2008년 8월
박사학위논문

Expression and characterization of amino acid transport system L in HTB-41 human salivary gland epidermoid carcinoma cells

조선대학교 대학원

치의학과

박 노 승

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사람 타액선 편평상피암세포주 *HTB-41*에서
아미노산 수송계 *L*의 발현 및 특성

2008 년 *8* 월 일

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이 논문을 치의학 박사학위신청 논문으로 제출함.

2008 년 *4* 월   일

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ABSTRACT

Expression and characterization of amino acid transport system L in HTB-41 human salivary gland epidermoid carcinoma cells

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Amino acids are required for protein synthesis and energy sources in all living cells. The amino acid transport system L is a major nutrient transport system that is responsible for Na^+-independent transport of neutral amino acids including several essential amino acids. The system L is divided into 2 major subgroups, the L-type amino acid transporter 1 (LAT1) and the L-type amino acid transporter 2 (LAT2). In malignant tumors, the LAT1 is highly expressed to support their continuous growth and proliferation. Although the salivary gland squamous cell carcinomas has an extremely poor prognosis, the expression and characterization of amino acid transporters for supplying nutrition to cells in the salivary gland tumors including the salivary gland squamous carcinomas cells are not known at all. In the present study, the expression and functional characterization of amino acid transport system L were, therefore, examined in the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

The expressions and functions of the system L amino acid transporters in the

HTB-41 cells were investigated using RT-PCR analysis, western blot analysis and amino acid transport measurements.

RT-PCR analysis and western blot analysis have revealed that the HTB-41 cells expressed the LAT1 together with its associating protein heavy chain of 4F2 antigen (4F2hc) in the plasma membrane, whereas the HTB-41 cells did not express the LAT2. The uptakes of [^{14}C]L-leucine by HTB-41 cells were Na$^+$-independent and completely inhibited by a system L selective inhibitor, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). The affinity of [^{14}C]L-leucine uptake and the inhibition profile of [^{14}C]L-leucine uptake by various L-amino acids in the HTB-41 cells were comparable with those for the LAT1 expressed in *Xenopus* oocytes. The majority of [^{14}C]L-leucine uptake is, therefore, mediated by LAT1 in the HTB-41 cells. In the MTT assay, the BCH inhibited the growth of the HTB-41 cells in the time- and concentration-dependent manners, indicating that the growth inhibition of HTB-41 cells by BCH is induced by the blocking of neutral amino acid transport mediated by LAT1.

These results suggest that the transports of neutral amino acids including several essential amino acids into the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells are mediated by LAT1. Therefore, the HTB-41 cell is proposed to be an excellent tool for examine the properties of LAT1. Moreover, the specific inhibition of LAT1 in tumor cells including the salivary gland tumor cells might be a new rationale for anti-tumor therapy.

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Key Words: Amino acid transport system L, LAT1, LAT2, Salivary gland carcinoma cells, Essential amino acids, Anti-tumor therapy

Ⅰ. *INTRODUCTION*

Amino acids are indispensable for protein synthesis, which is essential for cell growth and proliferation in both normal and transformed cells (1,2). Amino acid transport across the plasma membrane is mediated via amino acid transporters located on the plasma membrane (1,2). Among the amino acid transport systems, the amino acid transport system L, which is a Na^+-independent neutral amino acid transport system, is a major route for providing living cells, as well as tumor cells, with neutral amino acids including several essential amino acids (1,3). Because of its broad substrate selectivity, system L amino acid transporters are regarded as the drug transporters which transport not only naturally occurring amino acids but also amino acid-related drugs such as L-dopa, a therapeutic drug for Parkinsonism; melphalan, an anticancer phenylalanine mustard; triiodothyronine and thyroxine, two thyroid hormones; gabapentin, an anticonvulsant (1,4-9).

Recently, the system L-type amino acid transporters 1 and 2 (LAT1 and LAT2), the first and second isoforms of system L amino acid transport system, were isolated (10-12). They are predicted to be 12-membrane-spanning proteins that mediate Na^+-independent amino acid exchange (10-12). They require an additional single-membrane-spanning protein, a heavy chain of 4F2 antigen (4F2hc), for their functional expression in the plasma membrane (10-16). LAT1/4F2hc or LAT2/4F2hc forms a heterodimeric complex via a disulfide bond, respectively. LAT1 mRNA is only expressed in restricted organs such as brain, spleen, placenta and testis (10,11,16,17). However, the mRNAs of LAT2 and 4F2hc are ubiquitously expressed in all normal embryonic and normal adult tissues (10-12,16). In addition, LAT1 is highly expressed in malignant tumors presumably to support their continuous growth and proliferation (10,11,18,19). The LAT1

prefers large neutral amino acids for its substrates (10,11,20), while the LAT2 transports not only large neutral amino acids, but also small neutral amino acids, in a fashion that appears to have broader substrate selectivity than LAT1 (12,21-23).

As mentioned above, it has been proposed that the manipulation of system L activity in particular that of LAT1 could have anti-tumor therapeutic implications. The inhibition of LAT1 activity in tumor cells could suppress the growth of tumor cells by depriving of essential amino acids (9). When the LAT1 activity in tumor cells is completely blocked, the growth and proliferation of normal cells could be possible due to the presence of LAT2.

Up to now, the functional properties of amino acid transporters including LAT1 and LAT2 have been studied formerly by injecting cRNAs of amino acid transporters into *Xenopus* oocytes (10-12,20). However, the *Xenopus* oocytes experimental system is not well suited for the exact investigation the characteristics of mammalian amino acid transporters and makes many demands of time and money. So, it is essential for the development of mammalian cell experimental system for the research of amino acid transporters including LAT1 and LAT2.

Salivary gland tumors are a morphologically and clinically diverse group of neoplasms, which may present considerable diagnostic and management challenges to the pathologist or surgeon (24). Salivary gland tumors are rare with an overall incidence in the Western world of about 2.5 – 3.0 per 100,000 per year (25). Among salivary gland tumors, the salivary gland squamous cell carcinomas are more rare (25). However, although this tumor has an extremely poor prognosis, unlike most other salivary gland malignancies, survival at 5 years is prognostically significant (25). Furthermore, the expression and functional characterization of amino acid transporters such as the system L amino acid transporters for

supplying nutrition to cells in the salivary gland tumors including the salivary gland squamous carcinomas cells are not known at all.

In the present study, therefore, the expressions and functional characterization of system L amino acid transporters were investigated in the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

II. MATERIALS AND METHODS

1. Materials

[^{14}C]L-leucine was purchased from PerkinElmer Life Sciences Inc. (Boston, MA, USA). Affinity-purified rabbit anti-LAT1, anti-LAT2 and anti-4F2hc polyclonal antibodies were supplied by Kumamoto Immunochemical Laboratory, Transgenic Inc. (Kumamoto, Japan). Anti-rabbit-horseradish peroxidase conjugated-secondary antibody and envision (+) rabbit peroxidase were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and DAKO (Glostrup, Denmark), respectively. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) and other chemicals were purchased from Sigma (St Louis, MO, USA).

2. Cell line and cell culture

HTB-41 human submaxillary salivary gland epidermoid carcinoma cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA). The HTB-41 cells were grown in modified McCoy's media with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate with 10% FBS in accordance with ATCC's instruction. The cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO_2.

3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from HTB-41 cells maintained in the growth medium at 37°C by RNA preparation kit (Isogen, Nippon-Gene, Japan) in

accordance with the manufacturer's instruction.

For RT-PCR analysis, the first-strand cDNA was prepared from HTB-41 cell total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Inc., CA, USA) with an oligo dT primer, and used as a template for PCR amplification. The PCR amplification was performed using Taq polymerase Amplitaq Gold (Roche Molecular Systems, Inc., Germany) in the following protocol: 94°C for 12 min, followed by 25 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec and a final extension step of 72°C for 10 min. A pair of primers, 5'-TTCATCGCAGTACATCGTGG-3' (491-510 bp) and 5'-CCCAGGTGATAGTTCCCGAA-3' (1008-1027 bp), was used for PCR amplification of human LAT1. A pair of primers, 5'-AGCCCTGAAGAAAGAGAT CG-3' (811-830 bp) and 5'-TGCATATCTGTACAATCCCC-3' (1321-1340 bp), was used for PCR amplification of human LAT2. A pair of primers, 5'-TCGATTACCTGAGCTCTCTG-3' (551-570 bp) and 5'-GGGATTTTGTATGC TCCCCA-3' (1041-1060 bp), was used for PCR amplification of human 4F2hc.

4. Western blot analysis

Protein samples from HTB-41 cells were prepared as described elsewhere (26,27), with minor modifications. Briefly, HTB-41 cells were washed with PBS and prepared by centrifugation 5 min at 1000 × g. The HTB-41 cells were homogenized in 9 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose) with 15 strokes of Dounce homogenizer. The homogenate was centrifuged for 10 min at 8,000 × g, and the supernatant was centrifuged further for 1 h at 100,000 × g. After centrifugation, the membrane pellet was resuspended in resuspend buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose). The

protein samples were heated at 100°C for 5 min in the SDS sample buffer (60 mM Tris-HCl, pH 6.8, 4% SDS, 25% glycerol, 0.1% Bromophenol blue) in the presence of 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to a Hybond-P polyvinylidene difluoride transfer membrane. The membrane was treated with non-fat dried milk and diluted anti-LAT1 (1:1000), LAT2 (1:1000) and 4F2hc (1:1000) affinity-purified antibodies (26,27) and then with horseradish peroxidase-conjugated antirabbit IgG as a secondary antibody. The signals were detected with an ECL plus system (Amersham Pharmacia Biotech, NJ, USA).

5. Uptake measurements

To characterize the function of the endogenously expressed system L amino acid transporters in the HTB-41 cells, uptake experiments were performed as described elsewhere (28). The HTB-41 cells were maintained in the growth medium at 37°C. The cells were collected and seeded on 24-well plates (1 X 10^5 cells/well) in fresh growth medium. The uptake measurements were performed when the cells were approximately 85 – 95% confluent on 24-well plates.

After the removal of the growth medium, the cells were washed 3 times with the standard uptake solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM HEPES, 1.2 mM KH$_2$PO$_4$, 5.6 mM Glucose, pH 7.4) or Na$^+$-free uptake solution (125 mM choline-Cl, 4.8 mM KCl, 1.3 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM HEPES, 1.2 mM KH$_2$PO$_4$, 5.6 mM Glucose, pH 7.4), and preincubated for 10 min at 37°C. Then, the medium was replaced by the uptake solution containing [^{14}C]L-leucine. The uptake was terminated by removing the uptake solution, followed by washing three times with ice-cold uptake solution (28). Then, the cells were solubilized with 0.1 M NaOH and the radioactivity was counted by liquid

scintillation spectrometry. The values are expressed as pmol/mg protein/min. For the measurement of the uptake of [^{14}C]L-leucine, four to six wells of HTB-41 cells were used for each data point. To confirm the reproducibility of the results, three or four separate experiments were performed for each measurement. Results from the representative experiments are shown in figures.

The K_m and V_{max} values were determined using Eadie-Hofstee plots based on the [^{14}C]L-leucine uptakes measured for 1 min at 1, 3, 10, 30, 100, 300 and 1000 μM for the HTB-41 cells. The IC_{50} values for BCH on the L-leucine transport were determined on the 1 μM [^{14}C]L-leucine uptake measured for 1 min in the presence of 0, 1, 3, 10, 30, 100, 300, 1000 and 3000 μM BCH. To measure the K_i values for BCH, the uptake rates of [^{14}C]L-leucine were measured for 1 min at various concentrations of [^{14}C]L-leucine (1, 3, 10, 30, 100, 300 and 1000 μM) with or without the addition of 100 μM BCH. The K_i values were determined by double reciprocal plot analysis where 1/uptake rate of [^{14}C]L-leucine was plotted against 1/L-leucine concentration. The K_i values were calculated from the following equation when competitive inhibition was observed: K_i = concentration of inhibitor/((K_m of L-leucine with inhibitor/K_m of L-leucine without inhibitor)-1) (20,27). For the inhibition experiments, the uptake of 30 μM [^{14}C]L-leucine uptake was measured in the presence or absence of the 3 mM non-labeled L-amino acids and BCH.

6. MTT assay

The MTT assay was performed according to a previously described method (29), with minor modifications. The HTB-41 cells were seeded at a concentration of 5 X 10^3 cells/well in a 24-well plate. After 24 h growth, the cells were treated with BCH at various concentrations and incubation times. Then, cell viability was

assessed using MTT assay. Briefly, the cells were grown in the medium in the absence or presence of BCH for various incubation times. Following the culture, 0.5 mg/ml of MTT was added to each well. After 4 h incubation at $37^{\circ}C$, isopropanol with 0.04 M HCl was added to each well to dissolve precipitates. Then, the absorbance was measured at 570 nm using a spectrophotometer (Ultrospec 2000; Amersham Pharmacia Biotech, NJ, USA). Three or four separate experiments were performed for each concentration/exposure time combination.

7. Data Analysis

All experiments were performed at least in triplicate. Results were presented as mean ± S.E.M. Statistical significance was analyzed using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. $P<0.05$ was considered statistically significant.

Ⅲ. *RESULTS*

1. Detection of system L amino acid transporters in HTB-41 cells

In the RT-PCR analysis, the PCR products for LAT1 and its associating protein 4F2hc were detected in the HTB-41 cells, whereas the LAT2 was not detected in HTB-41 cells (Fig. 1). Western blot analysis was performed on the membrane fractions prepared from HTB-41 cells. The antibodies raised against LAT1 and 4F2hc recognized the 40 kDa protein band and 85 kDa protein band under the reducing condition, respectively (Fig. 2). The protein band of LAT2 did not recognize on the membrane fractions prepared from HTB 41 cells (Fig. 2). The results from the RT-PCR and western blot analyses indicate that LAT1 but not LAT2 is present together with 4F2hc in HTB-41 cells.

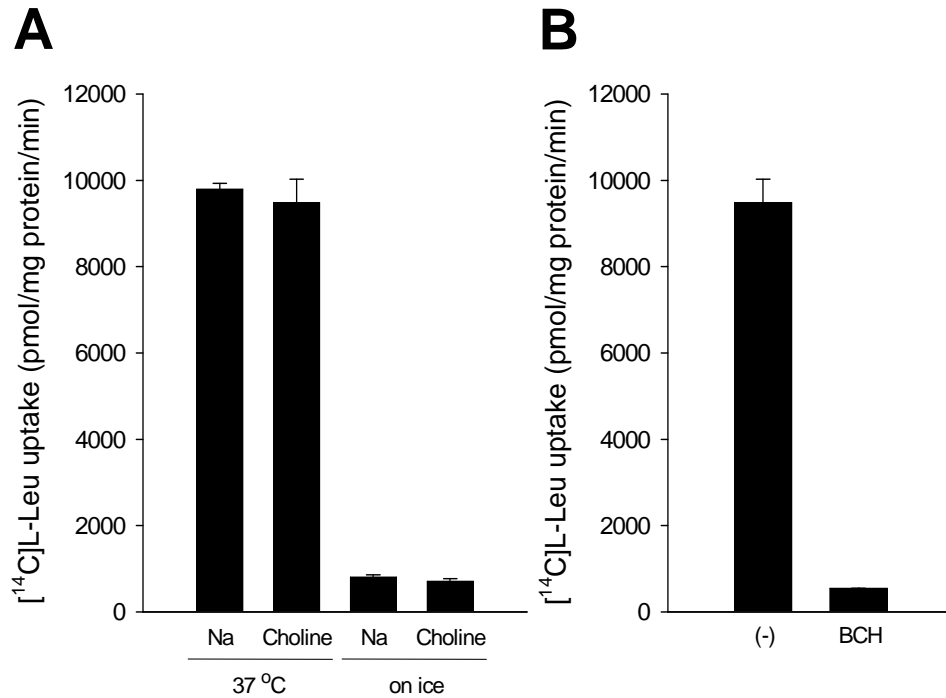## *2. Properties of [^{14}C]L-leucine uptake by HTB-41 cells*

The properties of [^{14}C]L-leucine transport were examined in HTB-41 cells. As shown in Fig. 3A, the level of [^{14}C]L-leucine uptake (30 μM) by HTB-41 cells measured in the standard uptake solution (Na) was not altered by replacing NaCl of the uptake solution with choline-Cl (Choline), indicating that L-leucine uptake by HTB-41 cells is primarily Na^{+}-independent. In the subsequent experiments, the transport measurements were performed under Na^{+}-free conditions. When the uptake measurements were performed on ice, the [^{14}C]L-leucine uptake was not detected, confirming that the [^{14}C]L-leucine uptake by HTB-41 cells was due to the transporter-mediated transport (Fig. 3A). As shown in Fig. 3B, the uptake of [^{14}C]L-leucine (30 μM) was almost completely inhibited by 3 mM BCH, a specific inhibitor of system L amino acid transporters, indicating that the system L amino acid transporters are responsible for the [^{14}C]L-leucine uptake in the HTB-41 cells.

Fig. 1. Detection of LAT1, LAT2 and 4F2hc by RT-PCR in HTB-41 human submaxillary salivary gland epidermoid carcinoma cells. The PCR products were subjected to electrophoresis on a 1.2 % agarose gel and visualized with ethidium bromide. The LAT1-specific PCR product (536 bp), LAT2-specific PCR product (529 bp) and 4F2hc-specific PCR product (509 bp) were obtained from HTB-41 cells.
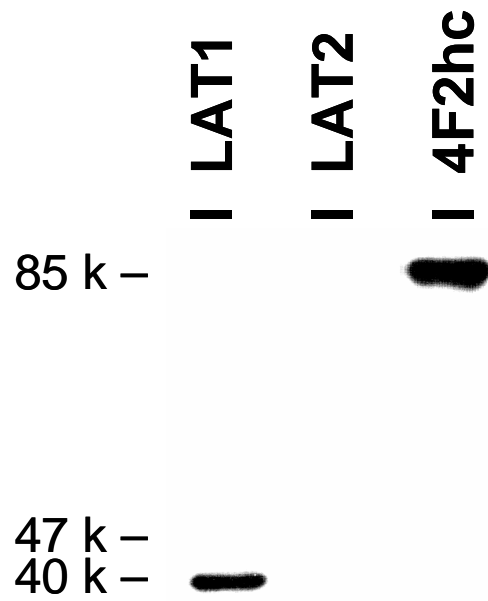
Fig. 2. Western blot analysis of LAT1, LAT2 and 4F2hc in HTB-41 cells. Western blot analyses were performed on the membrane fractions prepared from HTB-41 cells in the presence of 2-mercaptoethanol using anti-LAT1, anti-LAT2 and anti-4F2hc antibodies. For LAT1, LAT2 and 4F2hc, the 40 kDa- , 47 kDa- and 85 kDa-protein bands detected, respectively.

Fig. 3. [^{14}C]L-leucine uptake by HTB-41 cells. (A) Ion dependence of [^{14}C]L-leucine transport. [^{14}C]L-leucine (30 μM) uptake measured in the standard uptake solution (Na) was compared with that measured in the Na$^+$-free uptake solution (Choline). The [^{14}C]L-leucine transport measurement was performed at 37℃ and on ice. *(B)* Inhibition of [^{14}C]L-leucine transport by BCH, a specific inhibitor of amino acid transport system L. The [^{14}C]L-leucine (30 μM) uptake was measured in the presence (BCH) or absence ((-)) of 3 mM BCH.

To determine the time-course of [^{14}C]L-leucine uptake by HTB-41 cells, the level of [^{14}C]L-leucine (30 µM) uptake was measured for 0.5, 1, 2, 2.5, 5, 10, 15 and 20 min. The uptake of [^{14}C]L-leucine was time-dependent and exhibited a linear dependence on the incubation time up to 1 min (Fig. 4). So all the subsequent uptake measurements were conducted for 1 min and the values are expressed as pmol/mg protein/min. As shown in Fig. 5, the [^{14}C]L-leucine uptake was saturable and followed Michaelis-Menten kinetics with a K_m value of 47.7 ± 5.2 µM (mean ± S.E.M. of three separate experiments) for [^{14}C]L-leucine uptake.

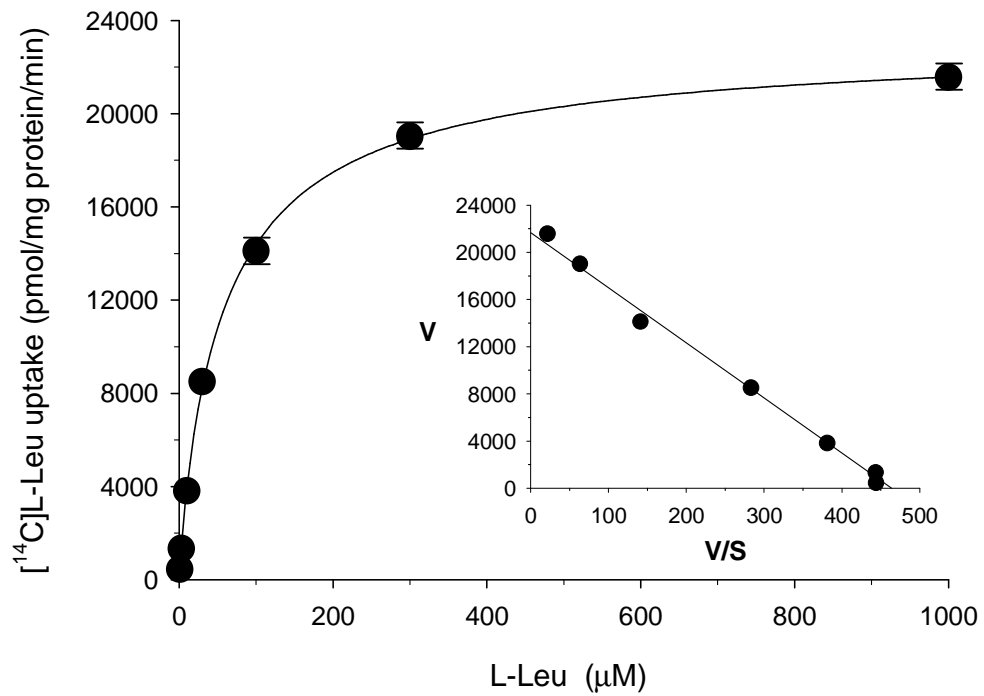## 3. Inhibition of [^{14}C]L-leucine uptake by BCH in HTB-41 cells

In order to examine which BCH concentration interacts with the L-leucine uptake mechanism in the HTB-41 cells, the [^{14}C]L-leucine (1 µM) uptake was measured in the presence of various BCH concentrations (0, 1, 3, 10, 30, 100, 300, 1000 and 3000 µM) and the uptake rate was measured at various [^{14}C]L-leucine concentrations (1, 3, 10, 30, 100, 300 and 1000 µM), with or without the addition of 100 µM BCH. As shown in Fig. 6, the BCH (1 – 3000 µM) inhibited the [^{14}C]L-leucine (1 µM) uptake in a concentration-dependent manner with an IC_{50} value of 65.3 ± 5.2 µM (mean ± S.E.M. of three separate experiments). In the HTB-41 cells, the inhibition of [^{14}C]L-leucine uptake by BCH was shown to be competitive in a double reciprocal plot analysis with a K_i value of 41.1 ± 3.8 µM (mean ± S.E.M. of four separate experiments) (Fig. 7).
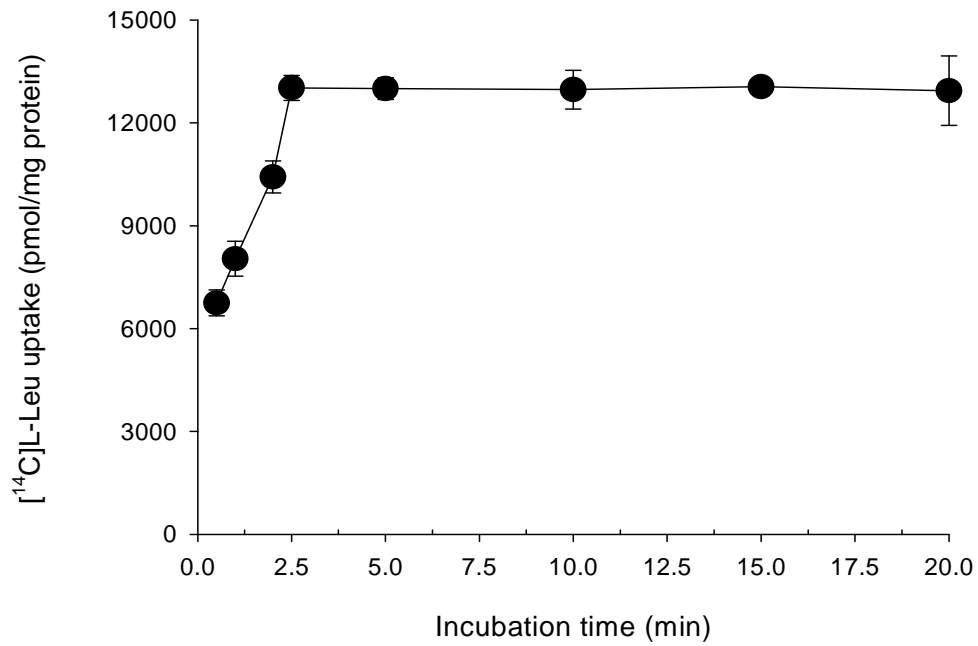
Fig. 4. Time-course of [¹⁴C]L-leucine uptake by HTB-41 cells. The HTB-41 cells were incubated in the Na⁺-free uptake solution containing 30 μM [¹⁴C]L-leucine for 0.5, 1, 2, 2.5, 5, 10, 15 and 20 min.

Fig. 5. Concentration-dependence of [^{14}C]L-leucine uptake by HTB-41 cells.
The uptake of [^{14}C]L-leucine by HTB-41 cells was measured for 1 min and plotted against L-leucine concentration. The L-leucine uptake was saturable and fit to the Michaelis-Menten curve (K_m = 47.7 μM). The inset shows an Eadie-Hofstee plot of L-leucine uptake that was used to determine the kinetic parameters.
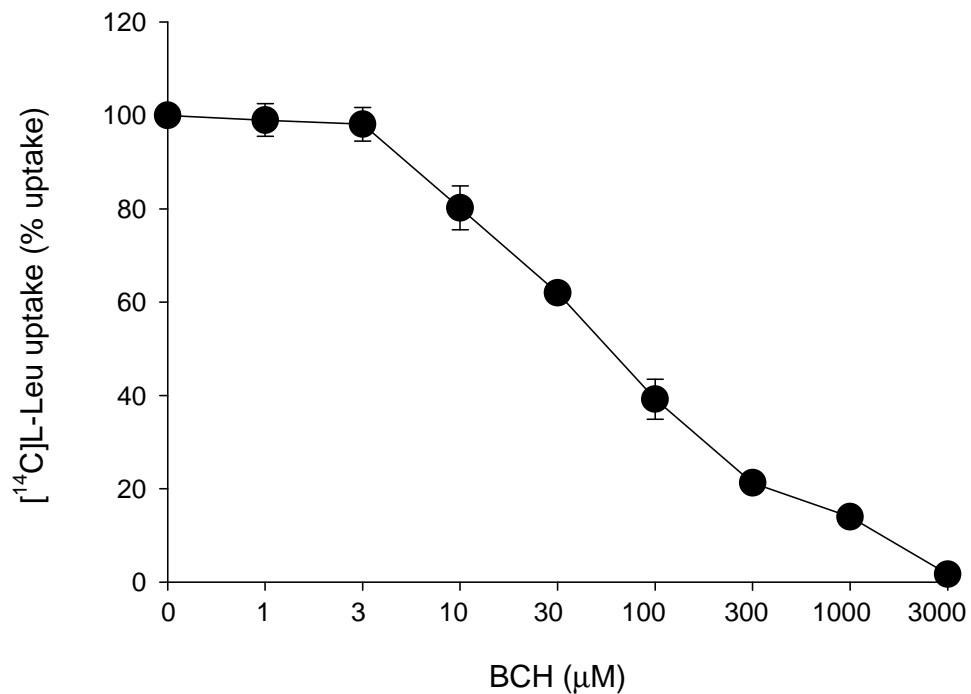
Fig. 6. Concentration-dependent inhibition of [^{14}C]L-leucine uptake by BCH in HTB-41 cells. The [^{14}C]L-leucine uptake (1 μM) was measured for 1 min in the presence of various BCH concentrations in HTB-41 cells, and was expressed as a percentage of the control L-leucine uptake in the absence of BCH.
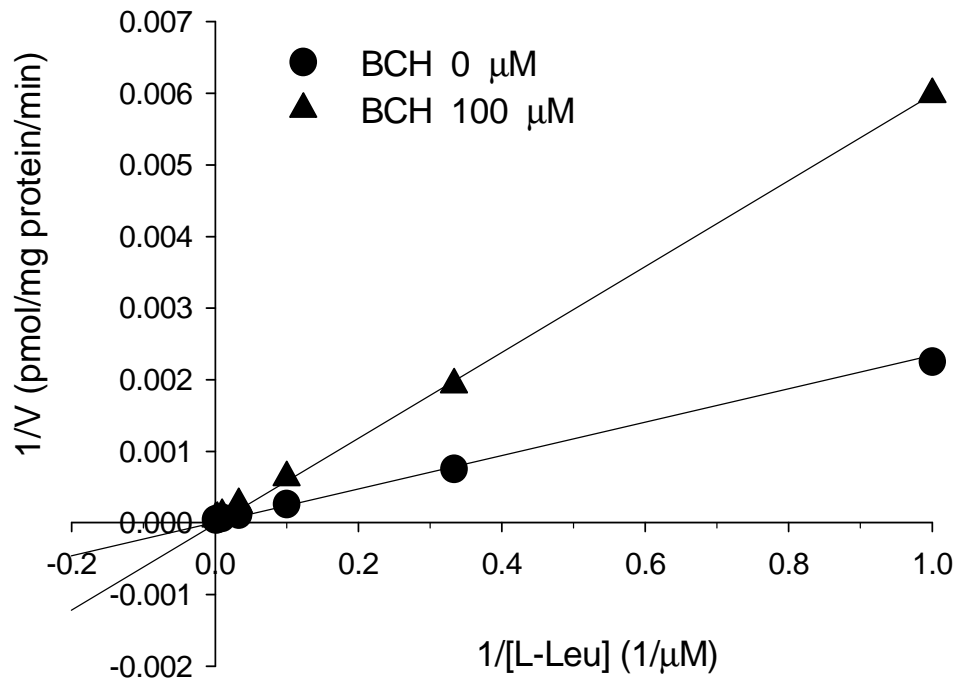
Fig. 7. Double reciprocal plot analysis of inhibitory effect of BCH on the [^{14}C]L-leucine uptake in HTB-41 cells. The [^{14}C]L-leucine uptakes (1, 3, 10, 30, 100, 300 and 1000 μM) were measured in the Na$^+$-free uptake solution in the presence (filled triangle) or absence (filled circle) of 100 μM BCH in HTB-41 cells.
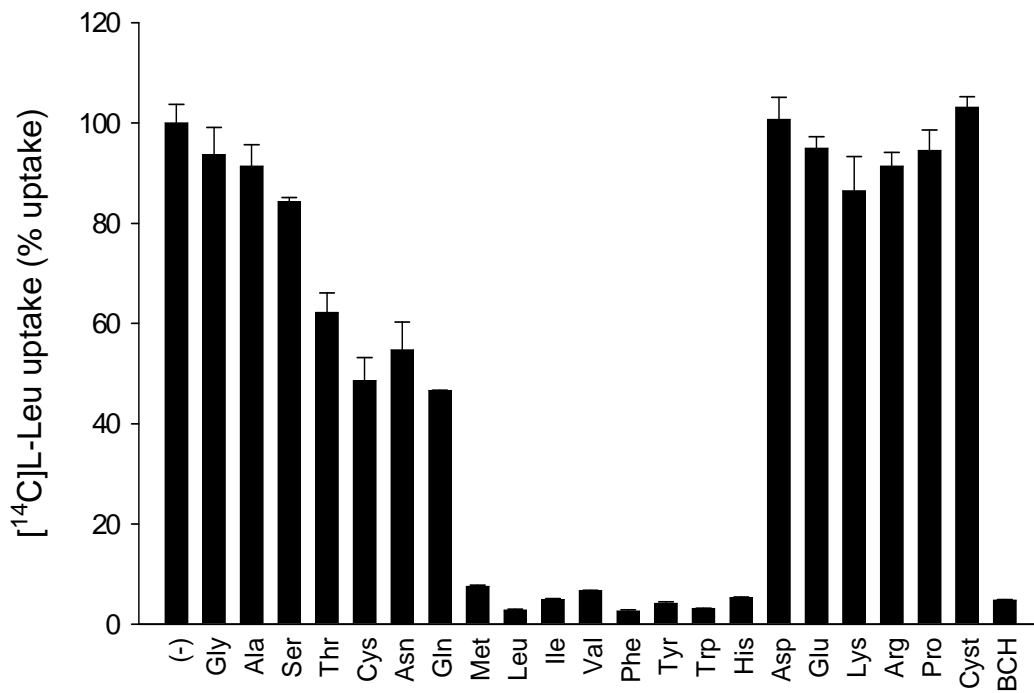
4. Inhibition of [^{14}C]L-leucine uptake by various amino acids in HTB-41 cells

In order to examine which various L-amino acids interact with L-leucine uptake mechanism in the HTB-41 cells, the [^{14}C]L-leucine (30 μM) uptake was measured in the presence of 3 mM nonlabeled L-amino acids in the Na$^+$-free uptake solution. The [^{14}C]L-leucine uptake was strongly inhibited by the L-isomers of methionine, leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan and histidine (Fig. 8). In the HTB-41 cells, threonine, cysteine, asparagine and glutamine exhibited weaker inhibitory effects on [^{14}C]L-leucine transport and glycine, alanine, serine, aspartate, glutamate, lysine, arginine, proline and cystine have not revealed any inhibitory effects on [^{14}C]L-leucine transport (Fig. 8), consistent with the properties of LAT1 expressed in *Xenopus* oocytes (10,11,20).

5. Growth inhibition of HTB-41 cells by BCH

To analyze the effect of BCH on the viability of HTB-41 cells, the cells were treated with BCH at various concentrations and incubation times, then the MTT assay was performed. From 3 to 50 mM treatment of BCH, the growth inhibition of HTB-41 cells depended on the BCH treatment time (Fig. 9). When the BCH treated to the cells at 1, 3, 10, 20 and 50 mM, the BCH inhibited the proliferation of HTB-41 cells in a dose-dependent manner (Fig. 10).

Fig. 8. Inhibition of [¹⁴C]L-leucine uptake by amino acids in HTB-41 cells.
The [¹⁴C]L-leucine (30 μM) uptake was measured in the presence of 3 mM
nonradiolabeled indicated L-amino acids and system L specific inhibitor BCH in the
Na⁺-free uptake solution.
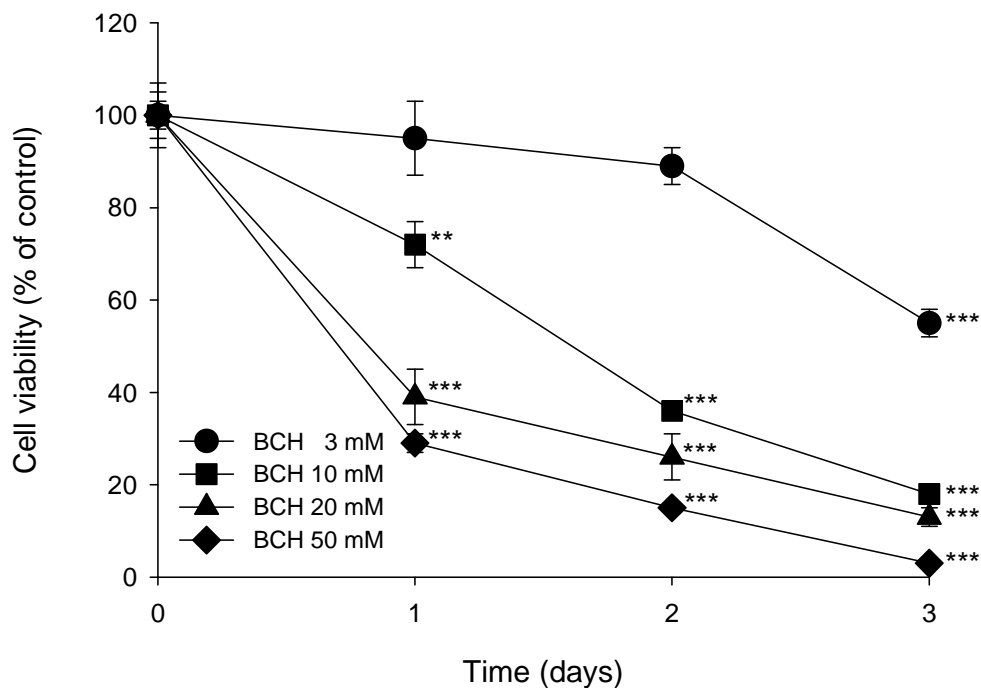
Fig. 9. Time-dependent effect of BCH on the cell viability in HTB-41 cells.
The HTB-41 cells were treated with 0, 3, 10, 20 and 50 mM BCH for 0 – 3 days.
The cell viabilities were determined by the MTT assays. The percentage of cell
viability was calculated as a ratio of A570 nms of BCH treated cells and untreated
control cells. Each data point represents the mean ± SEM of three experiments.
**P<0.01 vs. control and ***P<0.001 vs. control (the control cells measured in the
absence of BCH).
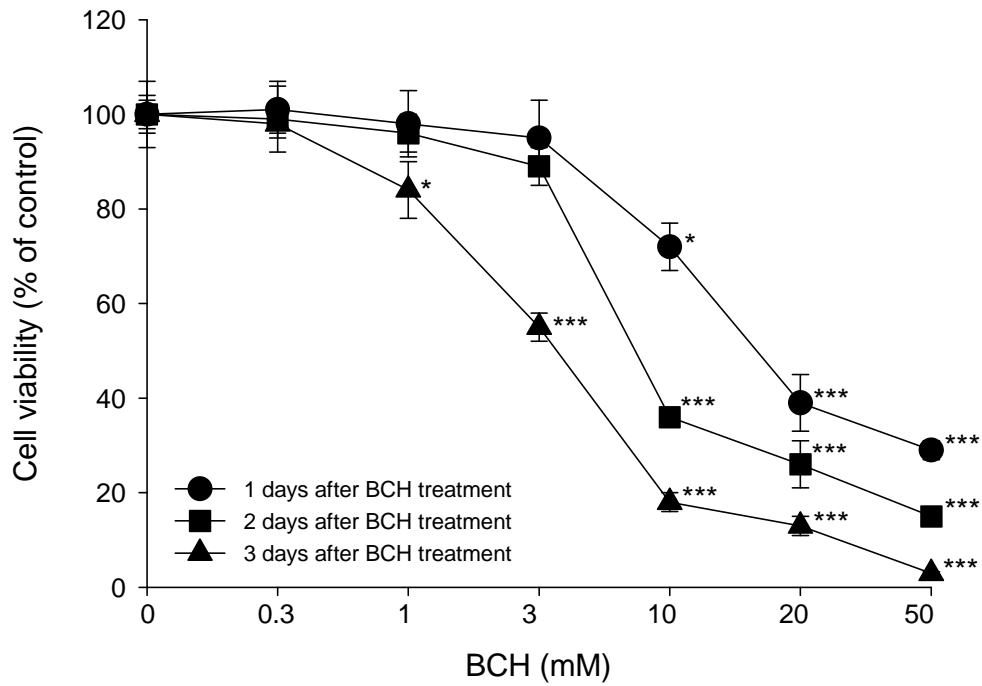
Fig. 10. Concentration-dependent effect of BCH on the cell viability in HTB-41 cells. The HTB-41 cells were treated with various concentrations of BCH or without BCH for 3 days. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of A570 nms of BCH treated cells and untreated control cells. Each data point represents the mean ± SEM of three experiments. $^{*}P<0.05$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of BCH).

Ⅳ. DISCUSSION

It has been known that the system L amino acid transporters play an important role in the proliferation and continuous growth of tumor cells (1,2). However, the expression and functional characterization of the system L amino acid transporter in the salivary gland tumor cells have not been clarified. In the present study, therefore, the expressions of the system L amino acid transporters and the properties of L-leucine transport were examined in the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells.

By RT-PCR analysis, the HTB-41 cells were shown to express LAT1, an isoform of system L amino acid transporter, together with its associating protein 4F2hc, but do not express the other system L isoform LAT2 (Fig. 1). In the western blot analysis on the membrane fraction prepared from HTB-41 cells, the proteins for LAT1 and 4F2hc were detected, but not LAT2 (Fig. 2). In the previous study, it was reported that the KB human oral cancer cells express LAT1 and 4F2hc, but not LAT2, and that the transport of neutral amino acids into the KB cells is mediated mainly by LAT1 (26). In addition, the LAT1 is highly expressed in malignant tumors, presumably to support their continuous growth and proliferation (10,11,18,19). As the results in this study correspond with that of the previous study (26), it can be concluded that the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells express the LAT1 with 4F2hc, but not LAT2.

The [^{14}C]L-leucine uptake measured in the HTB-41 cells was Na$^+$-independent and was almost completely inhibited by the selective inhibitor of the system L amino acid transporters, BCH (Fig. 3). This suggests that the majority of L-leucine uptake by HTB-41 cells is mediated by amino acid transport system L. Because, as already mentioned, the HTB-41 cells express LAT1, the

LAT1 is proposed to be responsible for the L-leucine uptake by the HTB-41 cells.

In this study, to determine the time-course of [^{14}C]L-leucine uptake by HTB-41 cells, the level of uptake was measured for 0.5 – 20 min. The uptake of [^{14}C]L-leucine (30 µM) was time-dependent and exhibited a linear dependence on the incubation time up to 1 min (Fig. 4). Therefore, all the uptake measurements were conducted for 1 min.

The [^{14}C]L-leucine uptake by the HTB-41 cells was saturable and followed Michaelis-Menten kinetics (Fig. 5). The K_m value of the HTB-41 cells for the [^{14}C]L-leucine uptake was approximately 48 µM. The K_m value of the HTB-41 cells was relatively higher than those for the human LAT1 (20 µM) (11) and rat LAT1 (18 µM) (10,20) expressed in the *Xenopus* oocytes. This difference may be due to the different cell systems and conditions used. In spite of this small difference, the K_m value for the HTB-41 cells was similar to those of human and rat LAT1s expressed in the *Xenopus* oocytes (10,11,20). In addition, when the K_m values of the HTB-41 cells (48 µM) and KB cells (65 µM) (26), as already mentioned, for [^{14}C]L-leucine uptake compared, the K_m value of the HTB-41 cells was apparently more similar to those of human and rat LAT1s expressed in the *Xenopus* oocytes. This result also suggests that the majority of L-leucine uptake by HTB-41 cells is mediated by LAT1.

BCH is an amino acid-related compound that has been used as a selective inhibitor of the system L amino acid transporters, including LAT1 and LAT2 (1,30). Although BCH is the selective inhibitor of system L, BCH also inhibits the transport of amino acids mediated by a Na$^+$-dependent neutral and basic amino acid transporter, ATB$^{0,+}$ (31). In the Na$^+$-free condition, however, BCH selectively inhibits the system L-mediated transport of amino acids (1,30,31). In this study, BCH completely inhibited the [^{14}C]L-leucine uptake measured in the HTB-41 cells (Fig. 3, Fig. 6 and Fig. 7). The [^{14}C]L-leucine uptake by the HTB-41 cells was

inhibited by BCH in a concentration-dependent fashion (Fig. 6). The inhibition was shown to be competitive with a K_i value of 41 μM in the HTB-41 cells (Fig. 7). The K_i value for the inhibition of [^{14}C]L-leucine uptake by BCH was very similar to the K_m value for [^{14}C]L-leucine uptake in the HTB-41 cells.

The [^{14}C]L-leucine uptake by the HTB-41 cells was markedly inhibited by the L-isomers of only the large neutral amino acids (Fig. 8). The profiles of inhibition of [^{14}C]L-leucine uptake by L-amino acids in the HTB-41 cells are, thus, in principle, comparable to the previous reports for human LAT1 (11) and rat LAT1 (10,20) expressed in *Xenopus* oocytes and those performed for the profiles of inhibition of [^{14}C]L-leucine uptake by L-amino acids in KB cells (26). As already mentioned, the LAT1 is the predominant system L amino acid transporter in HTB-41 cells. Taken together, it is concluded that the majority of [^{14}C]L-leucine uptake is mediated by LAT1 with its associating protein 4F2hc in the HTB-41 cells. These results also suggest that the HTB-41 cell system is an excellent tool to investigate the transport properties of various compounds via LAT1.

In this study, with the effect of BCH, the transport of [^{14}C]L-leucine in the HTB-41 cells was inhibited. So, the MTT assay was conducted to confirm how such intracellular amino acid changes affected to the HTB-41 cell growth. In MTT assay for inhibitory effect in cell growth, the BCH inhibited the HTB-41 cell growth in the time- and dose-dependent manners (Fig. 9 and Fig. 10). This result corresponded with the results of several compounds ([6]-paradol, norcantharidin and baccatin) having antitumor effect that suppressed the cancer cell growth in the time- and concentration-dependent manners (29,32,33). The LAT1 is upregulated in tumor cells to support their continuous growth and proliferation (10,11,18,19). The LAT1 was mainly expressed in the HTB-41 cells, too, and then neutral amino acids were transported for cell growth and proliferation. However, the blockade of LAT1 by BCH in the HTB-41 cells may induce intracellular depletion of neutral

amino acids containing essential amino acids being necessary for cell growth. As the results, it was thought that the inhibition of HTB-41 cell growth was induced.

In the present study, the expressions of the system L amino acid transporters and the properties of the L-leucine transport have characterized in the HTB-41 cells. The results suggested that the transport of neutral amino acids, including several essential amino acids, into the HTB-41 mediate mainly by LAT1 and also proposed that the HTB-41 cell system is an excellent tool to investigate the characterization of LAT1. The LAT1 is upregulated in tumor cells to support their continuous growth and proliferation (10,11,18,19), and is a major route through which the HTB-41 cells, gather up large neutral amino acids to support their continuous growth and proliferation. Although the LAT1 activity in tumor cells is completely blocked, the growth and proliferation of normal cells is possible because the LAT2 is present. Therefore, the specific inhibition of LAT1 would be a new rationale for the suppression of tumor cell growth including the salivary gland tumor cell growth.

Overall, the results of the present study demonstrate that the majority of transport of neutral amino acids into the HTB-41 human submaxillary salivary gland epidermoid carcinoma cells is mediated by LAT1 with its associating protein 4F2hc. Conclusively, these facts propose that the HTB-41 cell system is an excellent cell system to investigate the characterization of LAT1 and the LAT1 would be a new target to inhibit the salivary gland tumor cell growth.

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ABSTRACT in KOREAN

사람 타액선 편평상피암세포주 *HTB-41*에서
아미노산 수송계 *L*의 발현 및 특성

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사람 타액선 편평상피암세포주 HTB-41에서 아미노산 수송계 L의 발현 및 이들 수송계 L을 통한 이미노산 수송특성을 밝히기 위하여, HTB-41 세포에서 RT-PCR, analysis, western blot analysis, uptake 실험 및 MTT 분석을 시행하였다.

HTB-41 세포에서 아미노산 수송체 LAT1 및 그 보조인자 4F2hc의 발현은 확인할 수 있었으나, LAT2의 발현은 관찰할 수 없었다. HTB-41 세포에서 L-leucine 수송은 Na^+-비의존적이었으며, 아미노산 수송계 L의 선택적 억제제인 BCH에 의해 완전히 차단되었다. HTB-41 세포에서 $[^{14}C]$L-leucine의 수송친화력은 *Xenopus* oocyte에서 시행한 LAT1의 수송친화력과 유사하였으며, 아미노산들에 의한 $[^{14}C]$L-leucine의 수송 억제는 *Xenopus* oocyte에서 시행한 LAT1의 실험결과와 유사하였다. BCH는 HTB-41 세포의 성장을 시간- 및 농도-의존적으로 억제하였다.

본 연구의 결과로 사람 타액선 편평상피암세포주 HTB-41에서는 중성아미노산 수송계 L 중에서 주로 LAT1을 통해 L-leucine을 포함한 중성아미노산의 수송이 이루어

지고 있다는 것을 확인 할 수 있었으며, BCH는 이 LAT1을 차단하여 중성아미노산들의 세포 내 고갈을 유도함으로서 HTB-41 세포성장의 억제를 유도하는 것으로 사료된다. 또한 본 연구의 결과로 LAT1의 연구에 HTB-41 세포 유용성의 제시 및 이 LAT1의 억제제를 이용하여 타액선 암세포의 성장억제에 관한 또 하나의 방향성을 제시할 수 있을 것으로 사료된다.

저작물 이용 허락서

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논문제목		한글: **사람 타액선 편평상피암세포주 *HTB-41*에서 아미노산 수송계 *L*의 발현 및 특성** 영문: *Expression and characterization of amino acid transport system L in HTB-41 human salivary gland epidermoid carcinoma cells*			

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다   음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2008년 8월   일

저작자: 박 노 승   (서명 또는 인)

조선대학교 총장 귀하