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Research Paper

Decreased Proliferation and Erythroid Differentiation of K562 Cells by siRNA-induced Depression of OCTN1 (SLC22A4) Transporter Gene

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Purpose. Recently, it was reported that OCTN1 transporter (SLC22A4) is associated with rheumatoid arthritis (RA) and Crohn's disease. Additionally, we reported that OCTN1 is expressed in hematopoietic cells, preferentially in erythroid cells. Accordingly, we assessed the physiological role of OCTN1 by examining the effect of knockdown of *OCTN1* in blood cells using siRNA method.
Materials and Methods. Vector-based short hairpin RNA (shRNA) was used to establish K562 cell line which shows stably decreased expression of OCTN1. The characteristic of knockdown of *OCTN1* in K562 cells was investigated by cell proliferation, cell differentiation, and uptake of ergothioneine that is a good substrate of OCTN1.
Results. Several clones of K562 cells exhibited significantly reduced expression of *OCTN1* mRNA and protein. They also showed a decreased growth rate and butyrate-dependent differentiation to erythrocytes compared with control-vector transfected cells. In addition, uptake of [^{3}H]ergothioneine by K562 cells suggested that Na^+-dependent and high-affinity transporter which is similar to the characteristics of OCTN1 is functional. Moreover, uptake of ergothioneine by K562 cells which exhibit decreased-expression of OCTN1 was decreased in comparison with wild type K562 cells.
Conclusions. It was suggested that OCTN1 is involved in the transport of physiological compounds that are important for cell proliferation and erythroid differentiation.

KEY WORDS: ergothioneine; erythrocyte; K562; OCTN1; siRNA; transporter.

INTRODUCTION

Organic cation/carnitine transporter, OCTN1 (SLC22A4) shows a relatively broad tissue distribution and was firstly found as a pH-dependent organic cation transporter (1,2). The OCTN family consists of three members, that are OCTN1 and OCTN2 (SLC22A5) in mice, rats, and humans, and octn3 in mice. Among them, OCTN2 and octn3 exhibited predominant carnitine transport activity, while OCTN1 had relatively low carnitine transport activity, suggesting a different physiological role for OCTN1 (3–5). OCTN1 is located on the apical membrane of renal proximal tubular epithelial cells and has been suggested to be involved in the tubular secretion of cationic compounds (6), since OCTN1-mediated transport of organic cations was very similar to those of a proton/organic cation exchanger transport system observed in the renal apical membranes (1,2,6). Furthermore, since human OCTN1 is expressed in bone marrow and fetal liver but not in the adult liver, it was suggested that OCTN1 might be related to the differentiation and/or growth of blood cells (1). Based on characteristic tissue expression profile, we reported that OCTN1 is expressed preferentially in erythroid cells (7), whereas its physiological role remains to be clarified.

Recently, it was reported that OCTN1 is associated with chronic inflammatory diseases such as rheumatoid arthritis (RA) (8) and Crohn disease (9,10). The expression of OCTN1 is specific to hematological and immunological tissues and OCTN1 is also highly expressed in inflammatory joints of mice with collagen-induced arthritis (8). Moreover, ergothioneine was recently reported to be a good substrate of OCTN1 (11), and ergothioneine exhibited stimulatory effects on proliferation of OCTN1 expressing cells (12). Ergothioneine is a unique naturally occurring antioxidant that is abundant in most plants and animals. Ergothioneine cannot be synthesized by human and therefore is available only from dietary sources (13). It has been shown that ergothioneine

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ABBREVIATIONS: ET, ergothioneine; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NMG, *N*-methyl-D-glucamine; OCTN, organic cation/carnitine transporter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RA, rheumatoid arthritis.

plays a dual role both energy regulation and protecting cells from oxidative damage (14). On the other hand, we recently reported that L-arginine, which is a substrate of cationic amino acid transporter 1 (CAT1), is essential for the differentiation to erythrocyte by *in vitro* cell culture study (15) and the inhibition of CAT1 caused altered cell growth and differentiation (16). Therefore, it was thought that the small molecules such as vitamins, nucleosides and nucleobases, amino acids, and others should be controlled well by selective transporters to maintain normal cell function and the alteration of those transporter activities should lead to the abnormal differentiation of hematopoietic stem cells to matured cells.

Accordingly, in the present study we assessed the physiological role of OCTN1 by examining the effect of knockdown of *OCTN1* in blood cells using siRNA method.

MATERIALS AND METHODS

Chemicals

[^{3}H]Ergothioneine (3.7 GBq/mmol, custome made) was purchased from Moravec Biochemicals (Brea, CA). Unlabeled ergothioneine and other reagents for cell culture and transport experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO).

Cell Culture

K562 cells (human chronic myelogenous leukemia, American Type Culture Collection) were grown in RPMI1640 medium containing 10% (v/v) fetal bovine serum (FBS) (Invirogen, Carlsbad, CA), 5% CO_2 supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin. In testing differentiation to erythrocyte, chemical differentiating agent sodium butyrate (1 mM) was added to the culture medium (15).

Construction of Short Hairpin RNA Expression Plasmids

Three short hairpin RNA (shRNA) targeted to cDNA sequences corresponding to positions 675–695, 1,355–1,375, and 1,765–1,785 of the sequence of human *OCTN1* (Genbank accession number NM_003059; ORF region 166–1,821) were selected and BLAST-confirmed for specificity. Synthetic forward and reverse 62-nucleotide oligonucleotides (N510 sense, 5′-CACC**GAACGTTCTCTTCGCAACCAT***GTGTGCTG TCC***ATGGTTGCGAAGAGAACGTTC**TTTTT-3′; N510 antisense, 5′-GCATAAAAA**GAACGTTCTCTTCGCAAC CAT***GGACAGCACAC***ATGGTTGCGAAGAGAAC GTTC**-3′; N1190 sense, 5′-CACC**GGCGTTATATCAT AGCTGCAG***GTGTGCTGTCC***CTGCAGCTATGATAT AACGCC**TTTTT-3′; N1190 antisense, 5′-GCATAAA AA**GGCGTTATATCATAGCTGCAG***GGACAGCA CAC***CTGCAGCTATGATATAAC GCC**-3′; N1600 sense, 5′-CACC**ACAAGAGACTCAATGGAGACA***GTGTGC TGTCC***TGTCTCCATTGAGTCTCTTGT**TTTTT-3′; N1600 antisense, 5′-GCATAAAAA **ACAAGAGACTCAATGGA GACA***GGACAGCACAC***TGTCTCCATTGAGTCTC TTGT**-3′; bold, target sequence; italic, hairpin loop region) were annealed respectively and ligated to a large *Bsp*MI fragment of phU6/Zeo. The phU6/Zeo, piGENE hU6 (iGENE, Tsukuba, Japan) derivative contains a human U6 RNA polymerase III promoter and Zeocin resistance gene.

Establishment of OCTN1-Knockdown K562 Cell Line and OCTN1-Expressing HEK293 Cell Line

To generate stable knockdown transfectants, K562 cells (2×10^6) were electroporated with shRNA expression vector DNAs as described above by a Cell-Porator (Invitrogen) at 880 μF, 250 V, and selected with 100 μg/ml Zeocin (Invitrogen). Single clones were transferred to 96-well plates and grown until confluent and transferred to 10 cm cell plates. Cell growth was assessed by counting the number of live cells using the trypan-blue dye exclusion.

HEK293 cells were transfected with *OCTN1* (accession number AB007448) using plasmid vector pcDNA3 (Invitrogen) (pcDNA3/*OCTN1*) to establish stably OCTN1-expressing cells according to the calcium phosphate precipitation method (17). After 3 weeks of G418 selection (600 μg/ml), single colonies were screened for *OCTN1* expression by a polymerase chain reaction method and also for [^{3}H]ergothioneine transport activity. Transfected HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% FBS, 100 u/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 in a humidified incubator at 37°C under 5% CO_2.

K562 cells and HEK293 cells transfected with vector alone were used to obtain the background activity (termed MOCK).

Real-time Reverse Transcription Polymerase Chain Reaction

Total RNA was prepared from K562 cells using ISOGEN (Nippon Gene, Toyama, Japan). The total RNA content was determined by measuring the absorbance at 260 nm. mRNA level was analyzed using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Single-strand cDNAs were constructed using an oligo(dT) primer (Invitrogen) and Improm-II™ reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed using TaqMan Universal PCR Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA), used according to the manufacturer's instructions. The quantity of OCTN1 mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Amplification and detection were performed using Mx3000p Real-Time PCR system (Stratagene, Cedar Creek, TX) with the following profile: 1 cycle at 95°C for 10 min, 50 cycles each at 95°C for 15 s and 60°C for 1 min.

Western Blot Analysis

For Western blot analysis, rabbit polyclonal antibody against a synthetic polypeptide corresponding to amino acid residues 527-545 of human OCTN1 (1), and anti-Na^+/K^+ ATPase a-1 antibody (Upstate, Lake Placid, NY) were used. The cells were harvested and cell membrane was prepared as

described previously (18). Each sample (15 μg of proteins) was separated by 10% polyacrylamide gel electrophoresis, and proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane, Immobilon P (Millipore, Bedford, MA). The membrane was incubated with an anti-human OCTN1 antibody or anti-Na^+/K^+ ATPase a-1 antibody at a dilution of 1:10 or 1:1,000, respectively. It was then reacted with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Ig)G or anti-mouse IgG (GE Healthcare, Little Chalfont, UK) at a dilution of 1:10,000, and signals were visualized using the enhanced chemiluminescence detection method with the ECL-plus Western-blotting detection system (GE Healthcare). Quantitative analysis was made by densitometry using a Light capture apparatus (Atto, Tokyo, Japan).

Induction of Differentiation of K562 Cells

Butyrate-dependent differentiation of K562 cells to erythroid was evaluated by formation of heme or hemoglobin by the selective staining of them with a benzidine/hydrogen peroxide solution as reported elsewhere (15). Briefly, the K562 cells were seeded in 6-well plates at a density of 1×10^5 cells/ml and cultivated for 4 days in the presence of 1 mM sodium butyrate. The resultant cells were fixed on the slide glasses with methanol and dried. The slide glasses attached with the cells were immersed in benzidine (0.025% benzidine and 0.45% hydrogen peroxide in phosphate-buffered saline (PBS)) for 1 hr. After washing the slide twice with PBS, benzidine-positive and negative cells were counted using microscope and fraction of benzidine-positive cells were detected as the erythroid-differentiated cells.

Detection of Apoptotic Cell

Flow cytometry was performed to detect cells undergoing apoptosis. The K562 cells were seeded at a density of 2×10^4 cells/ml and cultivated for 1–3 days. The cells were harvested and washed with PBS containing 2% FBS. Then 2×10^5 cells suspended in 0.5 ml PBS containing 2% FBS and 0.02% NaN_3 were incubated with PE-conjugated APO2.7 (Immunotech, Marseilles, France) for 30 min at 4°C to detect 7A6 antigen, which is expressed by cells undergoing apoptosis (19). The cells were then washed twice with PBS and evaluated by flow cytometry (CytoACE-150, Japan Spectroscopic Co. Ltd., Tokyo, Japan). The ratios of APO2.7-positive cells to total cells are represented. Three different wells were prepared for each point, and the experiments were performed in triplicate.

Transport Experiments

For the transport experiments of [^{3}H]ergothioneine by K562 cells or HEK293 cells, the cells were harvested and suspended in transport medium containing 3 mM K_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 130 mM NaCl, and 20 mM Tris adjusted to pH 7.4 by HCl. Cell suspension was pre-incubated at 37°C for 20 min in the transport medium, then it was centrifuged and the resultant cell pellets were re-suspended in transport medium containing [^{3}H]ergothioneine. After a designated time, the cells were washed twice rapidly with ice-cold transport medium. Then, cells were solubilized in 1 M NaOH and the cell-associated radioactivity was measured by means of a liquid scintillation counter (ALOKA, Tokyo, Japan) using Cleasol-1 (Nacalai tesque, Kyoto, Japan) as a liquid scintillation fluid.

Analytical Methods

Cellular protein content was determined according to the method of Lowry with bovine serum albumin as the standard (20). Cell-to-medium ratio (μl/mg protein) was obtained by dividing the uptake amount by the cells by the concentration of test compound in the transport medium. The apparent kinetic parameters, Km (Michaelis constant) and Vmax (maximal transport rate) of ergothioneine uptake by K562 cells and HEK293 expressed with OCTN1, were calculated by non-linear least-squares regression analysis using MULTI program (21) according to the following Michaelis–Menten type equations, where v and $[s]$ are the velocity of substrate uptake and substrate concentration, respectively.

$$v = \mathrm{Vmax} \cdot [s]/(\mathrm{Km} + [s]) \quad (1)$$

All data were expressed as the mean ± S.E.M., and statistical analysis was performed by Student's t test. The criterion of significance was taken to be $p < 0.05$.

RESULTS

Characteristics of Ergothioneine Uptake by K562 Cells

To determine whether OCTN1 is functional in K562 cells, first of all the uptake of [^{3}H]ergothioneine was examined. The time course for the uptake of ergothioneine (3 μM) by the K562 cells is shown in Fig. 1. Uptake of [^{3}H]ergothioneine was increased linearly up to 5 min in K562 cells. Thus, uptake at 3 min was routinely used for initial uptake rate measurement in the subsequent studies. When extracellular Na^+ was replaced with N-methyl-D-glucamine (NMG^+) at equimolar concentration, the uptake was decreased. In addition, uptake of [^{3}H]ergothioneine by K562 cells was significantly decreased by lowering temperature to 4°C (Fig. 1).

Figure 2 shows the relationship between the initial uptake rate and concentrations of ergothioneine. The uptake was saturable at concentration up to 50 μM (Fig. 2a). An Eadie–Hofstee plot for the uptake by K562 cells, after correction for nonsaturable uptake which was estimated from the first-order rate constant obtained by nonlinear least-squares regression analysis of the result in Fig. 2b, indicated the involvement of one saturable transport system (Fig. 2b); the kinetic parameters for Michaelis constant (Km) and maximum uptake rate (Vmax) for saturable component and the first-order rate constant (kd) for nonsaturable component were 1.41 ± 0.40 μM, 7.95 ± 0.74 pmol/mg protein/3 min, and 1.41 ± 0.06 μl/mg protein/3 min, respectively.

In the present study, we evaluated the characteristics of ergothioneine transport via human OCTN1 using HEK293 cells transfected with *OCTN1* gene to compare the observed characteristics in K562 cells as described above. The kinetic

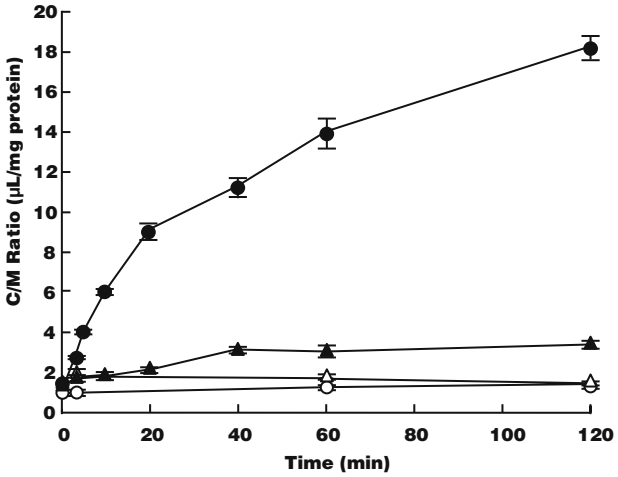

Fig. 1. Na^+- and temperature-dependence of [^{3}H] ergothioneine uptake by K562 cells. Uptake of [^{3}H] ergothioneine (3 μM) by K562 cells was measured for 120 min at 37°C. Cellular uptake was expressed as cell-to-medium ratio. Uptake of ergothioneine by K562 cells in presence or absence of Na^+ at 37°C. In the Na^+-free condition, all Na^+ was replaced with NMG^+. *Closed circle, open circle, closed triangle, and open triangle* show the uptakes at 37°C with Na^+, 4°C with Na^+, 37°C with NMG^+, and 4°C with NMG^+, respectively. Each result represents the mean ± S.E.M. ($n = 3$ or 4).

parameters for the initial uptake at 30 s of ergothioneine by OCTN1 were Km = 7.16 ± 0.51 μM and Vmax = 158.13 ± 4.76 pmol/mg protein/30 s. The uptake was significantly decreased by replacing the Na^+ in the uptake medium to NMG^+, showing the sodium dependent transport. Km and sodium dependence of OCTN1-mediated uptake were comparable with those observed in K562 cells and previous report by Grundemann *et al.* which showed firstly ergothioneine as the sübstrate of OCTN1 (11,12). Those results suggested that uptake of ergothioneine in K562 cells was mainly accounted for by OCTN1.

Suppression of OCTN1 Gene and Transport Activity of OCTN1 by shRNA

To identify an efficient RNAi-target sequences for the generation of stable vector-based shRNA-transfected cell clones, shRNA constructs designed against three different parts of the OCTN1 mRNA sequence were synthesized and ligated to the expression vector. Two oligonucleotides (N510 and N1600) among the three oligonucleotides were shown to significantly reduce mRNA levels of the *OCTN1* (Fig. 3a). In the Western blot analysis, the main bands of OCTN1 were observed at approximately 70 kDa and 100 kDa (Fig. 3b). The both bands linearly increased with increasing amount of membrane fraction (data not shown) and were comparable with that previously reported (22). The intensity of 100 kDa and 70 kDa bands from the cells transfected with N1600 was significantly decreased to about 20% and 60% of the control, respectively, while those bands of N510-transfected cells slightly decreased (Fig. 3b). Accordingly, we used the two oligonucleotides in the subsequent study. In addition, to estimate whether endogenous expression of OCTN1 was suppressed or not, transport activity of [^{3}H]ergothioneine was measured by the cells transfected with those oligonucleotides. In the K562 cells transfected with oligonucleotide N1600, the uptake of ergothioneine was significantly decreased (Fig. 3c). K562 cells transfected with knockdown vector with oligonucleotide N510 also showed decreased uptake, while the decrement was smaller than that observed by N1600 (Fig. 3c). The uptake of guanine that is not a substrate of OCTN1 did not show any difference between OCTN1 knockdown cells and mock cells (data not shown). The order of decrement of ergothioneine uptake activity was comparable with the order of the decrease of the expression of *OCTN1*-mRNA.

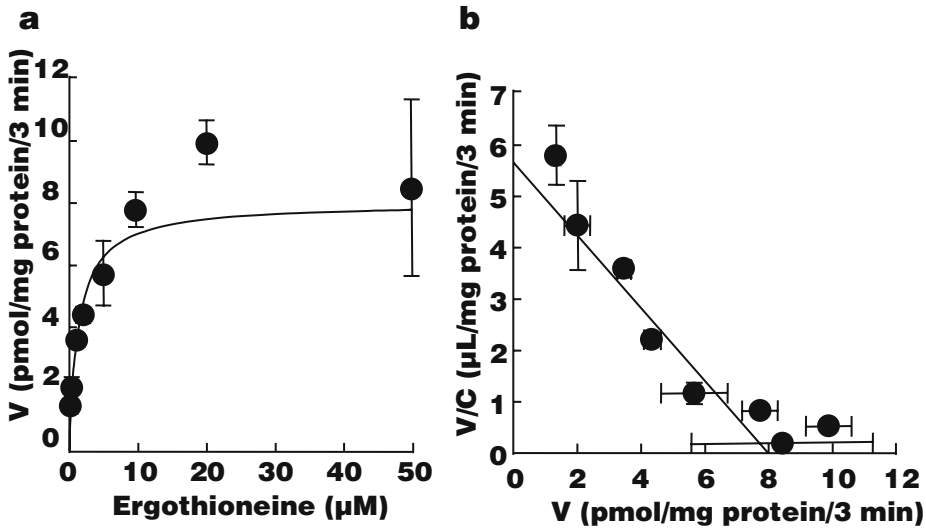

Fig. 2. Concentration dependence of ergothioneine uptake by K562 cells. **a** Total uptake of ergothioneine by K562 cells in the presence of Na^+ at 37°C for 3 min. **b** Eadie–Hofstee plots of ergothioneine uptake after correction for nonsaturable uptake evaluated from first-order rate constant obtained by nonlinear least-squares regression analysis as described in "Results." Each result represents the mean ± S.E.M. ($n = 3$ or 4).

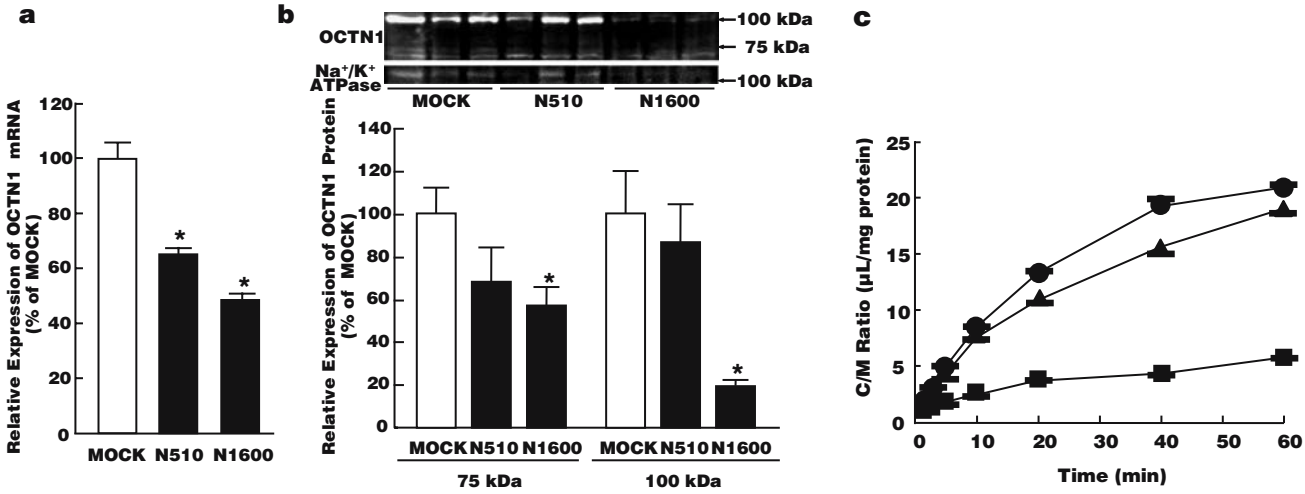

Fig. 3. Effects of knockdown of OCTN1 by RNA interference on *OCTN1* expression and transport activity in K562 cells. **a** Expression level of *OCTN1* mRNA in shRNA expressed K562 cells. Expression levels of *OCTN1* were quantitated by real-time PCR assay and normalized by G3PDH and were shown as the control of MOCK-transfected cells. **b** Western blot analysis of OCTN1 in OCTN1 knockdown-K562 cells. The membrane fraction of K562 cells (15 μg of protein) was subjected to Western blot analysis with anti-human OCTN1 antibody or anti-Na^+/K^+ ATPase antibody. The positions of molecular mass markers are shown at *right*. Protein amounts were normalized by Na^+/K^+ ATPase. Each result represents the means ± S.E.M. ($n = 3$). **c** Time courses of the uptake of [^{3}H] ergothioneine (4.5 μM) in OCTN1 knockdown K562 cells. [^{3}H] Ergothioneine uptake was measured at pH 7.4 and 37°C. *Circles, triangles, and squares* show the results of control-MOCK, N510-oligonucleotide, and N1600-oligonuecleotide transfected cells, respectively. Each result represents the means ± S.E.M. ($n = 4$).

Effect of shRNA on Proliferation and Differentiation of K562 Cells

The effects of *OCTN1* knockdown on proliferation and differentiation of K562 cells to erythrocytes were investigated. The growth of the K562 cells for five days were evaluated and the cells that stably expressing oligonucleotide N1600 showed retardation of the growth, while the cell number at day fifth was not changed significantly. In the case of the cells transfected with N510 oligonucleotide, although the extent of decrease was smaller than those of N1600, a decrease in growth rate was observed (Fig. 4a), while no significant decrease in the cell number was observed at the day fifth as the same as the case of N1600-transfected cells. In order to

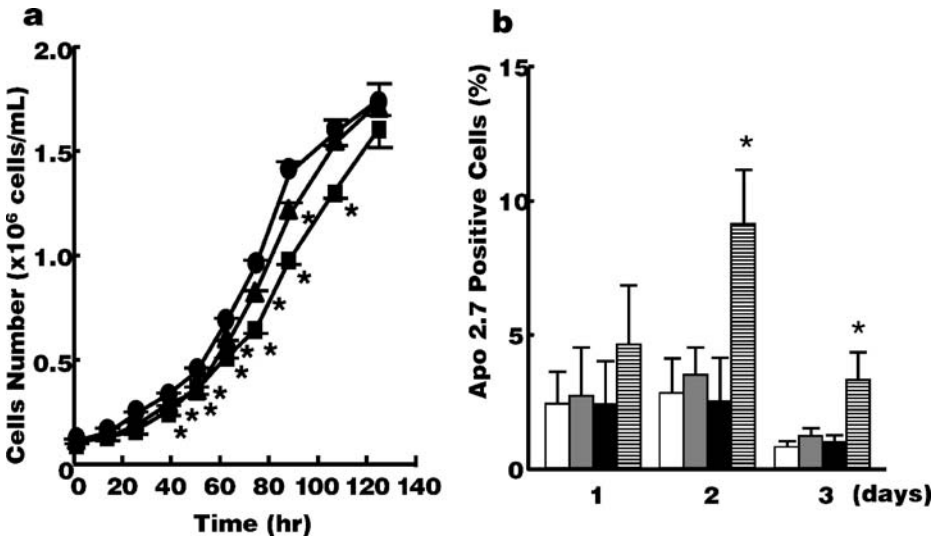

Fig. 4. Effects of knockdown of ***OCTN1*** by RNA interference on the proliferation of K562 cells. **a** Cells were plated at a density of 1×10^5 cells/ml in RPMI1640 medium. The number of viable cells was calculated at each time point. *Circles, triangles, and squares* show the results of control-MOCK, N510-oligonucleotide, and N1600-oligonuecleotide transfected cells, respectively. **b** The K562 cells were seeded at a density of 2×10^4 cells/ml and cultivated for 1–4 days. *White, gray, block,* and *horizontally striped* show the results of wild type K562 cells, control-MOCK, N510-oligonucleotide, and N1600-oligonuecleotide transfected cells, respectively. Each result represents the mean ± S.E.M. ($n = 4$). Significantly different between the clones: $*p < 0.05$.

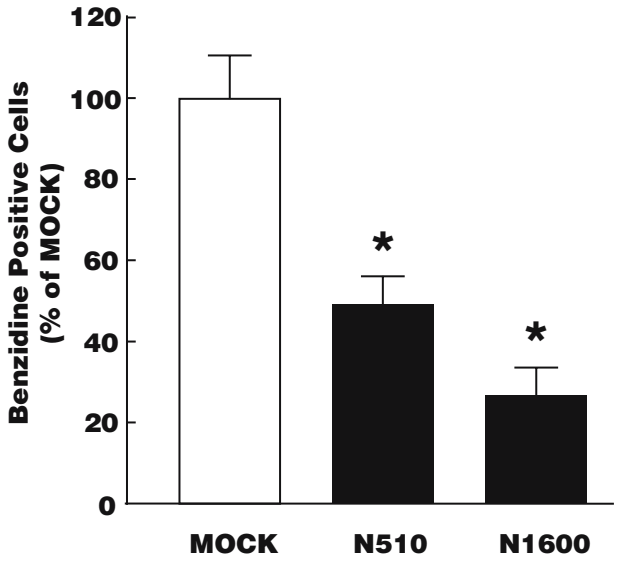

Fig. 5. Effect of knockdown of ***OCTN1*** by RNA interference on butyrate-induced erytroid differentiation of K562 cells. Data are expressed as the ratio of benzidine positive cells relative to that of control MOCK cells. Cells were treated with 1 mM butyrate for 4 days. After cultivation for 4 days, cells were stained with benzidine and the stained cells were counted under microscope. The results represent mean ± S.E.M. of four independent experiments. Significantly different between the clones: $*p < 0.05$.

assess the mechanisms of the decrease in the cell growth of N1600, the expression of 7A6 antigen on these cells was assayed to monitor cells undergoing apoptosis. Cells were collected on 1, 2 and 3 days of culture, and the expression of 7A6 antigen on their surfaces was analyzed using APO2.7 mAb. The apoptotic cells were observed in N1600 transfected cells, while such induction of apoptosis was not observed in N510 transfected cells in comparison with wild type (Fig. 4b).

K562 cells are committed to erythrocyte differentiation in the presence of 1 mM sodium butyrate and about 50% of them became benzidine-positive after 4 days, whereas less than 10% became benzidine-positive without sodium butyrate (15). In knockdown K562 cells, the number of K562 cells remained at about 1×10^5 cells/ml and benzidine-positive cells were significant decreased after 4 days of cultivation for the K562 cells transfected with N510 and N1600 oligonucleotides to 50% to 30% of control, respectively (Fig. 5). These results suggested that decreased expression of *OCTN1* affects both the proliferation and erythroid differentiation, however, the effect on the differentiation is much remarkable.

DISCUSSION

In the present study, physiological role of OCTN1 was assessed using K562 cells, that are derived from human chronic myelogenous leukemia cells and are often used as a model of blood cells, since OCTN1 is abundantly expressed in the blood cells as observed in our previous studies (7). Here, we first of all established K562 cell lines that exhibited lower expression of *OCTN1* by vector-based siRNA. When expression levels of *OCTN1* were quantitated by real-time PCR assay and Western blot analysis, several stable shRNA vector-transfected K562 clones exhibited significantly reduced mRNA levels and protein levels of OCTN1 (Fig. 3a, b). The estimated molecular weight from the amino acid sequence of OCTN1 was 62 kDa. The obtained bands (70 kDa and 100 kDa) were larger than the estimated molecular weight, suggesting the post-translational modification such as glicosylation as has been reported previously (22). Among them, we chose two cell lines that were transfected with oligonucleotides (N510 and N1600) and performed further experiments.

For the functional analysis of OCTN1 using K562 cells, we examined whether the OCTN1 activity can be assessed by uptake of ergothioneine, which is a good substrate of OCTN1 (11). Uptake of ergothioneine by K562 cells exhibited significant temperature- and Na^+-dependence and was saturable, demonstrating an involvement of a carrier-mediated transport mechanism (Figs. 1, 2). These characteristics are comparable with those reported previously and confirmed in the present study using HEK293 cells transfected with *OCTN1* gene (11). Moreover, since kinetic analysis of ergothioneine uptake showed a monophasic pattern in Eadie–Hofstee plot, it was suggested that ergothioneine uptake was mediated by a single saturable transport system with Km values of 1.41 ± 0.40 μM (Fig. 2). This Km value was close to the value (7.16 ± 0.51 μM) observed in the present study by HEK293 cells expressed with *OCTN1*. Accordingly, these results suggested that OCTN1 is functional in K562 cells and the apparent uptake of ergothioneine was considered to be due to OCTN1. High concentrations of ergothioneine are found in a number of tissues, including liver, kidney, eyes, seminal fluid, and erythrocytes (23). In addition, OCTN1 was strongly expressed in fetal liver, kidney, and erythrocytes (1,7). Therefore, it is possible that OCTN1 transports ergothioneine to provide it to those tissues, while there is no direct evidence that ergothioneine is involved in any physiological functions of those tissues.

Effectiveness of shRNA used in the present study was confirmed by the uptake of ergothioneine in those cells transfected with shRNA vector (Fig. 3c). The oligonucleotide N1600-transfected cell line exhibited significantly decreased uptake of ergothioneine, showing that the cell line can be used as the model that shows lowered expression and function of OCTN1 and the other cell line transfected with oligonucleotide N510 shows intermediated expression between MOCK and the cell line transfected with oligonucleotide N1600.

Cell growth of OCTN1-knockdown clones were significantly slow compared with MOCK clones, while the cell numbers reached comparable among all the cell lines in 5 days after cultivation (Fig. 4a). Although the mechanism is not clear, in general, it is thought that the decrease of growth was caused by inhibition of cell cycle or induction of apoptosis. Moreover, it was reported that ergothioneine is associated with cell proliferation and apoptosis (12,24). In the present study, we examined apoptotic changes of N1600 and N510 transfected cells using 7A6 antigen as a marker of apoptosis. As the result, 7A6 was detected in N1600-transfected cells but not in N510 transfected cells (Fig. 4b). Accordingly, the decrease of proliferation of cells was partially explained to apoptosis. Since *OCTN1*-knockdown cells were prepared using oligonucleotides N510 and N1600 that were designed for different parts of the human *OCTN1* gene, it was suggested that significantly slow growth of those cells was

specific to the decrease of *OCTN1* expression. Moreover, a significant decrease of the number of benzidine-positive cells, as the erythroid-differentiated cells, was observed in *OCTN1*-knockdown cell clones compared with MOCK clone (Fig. 5).

We previously reported that OCTN1 was strongly expressed in glycophorin A-positive erythroid cells and was associated with erythroid-lineage cells at the transition stage from immature erythroid cells to peripheral mature erythrocytes (7). In addition, the small molecules that are transported by transporters such as, L-arginine, polyamine, and thiamine are associated with cell differentiation (15,25,26). Accordingly, it is considered that OCTN1 physiologically transports small molecule(s) that are essential for normal cell growth and differentiation to erythrocytes. Ergothioneine might be one of those substrates but further direct evidence that ergothioneine is involved in the erythrocytes formation will be essential.

Recently, it was suggested that OCTN1 is associated with RA (10). We demonstrated that an expression of OCTN1 was regulated by cytokine and transcription factor that are related to RA (27). RA is chronic inflammatory disease characterized by the proliferation of the synovial membrane into a highly vascularized tissue known as pannus and inflammation. In addition, the mouse octn1 was expressed in inflammatory joints of mice with collagen-induced arthritis, a model of human arthritis, but not in the joint of normal mice (8). Therefore, it is possible that OCTN1 is associated with a proliferation of synovial membrane and fibroblast-like synoviocyte cells. Moreover, it was reported that SNP of OCTN1, such as 1672GT transversion resulting in the amino acid substitution L503F, is associated with Crohn's disease (9,12). K562 cells we used were shown to be 503F allele (data not shown) and to express high levels of *OCTN1* mRNA (1). Furthermore, it was reported that the cells with the 503F allele accumulate higher ergothioneine concentrations compared with those with the wild-type 503L (12). The results in this study suggest that the decrease of OCTN1 expression led to a decreased accumulation of ergothioneine, and then the cell proliferation was suppressed by inducing of apoptosis. In contrast, an increase of OCTN1 expression led to an increased accumulation of ergothioneine, and then proliferation of cells may increases and then causes to pannus and inflammation. Accordingly, it might be important to maintain the normal expression level of OCTN1 to prevent these kinds of diseases.

In conclusion, the present study suggested using *OCTN1*-gene knockdown cells by shRNA that OCTN1 contributed to the growth and erythroid differentiation of K562 cells. Therefore, the role of OCTN1 in blood cells should be to transport compounds which are essential for erythroid differentiation, maturation, and/or growth of the cells and ergothioneine could be one of compounds involved as the OCTN1 substrates.

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