

Treatment of Shwachman Syndrome by Japanese Herbal Medicine (Juzen-Taiho-To): Stimulatory Effects of Its Fatty Acids on Hemopoiesis in Patients

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ABSTRACT

Juzen-taiho-to (a Japanese herbal medicine) has been traditionally administered to patients with anemia, neutropenia, or wasting syndrome. We previously attempted to isolate and purify the hemopoiesis-stimulatory components in Juzen-taiho-to extracts using an *in vitro* hemopoietic stem cell (HSC) assay method in which mouse HSCs can proliferate on a stromal cell line (MS-5). We have found that fatty acids (particularly oleic acid and linolenic acid) actively promote the proliferation of HSCs, and that the effect is mediated by stromal cells, rather than by any direct action on the HSCs.

In the present study, we show, using human normal bone marrow cells (BMCs) and umbilical cord blood

cells, that similar stimulatory effects are due to the presence of oleic acid and linolenic acid, which stimulate the proliferation of HSCs in stroma-based culture systems.

Furthermore, a marked stimulatory effect was noted on BMCs from patients with Shwachman syndrome, which shows pancreatic and bone marrow dysfunctions. We also show the data on hemopoietic recovery after the administration of Juzen-taiho-to to a patient with Shwachman syndrome. These findings suggest that decreased fatty acid levels in the blood, caused by exocrine pancreatic insufficiency, induce bone marrow dysfunction in Shwachman syndrome. *Stem Cells* 2002;20:311-319

INTRODUCTION

It has been reported that Juzen-taiho-to (TJ-48; Shi-Quan-Da-Bu-Tang in Chinese), a traditional Kampo (Japanese herbal) medicine composed of 10 different herbs, shows a

profound stimulatory effect on lymphohemopoiesis in humans and mice [1-5]. This suggests that Juzen-taiho-to contains substances that act at the level of the hemopoietic stem cell (HSC) or progenitor cell. We previously attempted to

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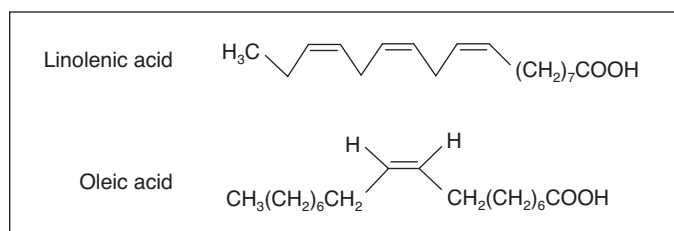


Figure 1. Chemical structures of linolenic acid and oleic acid.

isolate the HSC-stimulating substances in Juzen-taiho-to and discovered that at least some of the active substances are unsaturated fatty acids (oleic acid and linolenic acid), which are present at a concentration of 0.1% (Fig. 1) [6]. Fatty acids are commonly found in both animals and plants and are essential for the maintenance of homeostasis and cell growth: oleic acid appears to be essential for cell growth, since it can be added to a culture medium together with dipalmitoyl lecithin and cholesterol as a substitute for fetal bovine serum (FBS).

We have previously found that these standard fatty acids, obtained commercially, show a significant stimulatory effect on HSCs in both an HSC proliferation assay and a long-term bone marrow (BM) culture, and that the administration of oleic acid to mitomycin C-treated mice enhanced colony-forming unit-spleen (CFU-S) counts on day 14 to twice the control group [6]. In addition, we have found that the stimulatory effect of these fatty acids is mediated by stromal cells, rather than by any direct action on the HSCs [6].

In the present study, we investigate not only the stimulatory activity of the fatty acids on normal human HSCs, but also their effects on HSCs from patients with Shwachman syndrome. Patients with Shwachman syndrome show not only diarrhea due to exocrine pancreatic insufficiency but also neutropenia and anemia due to BM hypoplasia [7]. This syndrome is also characterized clinically by short stature, skeletal abnormalities, and various other less common findings, although dysfunctions in both the pancreas and BM are most commonly observed [8]. Up to now, why both pancreatic insufficiency and BM hypoplasia occur concurrently in this syndrome has not been well elucidated. Here, we show the stimulatory effects of the fatty acids on BM cells (BMCs) from two children with the syndrome. One of the patients potentially benefited from the therapy using Juzen-taiho-to, because neutrophil counts increased significantly after its administration or after an increase in the dose. The other patient was not administered Juzen-taiho-to, and the concentrations of fatty acids in the blood were lower than normal levels. We discuss the relationship between fatty acids and Shwachman syndrome.

MATERIALS AND METHODS

Chemical Substances

Oleic acid (NCP 53-2600-46) and linolenic acid (NCP 53-2600-62) were purchased from Funakoshi (Tokyo, Japan) (Fig. 1). Pharmaceutical preparation of Juzen-taiho-to was obtained from Tsumura & Co. (Tokyo, Japan; <http://www.tsumura.co.jp/english>). Juzen-taiho-to is made from the following ten herbs: astragali radix (3.0 g, root of *Astragalus membranaceus* Bunge), cinnamomi cortex (3.0 g, bark of *Cinnamomum cassia* Blume), rehmanniae radix (3.0 g, root of *Rehmannia glutinosa* Libosch var. *purpurea* Makino), paeoniae radix (3.0 g, rhizome of *Paeonia lactiflora* Pall), cnidii rhizoma (3.0 g, rhizome of *Cnidium officinale* Makino), atractylodis lanceae rhizoma (3.0 g, rhizome of *Atractylodes lancea* DC), angelicae radix (3.0 g, root of *Angelica acutiloba* Kitagawa), ginseng radix (3.0 g, root of *Panax ginseng* C.A. Meyer), hoelen (3.0 g, fungus of *Poria cocos* Wolf.), and glycyrrhizae radix (1.5 g, root of *Glycyrrhiza uralensis* Fisch. et DC.).

Bone Marrow Cells

After fully informed consent, human BMCs were collected from healthy volunteers or patients (Shwachman syndrome, aplastic anemia, and myelodysplastic syndrome [MDS]) and used for the present study.

BMCs from two patients with Shwachman syndrome (Patient No. 1 [Kansai Medical University Hospital] and Patient No. 2 [Mie University Hospital]) were examined in this study. These patients fulfilled the criteria for Shwachman syndrome: exocrine pancreatic dysfunction, pancytopenia, and clinical features commonly associated with this syndrome (diarrhea, growth failure, etc.). Table 1 shows their clinical data.

BMCs of a patient with aplastic anemia (22 years old, Kansai Medical University Hospital) were examined in methylcellulose assay. He showed severe pancytopenia (RBCs: $198 \times 10^4/\mu\text{l}$; WBCs: $2,000/\mu\text{l}$; neutrophil count: $700/\mu\text{l}$; hemoglobin: 7 g/dl; and platelet count: $1.9 \times 10^4/\mu\text{l}$). Bone marrow cellularity was as follows: total mononuclear cells (MNCs): $3,370/\mu\text{l}$; myeloid cells: 40.8%; erythroid cells: 27.0%; and lymphoid cells: 27.8%. Hypoplasia in megakaryoid-lineage cells was observed in his BM. He was treated with steroid pulse therapy and multiple blood transfusions.

BMCs of a patient with MDS (54 years old, Kansai Medical University Hospital) were used in long-term culture assay (data not shown). Her blood cell counts were as follows: RBCs: $146 \times 10^4/\mu\text{l}$; WBCs: $1,700/\mu\text{l}$; neutrophils: $816/\mu\text{l}$; hemoglobin: 5.5 g/dl; and platelets: $14.1 \times 10^4/\mu\text{l}$. Analyses of BM cellularity showed that only erythroid lineage cells were hyperplastic, but myeloid- and megakaryoid-lineage

Table 1. Clinical features in patients with Shwachman syndrome

	Patient No. 1	Patient No. 2	Normal
Sex	Female	Male	
Age	7 years	8 years	
Clinical presentation	Pancreatic insufficiency Pancytopenia Diarrhea Growth failure	Pancreatic insufficiency Pancytopenia Diarrhea Growth failure	
Blood			
RBCs	309 × 10 ⁴ /μl ↓	379 × 10 ⁴ /μl	380-550
WBCs	2,800/μl ↓	3,100/μl ↓	4,000-9,000
(Neutrophils)	(960/μl) ↓	(1,100/μl) ↓	
Hemoglobin	10.0 g/dl ↓	11.5 g/dl ↓	12-18
Platelet	6.0 × 10 ⁴ /μl ↓	9.7 × 10 ⁴ /μl ↓	15-35
Serum			
Trypsin	Not detected ↓	Not done	100-400
Amylase	7 IU/l ↓	61 IU/l	42-125
Lipase	5 IU/l ↓	15 IU/l ↓	11-53
Free fatty acid	1.14 mEq/l (0.18 mEq/l*)	0.08 mEq/l ↓	0.14-0.85
Triglyceride	52 mg/dl	25 mg/dl ↓	36-160
Phospholipid	Not done	126 mg/dl ↓	140-240
Cholesterol	Not done	105 mg/dl ↓	130-240
Bone marrow			
Total MNCs	12.0 × 10 ⁴ /μl	35.2 × 10 ⁴ /μl	10-25 × 10 ⁴
Myeloid	39.0% ↓	20.0% ↓	43-53
Erythroid	21.0%	42.6% ↑	14-25
Lymphoid	40.0% ↑	37.4% ↑	15-25
Treatment	Administration of Juzen-taiho-to and pancreatic enzymes	Administration of pancreatic enzymes and digestive enzymes	

*Before administration of Juzen-taiho-to (1 year and 10 months old)

cells were hypoplastic: total MNCs: 11.9 × 10⁴/μl; myeloid cells: 31.6%; erythroid cells: 53.2%; and lymphoid cells: 11.2%. She received multiple blood transfusions.

Cell Preparations

In some experiments, lineage-negative cells (Lin⁻ cells) were purified from whole BMCs (WBMCs). Briefly, mononuclear cells (low-density [LD] cells) were separated from the BM cell suspensions by Lymphoprep ($p = 1.077$; Nycomed Pharma; Oslo, Norway; <http://www.nycomed-amersham.com>) density gradient centrifugation. The LD cells were then incubated with monoclonal antibodies (mAbs) (mouse anti-human CD3, CD11b, CD14, CD19, CD56, glycophorin A; PharMingen; San Diego, CA; <http://www.bd-biosciences.com/pharmingen>) and removed using magnetic beads (Dynabeads M-450 SH/MS IgG) to obtain the Lin⁻ cells.

Lin⁻ cells were incubated with microbead-conjugated anti-CD34 class II mAb (Miltenyi Biotec; Bergisch Gladbach, Germany; <http://www.miltenyibiotec.com>) at 4°C for 20 minutes and processed through a MiniMACS

magnetic separation column (Miltenyi Biotec) to obtain a CD34⁺-cell-enriched fraction (CD34⁺ cells).

Human umbilical cord blood was collected and suspended in ACD (acid-citrate-dextrose) according to the guidelines of the Cord Blood Bank, Kansai Medical University. MNCs were separated from the ACD solutions by Lymphoprep density gradient centrifugation. Lin⁻ cells and CD34⁺ cells were obtained from the MNCs as described above.

Methylcellulose Assay

The colony-forming abilities of WBMCs, LD cells, Lin⁻ cells, and CD34⁺ cells were assessed in the methylcellulose assay. Appropriate numbers of these cells were plated in 12-well plates (Flow Laboratories, Inc.; Aurora, OH) in a volume of 1 ml MethoCult GF H4434 (StemCell Technologies Inc.; Vancouver, BC, Canada), consisting of optimal concentrations of cytokines (recombinant human stem cell factor [rhSCF], erythropoietin [EPO], interleukin-3 [IL-3], GM-CSF, and G-CSF), 30% FBS, 1% bovine serum albumin, 2 mM L-glutamine, 100 μM 2-mercaptoethanol (2-ME), and

0.9% methylcellulose. When normal human BMCs were cultured in this assay system, colonies composed of multilineage cells were formed in addition to single-lineage colonies. Standard fatty acids were added to the MethoCult in various concentrations. These substances were dissolved in MeOH/dimethyl sulfoxide (DMSO) (1:1, vol/vol) and were appropriately diluted in culture medium. In all the cultures, the final concentration of the solvent was less than 0.025%, a concentration that does not affect cell proliferation. The plates were incubated for 14-20 days at 37°C in 5% CO₂, and the numbers of colonies were counted under an inverted microscope. The average number of colonies and standard error were calculated from triplicated wells.

HSC-Proliferation Assay

Lin⁻ cells were cultured on MS-5 (a mouse stromal cell line derived from C3H mouse BMCs) [9], which had been preincubated with standard fatty acids. Their proliferation was then measured using a method similar to that reported previously [6]. Briefly, the MS-5 (in 96-well plates) was preincubated with various concentrations of standard fatty acids for 2 days, at which point it had become confluent. The cell line was then irradiated with 20 Gy, and the culture medium was removed. The cells were then washed twice, and the medium was replaced with fresh Iscove's modified Dulbecco's medium (IMDM) (GIBCO; Grand Island, NY; <http://www.lifetech.com>) supplemented with 10% FBS (lot No. HCC 6450, StemCell Technologies Inc.) and 10% horse serum (HS; lot No. HCC 6750) and 50 μM 2-ME (in triplicate). Low concentrations of human cytokines (IL-3, IL-6, G-CSF, SCF: 0.5 ng/ml, and FLT-3 ligand: 5 ng/ml) were added to the culture medium because the MS-5 cell line is derived from mice. Two thousand cells per well (normal BMCs) or 3,000 cells per well (Shwachman BMCs) were then added to the thus-treated MS-5. As a control, the Lin⁻ cells were cultured on the MS-5 preincubated in culture medium containing solvent alone. These cells were cultured for 18 days (normal BMCs) or 20 days (Shwachman BMCs), after which ³H-thymidine (³H-TdR) was introduced into each well (18.5 kBq/well). After 20 to 24 hours, the cells were harvested, and the incorporation of ³H-TdR was measured using Matrix[™] 96 (Packard; Meriden, CT; <http://www.packardbio.com>). The stimulation indices of samples (standard fatty acids) were expressed as ratios to the control: ³H-TdR incorporation on the sample well/that on the control well. We considered that the test sample showed a stimulatory activity when the value was more than 1.3.

Long-Term Culture

The MS-5 was preincubated with 1 or 5 μg/μl of oleic acid or linolenic acid for 3 days, irradiated (20 Gy), and

then washed twice with medium before the inoculation of Lin⁻ cells. The Lin⁻ cells (8 × 10³-10⁵ cells/25 cm² flask) were suspended in IMDM supplemented with 10% FBS, 10% HS, and 1 μM hydrocortisone (in triplicate). Low concentrations of human cytokines (IL-3, IL-6, G-CSF, SCF, LIF: 0.5 ng/ml, and FLT-3 ligand: 5 ng/ml) were added to the culture medium. The culture medium of these flasks was replaced with fresh medium at 1-week intervals, and the numbers of nonadherent cells and cobblestone colonies per flask were counted. The nonadherent cells were then collected and used for methylcellulose assays (MethoCult GF H4434).

RESULTS

Stimulatory Effects of Fatty Acids on Hemopoiesis of Normal Human BMCs

The stimulatory effect of linolenic or oleic acid (Fig. 1) on hemopoietic colony formation of normal BMCs was examined in a semisolid culture system: MethoCult GF H4434. The two fatty acids had no stimulatory effect on WBMCs, Lin⁻ cells (HSC-enriched fraction), or CD34⁺ cells (Fig. 2). However, when Lin⁻ cells were incubated in a stroma-based culture system (the cells were cultured on MS-5 [a stromal cell line] preincubated with the fatty acids), the fatty acids had a stimulatory effect on HSC proliferation (Fig. 3). A higher stimulation index was obtained when the MS-5 was preincubated in the presence of both linolenic acid and oleic acid. These fatty acids also showed the capacity to simulate hemopoiesis on a long-term culture system of Lin⁻ cells in which the cells were cultured on MS-5 preincubated with the fatty acids (Fig. 4). Greater numbers of cobblestone colonies, nonadherent cells, and CFU cultures (CFU-C) were observed, and these were more evident at the concentration of 5 μg/ml. These observations are consistent with the findings in the murine system that the fatty acids act on the proliferation of pluripotent-hematopoietic stem cells (P-HSCs) through stromal cells, rather than direct interaction, as we previously reported [6].

When Lin⁻ and CD34⁺ cells from umbilical cord blood were cultured in a semisolid culture system (MethoCult GF H4434) with linolenic acid or oleic acid, there was no stimulatory effect, as was the case with BMCs (data not shown). However, in a feeder layer (MS-5) culture system, in which the cells were preincubated with these fatty acids, the proliferation of HSCs was enhanced: cobblestone colonies by 1.4 times and CFU-C by 1.5 times the control at 3 weeks of culture.

Clinical Outcome after Administration of Juzen-Taiho-To to a Patient with Shwachman Syndrome

Patient No. 1, hospitalized in Kansai Medical University Hospital, was diagnosed as having Shwachman syndrome at

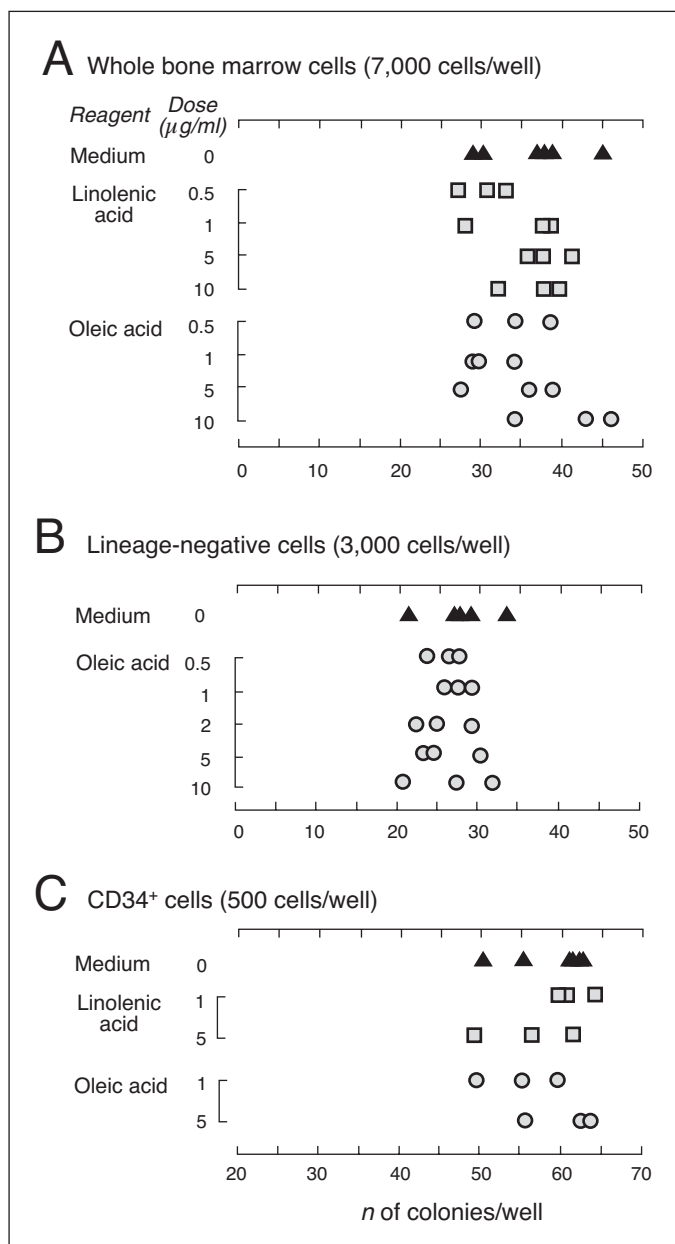


Figure 2. No effect of fatty acid on colony formation in methylcellulose assay of **A)** whole BMCs; **B)** lineage-negative cells, and **C)** CD34⁺ cells (HSCs) from a normal donor. Normal BMCs were cultured in methylcellulose containing various cytokines (EPO, SCF, GM-CSF, IL-3, and G-CSF), with or without linolenic or oleic acid. Fourteen days later, the colonies (composed of >50 cells) were counted.

the age of 1.5 years. The diagnosis was based on the presence of pancytopenia and diarrhea. Hypoplasia in myeloid-lineage cells was also observed in her BM. When she showed severe pancytopenia (neutrophils: 280/ μl ; platelets: $4.7 \times 10^4/\mu\text{l}$; and hemoglobin: 8 g/dl) at the age of 1 year and 10 months, the oral administration of Juzen-taiho-to (2.5 g/day) was started in addition to treatment with pancreatic enzymes (Fig. 5). Three months later, increases in her neutrophil counts and

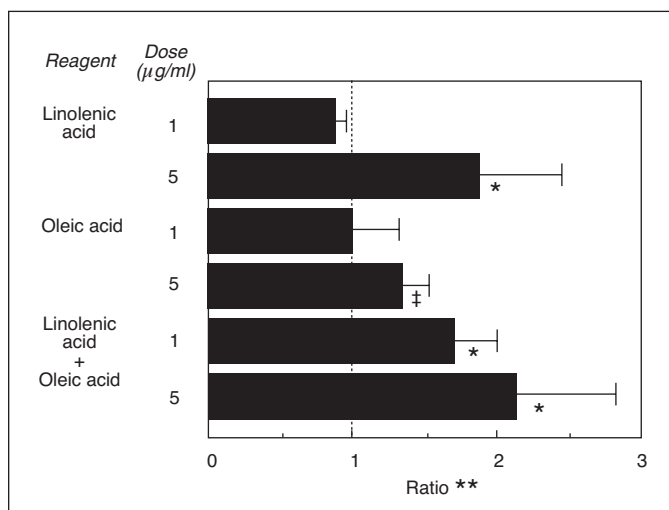


Figure 3. Stimulatory effects of fatty acids on normal BMCs in HSC-proliferation assay. MS-5 were preincubated for 2 days with various concentrations of fatty acids, and Lir⁻ cells were cocultured on the thus-treated MS-5 for 18 days. The cultured cells were pulsed with ³H-TdR for the last 20 hours of the culture period, and ³H-TdR uptake was counted. The stimulation indices of samples (fatty acids) were expressed as ratios to the control. We consider that the test sample showed a stimulatory activity when the value was more than 1.3. **Ratio = ³H-TdR incorporation on sample well/³H-TdR incorporation on control well. *Significantly different from control ($p < 0.05$). ‡Not significant ($p > 0.05$).

hematocrit values were observed, and stable hemopoiesis was achieved without severe infections (neutrophils: 500-1,400/ μl ; platelets: $5-10 \times 10^4/\mu\text{l}$; and hemoglobin: 11-13 g/dl). However, the pancytopenia (neutrophils: 744/ μl ; platelets: $5.4 \times 10^4/\mu\text{l}$; and hemoglobin: 5.3 g/dl) recurred without any obvious cause at the age of 5 years and 5 months. The symptoms were ameliorated with an increase in the dose of Juzen-taiho-to (2.5 to 7.5 g/day), and marked increases in neutrophil counts and hemoglobin were thereafter observed.

Table 1 shows the clinical data for Patient No. 1 and Patient No. 2 (Mie University Hospital), who were diagnosed as having Shwachman syndrome. Both show exocrine pancreatic dysfunction and pancytopenia. The pancreatic enzyme levels were low in their sera. The notable difference between these patients was the levels of free fatty acids.

Marked Stimulatory Effects of Fatty Acids on BMCs from Patients with Shwachman Syndrome

When linolenic acid was added to the methylcellulose assay (MethoCult GF H4434) of LD cells from Patient No. 1 (6 years and 11 months old), an increase in the colony counts was observed (Fig. 6, Exp. 1). However, there was no stimulatory effect on LD cells from the patient with aplastic anemia. As shown in Exp. 2 of Figure 6, a significant increase in colony formation was observed in Patient No. 2 (8 years and 7 months old) after the addition of fatty

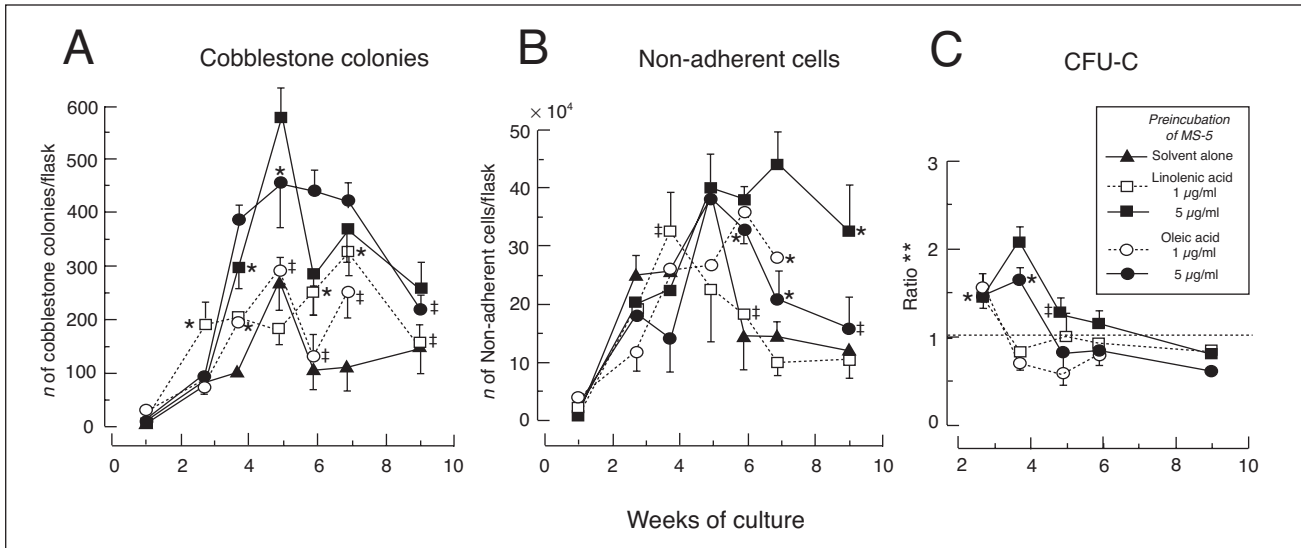


Figure 4. Stimulatory effects of fatty acids on normal BMCs in long-term cultures. Lin^- cells were cultured on MS-5 preincubated with fatty acids. Numbers of cobblestone colonies per flask were counted every week. Nonadherent cells were collected and incubated in methylcellulose containing human cytokines to obtain the number of progenitor cells (CFU-C). **Ratio = number of CFU-C per 10^4 cells collected from sample well/number of CFU-C per 10^4 cells collected from control well. *Significantly different from control ($p < 0.05$). #Not significant ($p > 0.05$).

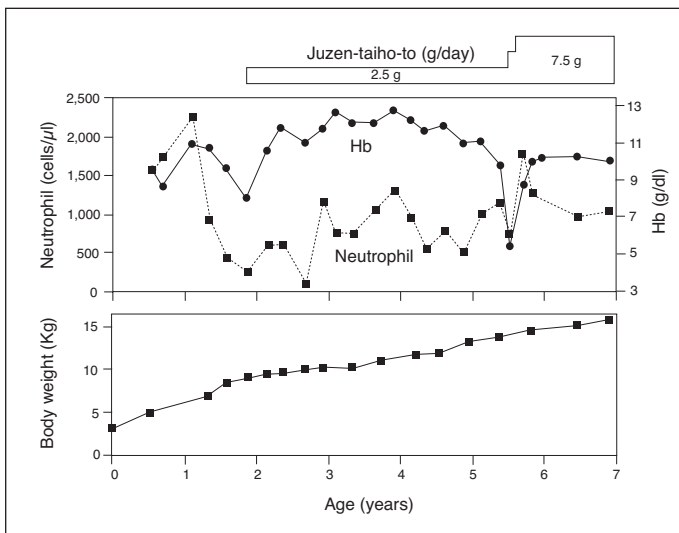


Figure 5. Hemopoietic recovery by administration of Juzen-taiho-to to Patient No. 1 with Shwachman syndrome.

acids. The colony counts obtained in Patient No. 2 (about $10/10^4$ LD cells) were higher than those in Patient No. 1, but were still one-tenth that of the normal control. These colonies were composed of granulocytes and/or macrophages, and no BFU-E or megakaryocytes were detected. The colony size was also smaller than normal BMCs.

As shown in Table 1, the concentrations of free fatty acids in the blood of Patient No. 2 were lower than normal levels, whereas the concentrations of free fatty acids in Patient No. 1

were higher than normal, probably because of the administration of Juzen-taiho-to. Therefore, we expected that the stimulatory effect of fatty acids might be more evident in this patient than in Patient No. 1. Lin^- cells from Patient No. 2 were examined to determine whether their proliferation was stimulated by the addition of fatty acids in stroma-based culture systems (HSC-proliferation assay and long-term culture).

As shown in Figure 7, a stimulation index of over 1.5 was obtained when the MS-5 was preincubated with 5 $\mu\text{g/ml}$ of linolenic acid. In contrast to normal BMCs, no additional effect was observed even if both linolenic acid and oleic acid were added to the preincubation of MS-5.

When Lin^- cells from Patient No. 2 were cultured with MS-5 pretreated with the fatty acids in long-term culture, the numbers of cobblestone colonies, nonadherent cells, and CFU-C significantly increased (Fig. 8). The stimulatory effect of linolenic acid was observed for up to 10 weeks of culture, whereas that of oleic acid was more evident after 10 weeks.

DISCUSSION

The link between the pancreatic dysfunction and BM hypoplasia observed in Shwachman syndrome remains unknown, although more than 35 years have passed since the first report by Shwachman [7]. There are, however, several hypotheses. First, several genes that control both the functions of the pancreas and hemopoiesis are shared or exist near each other. Therefore, a mutation or deletion of one locus, caused by chromosome breakage [10] or reciprocal translocation [11], elicits both dysfunctions. Second,

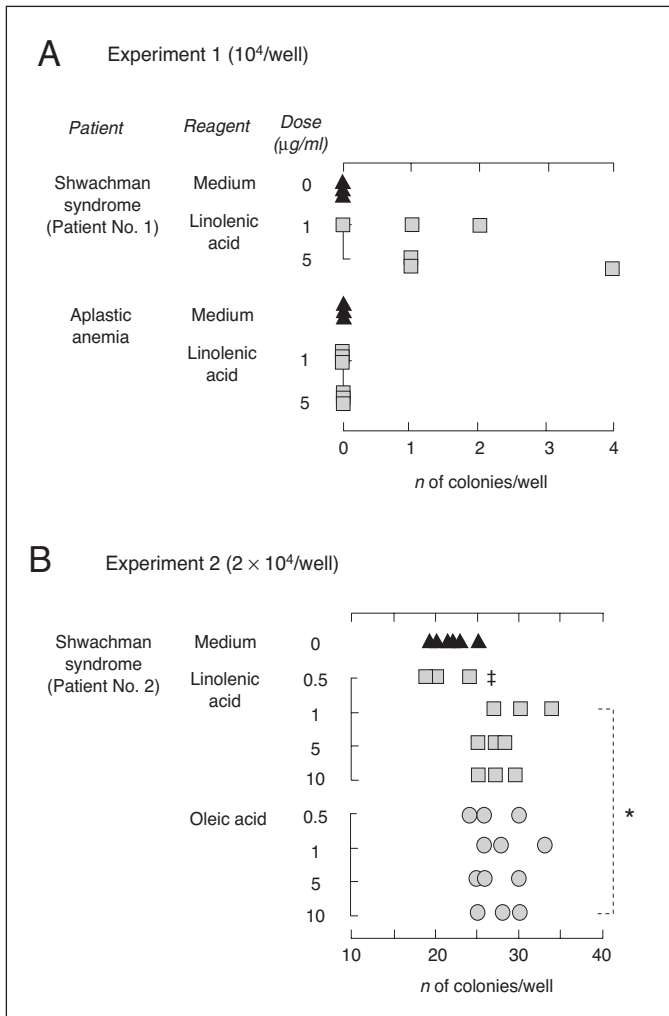


Figure 6. Stimulatory effects of fatty acid on colony formation in methylcellulose assay of BMCs from patients with Shwachman syndrome. LD cells were cultured in methylcellulose containing various cytokines (EPO, SCF, GM-CSF, IL-3, and G-CSF), with or without linolenic or oleic acid ($1-2 \times 10^4$ /well). Twenty days later, the colonies were counted. *Significantly different from control ($p < 0.05$). †Not significant ($p > 0.05$).

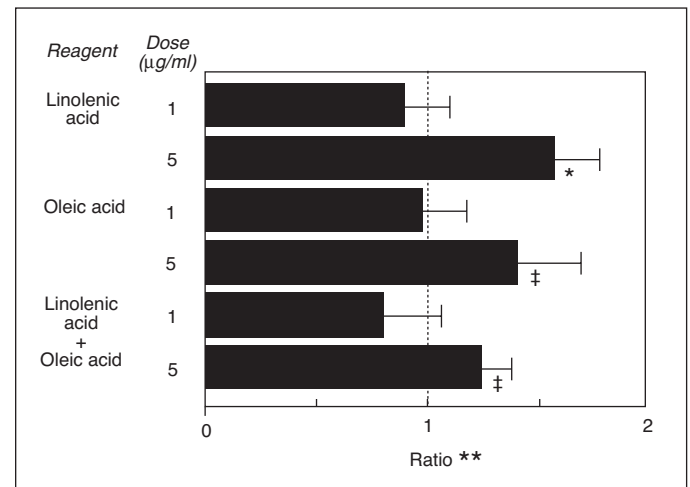


Figure 7. Stimulatory effects of fatty acids on BMCs from Patient No. 2 with Shwachman syndrome in HSC-proliferation assay. MS-5 were preincubated for 2 days with various concentrations of fatty acids, and Lin^- cells were cocultured on the thus-treated MS-5 for 20 days. The incubated cells were pulsed with $^3\text{H-TdR}$ for the last 24 hours of the culture period, and $^3\text{H-TdR}$ uptake was counted. The stimulation indices of samples (fatty acids) were expressed as ratios to the control. We considered that the test sample showed a stimulatory activity when the value was more than 1.3. **Ratio = $^3\text{H-TdR}$ incorporation on sample well/ $^3\text{H-TdR}$ incorporation on control well. *Significantly different from control ($p < 0.05$). †Not significant ($p > 0.05$).

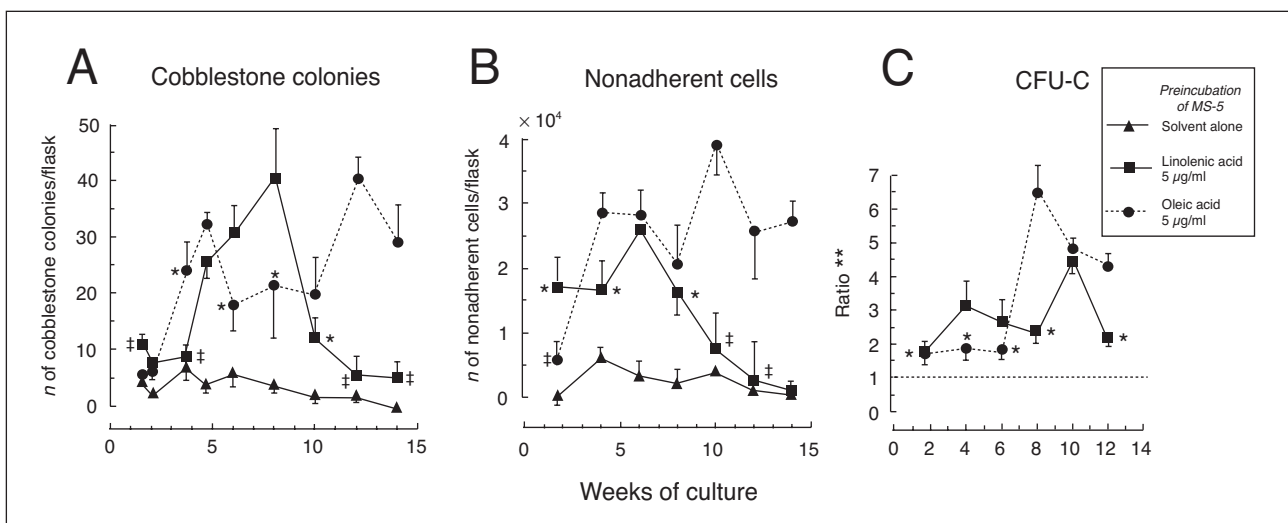


Figure 8. Marked stimulatory effects of fatty acids on long-term cultures of BMCs from Patient No. 2 with Shwachman syndrome. Lin^- cells were cultured on MS-5 preincubated with fatty acids. Numbers of cobblestone colonies per flask were counted every week. Nonadherent cells were collected and incubated in methylcellulose containing human cytokines to obtain the number of progenitor cells (CFU-C). **Ratio = number of CFU-C per 10^4 cells collected from sample well/number of CFU-C per 10^4 cells collected from control well. *Significantly different from control ($p < 0.05$). †Not significant ($p > 0.05$).

copper deficiency in the newborn elicits this syndrome, because a similar syndrome can be induced in rats by administering food lacking copper [12]. To date, there are about 50 reports concerning Shwachman syndrome, and in some cases, exocrine pancreatic insufficiency and diarrhea are ameliorated as the patients grow older, though neutropenia remains until adulthood [8]. Several reports show that Shwachman syndrome gradually shifts to acute myelogenous leukemia and MDS [8, 13]. Recently, Klupp *et al.* reported that the percentage of abnormal immature B cells increased to 40% of total BMCs in a patient with fatal Shwachman syndrome [14]. Suda *et al.* demonstrated that there were no inhibitory factors for colony formation in the blood of a patient with Shwachman syndrome [15]. Production of colony-stimulating activity from peripheral blood leukocytes appeared normal in this syndrome [16]. Thus, it seems that BM dysfunction is caused by disorders at the hemopoietic stem and/or progenitor cell levels, but not at the humoral factor levels.

The general treatment for Shwachman syndrome is the administration of pancreatic enzymes, such as pancreatin. In some cases, G-CSF is administered for severe neutropenia and infections, although its effect is short lived [17, 18]. In the present study, we found, for the first time to our knowledge, that Juzen-taiho-to can induce marked hemopoietic recovery in a patient with Shwachman syndrome, as shown in Figure 5. Pancreatic enzymes were also administered to Patient No. 1, and the dose was increased gradually to the maximum level. However, the possibility that pancreatic enzymes have some effect on hemopoiesis can be ruled out, since there was no change in the dose of these enzymes after the administration of Juzen-taiho-to. Table 1 shows that the blood cell count and BM cellularity were similar in both patients, although Patient No. 1 received Juzen-taiho-to but Patient No. 2 did not. Shwachman syndrome is a heterogeneous disorder, and indeed, Patient No. 1 has twice suffered severe pancytopenia (Fig. 5). Her hematological condition might, therefore, deteriorate without the administration of Juzen-taiho-to. In contrast, Patient No. 2 is not in such serious condition although he receives only pancreatic and digestive enzymes. However, the *in vitro* culture data of Patient No. 2 (Figs. 6-8) indicate the possibility that the administration of Juzen-taiho-to might greatly ameliorate the pancytopenia. These results indicate that Juzen-taiho-to can be administered to patients with Shwachman syndrome as a safe and effective drug, although it seems unlikely that this drug would be effective in all cases.

As shown in Figure 8, a marked stimulatory effect of fatty acids was observed when the BMCs of Patient No. 2 were cultured long term on MS-5 preincubated with the fatty acids. Therefore, it is conceivable that low levels of fatty acids in the patient's blood, caused by pancreatic insufficiency, exacerbated the BM hypoplasia in Shwachman syndrome. However,

the possibility that BM dysfunction is induced by the low blood levels of fatty acids cannot be ruled out in some cases of Shwachman syndrome, as in Patient No. 1, in whom the initial pancreatic dysfunction advanced to BM dysfunction. Low blood levels of fatty acids in neonates may cause profound damage to hemopoiesis, which cannot be recovered even when fatty acids are subsequently supplied by the administration of pancreatic enzymes, etc. It is important to examine the stimulatory effects of fatty acids on BMCs before and after treatments with Juzen-taiho-to. However, as we did not know the stimulatory effects of fatty acids on hemopoiesis before the administration of Juzen-taiho-to, we had not performed any experiments to examine the stimulatory effects of the fatty acids using her BMCs. The present findings may give us important clues about the relationship between fatty acids and hemopoiesis.

Linolenic acid stimulated the hemopoietic colony formation of BMCs from Patients No. 1 and 2, but not from a patient with aplastic anemia (Fig. 6). Moreover, no stimulatory effects were observed when BMCs from a patient with MDS were cultured for 7 weeks on MS-5 treated with 5 μ g/ml of linolenic acid or oleic acid (data not shown). Thus, these fatty acids cannot ameliorate hemopoietic diseases other than Shwachman syndrome, suggesting that it is indeed these fatty acids that are responsible for the hemopoietic dysfunction in Shwachman syndrome.

Fatty acids in blood are divided into two groups: esterified fatty acids (triglycerides, phospholipids, and cholesterol) and nonesterified (free) fatty acids. The clinical data of Patient No. 2 (Table 1) show that the levels of the free fatty acids were markedly lower than normal in contrast to the esterified fatty acids. Triglycerides serve mainly as storage for lipids, phospholipids serve as cell-surface-active substances, and cholesterol serve as precursors of cell surface substances and hormones. We believe that most of the fatty acids added to the culture medium in the present experiments exist as free fatty acids (binding to serum albumin), not as esterified fatty acids. Free fatty acids may contribute more to the stimulation of hemopoiesis than esterified fatty acids.

The stimulatory effects of these fatty acids were also observed in the case of normal BMCs (Figs. 3 and 4) and umbilical cord blood cells in the stroma-based culture systems, although the concentrations and time courses at which they were effective differed greatly between the BMCs of normal and Shwachman syndrome patients (compare Fig. 3 with Fig. 7 and Fig. 4 with Fig. 8). Our previous study has shown that the incubation of MS-5 with oleic acid or linolenic acid increases the expression of adhesion molecules (CD54: ICAM-1 and CD106: vascular cell adhesion molecule 1) and major histocompatibility complex class I as well as II antigens on the MS-5 [6]. Linolenic acid

is known as an essential fatty acid, and oleic acid is a fatty acid commonly found in both animals and plants. There are many papers showing their widespread activities on various cells, such as mouse neuroblastoma cells [19] and neonatal rat cardiac monocytes [20]. Recently, it has been shown that oleic acid induces an intracellular release of Ca^{2+} in human platelets [21]. Diepoxides of linolenic acid are found to be chemoattractants of human neutrophils [22]. Thus, these acids act as intra- and intercellular modifiers, indicating that they are very important for cell growth and the maintenance of homeostasis.

There is a possibility that other substances contained in Juzen-taiho-to stimulate the proliferation of HSCs and accelerate the hemopoiesis-stimulatory activity of the fatty

acids. Indeed, we find a fraction of acidic polysaccharides shows a stimulatory activity on HSC proliferation (data not shown). Experiments to purify and identify such substances are now in progress.

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**Treatment of Shwachman Syndrome by Japanese Herbal Medicine
(Juzen-Taiho-To): Stimulatory Effects of Its Fatty Acids on Hemopoiesis in
Patients**

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