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Physiological Role of L-type Amino Acid Transporter 1 in Two Lines of Cultured Choriocarcinoma Cells, JAR and JEG-3

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SUMMARY

In cells that proliferate abundantly, such as cancer cells, the expressions of many nutrient transporters increase in order to drive the uptake of required nutrients. The system L transporters are the main sodium-independent neutral amino acid transporters. They have four isoforms : L-type amino acid transporter (LAT) 1, 2, 3, and 4. The expression of LAT1 has been reported in lines of cultured choriocarcinoma cells. However, the role of LAT1 in choriocarcinomas has not been adequately clarified. This study examined the role of LAT1 in these cell lines JAR and JEG-3.

Based on an examination of mRNA and protein expression, it was determined that both JAR and JEG-3 cells express LAT1. The uptake of leucine, a substrate of LATs, by JAR and JEG-3 cells showed that there are sodium-independent leucine transport. To examine whether leucine transport is mediated by LAT1, a leucine uptake was examined with loading of JPH203, a LAT1 specific inhibitor, and transport inhibition was clearly seen. Cell counts were measured to examine whether cell proliferation was decreased by JPH203 treatment. Inhibition of JEG-3 cell proliferation was seen on day 4 of the treatment. No inhibition of JAR cell proliferation was seen by low concentrations of JPH203, however inhibition was seen on a higher concentration of JPH203. JPH203 decreased cell activity concentration-dependently in JEG-3 cells. JAR cell, however, showed an increased activity following JPH203 treatment at low concentrations. Thus, the study found that LAT1-mediated amino acid transport occurs in these two different lines of cultured choriocarcinoma cells. Moreover, it showed that the two types of cells responded differently with respect to inhibition of cell proliferation and changes in cell activity as a result of inhibition of LAT1-mediated amino acid transport. The results showed that LAT1-mediated amino acid transport in choriocarcinoma is complex and has a variety of functions.

Key Words : cancer, transporter, amino acids, leucine, choriocarcinoma

INTRODUCTION

Amino acids and other nutrients are supplied to the inside of cells via transporters on the cell membrane. The incorporation of required nutrients is increased in cells that proliferate abundantly, such as cancer cells.

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The expression of transporters of many nutrients has been reported to be increased in cancer cells[1]. The main sodium-independent neutral amino acid transporters are the system L[2]. System L has four isoforms : L-type amino acid transporter (LAT) 1, 2, 3, and 4. LAT1 was discovered in 1998[3,4], and LAT1 and LAT2 form heterodimers with the ancillary subunit 4F2hc (CD98) when transporting amino acids through the cell membrane[3,5]. LAT3 and LAT4, on the other hand, transport amino acids independently. However, LAT1 and LAT2 have a greater capacity for transport[2,6,7].

JPH203 (KYT0353) is a tyrosine analog that selectively inhibits amino acid transport by LAT1[8]. The specific inhibition of LAT1 has been reported to potently inhibit the proliferation of various cancer cell culture lines, particularly human enterocytes, in both in vitro and in vivo models[8]. JPH203 has recently been found to inhibit leucine uptake and cell proliferation and thereby exhibit anticancer activity in human oral cancer cells[9] and leukemia cells[10]. JPH203 has shown little toxicity for normal mouse thymocytes and human peripheral blood cells[10], so, cancer therapies using JPH203 are therefore anticipated. Consequently, clinical trials are currently being conducted using JPH203 as an anticancer drug.

Choriocarcinomas are tumors that occur as a result of malignant conversion of trophoblastic cells, and BeWo cells, a line of cultured choriocarcinoma cells, have been found to express LAT1[11]. Treatment of BeWo cells with 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which is an inhibitor of LATs, has been found to induce apoptosis and autophagy[12]. However, the role of LAT1 in individual choriocarcinoma cells has not been adequately clarified. Moreover, although chemotherapy is often used to treat choriocarcinomas, the prognosis is poor for chemoresistant patients[13]. Consequently, it is important to develop an anticancer agent that is effective against choriocarcinomas, and examining whether JPH203 inhibits choriocarcinoma cell proliferation may yield findings of value for the treatment of choriocarcinomas in the future.

As a preliminary step in investigating the role of LAT1 in choriocarcinomas, this study examined the physiological role of LAT1 in JAR and JEG-3 cells, which are lines of cultured choriocarcinoma cells.

METHODS

Reagents

JPH203 and anti-LAT1 antibodies were provided by J-Pharma Co., Ltd (Tokyo, Japan). JPH203 was dissolved in 10% DMSO/EtOH for use in the tests.

Culture cells

Human gestational choriocarcinoma JAR cells (HTB-144) and JEG-3 cells (HTB-36) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The JAR cells were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 23.8 mM $NaHCO_3$ and 10% fetal bovine serum (FBS). The JEG-3 cells were grown in Minimum Essential Medium Eagle (Sigma-Aldrich) supplemented with 26.2 mM $NaHCO_3$ and 10% FBS. The cell lines were cultured in an incubator at 37℃ under 5% CO_2.

Reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR

The RNeasy Mini kit (QIAGEN, Hilden, Germany) was used to extract mRNA from the JAR and JEG-3 cells. The experimental method is described in the package insert. Genomic removal was applied to the extracted mRNA (2 μg), and cDNA synthesis was performed using reverse transcriptase. The genomic removal and cDNA synthesis were performed as described in the package insert using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Shiga, Japan).

RT-PCR was performed using a Thermal Cycler Dice Touch (Takara Bio Inc.) with PCR Master Mix (Promega, Madison, WI, USA). Primer was added to a final concentration of 0.5 μM in the reaction solution. The primer sequences used are shown in Table 1. The PCR conditions consisted of initial denaturation for 2 min at 95℃, followed by three steps that were repeated for 30 rounds : 30 s at 95℃, 30 s at 55℃, and 30 s at 72℃. Finally, the reaction was incubated for 5 min at 72℃. Beta-actin was used as the internal control.

The quantitative RT-PCR reaction was performed using SYBR® Fast qPCR Mix (Takara Bio Inc.) with

Table 1 Sequence of primers used for RT-PCR

Primer	Forward (5' to 3')	Reverse (5' to 3')
LAT1	tgcctgtgttcttcatcctg	cctcctggctatgtctcctg
LAT2	gccctcaccttctccaacta	aatgcattctttggctccag
LAT3	cacgctactgcaagatccaa	agaagggctctcctttcagg
LAT4	aaatttggccttcactgtgg	acgacgatgaaggagacacc
4F2hc	cagaaggatgatgtcgctca	ccagtggcggatataggaga
β-actin	gctgcttttaactctggtaa	cgcggccatcacgccacagt

Table 2 The primer sequences used for quantitative RT-PCR

Primer	Forward (5' to 3')	Reverse (5' to 3')
LAT1	actcaggaccagatgtccgtctc	cgctgtgaagtctgtccatgtg
LAT2	tgctggacagatagtccttcg	ggaacagcaggttgatcttg
LAT3	tgtgttcgccttgcttcag	gagtgagaatagcaggaggc
LAT4	ctgaaggagtgtgaagacgc	Tgatcttctggatctgccg
4F2hc	atgagattggcctggatgc	aagctggactcatcccacag
β-actin	ctggcatcgtgatggactccgg	gtggatgccacaggactccatg

the Thermal Cycler Dice Real Time System II (Takara Bio Inc.). Primer was added to a final concentration of 0.4 μM in the reaction solution. The primer sequences used are shown in Table 2. The PCR conditions consisted of initial denaturation for 30 s at 95℃, followed by incubation of the reaction for 40 cycles, with each cycle consisting of 5 s at 95℃ and 10 s at 60℃. Using beta-actin as the internal control, relative quantitation was performed using the standard curve method. The level of beta-actin expression was assumed to be the same in JAR and JEG-3 cells, and the relative ratio of gene expression in JEG-3 cells was determined with gene expression in JAR cells assigned at a value of 1.

Western blot analysis

The JAR and JEG-3 cells were seeded on 24-well plates (2×10^5 cells/well) and dissolved 2 days later by adding 1×SDS loading buffer. The cell lysate was boiled for 5 min at 95℃, then electrophoresed through Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Hercules, CA, USA), and transcription was performed on a polyvinylidene difluoride membrane. Blocking was performed using 5% skim milk, and the membrane was soaked in primary antibody diluted with blocking buffer and incubated overnight at 4℃. Anti-LAT1 antibodies were diluted 1,000-fold, and anti-beta-actin, clone C4 antibody (EMD Millipore, Billerica, MA, USA) was diluted 3,000-fold for use. Anti-mouse IgG-HRP antibody (GE Healthcare, Chicago, IL, USA) was diluted 3,000-fold and used as the secondary antibody. The membrane was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and images were obtained with an ImageQuant LAS 4000 imager (Fuji Film, Tokyo, Japan). A solution of cells that persistently expressed LAT1 (S2-LAT1) was used as the positive control for LAT1[8]. A solution of cells that persistently expressed LAT2 (S2-LAT2) was used as the negative control[8].

[^{14}C] *Leucine uptake test*

JAR and JEG-3 cells were seeded (3×10^5cells/well) on 24-well plates coated with poly-L-lysine (Corning Incorporated, Corning, NY, USA) and grown for 2 days. Hank's balanced salt solution (HBSS) [125 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 5.6 mM glucose (pH 7.4)] or sodium-free HBSS with 125 mM $C_5H_{14}ClNO$ added instead of NaCl was used in the

uptake test. The cells were washed twice in 1 mL of HBSS warmed to 37℃ and preincubated for 10 min. HBSS supplemented with 1 μM of [^{14}C]-leucine (Moravek, Brea, CA, USA) was then added, and incubated for 1, 2, 5, 10, and 30 min. After incubation, the cells were washed twice with cold HBSS and dissolved with 0.1N NaOH. The radioactivity in the cell lysate was measured using an LSC-7200 liquid scintillation counter (Hitachi Aloka Medical America, Inc./ Hitachi Ltd., Tokyo, Japan). In addition, the protein content of the cell lysate was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.), and protein normalization was performed.

In the test of leucine uptake inhibition by JPH203, JPH203 (0.001, 0.01, 0.1, 1, and 10 μM) was added to 1 μM [^{14}C]-leucine solution using sodium-free HBSS, and the leucine uptake in 1 min was examined. A solution of 0.1% DMSO/1% EtOH was added as the control.

Cell proliferation test

To evaluate the proliferation-inhibiting effect of JPH203, cell proliferation over time was evaluated by measuring cell counts. JAR cells (1×10^4 cells/well) and JEG-3 cells (2×10^4 cells/well) were seeded on 24-well plates, and culture solutions containing 10, 30, and 100 μM JPH203 were substituted the following day. A culture solution containing 0.1% DMSO/1% EtOH was used as the control. Cell counts were measured using a TC20 Automatic Cell Counter (Bio-Rad Laboratories) immediately after, and also on 2 and 4 days after culture solution replacement.

Cell activity measurement

To evaluate cell activity after the addition of JPH203, cell activity was measured by the fluorescence method using Alamar Blue (Bio-Rad Laboratories). JAR cells (1×10^3 cells/well) and JEG-3 cells (2×10^3 cells/well) were seeded on 96-well plates, and 100 μL/well of JPH203 at different concentrations (10, 30, and 100 μM) and a control culture solution containing 0.1% DMSO/1% EtOH were substituted the following day. Ten microliters per well of Alamar Blue were added immediately after (0 days) and also on 2 and 4 days after culture solution replacement, the cells were incubated under 5% CO_2 at 37℃ for 3 h, and fluorescence intensity was measured (measurement wavelengths used : 544 nm excitation wavelength, 590 nm fluorescence emission wavelength). Measurements were also performed only for wells with culture solution that had JPH203 added, and the value yielded by subtracting the fluorescence intensity of wells with culture solution alone from the fluorescence intensity of the test wells was evaluated.

Statistical analysis

In the quantitative RT-PCR analysis, Student's *t*-test was used for JAR cell expression. A multigroup analysis of the results of the cell proliferation test and cell activity measurements was performed by analysis of variance (ANOVA) using SPSS Statistics version 24 (IBM Corp., Armonk, NY, USA), and the groups were compared using the Bonferroni method. The means and standard errors were shown for the data. A p-value <0.05 was considered significant.

RESULTS

Expressions of LATs and 4F2hc in cultured choriocarcinoma cells

RT-PCR was performed using mRNA extracted from JAR and JEG-3 cells to examine whether LATs were present in these lines of cultured choriocarcinoma cells using the expressions of LATs and 4F2hc in lines of cultured choriocarcinoma cells (Fig. 1A). RT-PCR of JAR cells showed clear bands for LAT1, LAT4, and 4F2hc. A weak band was seen for LAT2. With JEG-3 cell RT-PCR, clear bands were detected for LAT1, LAT2, LAT4, and 4F2hc. In addition, quantitative RT-PCR was performed using the same mRNA to assess whether there were differences between the cells in the expression of LAT mRNA (Fig. 1B). With the expression of LATs in JAR cells assigned at a value of 1, LAT1 expression in JEG-3 cells was 0.8-fold, which was significantly lower. The level of LAT2 expression in JEG-3 cells was 3.5-fold higher. The expressions of LAT3 and LAT4 were lower in JEG-3 cells than in JAR cells (LAT3 : 0.8-fold, LAT4 : 0.4-fold). No difference in 4F2hc expression were seen between JAR and JEG-3 cells. LAT1 expression in JAR and JEG-3 cells was also examined by Western blot analysis. Although the homology between LAT1 and LAT2 is 50%[5], it was deter-

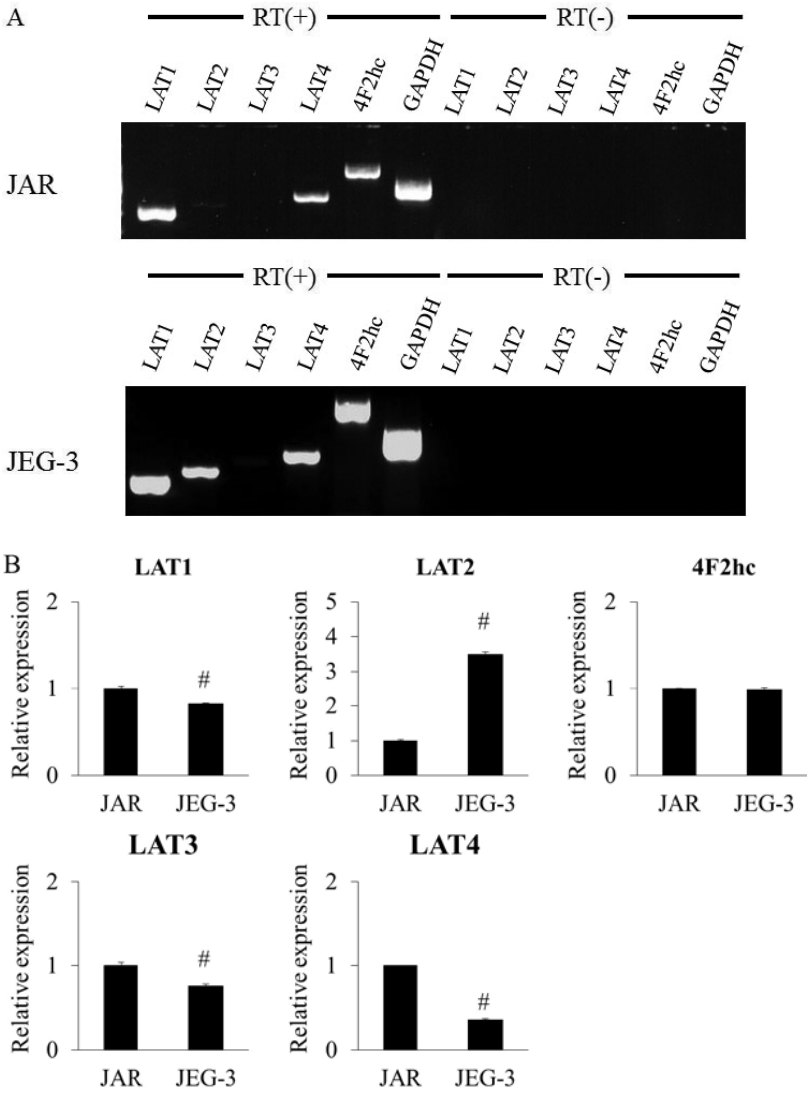

Figure 1 Expression of LATs and 4F2hc in JAR and JEG-3 cells

A：RT-PCR of LATs. RT-PCR of LATs was performed using collected mRNA from JAR and JEG-3 cells. In RT（+）side lane, synthesized cDNA was added to the reaction mixture. In RT（−）side lane, mRNA was added. Primers' sequence was shown in Table 1. **B**：Quantitative PCR of LATs. mRNA expression of LATs and 4F2hc was quantified by realtime PCR. Expression level was indicated as expression relative to JAR. Error bars represent ± standard error (SE) for triplicate experiments.

mined that the LAT1 antibody used in this study recognized only LAT1. Consequently, LAT1 protein expression was determined for both JAR and JEG-3 cells (Fig. 2).

Leucine uptake and its inhibition by JPH203 in a line of cultured choriocarcinoma cells

To examine whether LAT-mediated amino acid uptake occurs in JAR and JEG-3 cells, leucine uptake tests were performed using HBSS containing sodium and sodium-free HBSS, and whether sodium-independent amino acid transport occurs in these cells was examined. In both JAR and JEG-3 cells, no difference in leucine uptake was seen as a result of the difference in HBSS (Fig. 3A). That is, sodium-independent leucine transport was shown to exist in JAR and

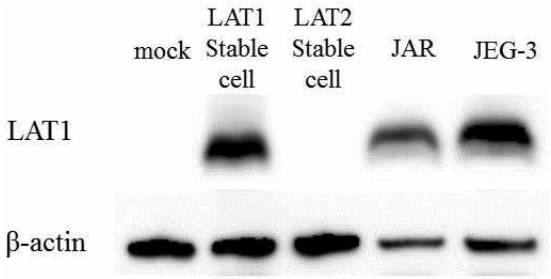

Figure 2 Western blotting of LAT1

Whole cell proteins were extracted from non-treated JAR and JEG-3 cells, LAT1 expressing cells (S2-LAT1), LAT2 expressing cells (S2-LAT2) and mock cells (S2-mock). Lysate of LAT1 expressing cells was considered a positive control. Blotting for β-actin serves as loading controls.

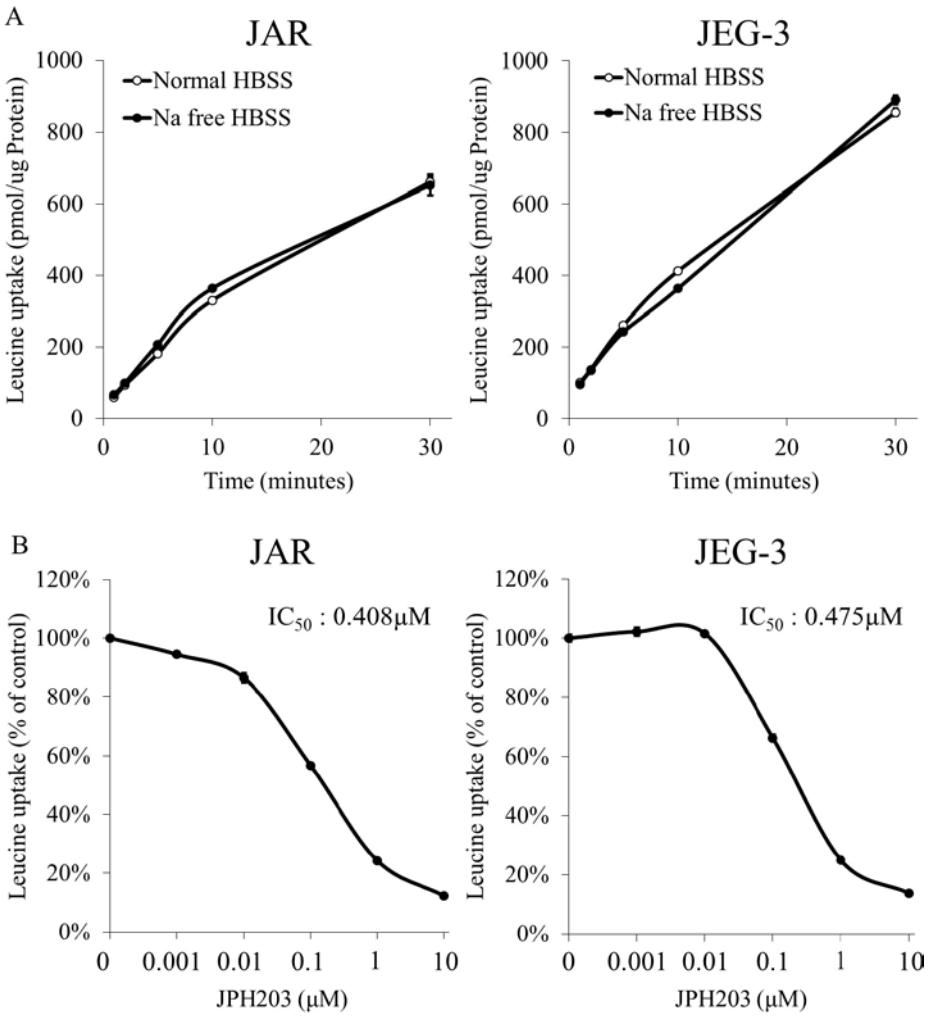

Figure 3 Leucine uptake of JAR and JEG-3 cells and inhibition by JPH203
A：Time courses of leucine uptake were examined with HBSS containing Na (Normal HBSS) and Na free HBSS. One μM [^{14}C]-Leucine uptake by JAR and JEG-3 cells was measured during a 30 minutes incubation. **B**：Inhibition of leucine uptake by JPH203 in JAR and JEG-3 cells. JAR and JEG-3 cells were incubated with [^{14}C]-Leucine and 0.001-10 μM JPH203 for 1 minute. Data are shown as means ± SE.

JEG-3 cells.

Next, a leucine uptake test with JPH203 loaded was performed to examine whether the sodium-independent leucine transport in JAR and JEG-3 cells is mediated by LAT1. JPH203-concentration-dependent inhibition of leucine uptake was seen in both JAR and JEG-3 cells (Fig. 3B). The IC_{50} was calculated to be 0.408 μM for JAR cells and 0.475 μM for JEG-3 cells. These results showed that LAT1-mediated leucine transport exists in JAR and JEG-3 cells.

Proliferation-inhibiting effect of JPH203

To examine whether JAR and JEG-3 cell proliferation is inhibited by the inhibition of LAT1-mediated amino acid transport, cell counts were measured after JPH203 loading (Fig. 4). No difference in the JAR cell count was seen immediately after culture solution replacement, but the cell count was significantly lower in the 100 μM group than in the control group 2 days after replacement. The cell count was also significantly lower in the 100 μM group than in the control group 4 days after replacement. However, cell counts in the 10 and 30 μM groups were nearly identical to those in the control group. With JEG-3 cells, no changes in the cell count were seen due to the addition of JPH203 immediately after or 2 days after cul-

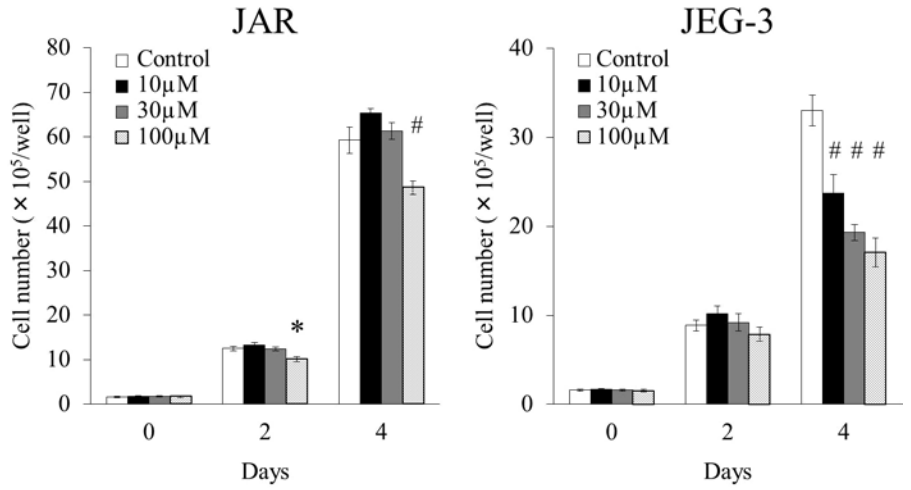

Figure 4 Growth inhibitory effect of JPH203
The cells were treated with 0, 10, 30 and 100μM JPH203 for 0, 2 and 4 days. After incubation, cells were trypsinized and counted by auto cell counter. Data are shown as means ± SE. (* : $p<0.05$, # : $p<0.01$ compared to the control group)

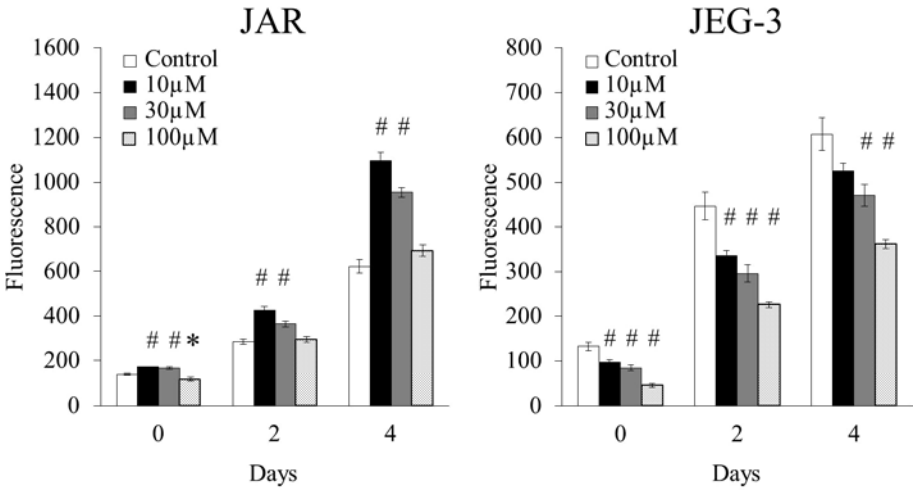

Figure 5 Metabolic activities of JPH203-treated JAR and JEG-3 cells
JAR and JEG-3 cells were treated with 0, 10, 30 and 100μM JPH203 for 0, 2 and 4 days. The metabolic activities were determined by Alamar Blue solution. Data are shown as means ± SE. (* : $p<0.05$, # : $p<0.01$ compared to the control group)

ture solution replacement. However, a JPH203 concentration-dependent decrease in cell count was seen 4 days after replacement.

Changes in cell activity due to JPH203

Cell activity following treatment with JPH203 was examined over time to determine whether amino acid transport by LAT1 is involved in the activity of JAR and JEG-3 cells (Fig. 5). The activity of the JAR cells in the groups with 10 and 30μM added increased on all of the measurement days. In the group with 100μM added, cell activity decreased immediately after culture solution replacement (3 h after), but it did not differ significantly compared with the control from 2 days after replacement onward. The activity of JEG-3 cells decreased significantly in the group with 10μM added immediately after and 2 days after culture solution replacement. The activity of cells in the groups with 30 and 100μM added decreased significantly immediately after and 2 and 4 days after replacement.

DISCUSSION

In this study, it was found that amino acids are transported by LAT1 in two kinds of choriocarcinoma cell lines, JAR cells and JEG-3 cells. Moreover, these cell lines showed different responses of cytostasis and cell activity changes to LAT1 inhibition.

The quantitative RT-PCR showed that the expressions of LAT1, 3 and 4 were slightly but significantly higher in the JAR cells than in the JEG-3 cells. On the contrary, the expression of LAT2 was higher in the JEG-3 cells than in the JAR cells. It is generally known that LAT2 and 4 are expressed in normal cells, while the expressions of LAT1 and 3 are increased in cancer cells[2]. Furthermore, it has been reported that the JAR cells proliferate faster and produce more human chorionic gonadotropin than the JEG-3 cells[14]. From these, it may be said that the JAR cells maintain the characteristics of choriocarcinoma in comparison with the JEG-3 cells. In the Western blotting, the LAT1 protein could be identified in the JEG-3 cells as well as the JAR cells. The LAT1/β-actin ratios were comparable between the two cell lines.

The leucine transport of the JAR cells or the JEG-3 cells was not affected by the existence or the absence of Na in HBSS. It is suggested that the leucine transport by LATs plays a substantial role in the amino acid transport by cultured choriocarcinoma cell lines. The IC_{50} levels of the leucine uptake were reportedly 0.06 μM in the human intestinal tract cancer cell line (HT29)[9] and 0.79 μM in the human oral cancer cell line (YD-38)[8]. In this study, the IC_{50} levels of JPH203 for the leucine uptake inhibition were lower in both the JAR cells and in the JEG-3 cells compared to the YD-38 cells. Thus, it is indicated that the inhibitory action of JPH203 on leucine uptake is higher in the choriocarcinoma cell lines than in the YD-38 cells.

In addition to the HT-29 cells, JPH203 is known to inhibit the proliferation of many other neoplastic cells[8~10]. For example, it has been reported that the 100 μM JPH203 reduced the number of HT-29 cells to 10% of the control after four days[8] and also reduced the number of YD-38 cells to 40% of the control[9]. However, more than half of the JAR and the JEG-3 cells had lived after four days giving 100 μM JPH203 as compared with the control (Figure 4), indicating that the suppressant effect of JPH203 is less potent in the JAR and the JEG-3 cells than in the HT-29 or the YD-38 cells. The role of LAT1 in cell proliferation may be different between these cell lines (HT-29 and YD-38) and the choriocarcinoma cell lines. It is known that amino acids are not only used as constituents or nutrients in cancer cells, but also work as a secondary messengers which promotes the proliferation of cancer cells[2]. A more detailed examinations are necessary to clarify whether the difference in the effect of JPH203 on cell proliferation is caused by the depletion of nutrients or by the signal transduction disturbance in the cancer cells.

The IC_{50} levels of JPH203 calculated by the leucine uptake experiment were comparable between the JAR cells and the JEG-3 cells. On the other hand, the cytostatic effect of JPH203 was concentration-dependent in the JEG-3 cells, while the effect was only seen by a high-concentration of JPH203 in the JAR cells (Figure 4). Considering that the JAR cells have higher proliferative capacity than the JEG-3 cells, it is imagined that the amino acid level necessary for cell proliferation is more in the JAR cells than in the JEG-3 cells (Figure 3A). However, because the amino acid uptake level was higher in the JEG-3 cells than in the JAR cells, it is suggested that these two cell lines have different demand characteristics for amino acids. The higher expression of LAT2 in the JEG-3 cells than in the JAR cells supports a higher demand of amino acids in the JEG-3 cells (Figure 1B). In addition, because JPH203 was added to the culture medium and the RPMI-1640 medium used for the JAR cells contains more amino acids than the MEM medium used for the JEG-3 cells, it is possible that the higher amino acid levels in the medium caused an increased amino acid uptake by the JAR cells than the JEG-3 cells even under the existence of JPH203. The experiments were performed in the environment that the cells are easy to multiply, but the examination under the condition of same amino acid availability for each cell lines may provide different results.

The cell activity evaluation using Alamar Blue reflecting the reduction power of viable cells is a quantitative measurement of the cell proliferation and is often performed in a purpose same as cell count[15].

However, we examined the cell count and the cell activity measurement separately to evaluate the effects of amino acid transport by LAT1 on cell activity in this study. Interestingly, the concentration-dependent decreasing effect of JPH203 was seen within a day in the JEG-3 cells. Whereas, the cell activity started to increase following the addition of JPH203 at concentrations of 10 and 30 μM on the day 0 in the JAR cell (Figure 5). It is thought that the JEG-3 cells could not maintain the metabolism of the cells when the amino acid transport was inhibited by JPH203, which resulted in the decrease of cell activity. On the other hand, the amino acid transport inhibition by JPH203 may have induced the autophagy mechanism which possibly increased the JAR cell activity. It is reported that the cell activity of the human cell line rises with the induction of autophagy mechanism[16], and it is speculated that the autophagy mechanism is easier to be induced in the JAR cells than in the JEG-3 cells. However, the decrease in cell count by 100 μM JPH203 in comparison with the control started to be seen on the 2nd day in the JAR cells, which was earlier than the occurrence of cytostatic effect by JPH203 in the JEG-3 cells. Thus, the high concentration JPH203 was shown to restrain cell proliferation irrespective of the kind of choriocarcinoma cell line. It became clear in this study that the cell activities of the JAR cells and the JEG-3 cells respond differently to the low concentration JPH203, but the high concentration JPH203 can restrain the cell proliferation of these two kinds of cultured choriocarcinoma cells.

CONCLUSION

The present study demonstrated that amino acid transport by LAT1 functions in two kinds of choriocarcinoma cell lines. In addition, highly-concentrated JPH203 showed an inhibitory effect on the proliferation of choriocarcinoma cell lines. Furthermore, it was suggested that the amino acid transport by LAT1 has complicated and various roles in the activity of choriocarcinoma cells.

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Conflict of Interest

No COI for all authors.

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