to be beneficial probably due to the ability to modify the internal environment of the endosomal/lysosomal compartment.[9–11] Thus, we hypothesized that CyDs are useful for treatment of GM1-gangliosidosis. However, there are few reports that examined the effects of CyDs on GM1-gangliosidosis, and the type of CyDs used were limited though their physicochemical properties greatly change depending on the type of cavity diameter and the substituent of CyDs. Therefore, in this study, we investigated the effects of various CyDs on the GM1-ganglioside level accumulated in EA1 cells, fibroblasts from patients with GM1-gangliosidosis.

Materials and Methods

Materials

2-Hydroxypropyl-α-cyclodextrin (degree of substitution (DS) 4.0), 2-hydroxypropyl-β-cyclodextrin (DS 4.4), 2-hydroxypropyl-γ-cyclodextrin (DS 5.7) were donated by Nihon Shokuhin Kako (Tokyo, Japan). Methyl-β-cyclodextrin (DS 12.2) was purchased from Junsei Chemical (Tokyo, Japan). Glucuronylglucosyl-β-cyclodextrin was obtained from Ensuiko Sugar Refining (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-labelled Cholera toxin B-subunit (FITC-CTB) was purchased from Sigma-Aldrich (St Louis, MO). Alexa488 conjugated CTB (Alexa-CTB) and LysoTracker Red DND-99 (LysoTracker) were obtained from Life Technologies Japan (Tokyo, Japan). Cyto-ID Autophagy Detection Kit was purchased from Enzo Life Sciences (Farmingdale, NY). Other chemicals and solvents were of analytical reagent grade.

Cell culture

EA1 cells, fibroblasts from GM1-gangliosidosis, were grown in Dulbecco's Modified Eagle's medium (DMEM) containing penicillin (1×10^5 mU/ml) and streptomycin (0.1 mg/ml) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

Cytotoxicity

Cytotoxicity of CyDs was assayed by the WST-8 method (a Cell Counting Kit, Wako Pure Chemical Industries, Osaka, Japan), which allows sensitive colorimetric assays for the determination of cell viability. WST-8 reagent is reduced by dehydrogenase activity in cells to give a yellow-colour formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activity of dehydrogenases in cells, is directly proportional to the number of living cells. Briefly, EA1 cells were seeded at 5×10^4 cells onto 24-well microplate (Iwaki, Tokyo, Japan) and incubated for 24 h in DMEM culture medium containing 10% FBS. Cells were washed with Hanks' balanced salt solution (HBSS, pH 7.4), and then incubated with 300 μL of culture medium containing 10 mM and 1 mM CyDs for 1 h and 24 h, respectively, at 37°C. After washing, 270 μL of HBSS and 30 μL of WST-8 reagent were added to the plates and incubated for 30 min at 37°C. The absorbance at 450 nm against a reference wavelength of 655 nm was measured with a microplate reader (Bio-Rad Model 550, Tokyo, Japan).

Extraction ability of cyclodextrins on cholesterol and phospholipids to the culture medium

EA1 cells (1×10^6/35 mm dish) were incubated with 10 mM and 1 mM CyDs in HBSS for 1 h and 24 h, respectively, at 37°C. After centrifugation (3000 rpm, 5 min, 4°C) of HBSS (700 μL), supernatant (300 μL) was recovered. Total cholesterol and phospholipids in the culture medium were determined using a Cholesterol E-test Wako and Phospholipids C-test Wako (Wako Pure Chemical Industries, Osaka, Japan) according to the instruction manual. Here, the Cholesterol E-test Wako and Phospholipids C-test Wako are enzymatic colorimetric methods for the quantitative determination of total cholesterol and phospholipids utilizing cholesterol oxydase and choline oxidase, respectively, in a reaction which can be measured photometrically.

Formation of autophagosome

EA1 cells (1×10^6/35 mm glass bottom dish) were incubated with DMEM culture medium for 24 h. After washing with phosphate buffered saline (PBS), the cells were incubated with 10 mM and 1 mM CyDs for 1 h and 24 h, respectively, and then the cells were treated with Cyto-ID Autophagy Detection Kit. Here, Cyto-ID Autophagy Detection Kit is applicable for the determination of autophagic vacuoles in live cells using a dye that selectively labels LC3-II on autophagic vacuoles. After washing with PBS, the cells were observed by a fluorescence microscope (KEYENCE Biozero BZ-8000, Tokyo, Japan).

GM1-ganglioside levels in EA1 cells

EA1 cells (1×10^6 cells/35 mm dish) were incubated for 24 h with DMEM culture medium containing 10% FBS. After washing with HBSS, the cells were treated with HBSS containing 10 mM and 1 mM CyDs for 1 h and 1 mM, 0.1 mM and 0.01 mM CyDs for 24 h, respectively, at 37°C. After washing with HBSS, 1 ml of FITC-CTB (5 μg/ml) were added to plates and incubated for 30 min at 37°C, and then the cells were suspended with 0.5 ml of PBS. After filtered through nylon mesh, the data were collected for 1×10^4 cells on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson, Mountain View, CA).

GM1-ganglioside levels in endolysosomes of EA1 cells

EA1 cells (5×10^4 cells/35 mm glass bottom dish) were incubated for 24 h with DMEM culture medium containing 10% FBS, and then pretreated with 1 ml of DMEM culture medium (FBS-free) containing 0.2 μM GM1-ganglioside for 48 h to increase the content of GM1-ganglioside in EA1 cells. After washing with PBS, cells were treated with 1 ml of DMEM culture medium (FBS-free) containing CyDs (10, 1, 0.1 mM). After washing with PBS, the cells were incubated with 200 μL of DMEM culture medium containing 50 nM LysoTracker for 30 min. After washing again, the cells were fixed by 200 μL of 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were permealized by the treatment with 200 μL of PBS containing 0.1% Triton X-100 for 10 min at room temperature. After washing again, 200 μL of PBS containing an Alexa-CTB (0.4 μg/ml) were added to plates and incubated for 30 min at 37°C. After the cells were washed, the fluorescence derived from Alexa Fluor 488 and LysoTracker in EA1 cells was detected by a confocal laser scanning microscopy. The fluorescence intensities of Alexa-CTB in endolysosomes were determined the area overlaid with that of LysoTraker by a BZ-II analyser software (Keyence, Osaka, Japan).

Data analysis

Data were given as the mean ± SEM. Statistical significance of mean coefficients for the studies was performed by analysis of variance followed by Scheffe's test. *P*-values for significance were set at 0.05.

Results and Discussion

Extracting effects of cyclodextrins on membrane components of EA1 cells

It is acknowledged that CyDs induce haemolysis and cytotoxicity through extraction of membrane components such as phospholipids, cholesterol and proteins from plasma membranes of cells, since CyDs enter cells only very slightly and include membrane lipids in the intramolecular cavity.[12,13] However, there are no data on extraction activity of CyDs on membrane components from EA1 cells. Therefore, we examined the extracting effects of CyDs on cholesterol and phospholipids of plasma membranes of EA1 cells. The treatment with 10 mM CyDs for 1 h induced cholesterol efflux from the cells, especially methyl-β-CyD (M-β-CyD), HP-β-CyD and glucuronylglucosyl-β-CyD (GUG-β-CyD) significantly induced the cholesterol efflux, compared with control (Figure 1a). Meanwhile, the treatment with 1 mM CyDs for 24 h did not induce cholesterol efflux from the cells, except for M-β-CyD (Figure 1b). Next, we examined the effects of CyDs on phospholipids efflux from EA1 cells. The treatment with 10 mM CyDs for 1 h induced phospholipids efflux from the cells, dimethyl-α-CyD (DM-α-CyD), not the other CyDs, significantly induced the phospholipids efflux, compared with control (Figure 1c). Meanwhile, the treatment with 1 mM CyDs for 24 h did not induce phospholipids efflux from the cells (Figure 1d). Here, the low levels of phospholipids were released from EA1 cells in not only control but also these CyDs, probably due to the 24 h incubation with HBSS (Figure 1d). These results suggest that the treatment with 1 mM CyDs for 24 h did not extract membrane components from the cells, although the treatment with 10 mM CyDs for 1 h did.

Cytotoxicity

To examine whether CyDs show cytotoxicity to A134 cells under the present experimental conditions, we evaluated cell viability using the WST-8 method. As shown in Figure 2a, there was no significant cytotoxicity in EA1 cells after treatments with 10 mM CyDs for 1 h except for M-β-CyD. Also, cytotoxicity was not observed after treatment with 1 mM CyDs for 24 h except for M-β-CyD (Figure 2b). Among CyDs used in this study, M-β-CyD is most likely to elicit the high cytotoxicity of A134 cells, since M-β-CyD possesses the strong extraction ability of cholesterol from erythrocytes and cell membranes.[12,14] However, the treatment with 10 mM CyDs for 24 h showed cytotoxicity, possibly due to the high extracting effect on membrane components (data not shown). These results indicate that CyDs excluding M-β-CyD show negligible cytotoxicity under the present experimental conditions.

Effects of cyclodextrin on GM1-ganglioside levels in EA1 cells

We examined the effects of CyDs on the GM1-ganglioside levels in EA1 cells using FITC-labelled cholera toxin B-subunit, which can bind to GM1-gangliosides specifically. The fluorescence intensity of FITC was determined by a flow cytometry. Herein, we used the two experimental conditions shown in Figures 1 and 2, that is, (1) 10 m mM CyD and 1 h treatment and (2) 1 mM CyD and 24 h treatment. Under the former conditions, CyDs extract membrane components from the cells (Figure 1) and are known to alter lipid rafts' function.[15,16] Under the latter conditions, CyDs except for M-β-CyD did not extract the membrane components (Figure 1), but the latter conditions CyDs are reported to decrease cholesterol in endolysosomes in NPC model cells.[17,18] As shown in Figure 3a, treatment with 10 mM CyDs for 1 h did not significantly change fluorescence intensity derived from FITC. Meanwhile, the treatment with 1 mM CyDs for 24 h decreased the fluorescent intensity, especially the prominent lowering effects of M-β-

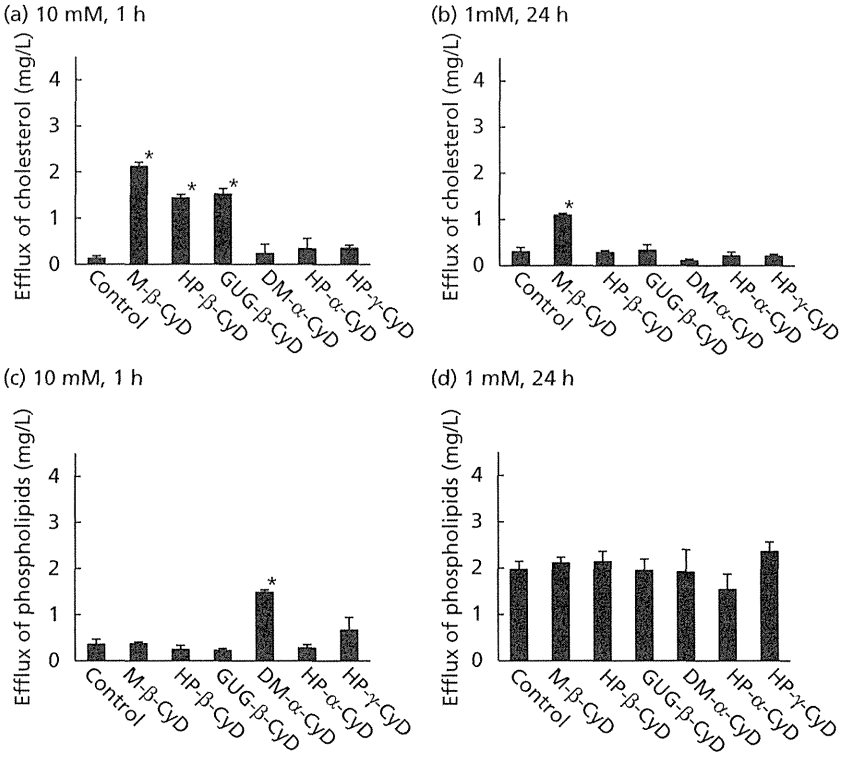

Figure 1 Effects of cyclodextrins on efflux of cholesterol and phospholipids from EA1 cells. EA1 cells were incubated with Hanks' balanced salt solution containing cyclodextrins (10 mM, 1 h or 1 mM, 24 h) at 37°C. The concentrations of cholesterol and phospholipids in supernatants were determined by Cholesterol E-test Wako and Phospholipid C-test Wako, respectively. Each value represents the mean ± SEM of four to five experiments. *$P < 0.05$, compared with control.

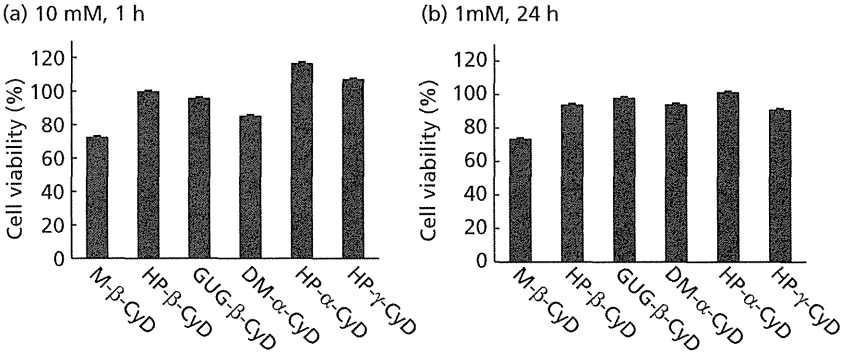

Figure 2 Cytotoxicity of cyclodextrins in EA1 Cells. EA1 cells were incubated with culture medium containing cyclodextrins (10 mM, 1 h or 1 mM, 24 h) at 37°C. After washing, 270 μL of fresh Hanks' balanced salt solution and 30 μL of WST-8 reagent were added. Each value represents the mean ± SEM of three to four experiments.

CyD and DM-α-CyD were shown with statistical difference, compared with that of the other CyDs (Figure 3b). The similar lowering effects of CyDs on the fluorescence intensity derived from Alexa-CTB were observed in EA1 cells using a confocal laser scanning microscopy (data not shown). Hence, the lowering effect of CyDs on the GM1-ganglioside levels in the cells is highly unlikely to be associated with the extracting ability of CyDs on plasma membrane components of the cells. These results suggest that CyDs impair the GM1-ganglioside levels in EA1 cells through the different way from the extraction ability of CyDs on membrane components. Here, in flow cytometry

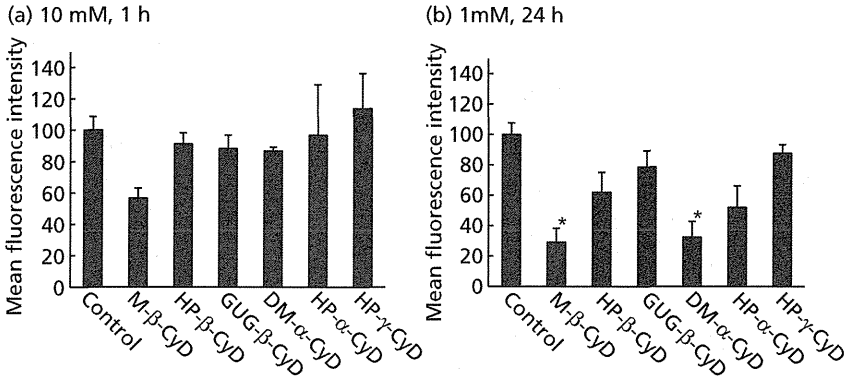

Figure 3 Cellular Association of FITC-CTB with GM1-ganglioside in EA1 cells after treatment with cyclodextrins. EA1 cells were incubated with medium containing cyclodextrins (10 mM, 1 h or 1 mM, 24 h) at 37°C. After washing, FITC-CTB was added to the plates and incubated for 30 min at 37°C. After washing, the fluorescence intensity of FITC-CTB in cells was determined by a flow cytometer. Each value represents the mean ± SEM of four to seven experiments. $*P < 0.05$, compared with control.

analysis, there is some risk to underestimate the total fluorescence by non-specific endocytic uptake of the Alexa-CTB. To eliminate this concern, we are planning to determine the GM1-ganglioside by a high performance TLC or LC-MS analysis after extraction from the cells.

Gangliosides of cellular surfaces reach luminal intra-endolysosomal vesicles or intra-endosomal membranes, which are generated during endocytosis.[1] In addition, Takamura *et al.* reported that GM1-gangliosides were mainly localized in the endolysosomes of GM1-gangliosodosis model mice.[19] Therefore, to confirm whether CyDs decrease the GM1-ganglioside levels in endolysosomes of EA1 cells, we observed the colocalization of Alexa-labeled CTB, which binds to GM1-gangliosides and LysoTracker, a fluorescent acidotropic probe for labelling and tracking acidic organelles in live cells, after treatment with CyDs in EA1 cells using a confocal laser scanning microscope. In this study, GM1-ganglioside-pretreated EA1 cells were used to obtain the high-resolution images after treatment with Alexa-CTB. As shown in Figure 4, in the absence of CyDs, the Alexa-CTB colocalized with LysoTracker, indicating that GM1-gangliosides accumulated in the endolysosomes of EA1 cells. Meanwhile, the treatment with 10 mM and 1 mM CyDs for 1 h reduced the fluorescence intensities derived from Alexa-CTB only slightly, compared with control (Figure 4a and 4b). In addition, the treatment with 1 mM and 0.1 mM CyDs, but not 0.01 mM CyDs, for 24 h drastically reduced the fluorescent intensity of Alexa-CTB in EA1 cells, compared with control (Figure 4c–4e). Actually, we determined the fluorescence intensity of Alexa-CTB in the endolysosomes using the BZ-II analyser software (Figure 5). As shown in Figure 5a and 5b, the treatment with 10 mM CyDs and 1 mM for 1 h tended to lower the fluorescence intensities derived from Alexa-CTB, compared with control. Meanwhile, the treatment with 1 mM and 0.1 mM CyDs, but not 0.01 mM CyDs, for 24 h significantly reduced the fluorescent intensity of Alexa-CTB in EA1 cells, compared with control (Figure 5c–5e). In addition, the treatment with 1 mM CyDs for 24 h showed down-regulation of the fluorescent intensity of Alexa-CTB, compared with that with 1 mM CyDs for 1 h (Figure 5b and 5c). These results suggest that the lowering effects of CyDs on the fluorescent intensity of Alexa-CTB were affected by not only the incubation time of CyDs at 1 mM but also the concentration of CyDs for 24 h treatment. Herein, the relatively high lowering effect of M-β-CyD may be associated with its cytotoxicity as shown in Figure 2. In addition, the slightly high lowering effect of DM-α-CyD is partially correlated with the results using a flow cytometry without the pretreatment with GM1-gangliosidie (Figure 3b). Thereby, it is highly possible that CyDs lower GM1-gangliosidie in endolysosomes of EA1 cells. However, the reduction of fluorescent intensity of Alexa-CTB with CyDs, at least in part, reflects changes in GM1-gangliosoide levels in plasma membranes, which has been reported in GM1-gangliosidosis fibroblasts by Tokuda *et al.*[20] Actually, it is still unclear how the CyDs reduced GM1-ganglioside levels. Therefore, further elaborate studies on whether GM1-ganglioside is released or not are required.

Effects of cyclodextrin on autophagy in EA1 cells

Sarkar *et al.* have recently shown impairment in autophagic flux in NPC1 disease associated with abnormal cholesterol sequestration, but cholesterol depletion treatment with HP-β-CyD impedes autophagy.[21] Thereby, we next

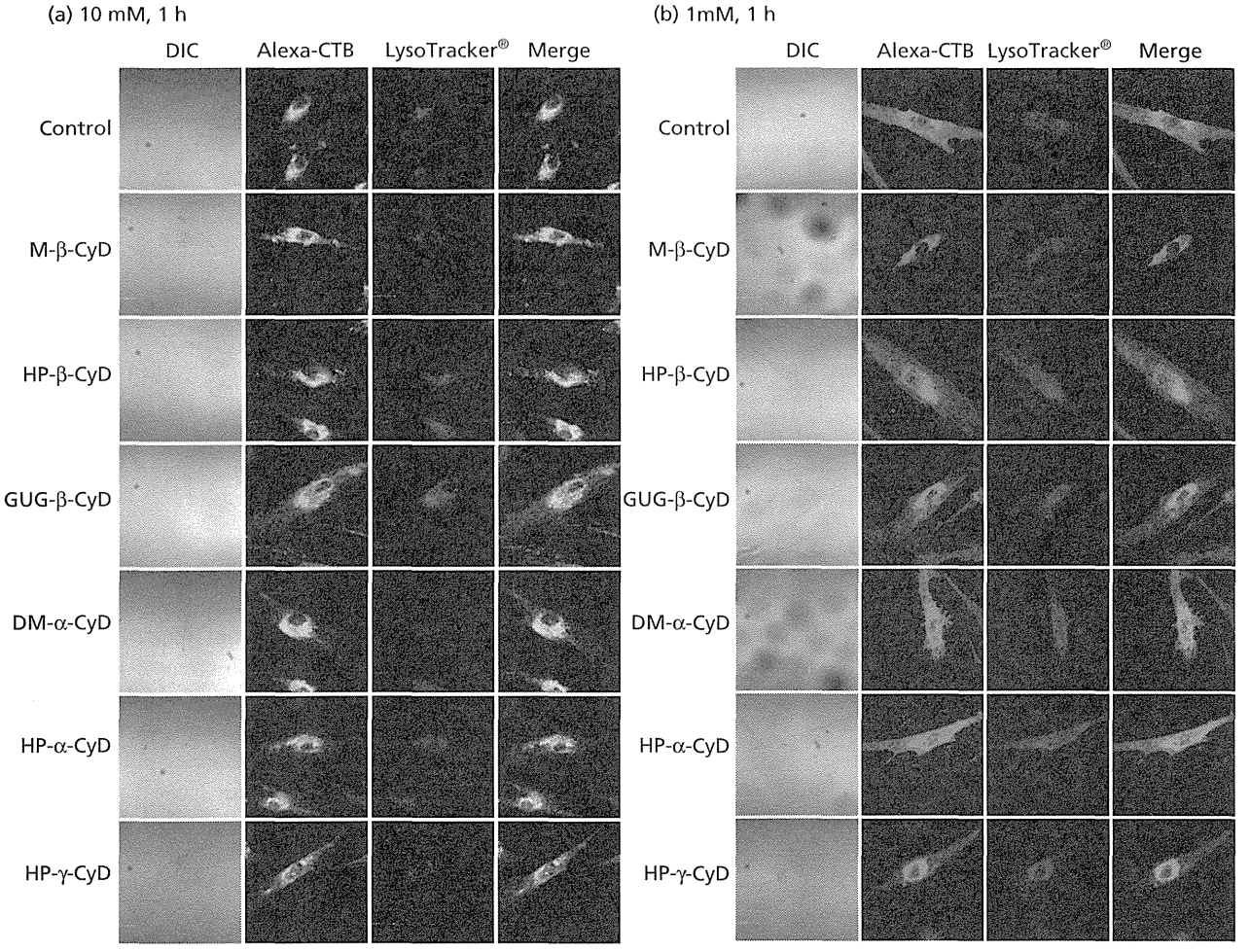

Figure 4 Effects of cyclodextrins on GM1-ganglioside levels in endolysosomes of EA1 cells. EA1 cells were incubated with culture medium containing cyclodextrins ((a) 10 mM, 1 h, (b) 1 mM, 1 h, (c) 1 mM, 24 h, (d) 0.1 mM, 24 h, (e) 0.01 mM, 24 h) at 37°C. Cells were incubated with DMEM culture medium containing 50 nM LysoTracker for 30 min. After fixation by 4% paraformaldehyde, the cells were permealized by 0.1% Triton X-100 for 10 min. After washing, 200 μL of PBS containing an Alexa-Cholera toxin B-subunit (0.4 μg/ml) were treated. The experiments were performed independently three times, and the representative images are shown.

examined the effects of CyDs on the LC3-II, an autophagosome marker in EA1 cells using the Cyto-ID green detection reagent. Herein, the fluorescence levels derived from LC3-II were determined using a BZ-II analyser. As shown in Figure 6a and 6b, the treatment with 1 mM CyDs for 24 h decreased the fluorescence levels only very slightly. These results suggest that CyDs did not affect autophagy anymore under the present experimental conditions.

The mechanisms by which the treatment with 1 mM CyDs for 24 h elicited higher lowering effects of GM1-gangliosides in endolysosomes in EA1 cells than that with 10 mM for 1 h still remains unclear. As described before, the lowering effects of CyDs cannot be explained by the extraction ability of CyDs on membrane components from plasma membranes, thereby the other hypothesis should be considered. Rosenbaum *et al.* reported that CyD-mediated enhanced cholesterol transport from the endocytic system can reduce cholesterol accumulation in cells with defects in either NPC1 or NPC2.[22,23] Therefore, endocytosis of CyDs might be involved in the lowering effects in EA1 cells. In addition, the evidence which the type of CyDs provided the different lowering effect makes us suggest that the intramolecular cavity of CyDs may somewhat act the lowering effect on GM1-gangliosides. However, it cannot be denied that CyDs decrease cholera toxin binding to GM1-gangliosides, not lowering the GM1-ganglioside levels in the endolysosomes, since α-CyD is reported to decreases cholera toxin binding to GM1-gangliosides.[24] Additionally, as far as I know, there are only a few reports regarding the interaction between CyDs

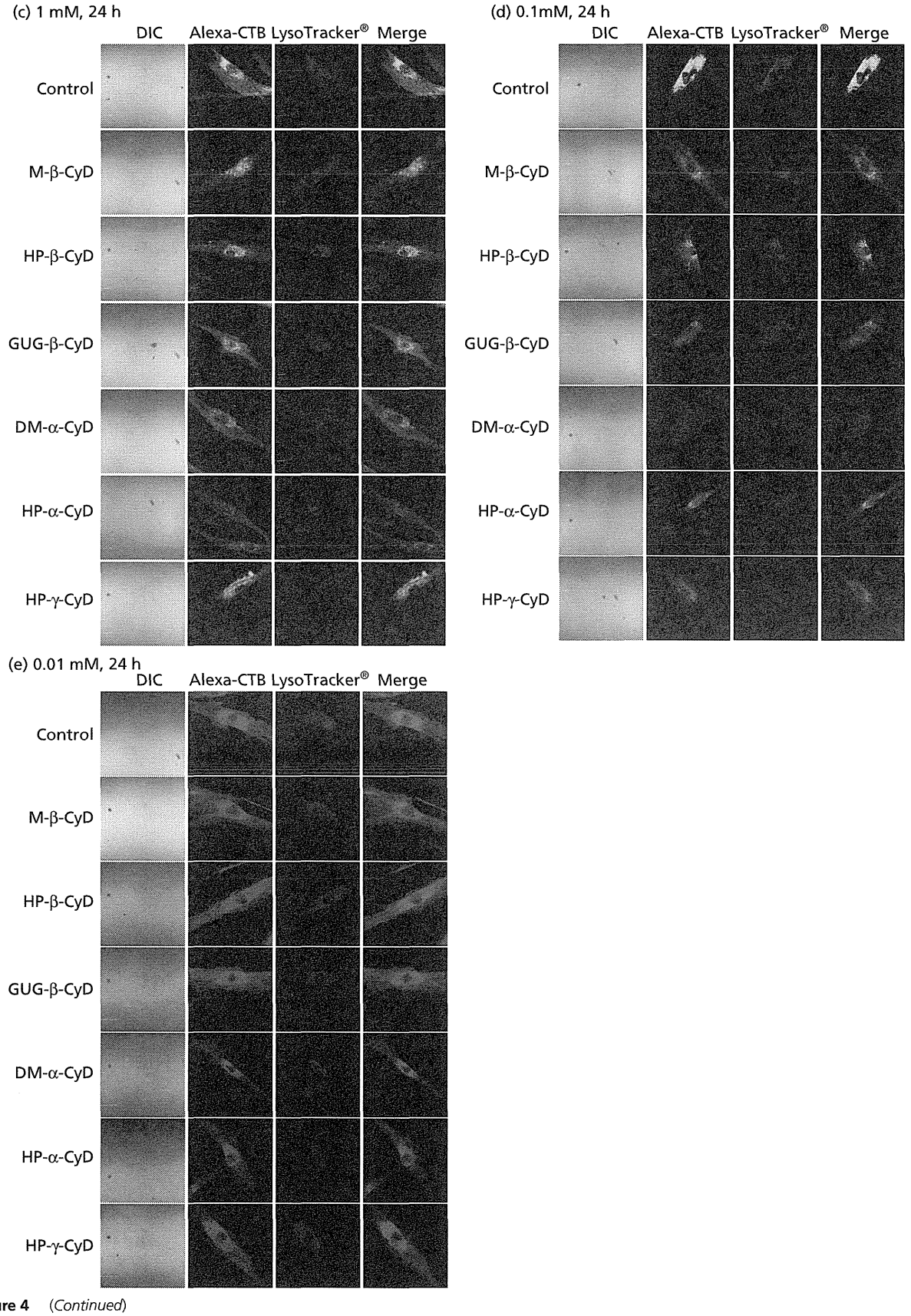

Figure 4 (*Continued*)

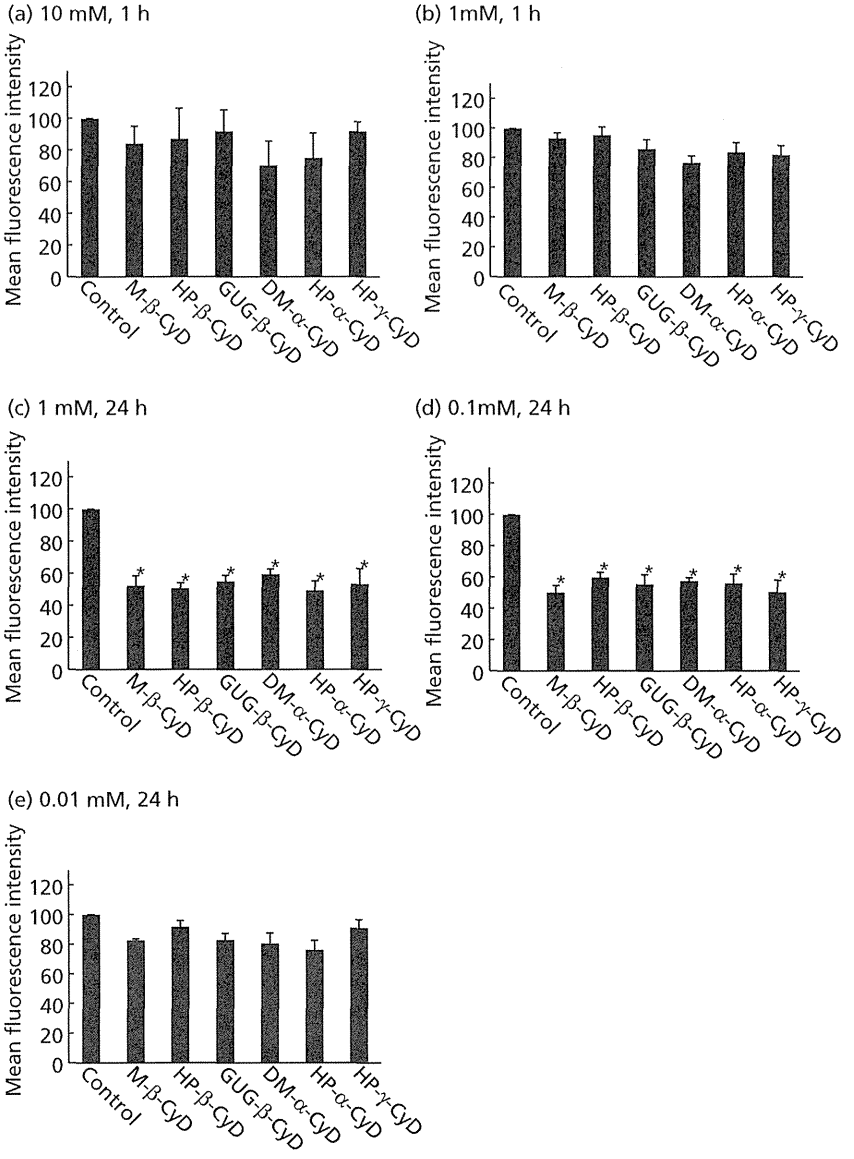

Figure 5 Effects of cyclodextrins on GM1-ganglioside levels in EA1 cells. EA1 cells were incubated with culture medium containing cyclodextrins ((a) 10 mM, 1 h, (b) 1 mM, 1 h, (c) 1 mM, 24 h, (d) 0.1 mM, 24 h, (e) 0.01 mM, 24 h) at 37°C. After washing, Alexa-Cholera toxin B-subunit was added to the plates and incubated for 30 min at 37°C. After washing, the samples were incubated in fresh culture medium for 30 min. The fluorescence intensities were determined by a BZ-II analyser. Each value represents the mean ± SEM of three to five experiments. $*P < 0.05$, compared with control.

and GM1-gangliosides, e.g. 16.5 mM α-CyD enhances selectivity and efficiency of separation of gangliosides in a capillary zone electrophoresis.[25] However, it remains obscure whether the other CyDs interact with GM1-gangliosides and why various CyDs lowered the GM-1 ganglioside levels in endolysosomes of EA1 cells. Hence, we now have many challenges to reveal the mechanisms. Thereafter, further studies are required to clarify the mechanism. In addition, Davidson *et al.* reported that the potential use of HP-β-CyD to treat late endosome-lysosomal accumulation of unesterified cholesterol in NPC disease after subcutaneous administration but not in GM1-gangliosidosis.[26] However, the present results trend to show some differences between each CyD, it remains conceivable that M-β-CyD and DM-α-CyD might show some in-vivo impact. Therefore, we are now investigating the in-vivo treatment effects of CyDs in GM1-gangliosidosis model mice with various administration

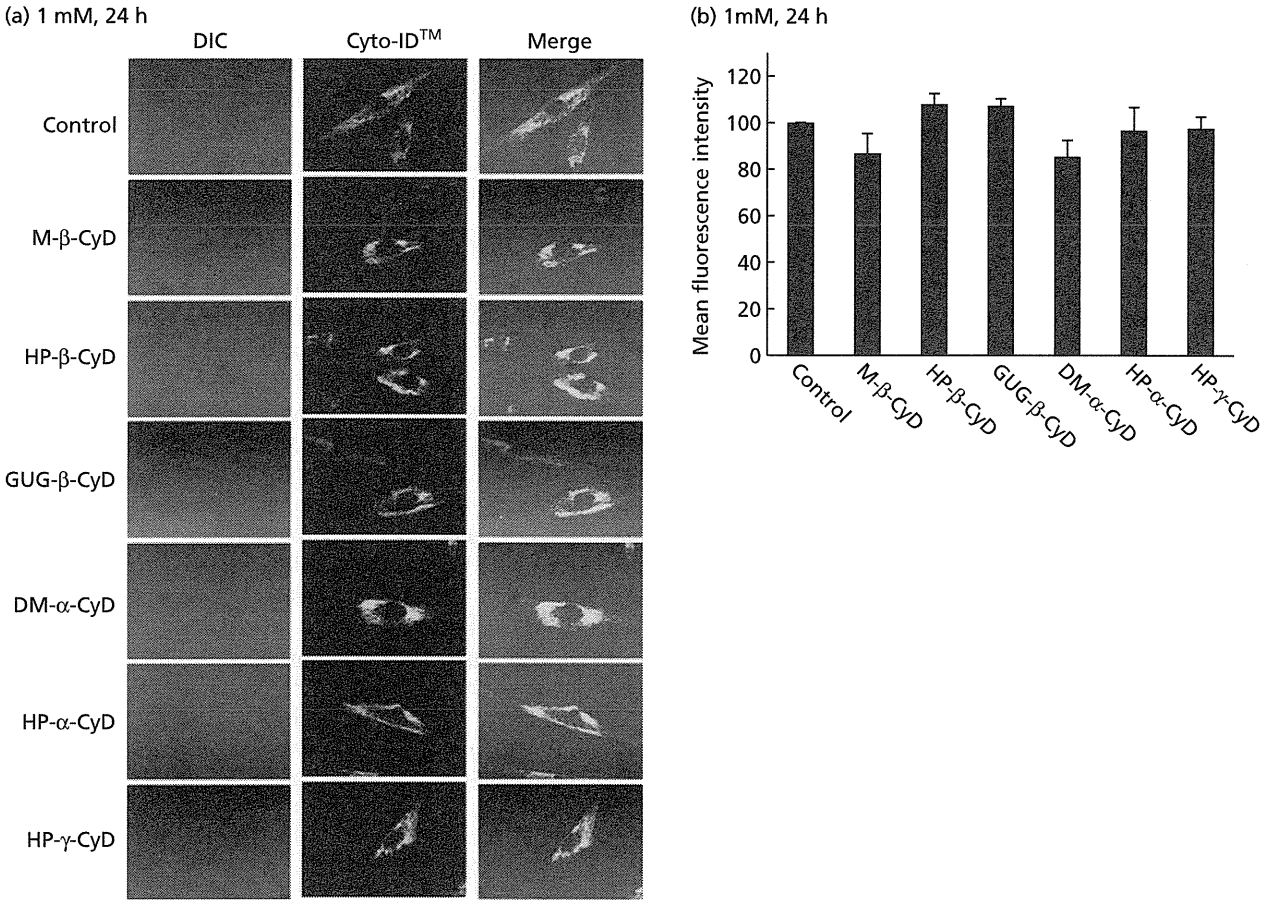

Figure 6 Effects of cyclodextrins on LC3-II Levels in EA1 cells. EA1 cells were incubated with medium containing cyclodextrins (1 mM, 24 h) at 37°C. After washing, 100 μL of Cyto-ID Green Detection Reagent was added and incubated for 30 min at 37°C. The experiments were performed independently three times, and the representative images are shown. Each value represents the mean ± SEM of three experiments.

routes. Taken together, these results suggest that cytotoxicity of CyDs is not involved in the reducing effect of CyDs on the GM1-ganglioside levels in endolysosomes of EA1 cells.

Conclusions

In this study, we investigated the effects of CyDs on GM1-gangliosides in endolysosomes of EA1 cells. As a result, the treatment of EA1 cells with 1 mM CyDs for 24 h was decreased the GM1-ganglioside levels in the endolysosomes, although the efflux of membrane components is not associated with the down-regulation. These results suggest that CyDs may have the potential as drugs for GM1-gangliosidosis, although the mechanism should be thereafter clarified.

Declarations

Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgements

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Genes to Cells

Herbal drug ninjin'yoeito accelerates myelopoiesis but not erythropoiesis *in vitro*

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Some Kampo medicines that are herbal and traditional in Japan have had beneficial effects when given to patients with anemia. However, molecular mechanisms underlying their effects are unclear. To address this question, four Kampo medicines used to treat anemia—ninjin'yoeito (NYT), shimotsuto (SMT), juzentaihoto (JTT), and daibofuto (DBT)—were tested separately using *in vitro* cultures of mouse bone marrow mononuclear cells. Among them, NYT was most effective in stimulating cell proliferation and up-regulating *Myc* expression. Flow cytometry analysis indicated that, among hematopoietic components of those cultures, myeloid cells expressing CD45/Mac-1/Gr-1/F4/80 increased in number, but Ter119/CD71 erythroid cells did not. Accordingly, real-time PCR analysis showed up-regulation of the myeloid gene *Pu.1*, whereas the erythroid genes *Gata1* and *Klf1* were down-regulated. Overall, these findings provide molecular evidence that NYT accelerates myelopoiesis but not erythropoiesis *in vitro*.

Introduction

Hematopoiesis is the process whereby functional, mature hematopoietic cells (red blood cells (RBCs), leukocytes, and platelets) are generated from hematopoietic stem cells in bone marrow (BM). Erythropoiesis is one aspect of hematopoiesis in which erythroid progenitors, such as burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) cells, are initially generated and then give rise to erythroblasts, reticulocytes, and finally RBCs, which contain hemoglobin functioning in oxygen transport (McGrath & Palis 2008). Failure of erythropoiesis results in a shortage of or damage to RBCs and underlies anemia.

Kampo is a Japanese herbal medicine and widely used to treat many kinds of diseases. Among them, the group shimotsuto, primarily in combination with other drugs, has been used clinically as a blood replenishment agent to treat anemia. Ninjin'yoeito (NYT), a member of shimotsuto group, reportedly antagonizes various forms of anemia, including iron-deficiency anemia (Yanagihori *et al.* 1995; Ando 1999), aplastic anemia (Ohmori *et al.* 1993; Miyazaki *et al.* 1994), refractory anemia (Ohmori *et al.* 1992; Nagoshi *et al.* 1993), renal anemia (Takemura 2000), and anemia resulting from anticancer therapies in humans (Motoo *et al.* 2005). In mice, oral administration of NYT improves 5-fluorouracil (5-FU) induced anemic conditions, as evidenced by the assessment of

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reticulocyte and RBC numbers, hemoglobin and hematocrit levels in peripheral blood, and increases in BFU-E and CFU-E in BM (Takano *et al.* 2009). However, molecular mechanisms underlying NYT's effect have not been clarified in human beings or mice.

To address those mechanisms, we examined the effect of herbal remedies on cell proliferation and hematopoietic differentiation of BM mononuclear cells (MNCs) in mice by testing four Kampo medicines, NYT, SMT, JTT, and DBT, all historically used to treat anemia. We found that one of those, NYT, enhanced cell proliferation and up-regulated *Myc* transcript levels, likely accounting for the enhanced proliferative state. Among hematopoietic cells, NYT did not increase percentage of erythroid cells but rather decreased expression of the erythroid genes *Gata1* and *Klf1*, whereas the number of macrophages and granulocytes in cultures increased, accompanied by up-regulation of *Pu.1* expression.

Results

Hematopoietic cell proliferation in the presence of Kampo medicines

To investigate the effect of Kampo medicines on hematopoietic cell proliferation, we cultured BM MNCs separately with four Kampo medicines—ninjin'yoeito (NYT), daibofuto (DBT), juzentaihoto (JTT), or shimotsuto (SMT) (Table S1 and Fig. S1 in Supporting information)—for 11 days. To evaluate a potential direct effect of Kampo medicines, no cytokines were added to the cultures. Both round-shaped and adherent cells were observed in the negative control at day 11, while NYT treatment resulted in variable sizes of round-shaped cells and significant proliferation of the round cells by day 11 (Fig. 1A). Cells treated with SMT, JTT, or DBT exhibited similar morphological changes but showed fewer round-shaped cells (T. Inoue, K. Kulkeaw and K. Muennu, unpublished data). The total number of viable cells in negative control samples (♦) increased slightly by day 4 ($3.1 \pm 1.4 \times 10^5$ cells) and then decreased at days 8 and 11 ($0.5 \pm 0.03 \times 10^5$ and $0.76 \pm 0.1 \times 10^5$ cells, respectively). By contrast, BM MNCs cultured in the presence of NYT, SMT, or DBT exhibited significantly increased proliferation by day 11 ($P < 0.05$) (Fig. 1B). Specifically, NYT-treated cells (■) showed a slight increase at day 4 ($3.1 \pm 0.9 \times 10^5$ cells) and at day 8 ($3.5 \pm 2.1 \times 10^5$ cells) and then showed a 2.55-fold increase by day 11 ($8.9 \pm 3.3 \times 10^5$ cells). JTT-treated cells (●) showed the same trend: Their numbers were $2.6 \pm 1.0 \times 10^5$ cells at day 4, $2.8 \pm 1.8 \times 10^5$ at day 8, and $4.1 \pm 3.0 \times 10^5$ at day 11. However, compared with the negative control, no significant difference was observed in JTT-treated cells at day 11. By contrast, DBT-treated cells (x) decreased in number by day 4 ($2.1 \pm 0.6 \times 10^5$ cells) but then consistently increased at days 8 ($4.5 \pm 2.5 \times 10^5$ cells) and 11 ($5.7 \pm 2.0 \times 10^5$ cells). SMT-treated cells (▲) showed the same trend as DBT-treated cells: Their numbers were $2.4 \pm 0.5 \times 10^5$ at day 4, $3.5 \pm 2.9 \times 10^5$ at day 8, and $4.9 \pm 1.1 \times 10^5$ at day 11 (Fig. 1B).

To assess molecular mechanisms underlying cell proliferation, we used real-time PCR to examine expression of the transcription factor *Myc*, which functions in cell proliferation (Fig. 1C). Regardless of the type of Kampo medicine used, *Myc* expression in cell cultures gradually increased, including that seen in the negative control. DBT- and JTT-treated cultures showed lower *Myc* expression than did the negative control, whereas NYT-treated cells showed the highest *Myc* expression (day 4: 0.52 ± 0.03, day 8: 0.70 ± 0.01 and day 11: 1.64 ± 0.21) among all culture conditions at all time points (Fig. 1C). Particularly at day 11, *Myc* expression in NYT-treated cells increased significantly relative to the negative control (1.64 ± 0.21 versus 0.49 ± 0.12; $P = 0.02$) (Fig. 1C).

NYT consists of 12 component medical plants, such as Japanese Angelica root, Rehmannia root, peony root, atractylodes rhizome, Poroa Sclerotium, ginseng, cinnamon bark, Polygala root, Citrus unshiu peel, Astragalus root, Glycyrrhiza and Schisandra fruit. To determine whether the herbal constituents of NYT act synergistically to stimulate cell proliferation, cultures were treated with individual NYT components and assessed for cell number and *Myc* expression. As shown in Fig. S2 and Fig. 1D, all NYT components except for Poria sclerotium and cinnamon bark enhanced proliferation relative to controls. *Myc* expression increased in the presence of Japanese Angelica root ($P = 0.0005$) and Rehmannia root ($P = 0.0002$) but remained lower than that stimulated by NYT (Fig. 1E), suggesting that NYT components act synergistically and that Japanese Angelica root and Rehmannia root are likely essential NYT components.

NYT does not alter erythropoiesis in bone marrow mononuclear cells

We next examined the effect of NYT on erythroid cell differentiation. Both $CD71^+/Ter119^+$ cells

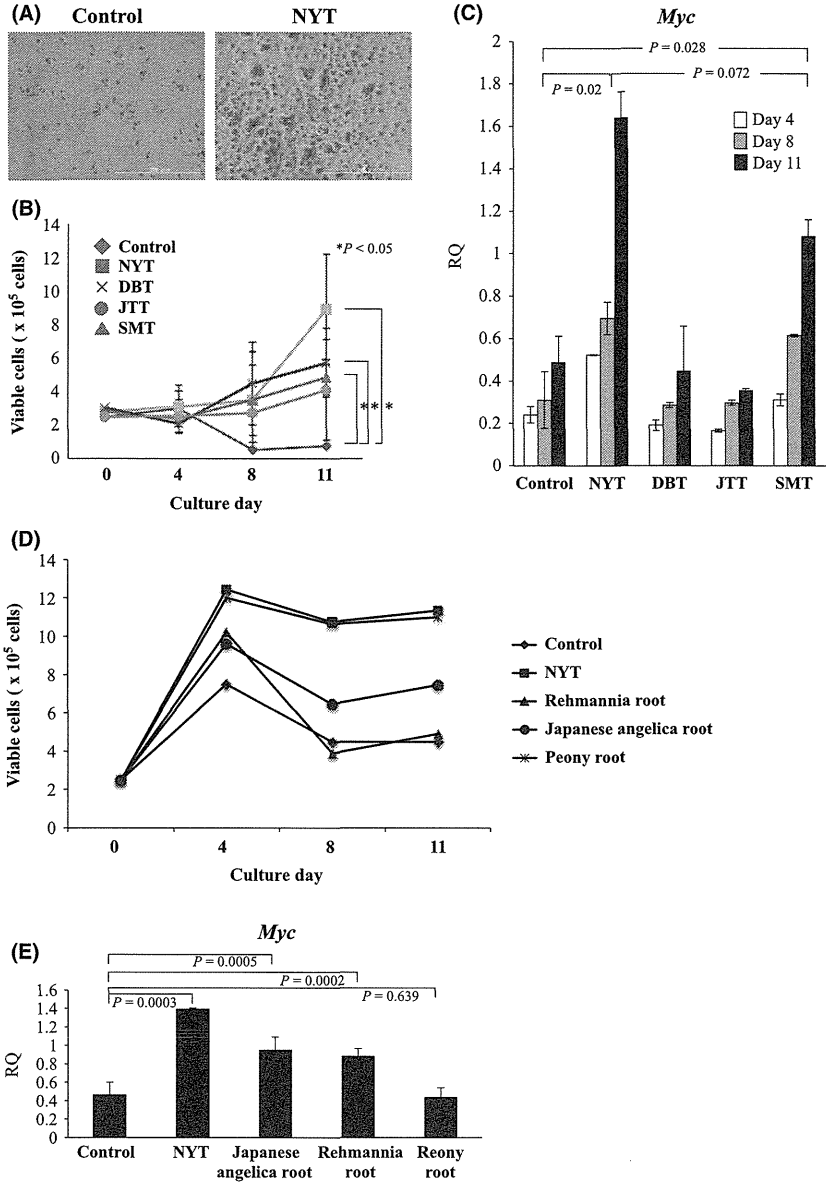


Figure 1 Hematopoietic cell proliferation in the presence of Kampo medicines. (A) Morphology of cells derived from cultured BM MNCs after 11 days of NYT treatment. Shown are phase-contrast images. Scale bars: 200 μm. (B) Total number of viable cells after Kampo medicine treatment. BM MNCs were collected, and viable cells were counted using trypan blue dye after 4, 8, and 11 days. (C), (E) Quantitative real-time PCR analysis of the cell proliferation marker *Myc* at days 4, 8, and 11 (day 11 in E) of cultured BM MNCs. Data are normalized to β-*actin* expression. Student's *t*-test ⋆ was used to calculate statistical significant difference ($P < 0.05$). (D) Total number of viable cells after treatment of NYT and components (Rehmannia root, Japanese Angelica root and Peony root). BM MNCs were collected, and viable cells were counted using trypan blue dye after 4, 8, and 11 days.

representing erythroblasts and $CD71^-/Ter119^+$ cells representing mature erythrocytes were analyzed at days 4, 8, and 11 by flow cytometry. $CD71^-/Ter119^+$ erythrocytes were generated at low efficiency (< 0.6%) in cell culture (Fig. 2A). A significant decrease in the number of $Ter119^+$ cells in NYT-treated culture (1.5 times lower) was observed at day 8, whereas there was no significant difference between $CD71^-/Ter119^+$ cells at days 4 and 11 (Fig. 2B). In agreement, real-time PCR analysis showed that expression of the erythropoietic transcription factor *Gata1* (Whitelaw *et al.* 1990) was

higher in control versus NYT-treated cells (day 4: 0.8 ± 0.16 in controls and 0.147 ± 0.09 in NYT-treated cells; and day 8: 0.419 ± 0.07 in controls and no expression in NYT-treated cells) (Fig. 2C). The transcription factor *Klf1* (Miller & Bieker 1993), whose expression is regulated by Gata1, was detected in controls and NYT-treated cells only on day 4 but not days 8 and 11. No expression of *Klf1* was seen after SMT, JTT, or DBT treatment (T. Inoue, K. Kulkeaw and K. Muennu, unpublished data). These findings indicate that NYT does not accelerate erythroid differentiation.

NYT accelerates myelopoiesis of bone marrow mononuclear cells

To investigate the effect of NYT on leukopoiesis, which consists of myelopoiesis and lymphopoiesis, we examined expression of the common leukocyte antigen CD45, a pan-leukocyte marker, at days 4, 8 (T. Inoue, K. Kulkeaw and K. Muennu, unpublished data), and 11 (Fig. S3A, left in Supporting information) by flow cytometry. Percentage of $CD45^+$ cell was slightly higher in NYT-treated cells (control: 95.1 ± 1.33%, NYT-treated cells: 97.6 ± 0.28%), but the difference was not statistically significant ($P = 0.118$) (Fig. S3A in Supporting information). When leukopoiesis was analyzed by flow cytometry using Mac-1 (CD11b), a marker of macrophages and granulocytes, and B220, a B-lymphocyte marker, NYT-treated BM MNCs differentiated into $CD45^+$/$Mac\text{-}1^+$ cells in numbers 2.23 times greater than controls ($P = 0.002$) (Fig. S3B in Supporting information) and into $CD45^+/Mac\text{-}1^-/B220^+$ B lymphocytes in numbers 3.00 times lower than control ($P = 0.002$) (Fig. S3B in Supporting information) by 11 days in culture. In agreement with the increase in $CD45^+/Mac\text{-}1^+$ cells, real-time PCR analysis showed that expression of *Pu.1 (Spi1)*, which positively regulates generation of macrophages and granulocytes, was higher in NYT-treated cells (1.00 ± 0.001 in controls and 5.55 ± 0.09 in NYT-treated cells) ($P = 0.007$) (Fig. S3C in Supporting information). Also, expression of colony-stimulating factor 1 receptor (*Csf1r*), the receptor for macrophage colony-stimulating factor, was higher in NYT-treated (1.12 ± 0.09 in controls and 1.53 ± 0.08 in NYT-treated cells) ($P = 0.06$) at day 11 (Fig. S3C in Supporting information). Taken together, these findings suggest that the primary effect of NYT on hematopoiesis is to accelerate myelopoiesis.

To investigate NYT activity using purified populations, we removed erythroid cells by sorting $CD45^+/Ter119^-$ cells from BM MNCs (Fig. 3A) and then

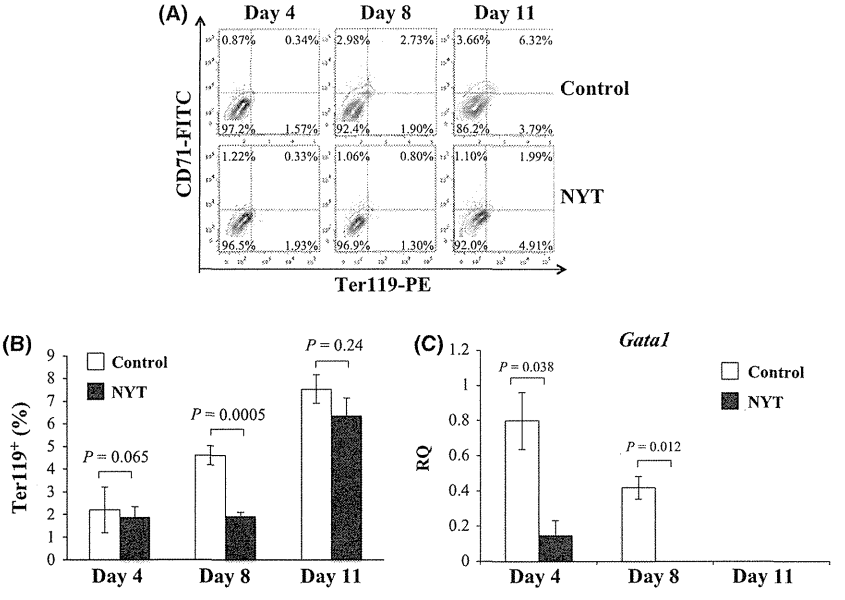


Figure 2 NYT treatment does not alter erythropoiesis. (A) BM MNCs cultured with and without NYT were collected at days 4, 8, and 11, and erythroid differentiation was assessed by flow cytometric analysis of cells stained positive for CD71 and Ter119. The percentage of $Ter119^+$ erythroid cells is shown in (B). (C) Quantitative real-time PCR analysis of the erythroid gene *Gata1* at days 4, 8, and 11. Data were assessed by normalized values to control at day 4.

cultured them in the presence of NYT for 11 days. The total number of cells in the untreated negative control group increased at day 4 ($2.93 \pm 0.11 \times 10^5$ cells) and then decreased at days 8 and 11 ($2.40 \pm 0.84 \times 10^5$ and $2.10 \pm 0.42 \times 10^5$ cells, respectively). By contrast, NYT-treated $CD45^+$ cells showed a 2.4-fold increase in total cell number at day 4 ($4.80 \pm 0.21 \times 10^5$ cells) ($P = 0.007$), decreased at day 8 ($4.20 \pm 0.42 \times 10^5$ cells) ($P = 0.116$), and then increased at day 11 ($6.75 \pm 1.06 \times 10^5$ cells) ($P = 0.029$) (Fig. 3B). Flow cytometric analysis (Fig. 3C) showed that $CD45^+$ cells were more abundant in NYT-treated (94.0 ± 0.46%) versus control (73.7 ± 1.89%) cells ($P = 0.0001$) (Fig. 3C). Among $CD45^+$ cells, the proportion of $Mac\text{-}1^+/F4/80^+$ macrophages differentiated from BM MNC $CD45^+$ cells was 8.05 times higher in NYT-treated than in control cells ($P = 0.01$) (Fig. 3D). Moreover, the proportion of $Mac\text{-}1^+/Gr\text{-}1^+$ granulocytes was 3.41 times higher in NYT-treated than control cells ($P = 7.07 \times 10^{-6}$) (Fig. 3E). Furthermore, real-time PCR analysis showed that expression of *Pu.1* was higher in cultures of NYT-treated compared with control cells (3.15-fold at day 4, 1.28-fold at day 8 and 5.43-fold at day 11) (Fig. 3F). *Csf1r* expression was 8.41 times lower in NYT-treated compared with control cells at day 4, 4.72 times higher at day 8, and 2.66 times higher at day 11 (Fig. 3F).

To determine which stage of myeloid differentiation is affected by NYT, we carried out CFU assays. No colonies were produced without addition of cytokines (T. Inoue, K. Kulkeaw and K. Muennu, unpublished data). In the presence of the myeloid cytokines SCF, IL-3, and IL-6, NYT increased total colony number 1.30-fold ($P = 0.528$). Among those colonies, the number of CFU-M, CFU-G, and CFU-GM, all indicative of committed myeloid progenitors, increased, implying that NYT accelerates myelopoiesis at the progenitor level (Fig. S4 in Supporting information).

Gene expression changes in bone marrow cells

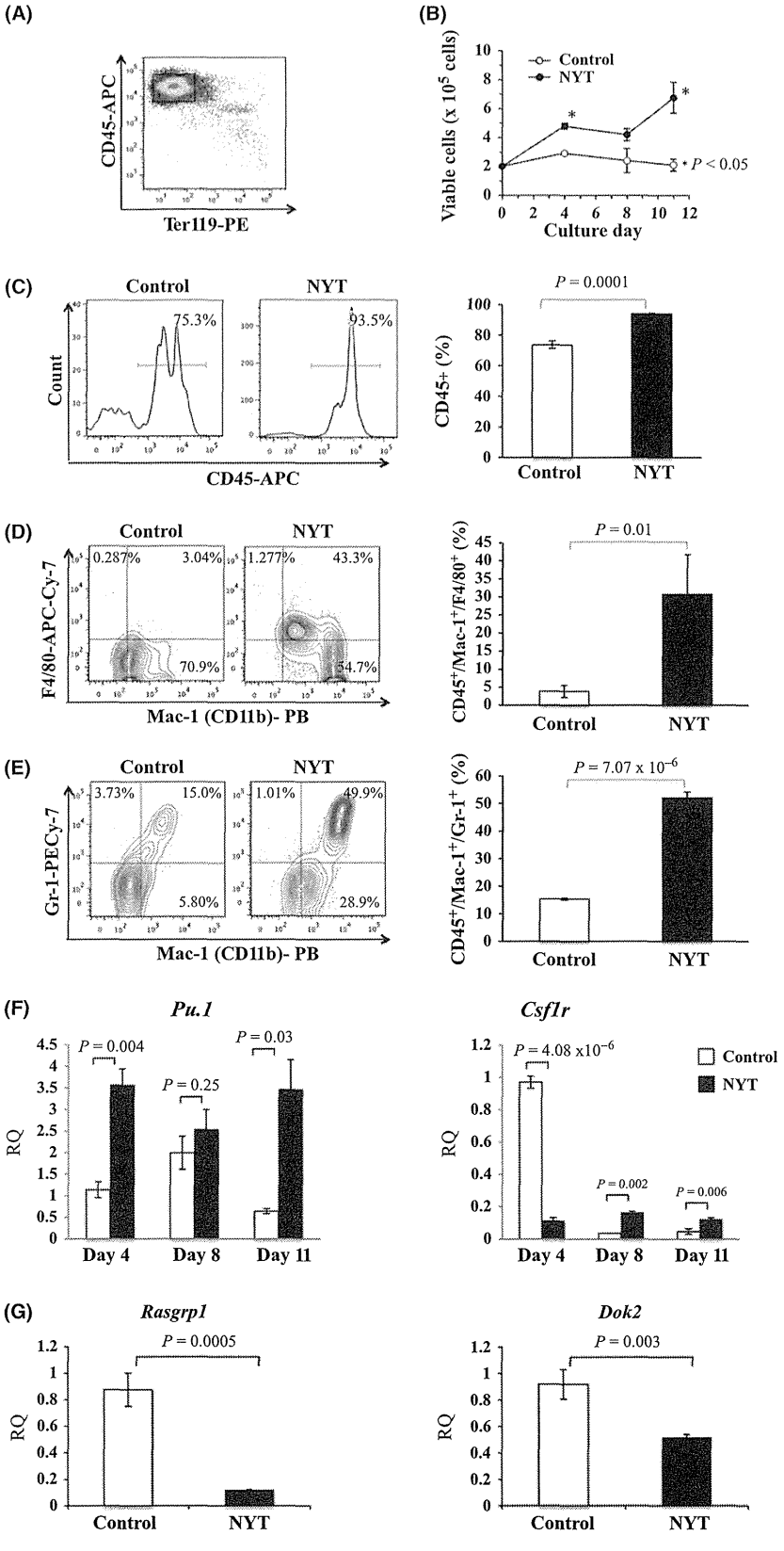
To identify hematopoietic genes regulated by NYT, microarray analysis was carried out to compare BM MNCs cultured with and without NYT for 11 days. Up-regulated ($P < 0.05$) and down-regulated ($P < 0.05$) genes were analyzed and categorized functionally (Fig. S5 in Supporting information). In agreement with results shown in Fig. 3F and Fig. S4, we identified factors affecting leukopoiesis. Among them, we observed down-regulation of *Rasgrp1* (de la Luz Sierra *et al.* 2010) and *Dok2* (Garcia *et al.* 2004) after 11 days in culture with NYT (7.31-fold decrease ($P = 0.0005$) and 1.78-fold decrease ($P = 0.003$), respectively) (Fig. 3G), a finding validated by real-time PCR.

Discussion

Here, we assessed molecular mechanisms underlying the effect of Kampo medicines on erythropoiesis. Among four such medicines tested, NYT showed the greatest stimulation of BM MNC proliferation accompanied by the highest up-regulation of *Myc*, a factor associated with up-regulated proliferation, by 11 days after culture (Fig 1B). Relevant to proliferation, our findings are in accordance with a report that NYT increased the number of primary rat oligodendrocyte precursors *in vitro* as assessed by BrdU incorporation studies (Kobayashi *et al.* 2003).

Previously, others had shown that after induction of anemia, NYT stimulates erythroid cell differentiation (Takatsuki *et al.* 1996; Takano *et al.* 2009). Thus, we initially hypothesized that Kampo medicines would accelerate erythropoiesis. However, in our cul-

Figure 3 Effect of NYT on myeloid differentiation of BM $CD45^+$ cells. (A) $CD45^+/Ter119^-$ leukocytes were isolated from BM MNCs to remove erythroid cells and cultured for 11 days. (B) Viable cells were collected at days 4, 8, and 11 and counted using trypan blue dye. (C) BM $CD45^+$ leukocytes cultured with and without NYT were collected at day 11, and leukocyte differentiation was assessed by flow cytometry based on CD45 staining. The percentage of $CD45^+$ leukocytes is shown at right. (D) Myeloid differentiation of $CD45^+$ cells was evaluated by flow cytometry at day 11 based on staining with Mac-1 (CD11b) and F4/80. The percentage of $CD45^+/Mac\text{-}1^+/F4/80^+$ macrophages is shown at right. (E) Granulocyte differentiation of $CD45^+$ cells was evaluated by flow cytometry at day 11 based on staining with Mac-1 (CD11b) and Gr-1. The percentage of $CD45^+/Mac\text{-}1^+/Gr\text{-}1^+$ granulocytes is shown at right. (F) Quantitative real-time PCR analysis of expression of the myeloid genes *Pu.1* and *Csf1r* at days 4, 8, and 11. Data were assessed by normalized values to control at day 4. (G) BM MNCs cultured with and without NYT *in vitro* were collected and analyzed by quantitative real-time PCR at day 11 for expression of *Rasgrp1*, a lymphopoietic gene, and *Dok2*, a myelopoietic gene. Data are normalized to β-*actin* expression.



ture conditions, we did not observe up-regulation of *Gata1* and *Klf1* mRNAs or of Ter119 proteins—markers of erythropoiesis—after NYT treatment (Fig. S3 in Supporting information). These discrepancies suggest that NYT activity may differ in normal homeostasis compared with anemic conditions. However, when we evaluated leukopoiesis, we found that culturing BM MNCs for 11 days in NYT accelerated myelopoiesis but not lymphopoiesis, based on flow cytometric and gene expression analysis (Fig. S3B in Supporting information). Previously, Okamura *et al.* reported that NYT dose dependently augmented production of GM-CSF but not G-CSF, as evaluated by ELISA analysis of human peripheral blood MNCs after 3 days of culture (Okamura *et al.* 1991). However, we did not observe expression of *GM-CSF* and *G-CSF* transcripts at days 4, 8, or 11 in $CD45^+$ cells derived from BM MNCs cultured with NYT (T. Inoue, K. Kulkeaw and K. Muennu, unpublished data). These discrepancies may be attributable to species differences or culture conditions. Miura *et al.* reported that intraperitoneal NYT administration in mice increased the number of macrophages in both the peritoneal cavity and spleen within 7 to 10 days (Miura *et al.* 1989).

Some bioactive ingredients of JTT, such as polysaccharides and fatty acids, reportedly have proliferative effect on hematopoietic cells. Polysaccharides obtained from Glycyrrhiza, a component of JTT exhibited mitogenic activity that influences the selective proliferation of B cells (Yamada & Saiki 2005). Fatty acids, such as oleic and linoleic acids, stimulate the proliferation of hematopoietic stem cells *in vitro* (Hisha *et al.* 1997). As Kampo medicines tested in this study originally include polysaccharides and fatty acids, we cannot deny the possibility that these bioactive ingredients of NYT might affect the myeloid cell proliferation from BM MNCs (Fig. 1B). It will be a topic in the future.

In summary, we provide the molecular evidence that NYT accelerates myelopoiesis *in vitro*.

Experimental procedures

Mice

C57BL/6 (2–4 months) mice were purchased from Nihon SLC (Hamamatsu, Japan) and Kyudo (Tosu, Japan). Animals were handled according to the Guidelines for Laboratory Animals of Kyushu University. This study was approved by the Animal Care and Use Committee, Kyushu University (Approval ID: A21-068-0).

Preparation of Kampo medicines

Kampo medicines including NYT, SMT, JTT, and DBT (Table S1 and Fig. S1 in Supporting information) (Tsumura & Co, Tokyo, Japan) were freshly prepared as follows. First, 0.25 g of each Kampo medicine extract powder was dissolved in 5 mL of hot distilled water (at a final concentration of 50 mg/mL). The solution was centrifuged at 2395 ***g*** for 10 min, and supernatants were filtered through 0.45-μm filters.

Primary cell culture

BM cells were harvested by flushing femurs of 2–4 months C57BL/6 mice with Iscove's Modified Dulbecco's Medium (IMDM, SIGMA-ALDRICH, St. Louis, MO) supplemented with 2% fetal bovine serum (FBS). After one PBS wash, cells were incubated with red blood cell lysis buffer (0.16 M NH_3Cl, 10 mM $KHCO_3$, 5 mM EDTA) on ice for 10 min, and then mononuclear cells (MNCs) were separated using Lympholyte-M (Density: 1.0875 + 0.0010 g/cm^3, CEDARLANE®) according to the manufacturer's instruction. BM MNCs were cultured with IMDM containing 10 μg/mL Kampo supernatant, 15% FBS, 0.1% 2-mercaptoethanol, and 10 U/mL penicillin/10 μg/mL streptomycin (SIGMA-ALDRICH). Negative controls were cultured in the same media without Kampo medicines. Cells were incubated at 37 °C, 5% CO_2, and 95% humidity. Culture media were changed at day 8. Cells were collected for counting and analysis at days 4, 8, and 11 of culture. The number of viable cells was determined using trypan blue staining. To assess the effect of Kampo medicines on $CD45^+$ leukocytes, BM MNCs were stained with anti-CD45-APC antibody (Ab) (eBioscience, San Diego, CA) and sorted using a FACS Aria cell sorter (BDIS, San Jose, CA). $CD45^+$ cells were cultured with respective Kampo medicines and noncytokine-containing medium according to methods described above.

Quantitative real-time PCR

Total RNA was extracted from cultures of BM MNCs and $CD45^+$ cells using a RiboPure™ Kit (Life Technologies, Carlsbad, CA). Total RNA was subjected to reverse transcription using a High-Capacity RNA-to-cDNA Kit (Life Technologies) according to established protocols. Gene expression levels were measured by StepOnePlus™ Real-Time PCR (Life Technologies) with TaqMan® Gene Expression Master Mix. All probes (*Myc, Gata1, Pu.1, Csf1r, Rasgrp1* and *Dok2*) were from TaqMan® Gene Expression Assays. PCR conditions were as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s (40 cycles), and extension at 72 °C for 20 s. A final dissociation stage was carried out consisting of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. β-*actin* served as internal control. Normalized values ($-2^{\Delta\Delta C_t}$, ddCT) were compared among samples, and experiments were carried out in triplicate.

Microarray analysis

BM MNCs cultured with NYT or negative controls were collected at day 11. Total RNA was extracted using RNeasy® Plus Micro Kit (QIAGEN). RNA concentration was measured by NANODROP 2000c (Thermo Fisher Scientific). cRNA was amplified, labeled, and hybridized to an Agilent Whole Mouse GE 4 × 44K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. All hybridized microarrays were scanned using an Agilent scanner, and signals of all probes were analyzed using Feature Extraction Software (9.5.1.1) (Agilent Technologies).

Flow cytometry

To analyze erythroid differentiation, cells were stained with anti-Ter119-PE Ab and anti-CD71-FITC Ab (BD Bioscience). To analyze myeloid and lymphoid differentiation, cells were stained with anti-Mac-1-PB Ab (Biolegend, San Diego, CA) and anti-B220-APC-Cy7 Ab (Biolegend). To isolate $CD45^+Ter119^-$ cells, BM MNCs were stained with anti-CD45-APC Ab (Biolegend) and anti-Ter119-PE Ab. To identify leukocytes, $CD45^+$ cultured cells were stained with anti-Mac-1-PB Ab, anti-F4/80-APC-Cy7 Ab (Biolegend) and anti-Gr-1-PE-Cy7 Ab (Biolegend).

Colony formation assay

BM MNCs were suspended in 4 mL of MethoCult® GF M3234 (Stemcell Technologies) supplemented with IL-3 (10 ng/mL), IL-6 (10 ng/mL), and SCF (50 ng/mL) and distributed into three 35-mm dishes (1×10^4 cells/dish). Cells were then incubated with 5% CO_2 at 37 °C. Colonies were counted on days 10–12 using an inverted phase-contrast microscope CKX41 (Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as means plus standard deviation (SD). Student's *t*-test was used to calculate statistical significance. A *P*-value <0.05 was considered statistically significance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1 3D HPLC pattern of Kampo medicines.

Figure S2 Hematopoietic cell proliferation in the presence of NYT and its components.

Figure S3 Effect of NYT on leukopoiesis of BM MNCs.

Figure S4 NYT accelerates hematopoietic colony formation.

Figure S5 Global changes in gene expression observed in NYT-treated BM cells.

Table S1 Components of four Kampo medicines