-biased NKT cells for Th1 cell immunotherapy, such as cancer cell therapy.

## Acknowledgements

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# Expression dynamics of drug resistance genes, multidrug resistance 1 (MDR1) and lung resistance protein (LRP) during the evolution of overt leukemia in myelodysplastic syndromes

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**Abstract**

It is well-known that leukemic cells of overt leukemia (OL) that have transformed from myelodysplastic syndromes (MDS) are more resistant to chemotherapy as compared with de novo AML cells. Thus, to examine the expression levels of drug-resistant genes and their alterations with the development of OL in MDS, the expression of mRNA for MDR1 and LRP was determined in bone marrow samples from control, de novo AML, MDS, MDS at the time of OL transformation (MDS → OL), and after transformation (OL) by quantitative real-time RT-PCR. The expression of MDR1 in MDS bone marrow at the time of initial diagnosis was as low as that for control subjects. However, the expression level was significantly elevated at the time of the development of OL (MDS → OL) compared with the initial MDS subjects ($P < 0.05$), while expression was relatively reduced after OL development (OL). The expression of LRP was significantly higher in MDS and MDS → OL samples than control subjects. However, the high expression of LRP in MDS → OL was significantly reduced after OL development (OL). The expression levels of drug-resistant genes in MDS → OL or OL were not significantly higher than those of de novo AML samples, although LRP expression in MDS or MDS → OL was relatively higher than that of de novo AML. Detecting increases in the expression of MDR1 would be useful for predicting OL development in MDS patients.


*Keywords:* MDR1; LRP; MDS; Overt leukemia; Bone marrow

## Introduction

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis with frequent apoptosis (Fontenay-Roupie et al., 1999; Gupta et al., 1999; Harris et al., 1999; Heaney and Golde, 1999; Kitagawa et al., 1997, 1998, 1999; Parcharidou et al., 1999; Sawanobori et al., 2003; Vardiman et al., 2002) and a high risk of progression to overt leukemia (OL) of acute myeloid leukemia (AML) type (Harris et al., 1999; Heaney and Golde, 1999; Kitagawa et al., 1989). Patients with OL following MDS have a low response rate to conventional chemotherapy compared with de novo AML patients. This could be due in part to a higher incidence of drug resistance gene expression in MDS than in de novo AML (Leith et al., 1997; Lepelley et al., 1994; Marie et al., 1991; Poulain et al., 2000; Wattel et al., 1998) and anti-apoptotic mechanisms present in $CD34^+$ cells of MDS when OL developed (Suárez et al., 2004).

In recent years, important advances have been achieved in the treatment of patients with AML. However, AML in the elderly continues to result in a dismal outcome (Nabhan et al., 2005; Pinto et al., 2001). It has been suggested that blast cells from elderly AML patients frequently show unfavorable karyotypes in association with high expression of multidrug resistance 1 (MDR1) (Del Poeta et al., 1999; Leith et al., 1997; Nabhan et al., 2005; Suárez et al., 2005) as well as increased resistance to apoptosis (Cascavilla et al., 2000; Van Stijn et al., 2003). MDS is also a disease that mainly affects the elderly. Thus, to focus on the contribution of drug-resistant genes to the

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chemotherapy-resistant nature of OL that has transformed from MDS, we collected bone marrow samples from MDS cases in the present study. In these cases, we could follow over the course of development of OL. Using quantitative RT-PCR, we traced the expression of MDR proteins during the course of OL development from MDS. Among the MDR proteins, MDR1, lung resistance protein (LRP) and multidrug-resistance-associated protein (MRP1), expression level of MRP1 did not correlate with the prognosis of MDS patients, while the expression levels of MDR1 as well as LRP did (Poulain et al., 2000). Therefore, we examined the expression of MDR1 and LRP.

The level of expression of MDR1 was relatively high in bone marrow cells from de novo AML samples compared with control bone marrow. However, the level of expression in MDS at the time of initial diagnosis was similar with that of control samples. At the time of OL evolution, the expression level showed a remarkable increase followed by a relative decrease thereafter. By contrast, the expression of LRP in MDS at the time of initial diagnosis was higher than that of control subjects and exhibited a slight increase at the time of OL evolution (MDS → OL). Then, it was significantly reduced after OL development (OL). The significance of expression dynamics of drug-resistant genes in the bone marrow cells of MDS to OL is discussed.

## Materials and methods

### *Patients*

Fresh frozen bone marrow samples from controls (7 cases, age, median 55, max. 74, min. 43; male:female, 1:6), MDS (7 patients, 3 with RA and 4 with RAEB; male:female, 5:2; age, median 68, max. 77, min. 55) who developed overt leukemia later in the course (duration, 3 months to 3 years) and de novo AML (12 patients of M2 by FAB classification, male:female, 5:7; age, median 62, max. 76, min. 49) patients were analyzed. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Diagnoses were based on standard clinical and laboratory criteria, including cell morphology (Harris et al., 1999; Heaney and Golde, 1999; Vardiman et al., 2002). All samples were collected at the time of the initial aspiration biopsy, and the samples from de novo AML contained more than 80% blasts. The patients were not infected with specific viruses including HTLV-I and had not been treated prior to the study. In MDS, we followed the bone marrow changes just before and after the transformation to OL and further chronological changes determined at more than three points were analyzed. During the course of OL development, patients were not treated with the specific anti-tumor chemotherapy. We determined the beginning of blast proliferation in MDS patients and followed the changes in the number of blasts in the bone marrow. MDS samples were collected at the time of initial diagnosis. Furthermore, MDS → OL samples were collected from bone marrow containing 20–30% blasts and OL samples were collected from bone marrow with blasts comprising beyond 30% of all bone marrow cells.

The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

### *Preparation of RNA and quantitative assay for MDR1 and LRP using TaqMan RT-PCR*

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, de novo AML patients and MDS patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Although the proportions of cell types were variable in the bone marrow samples of MDS, we examined the overall expression of MDR genes by total bone marrow cells because of the clonal nature of this disease entity.

For quantitative RT-PCR, fluorescent hybridization probes and the TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for MDR1, LRP, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes that were used were as follows. The sequence of the forward primer for MDR1 mRNA was 5′-GTCTACAGTTCGTAATGCTGACGT-3′ and that of the reverse primer was 5′-TGTGATCCACGGACACTCCTAC-3′; the TaqMan probe was 5′-CGCTGGTTTCGATGATGGAGTCATTG-3′. For LRP mRNA, the forward primer was 5′-CGCTGTGATTGGAAGCACCTA-3′ and the reverse primer was 5′-CGGGAGGCAGCTCTTTCTC-3′; the TaqMan probe was 5′-ATGCT-GACCCAGGACGAAGTCCT-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions for one-step RT-PCR were as follows: 2 min at 50°C (Stage 1, reverse transcription), 10 min at 95°C (Stage 2, RT inactivation and AmpliTaq Gold activation) and then 45 cycles of amplification for 15 s at 95°C and 1 min at 60°C (Stage 3, PCR). The expression of MDR1 and LRP was quantified according to a method described elsewhere (Yamamoto et al., 2004). Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the MDR1 and LRP were normalized using that for GAPDH in each sample.

### *Statistical analysis*

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney's *U* test for comparisons between control, de novo AML, MDS, MDS → OL, and OL samples except for the comparison of paired samples from MDS, MDS → OL, and OL, for which Wilcoxon's test was used.

## Results

### *Expression of mRNA for MDR1 determined by real-time quantitative PCR*

To quantitate the mRNA expression levels of the MDR1 in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow from control, de novo AML, MDS, MDS → OL, and OL samples. As shown in Fig. 1, the expression of MDR1 exhibited relative up-regulation in de novo AML compared with the controls. By contrast, the mRNA for MDR1 revealed similar levels of expression in MDS bone marrow and control marrow. At the time of OL development (MDS → OL), the MDR1 expression level became as high as that of de novo AML. The difference was significant between MDS and MDS → OL ($P < 0.05$). However, expression was slightly reduced after OL development (OL). Furthermore, we followed more precise dynamics for a couple of cases with MDS. Fig. 2 shows the expression intensity of MDR1 during the course and the proportion of blasts in the bone marrow at each point. As indicated, the MDR1 expression transiently increased when the blast population increased to circa 20%, and then expression was reduced in spite of the blast numbers still increasing in the bone marrow.

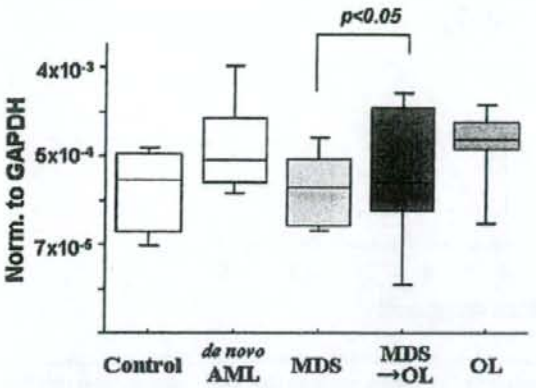


Fig. 1. Quantitative RT-PCR analysis of MDR1 in control ($n = 7$), de novo AML ($n = 12$), MDS ($n = 7$) at the time of initial diagnosis, at the time of OL development (MDS → OL), and after OL evolution (OL). The relative intensity was calculated as (intensity of reaction of MDR1 [total Raji RNA, ng])/(intensity of reaction of GAPDH [total Raji RNA, ng]). The box plot graphs indicate the values for AML and MDS cases, where the bars indicate the 90th and 10th percentile and the boxes indicate the 75th to 25th percentile. The difference was significant between MDS and MDS → OL ($^aP < 0.05$) by Wilcoxon's test.

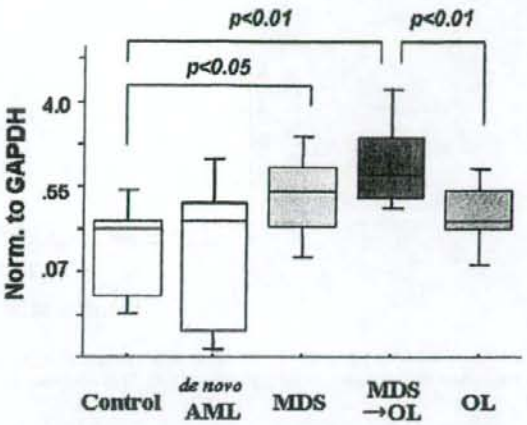


Fig. 3. Quantitative RT-PCR analysis of LRP in control ($n = 7$), de novo AML ($n = 12$), MDS ($n = 7$), MDS → OL, and OL samples. Differences were significant between the samples using Mann–Whitney's $U$ test or Wilcoxon's test: control and MDS ($^aP < 0.05$), control and MDS → OL ($^bP < 0.01$), and MDS → OL and OL ($^cP < 0.01$).

These results indicate that a high expression of MDR1 might not be characteristic of OL transformed from MDS because the level of expression was similar with that of de novo AML. However, a significant difference in the MDR1 expression in MDS and MDS → OL is a useful finding for predicting the evolution of OL in MDS.

*Expression of mRNA for LRP determined by real-time quantitative PCR*

Next, to quantitate the mRNA expression dynamics of LRP in MDS bone marrow cells, real-time quantitative RT-PCR was performed using bone marrow from control, de novo AML, MDS, MDS → OL, and OL samples. As shown in Fig. 3, the expression of LRP exhibited a significant up-regulation in MDS and MDS → OL samples compared with the controls. mRNA expression was significantly decreased after transformation to OL from MDS → OL samples. However, differences were not remarkable between de novo AML and MDS, AML and MDS → OL, or AML and OL. These results indicate that the expression dynamics of LRP during the course of OL development in MDS were not significant. However, expression was reduced after transformation to OL. Detailed dynamics on two cases are shown in Fig. 4. Similar to MDR1, LRP expression also revealed a transient peak at the time when the blast population increased and gradually decreased thereafter. However, the period for the peak expression of LRP was relatively earlier than that of MDR1 in both cases.

In any event, the expression dynamics of LRP did not explain the chemotherapy-resistant nature of MDS → OL or OL bone marrow compared with de novo AML because the increased expression of LRP was transient in MDS during the

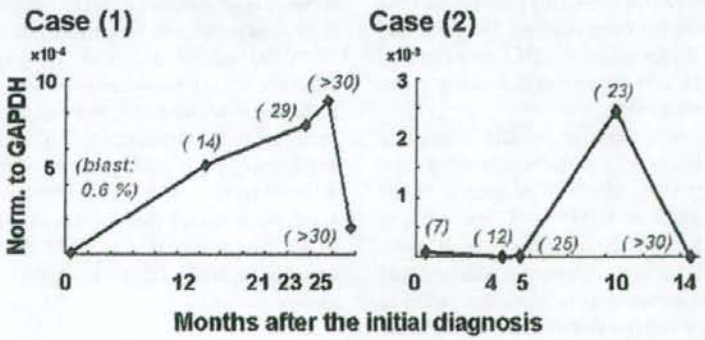


Fig. 2. Dynamics of the expression of mRNA for MDR1 in two cases of MDS during the course of OL development. Although the expression in seven cases was determined chronologically, representative two cases are shown. Case 1 was a 76-year-old male who was initially diagnosed with RA and Case 2 was a 55-year-old female who was initially diagnosed with RAEB. The percentage of blasts in the bone marrow is indicated at each point. Note the peak expression of MDR1 23 months after the initial diagnosis for Case 1 and 10 months for Case 2 when the evolution of OL occurred. The blasts accounted for 20 to 30% of all bone marrow cells.

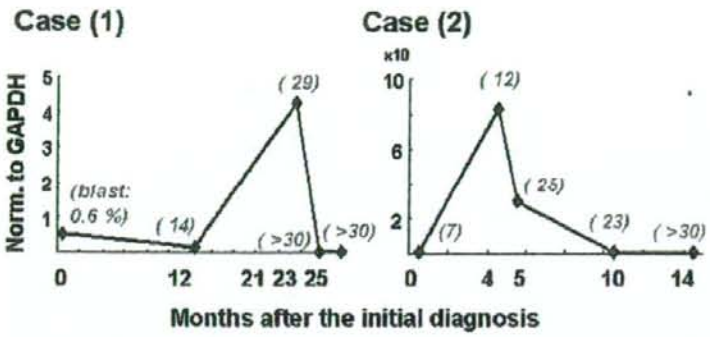


Fig. 4. Dynamics of the expression of LRP mRNA in two cases of MDS during the course of OL development. The representative cases are the same as those indicated in Fig. 2. Note that the peaks for the expression of LRP 21 months after the initial diagnosis for Case 1 and 4 months for Case 2 were relatively earlier than those of MDR1 in both cases.

course of OL development and expression in OL was not significantly higher than that of de novo AML.

## Discussion

Regarding the expression of MDR1 in myeloid neoplasms, previous studies revealed that over-expression occurred in de novo AML and MDS samples (Leith et al., 1999; Lepelley et al., 1994). Expression was more frequent in AML in the elderly than AML in younger people (Leith et al., 1999) and in "high risk" MDS than in "low risk" MDS (Lepelley et al., 1994). Similarly, LRP over-expression is more frequent in MDS than in de novo AML (Lepelley et al., 1998). However, little is known about the quantitative changes in the expression intensity of mRNA for these genes in particular patients. In other words, the expression dynamics of MDR1 and LRP have not been previously studied in association with the pathogenesis of MDS, MDS → OL, and OL.

The expression of MDR1 is associated with a CD34-positive stem cell phenotype in MDS (List et al., 1991; Sonneveld et al., 1993) as well as in de novo AML (Guerci et al., 1995). Therefore, in MDS, the number of CD34-positive immature cells in the bone marrow strongly influences the expression intensity of MDR1. Our data which show that MDR1 expression increased at the time of OL development (MDS → OL) might reflect the increase in immature cells in the bone marrow. However, the reduced expression after OL development (OL) could not be explained by the number of CD34-positive cells because it was still increasing after OL development.

LRP over-expression is more frequent in MDS than in de novo AML and, as in AML, is only partially correlated with MDR1 expression (Lepelley et al., 1998). In the present study, the expression of LRP in MDS or MDS → OL was stronger than that in control samples, but the expression in de novo AML was similar with that of control samples. Although the number of samples was inadequate to evaluate rather a heterogeneous entity of hematological malignancy, MDS, we found a significant elevation in expression in MDS samples even at the time of initial diagnosis. However, LRP is not a prognostic factor for response to chemotherapy and survival in MDS (Lepelley et al., 1998). Our findings were consistent with this observation in the sense that the MDS → OL or OL samples did not exhibit higher expression than the initial MDS samples.

The decline in MDR1 and LRP protein expression after the evolution of OL may be attributable to gene silencing, for example, by DNA hypermethylation. Although this interpretation is speculative, over-expression of MDR1 and LRP in MDS and MDS → OL would be a reversible change while the down-regulation in OL samples might be irreversible/reversible.

Recently, modulation of drug resistance transporters has been considered as a strategy for treating myelodysplastic syndromes (Ross, 2004). Several drugs capable of modulating and decreasing MDR1, such as quinine, tamoxifen, calcium channel blockers, and cyclosporine A, have been used for treating poor-risk AML (Advani et al., 1999; List et al., 1993; Solary et al., 1992; Tallman et al., 1999). Furthermore, a potent inhibitor of the MDR1 efflux pump, valspodar, is used for patients with relapsed or refractory AML and high-risk MDS with mitoxantrone, etoposide, and cytarabine (phase III trial) (Greenberg et al., 2004). The present data suggest that this type of chemotherapy is attractive for treating patients when OL occurs in MDS. Although our data indicate that patients with OL after transformation from MDS exhibit low level expression of MDR1, these drugs might be effective for preventing drug-induced up-regulation of MDR1 expression.

In conclusion, we investigated the transient over-expression of MDR1 mRNA during the progression from MDS to OL, although the expression was relatively decreased after transformation was completed. Clinically, it would be useful to detect increases in the expression of MDR1 mRNA for predicting the evolution of OL in MDS patients. Although the levels of mRNA do not directly reflect protein expression status, further study is warranted to clarify the mechanisms of up- and down-regulation of MDR1/LRP expression in MDS, MDS → OL, and OL in association with the chemotherapy-resistant nature of this disease.

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# GENE EXPRESSION PROFILING OF RAT LIVER TREATED WITH SERUM TRIGLYCERIDE-DECREASING COMPOUNDS

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**ABSTRACT** — We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for interpretation of plasma triglyceride (TG) decrease, we extracted 218 probe sets of rat hepatic genes from data of 15 drugs that decreased the plasma TG level but differentially affected food consumption. Pathway and gene ontology analysis revealed that the genes belong to amino acid metabolism, lipid metabolism and xenobiotics metabolism. Principal component analysis (PCA) showed that 12 out of 15 compounds were separated in the direction of PC1, and these 12 were separated in the direction of PC2, according to their hepatic gene expression profiles. It was found that genes with either large or small eigenvector values in principal component PC 2 were those reported to be regulated by peroxisome proliferator-activated receptor (PPAR)α or constitutive androstane receptor (CAR), respectively. In fact, WY-14,643, clofibrate, gemfibrozil and benzbromarone, reported to be PPARα activators, distributed to the former, whereas propylthiouracil, omeprazole, phenobarbital, thioacetamide, methapyrilene, sulfasalazine and coumarin did to the latter. We conclude that these identified 218 probe sets could be a useful source of biomarkers for classification of plasma TG decrease, based on the mechanisms involving PPARα and CAR.

**KEY WORDS:** Triglyceride, Liver, CAR, PPAR, Toxicogenomics

## INTRODUCTION

The toxicogenomics project was a 5-year collaborative project by the National Institute of Biomedical Innovation (NIBIO), the National Institute of Health Science (NIHS), and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, were selected, and gene expression in liver (also kidney in some cases) was comprehensively analyzed by using Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and the prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project, Japan).

In toxicity studies, plasma triglyceride (TG) decrease is often observed. Because plasma TG level can be influenced mostly by nutritional status, decrease in food consumption is one of the factors for its change. For activator of peroxisome proliferator-activated

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receptor (PPAR)α, it is a hot field of drug development, and this drug facilitates the expression of genes related to fatty acid β–oxidation (Schoonjans *et al.*, 1996), subsequently lowering plasma TG level. Phenobarbital (PB), an antiepileptic barbiturate derivative, decreases plasma TG level but increases plasma total cholesterol without decrease in food consumption in rats (Kiyosawa *et al.*, 2004; Hall *et al.*, 1990). Kiyosawa *et al.*(2004) proposed a mechanism of serum cholesterol elevation via up-regulation of hepatic cholesterol synthesis. So far, PB is not reported to be a PPARα activator, and the mechanism of plasma TG decrease by PB is not well understood. In these cases, plasma TG decrease could be a target of the drug in one case, or a sign of toxicity in another case, each with different mechanisms. Thus, identification of the mechanisms behind plasma TG decrease during drug treatment would enable both seed discovery and interpretation of toxicity.

One of the main purposes of TG-GATEs is to identify biomarkers for toxicity evaluation. Although there have been various reports describing strategies to extract marker genes from the transcriptome data (Hibbs *et al.*, 2004; Mutlib *et al.*, 2006; Tan *et al.*, 2006), the best way has not been established. In the present study, we have started to identify candidates of potential biomarker genes for interpretation of the fundamental mechanism(s) of plasma TG decrease, since our database contains several drugs that cause plasma TG decrease.

## MATERIALS AND METHODS

### Animals and treatments

Male Crj:CD(SD)IGS rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21-25°C with a relative humidity of 40-70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan). Rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution (MC) or corn oil according to their dispersibility. At the time when the present analysis was performed, 15 compounds in our database were found to decrease the plasma triglyceride level during repeated administration (Table 1). Of these, isoniazid (INAH, 50, 100, 200 mg/kg; MC), phenobarbital (PB, 10, 30, 100 mg/kg; MC), thioacetamide (TAA, 4.5, 15, 45 mg/kg; MC), benzbromarone (BBr, 20, 60, 200 mg/kg; MC), methapyrilene (MP, 10, 30, 100 mg/kg: MC), amiodarone hydrochloride (AM, 20, 60, 200 mg/kg; MC), gemfibrozil (GFZ, 30, 100, 300 mg/kg; corn oil) and sulfasalazine (SS, 100, 300, 1000 mg/kg; MC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-naphthylisothiocyanate (ANIT, 1.5, 5, 15 mg/kg; corn oil) was purchased from Kanto Chemical (Tokyo, Japan). Coumarin (CMA, 15, 50, 150 mg/kg; corn oil), propylthiouracil (PTU, 10, 30, 100 mg/kg; MC) and WY-14,643 (WY, 10, 30, 100 mg/kg; corn oil) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Carbon tetrachloride (CCL4, 30, 100, 300 mg/kg; corn oil), clofibrate (CFB, 30, 100, 300 mg/kg; corn oil) and omeprazole (OPZ, 100, 300, 1000 mg/kg; MC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing and expressed as g/day. The animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Blood samples were collected to heparinized tube under ether anesthesia from the abdominal aorta after which the animals were sacrificed.

### Blood chemistry analysis

Heparinized blood samples were centrifuged at 1,600 × g for 20 min to obtain plasma and the concentration of TG was determined using an automated clinical analyzer (Japan Bioassay Research Center and Anpyo Center; HITACHI 7070, Hitachi Ltd., Food and Drug Safety Center: COBAS MIRA plus, Roche Diagnostics, Bozo Research Center: TBA-120FR, Toshiba Lab Medical, Tokyo, Japan).

### Microarray analysis

After collecting the blood, the animals were euthanized by exsanguinations from abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater®(Ambion, Austin, TX, USA) overnight at 4°C, and frozen at −80°C until use. Liver samples were homogenized with the buffer RLT supplied in RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the

**Table 1.** List of compounds used in the present study.

| Compound (abbreviation) | No. of mobilized genes (ANOVA, $p<0.05$) | Usage | Hepatotoxicity | Proposed toxicological action |
|---|---|---|---|---|
| alpha-naphthylisothiocyanate (ANIT) | 1063 | hepatotoxins | cholestasis | toxic metabolite can injure epithelial cells in biliary duct |
| amiodarone hydrochloride (AM) | 640 | antiarrhythmic, antianginal agents | phospholipidosis | oxidative stress by free radical |
| benzbromarone (BBr) | 1720 | uricosuric agents | hepatomegaly | peroxisome proliferators-like action |
| carbon tetrachloride (CCL4) | 2262 | hepatotoxins | hepatocellular carcinoma fatty liver | production of toxic metabolite that leads to peroxidative injury of membrane lipids and membrane perturbation |
| clofibrate (CFB) | 1800 | antilipemic agents | hypertrophy, hepatocellular carcinoma | peroxisome proliferator |
| coumarin (CMA) | 1005 | hepatotoxins | necrosis | formation of coumarin 3,4-epoxide |
| gemfibrozil (GFZ) | 1943 | antilipemic agents | hepatomegaly | peroxisome proliferator |
| isoniazid (INAH) | 1482 | antituberculous agents | necrosis | generation of a reactive metabolite from acetylhydrazine |
| methapyrilene (MP) | 4910 | antiallergic, hypnotic, sedative agents | carcinoma | induction of hepatic cell proliferation, lipid peroxidation of the liver |
| omeprazole (OPZ) | 1441 | antiulcer agents | elevation of serum enzyme | unknown |
| Phenobarbital (PB) | 548 | hypnotics, sedatives, anticonvulsants | hepatocellular tumor | induction of c-fos gene expression |
| propylthiouracil (PTU) | 1544 | antithyroid agents | elevation of serum enzyme | thyroxin synthesis inhibitor |
| sulfasalazine (SS) | 860 | antiinflammatory, antirheumatic, antiinfective agents | genotoxicity, carcinogenicity | antiinflammatoric, antibacterial actions, inhibition of production of cytokines |
| thioacetamide (TAA) | 5712 | hepatotoxins | carcinogenicity | hepatocarcinogen |
| Wy-14,643 (WY) | 4307 | hepatotoxins | carcinoma hepatomegaly | peroxisome proliferator |

manufacturer's instructions. Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE 230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15,923 probe sets. The procedure was conducted basically according to the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and T7-(dT)24-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to a RAE230A probe array for 18 hr at 45°C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using Fluidics Station 400 (Affymetrix) and scanned by Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0. Microarray image data were analyzed with GeneChip Operating Software (Affymetrix).

**Statistical analysis**

Plasma TG and food consumption results were expressed as means ± SD. They were analyzed by Bartlett test that evaluates the homogeneity of variance. If the variances were homogenous, ANOVA was applied. If the variances were heterogeneous, Kruskal-Wallis test was performed. When ANOVA resulted in a statistical difference between the groups, Dunnett test was applied. When Kruskal-Wallis test resulted in statistically different groups, Dunnett type mean rank test was performed. Identification of genes related to plasma TG decrease and gene expression data were analyzed by Welch ANOVA for the dose level and applied with Benjamini and Hochberg False Discovery Rate as a multiple-testing correction. In these tests, a significant level at $p<0.05$ was considered acceptable (Snedecor and Cochran, 1989).

GeneChip data were normalized by the global median normalization method using GeneSpring version 7 (Agilent Technologies Inc., Palo Alto, CA, USA). Probe sets with present or marginal call in at least 1 of 48 samples (N=3 for 4 time points and 4 dose levels) were selected. Principal component analysis (PCA) of the GeneChip data was performed using Spotfire DecisionSite ver. 8.2 (Spotfire, Somerville, MA, USA).

**Pathway and Gene Ontology (GO) analysis**

The identified probe sets were subjected to analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and GO analysis by DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://apps1.niaid.nih.gov/david/) using Fisher's exact test (Dennis *et al.*, 2003). Level 5 analysis was adopted.

## RESULTS

**Plasma biochemistry and food consumption**

Rats were treated with each compound (ANIT, AM, BBr, CCL4, CFB, CMA, GFZ, INAH, MP, OPZ, PB, PTU, SS, TAA and WY) by gavage for up to 4 weeks. The TG concentration and food consumption results are shown in Fig. 1. All compounds that we selected showed a TG-decreasing property during the dosing period, while their effects on food consumption differed. In BBr-, CMA-, OPZ- and SS-treated animals, food consumption transiently dropped in the first 3 days while it returned to normal thereafter. In AM-, INAH- and TAA-treated rats, food consumption was depressed throughout the dosing period. PTU-treatment decreased food consumption from day 15 whereas MP-treatment decreased at day 29. ANIT-, CFB-, CCL4, GFZ-, PB-, and WY-treatment affected the food intake only slightly.

**Identification of genes related to the plasma TG-decreasing property**

After filtering the probe sets (with present or marginal call in at least 1 of 48 samples), Welch ANOVA with multiple testing correction was applied to each compound to extract significantly mobilized probe sets. As shown in Table 1, the numbers of extracted probe sets varied among the compounds, from 640 (AM) to 5,712 (TAA). We then selected the probe sets that were commonly changed from more than 10 out of 15 compounds, and 218 probe sets were obtained. KEGG pathway analysis revealed that pathways related to "proteasome", "fatty acid metabolism", "amino acid metabolism", and "bile acid biosynthesis" were mainly altered in liver treated with these compounds (Table 2). GO analysis showed that some groups (other than cellular lipid metabolism) related to xenobiotics metabolism, such as carboxylic acid metabolism, and glucuronosyltransferase and aldehyde dehdrogenase activity, were also affected in liver (Table 3).

**Principal component analysis (PCA)**

To assess the expression profiles of identified

probe sets, PCA with 218 probe sets for data of all 15 compounds were performed. As shown in Fig. 2, each sample was separated from control according to the expression of these probe sets. Each sample tended to have a smaller value in the component PC 1 as the treatment period increased, and each was distinctly separated into either direction in component PC 2. It should be noted that liver treated with WY, BBr, GFZ and CFB had a relatively large principal component PC 2, while PTU, OPZ, PB, TAA, MP, SS and CMA showed small PC 2. Liver treated with AM for 28 days had a near zero value in PC 2. Some compounds such as ANIT, CCL4 and INAH did not change their position very much.

To elucidate which genes contributed more for each principal component, eigenvector values of each probe sets were examined. As shown in Table 4, "vanin 1", "similar to Aig1 protein", "CD36 antigen", and "cell death-inducing DNA fragmentation factor, alpha subunit-like effecter A" had large eigenvector values for PC 2. Meanwhile, "glutathione *S*-transferase A5, aldehyde dehydrogenase family 1, member A1", "liver UDP-glucuronosyltransferase, phenobarbital-inducible form", "carbonic anhydrase 2" and "cytochrome P450, family 2, subfamily b, polypeptide 15" had small values for PC 2 (Table 5). "Aldehyde dehydrogenase family 1, member A1", "glutathione *S*-transferase A5", "vanin 1", "carboxylesterase 2 (intestine, liver)" and "CD36 antigen" had smaller eigenvector values for PC 1 (Table 6).

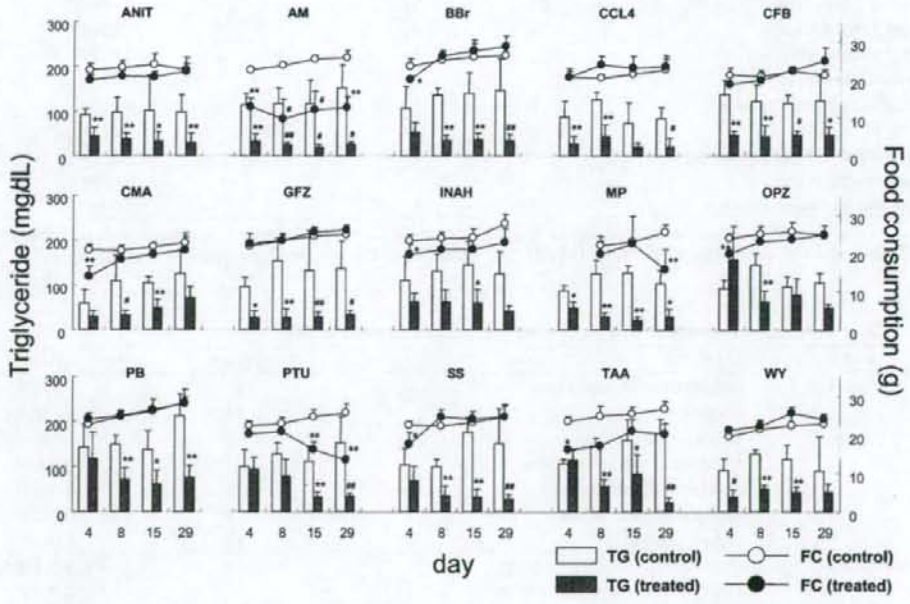


**Fig. 1.** Effects of TG-decreasing compounds on food consumption and plasma TG level.
Six-week-old male Sprague-Dawley rats were gavaged with each compound for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Food consumption was recorded every 4 days during dosing and blood samples were collected at sacrifice. Plasma TG concentrations were estimated as described in materials and methods. For simplicity, the data of the highest dose were presented for each compound. Open (control) and filled (treated) columns represent plasma TG concentration. Open (control) and filled (treated) circles represent food consumption. Values are expressed as mean ± SD of 5 rats each for each time and compound. Significant difference from control rat: (*$p<0.05$, **$p<0.01$: Dunnett test, #$p<0.05$, ##$p<0.01$: Dunnett type mean rank test). MP-food consumption on day 4 was not determined.

As PC 2 was considered to be indirectly related to cholesterol metabolism (see DISCUSSION), three compounds with the smallest values for both PC 1 and PC 2, i.e., PB, OPZ and PTU were selected, and their effects on plasma cholesterol level are shown in Fig. 3. It is obvious from the figure that OPZ and PTU, which had smaller PC 2 values than PB, significantly increased plasma cholesterol.

## DISCUSSION

Lowering of the plasma TG level is often observed in rat toxicity studies. It would be useful to elucidate its mechanism not only for safety evaluation of drugs but also for finding seeds of lipid-lowering agents. In the course of our trials to identify useful toxicity biomarkers from our large-scale database, we selected plasma TG decrease as a toxicological phenotype, and picked up 15 such compounds from 40 (the number of compounds available at the time when the

**Table 2.** KEGG pathways of the identified 218 probe sets related to plasma TG.

| Term | Count | p value |
|---|---|---|
| proteasome | 8 | 1.66E-06 |
| fatty acid metabolism | 10 | 4.61E-06 |
| tryptophan metabolism | 9 | 3.44E-05 |
| bile acid biosynthesis | 4 | 0.00997 |
| histidine metabolism | 4 | 0.00997 |
| propanoate metabolism | 4 | 0.0224 |
| fatty acid biosynthesis (path 2) | 3 | 0.0236 |
| pyruvate metabolism | 4 | 0.0289 |
| valine, leucine and isoleucine degradation | 4 | 0.0289 |
| nitrogen metabolism | 3 | 0.0364 |
| arginine and proline metabolism | 4 | 0.0492 |

Statistically extracted 218 probe sets related to plasma TG were categorized by KEGG pathway. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/ ) are presented in the table.

**Table 3.** Gene ontology of the identified 218 probe sets related to plasma TG.

| Category | Term | Counts | p value |
|---|---|---|---|
| Biological process | carboxylic acid metabolism | 15 | 5.25E-06 |
| | electron transport | 14 | 1.83E-04 |
| | fatty acid metabolism | 8 | 6.04E-04 |
| | response to chemical substance | 7 | 0.00164 |
| | cellular lipid metabolism | 10 | 0.0115 |
| | amino acid metabolism | 6 | 0.0154 |
| | protein catabolism | 11 | 0.0492 |
| Molecular function | glucuronosyltransferase activity | 6 | 5.01E-05 |
| | aldehyde dehydrogenase activity | 3 | 0.0249 |
| Cellular component | endoplasmic reticulum | 15 | 1.25E-05 |
| | microsome | 9 | 1.93E-04 |
| | micorobody | 5 | 0.00726 |
| | peroxisome | 5 | 0.00726 |
| | proteasome complex (SENSU EUKARYOTA) | 4 | 0.00829 |
| | proteasome core complex (SENSU EUKARYOTA) | 4 | 0.00829 |
| | mitochondrion | 15 | 0.0103 |

Statistically extracted 218 probe sets related to plasma TG were categorized by gene ontology. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/) are presented in the table.

present analysis was performed). Since our database has a broad variety of compounds for hepatic toxicity, each compound has different properties for drug efficacy and toxicity (Table 1).

As shown in Fig. 1, the effects of these compounds on food consumption were not similar. It is well known that plasma TG levels depend largely upon nutrition, and the results suggested that there should have been multiple factors other than a simple general toxicity. To clarify the multiple mechanisms in plasma TG homeostasis, we extracted 218 probe sets as commonly changed genes in more than 10 out of 15 compounds (commonly changed in two-thirds of the compounds). The fact that there were no probe sets commonly changed in all the compounds (data not shown) also suggested that there are multiple factors involved in the mechanism of plasma TG decrease.

KEGG pathway analysis suggested that the proteasome-, fatty acid metabolism-, tryptophan metabolism-, bile acid biosynthesis-, and histidine metabolism-related pathways were involved (Table 2). Since TG is an important source of energy, it is understood

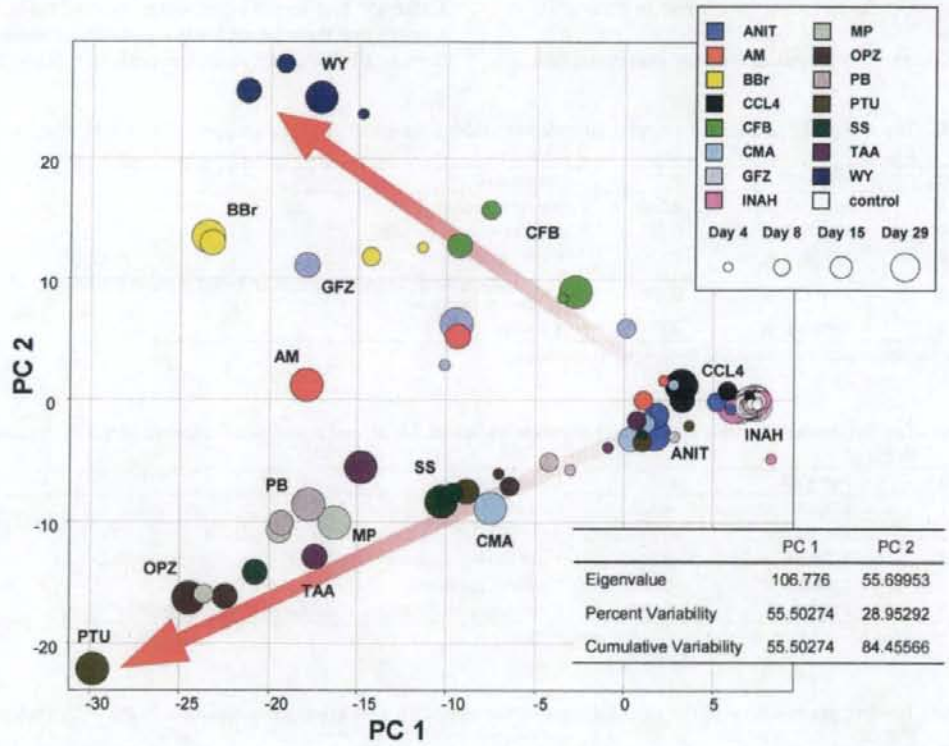


**Fig. 2.** Principal component analysis of gene expression profiles.
PCA of gene expression profiles was performed using identified 218 probe sets for the data of the highest dose of 15 compounds at various time points. The selection of the 218 probe sets related to plasma TG decrease is described in materials and methods. The values used in the analysis were the normalized signal values. Each spot represents the mean of the normalized gene expression value. The abbreviation for each drug is summarized in Table 1. The eigenvalue, percent variability, and cumulative variability for PC 1 and PC 2 are shown in the table on the lower right corner.