The RNA Interference of Amino Acid Transporter LAT1 Inhibits the Growth of KB Human Oral Cancer Cells

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Abstract. *Background: Amino acid transporters are essential for growth and proliferation in all living cells. Among the amino acid transporters, the system L amino acid transporters are the major nutrient transport system responsible for the Na^+-independent transport of neutral amino acids, including several essential amino acids. The L-type amino acid transporter 1 (LAT1) is overexpressed to support cell growth in malignant tumors. Double-stranded RNA-mediated RNA interference (RNAi) analysis can be used in a wide variety of eukaryotes to induce the sequence-specific inhibition of gene expression. The current study attempted to investigate the effects of silencing LAT1 expression with small interfering RNA (siRNA) on cell growth in the KB human oral squamous cell carcinoma. Materials and Methods: The effects of silencing LAT1 expression with siRNA KB on cell growth were examined using RT-PCR, Western blot analysis, amino acid transport measurement and the MTT assay. Results: In the RT-PCR and Western blot analyses, the siRNA of LAT1 inhibited the expressions of LAT1 mRNA and protein. The uptake of [^{14}C]L-leucine was also inhibited by the siRNA of LAT1. In the MTT assay, the siRNA of LAT1 inhibited the growth of the KB cells in a time-dependent manner, indicating that this growth inhibition was induced by the LAT1-mediated blocking of neutral amino acid transport. Conclusion: The transport of neutral amino acids, including several essential amino acids, into the KB human oral squamous cell carcinoma is mainly mediated by LAT1. Furthermore, LAT1 could be a new target for the inhibition of cancer cell growth.*

Amino acid transporters are essential for growth and proliferation in normal and transformed cells (1, 2). Among the amino acid transport systems, the system L amino acid transporter, an Na^+-independent neutral amino acid transport system, is a major route for providing living cells, including tumor cells, with neutral amino acids of which several are essential (1, 3).

Recently, the L-type amino acid transporter 1 (LAT1) was isolated (4, 5). It was predicted to be 12-membrane-spanning proteins that mediate the exchange of Na^+-independent amino acids (4, 5). It requires an additional single-membrane-spanning protein, which is a heavy chain of 4F2 antigen (4F2hc), for their functional expression in the plasma membrane (4-6). LAT1 and 4F2hc form a heterodimeric complex *via* a disulfide bond (4-6). The LAT1 mRNA is only expressed in restricted organs such as the brain, spleen, placenta and testis (4-7). In contrast, 4F2hc mRNA is ubiquitously expressed in all normal embryonic and normal adult tissues (4-6, 8). In addition, LAT1 is highly expressed in malignant tumors, presumably to support their continuous growth and proliferation (4, 5, 9, 10). Based on the above, it has been proposed that the manipulation of LAT1 activity would have anticancer therapeutic implications. The inhibition of LAT1 activity in tumor cells could be effective in the suppression of cell growth by depriving these cells of essential amino acids (11, 12).

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene (13). The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs (14).

It is known that oral cancer is the sixth most common cancer worldwide (15). Despite the introduction of novel

therapeutic modalities in the treatment of oral cancer, improvements in long-term survival rates have only been modest. Advances in the understanding of the underlying mechanisms of oral cancer are likely to be necessary to improve survival rates, which, despite the better early detection of oral cancer, have plateaued over the past two decades and remain among the worst of all cancer sites (16). Furthermore, there are few studies into the expression and functional characterization of amino acid transporters, including the system L amino acid transporters, for supplying nutrients to oral cancer cells.

In the present study, therefore, the effects of silencing LAT1 expression with siRNA on cell growth in KB human oral squamous cell carcinoma were investigated. The transport of neutral amino acids, including several essential amino acids, into KB cells is mediated mainly by LAT1. Furthermore, LAT1 could be a new target for the inhibition of cancer cell growth.

Materials and Methods

Materials. [^{14}C]L-leucine was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, USA). The affinity-purified anti-LAT1 polyclonal antibody was kindly provided by the Kumamoto Immunochemical Laboratory, Transgenic Inc. (Kumamoto, Japan). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell line and cell culture. The KB human oral carcinoma cells were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA) and were grown in MEM and NEAA (non-essential amino acid) at a ratio of 100:1, supplemented with 5% FBS, penicillin (100 IU/ml) and streptomycin (100 mg/ml). The cells were maintained as monolayers in plastic culture plates at 37˚C in a humidified atmosphere containing 5% CO_2.

LAT1 siRNA and transfection. The 19-nt human LAT1 siRNA duplex was designed and provided by Proligo (Boulder Location, CO, USA). The siRNA sequences were designed based on the human LAT1 mRNA sequence (GeneBank Accession No. AB018009). The LAT1 siRNA, beginning at nt 786 had the sequence: 5'-GGAACAUUGUGCUGGCAUUdTdT-3' (sense) and 5'-AAUGCCAGCACAAUGUUCCdTdT-3' (antisense). The control LAT1 siRNA (scrambled siRNA) had the sequence: 5'-GUUAAAGGUUUGA CUCGCGdTdT-3' (sense) and 5'-CGCGAGUCAAACCUUU AACdTdT-3' (antisense). The KB cells were transfected with LAT1 siRNA, at various concentrations and incubation times, using Lipofectamine 2000 (Invitrogen Life Technologies Inc., CA, USA) (17) when the cells were approximately 30-60% confluent on the plates.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNAs were prepared from the KB cells of each experimental group maintained in the growth medium at 37˚C using RNA preparation kits (Isogen, Nippon-Gene, Japan), in accordance with the manufacturer's instruction. For RT-PCR analysis, the first-strand cDNAs were prepared from KB cell total RNAs using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen

Life Technologies Inc.) 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) and the following protocol: 94˚C for 12 min, followed by 30 cycles of 94˚C for 1 min, 54˚C for 30 sec and 72˚C for 1 min, with 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), were used for the PCR amplification of LAT1.

Western blot analysis. Protein samples from the KB cells of each experimental group were prepared as described elsewhere (5,18), with minor modifications. The protein samples were heated at 100˚C for 5 min in the sample buffer in the presence of 5% 2-mercaptoethanol and were then 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 dilution) affinity-purified antibody (19), and then with horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody. The signals were detected using an ECL plus system (Amersham Pharmacia Biotech, NJ, USA) (18, 19).

Uptake measurements in KB cells. To characterize the effect of LAT1 siRNA on the function of the endogenously expressed system L amino acid transporter in the KB cells, uptake experiments were performed as described elsewhere (12,19). The KB cells were maintained in the growth medium at 37˚C in 5% CO_2. The cells were collected and seeded on 24-well plates (1x10^5 cells/well) in fresh growth medium. The cells were treated with LAT1 siRNA at various concentrations and incubation times when they were at 30-60% confluence on 24-well plates. The cells were grown in the medium in the presence or absence of LAT1 siRNA for various incubation times. Following culture, the uptake measurements were performed. After the removal of the growth medium, the cells were washed three times with 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_2PO_4 and 5.6 mM glucose, pH 7.4), and pre-incubated for 10 min at 37˚C. The medium was then replaced by the uptake solution containing [^{14}C]L-leucine. Because the transport of amino acids *via* system L amino acid transporters was not dependent on Na$^+$ (4, 5, 12, 19), all the transport experiments were performed in Na$^+$-free uptake solution. The uptake was terminated by removing the uptake solution followed by washing three times with ice-cold uptake solution (12, 19). The cells were then solubilized with 0.1 M NaOH and the radioactivity was counted by liquid scintillation spectrometry. The values are expressed as pmol/mg protein/min. To measure the uptake of [^{14}C]L-leucine, four to six wells of cells were used for each data-point. To confirm the reproducibility of the results, three or four separate experiments were performed for each measurement. The results shown in the figures are from these representative experiments. 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 3, 10, 30, 100, 300, 1000 and 3000 μM for the KB cells in the presence or absence of LAT1 siRNA.

Growth inhibition of KB cells by LAT1 siRNA (MTT assay). The KB cell growth inhibition experiments in the presence or absence of LAT1 siRNA were performed according to a previously described
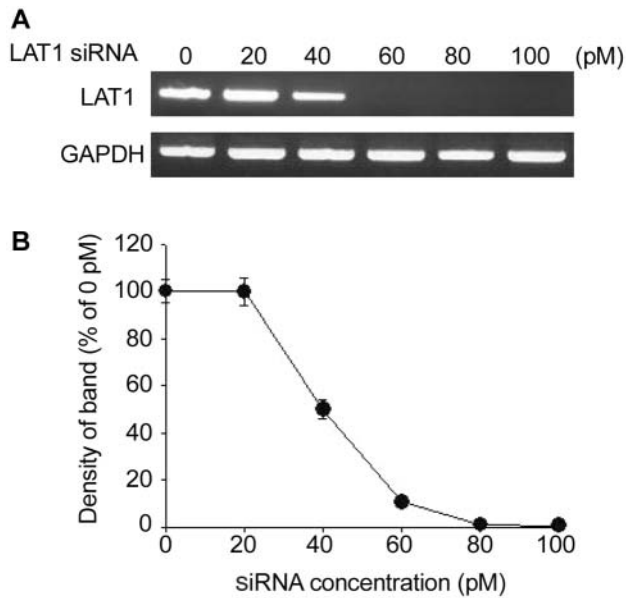
Figure 1. *The concentration-dependent inhibition of LAT1 mRNA expression by LAT1 siRNA in KB human oral cancer cells. (A) The first strand of cDNA, prepared from the total RNA of KB cells treated with various concentrations of LAT1 siRNA for 12 h, was used as the template for PCR amplification. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and were visualized with ethidium bromide. LAT1 expression was completely inhibited by LAT1 siRNA at 60 pM. (B) The percentage of LAT1 mRNA expression was calculated as a ratio of LAT1 siRNA-treated cells and untreated control cells.*

Figure 2. *The time-dependent inhibition of LAT1 mRNA expression by LAT1 siRNA in KB cells. (A) Agarose gel electrophoresis was performed on the RT-PCR products from KB cells (LAT1; 536 bp, GAPDH; 349 bp) (C; scrambled siRNA-60-pM-treated control cells, S; LAT1 siRNA-60-pM-treated cells). (B) The percentage of LAT1 mRNA expression was calculated as a ratio of LAT1 siRNA-treated cells and scrambled siRNA-treated control cells.*

method (20), with minor modifications. The KB cells were seeded at a density of 5×10^3 cells/well in a 24-well plate. The cells were treated with LAT1 siRNA at various concentrations and incubation times when they were approximately 30-60% confluent on 24-well plates. Cell viability was assessed using the MTT assay. Briefly, the cells were grown in the medium in the presence or absence of LAT1 siRNA for various incubation times. Following the culture, 0.5 mg/ml of MTT was added to each well. After 4-h incubation at 37˚C, isopropanol with 0.04 M HCl was added to each well to dissolve the precipitates . The absorbance was then 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.

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

Results

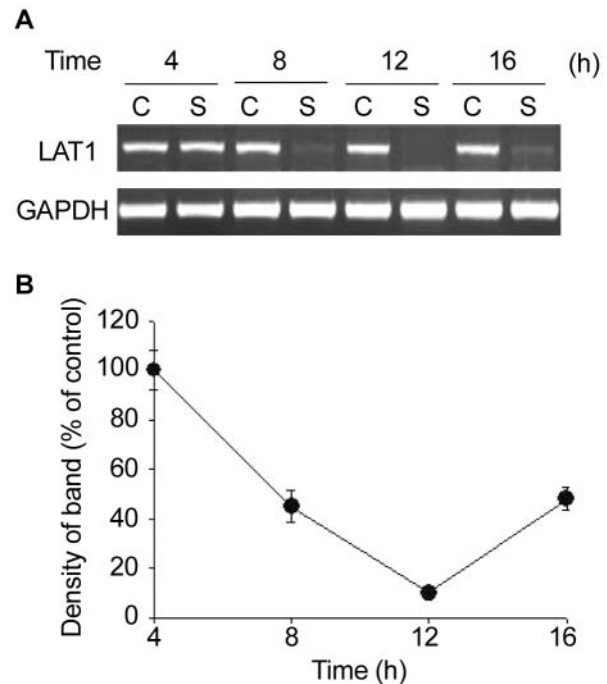*Inhibition of LAT1 expression by LAT1 siRNA in KB cells.* After LAT1 siRNA transfection into KB cells, RT-PCR analysis was performed to examine and compare the LAT1 expression level and then the results were transformed numerically by means of a video-based densitometry method with image analyzer. To determine the LAT1 siRNA concentration, the RT-PCR analysis was first performed after 12 h in the control KB cells (0 pM) and LAT1 siRNA-(20 pM, 40 pM, 60 pM, 80 pM and 100 pM) treated KB cells. The expression of LAT1 mRNA was inhibited by LAT1 siRNA in a concentration-dependent manner and was almost completely inhibited by LAT1 siRNA 60 pM (Figure 1); all the subsequent LAT1 siRNA transfection experiments were performed under LAT1 siRNA 60 pM. It was observed that the expression of LAT1 mRNA was significantly decreased after 8 h of 60 pM LAT1 siRNA exposure and was completely inhibited at 12 h (Figure 2). The LAT1 mRNA was expressed after16 h (Figure 2), thus the LAT1 siRNA fragments were probably consumed. Following transfection of 60 pM LAT1 siRNA into KB cells, Western blot analysis was conducted to confirm the LAT1 protein expression level. The expression of the LAT1 protein was reduced after 12 h of LAT1 siRNA transfection (Figure 3).

Inhibition of [^{14}C]L-leucine transport by LAT1 siRNA in KB cells. After LAT1 siRNA transfection into KB cells,
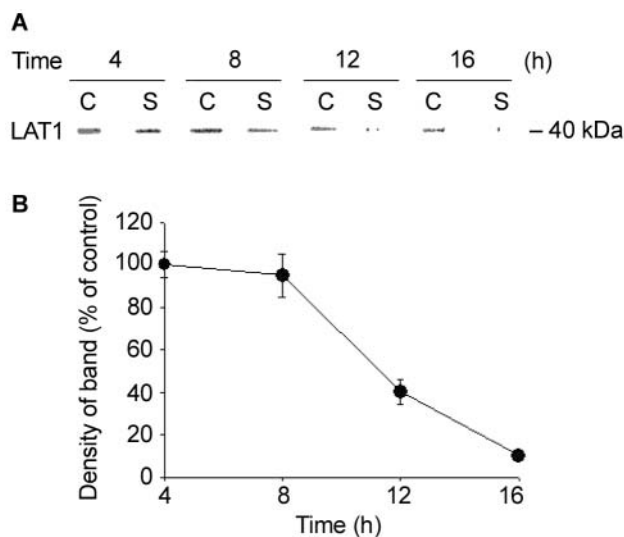
Figure 3. *The time-dependent inhibition of LAT1 protein expression by LAT1 siRNA in KB cells. (A) Western blot analyses were performed on the protein extracts prepared from KB cells in the presence of 2-mercaptoethanol using anti-LAT1 antibodies. For LAT1, the 40 kDa-protein band was detected (C; scrambled siRNA-60-pM-treated control cells, S; LAT1 siRNA-60-pM treated cells). (B) The percentage of LAT1 protein expression was calculated as a ratio of LAT1 siRNA-treated cells and scrambled siRNA-treated control cells.*

Figure 4. *The time-dependent inhibition of [^{14}C]L-leucine uptake by LAT1 siRNA in KB cells. The [^{14}C]L-leucine uptake (30 μM) was measured for 1 min in the Na$^+$-free uptake solution in the presence of LAT1 siRNA 60 pM and is expressed as a percentage of control L-leucine uptake in the presence of scrambled siRNA 60 pM. Each data point represents the mean±SEM for three experiments. **p<0.01 vs. control (the control cells measured in the presence of scrambled siRNA 60 pM) and *** p<0.001 vs. control.*

[^{14}C]-labeled L-leucine (30 μM) uptake experiments were carried out in the presence of 60 pM LAT1 siRNA for L-leucine transport investigations. Subsequent to transfection, the uptake experiments were carried out using a solution containing [^{14}C]L-leucine 30 μM at 37°C. Sixty pM LAT1 siRNA inhibited the uptake of [^{14}C]L-leucine in a time-dependent manner (Figure 4). The K_m and V_{max} values were determined based on the [^{14}C]L-leucine uptakes measured for 1 min at 3, 10, 30, 100, 300, 1000 and 3000 μM for the KB cells at 12 h and 16 h in the presence or absence of 60 pM LAT1 siRNA. The K_m and V_{max} values for [^{14}C]L-leucine uptake in the control KB cells were 65.9±7.1 μM and 2725.8±187.7 pmol/mg protein/min, respectively (Figure 5). The K_m and V_{max} values in the LAT1 siRNA-treated cells (12 h) were 59.5±5.8 μM and 1473.0±121.8 pmol/mg protein/min, respectively (Figure 5). The K_m and V_{max} values in the LAT1 siRNA-treated cells (16 h) were 56.3±6.4 μM and 1158.2±118.4 pmol/mg protein/min, respectively (Figure 5), indicating that the LAT1 siRNA decreased the V_{max} value for [^{14}C]L-leucine uptake without affecting the K_m value in the KB cells.

Cell growth inhibition by LAT1 siRNA in KB cells. To study the cell toxicity of LAT1 siRNA in KB cells, the LAT1 siRNA was transfected into KB cells and the MTT assay was performe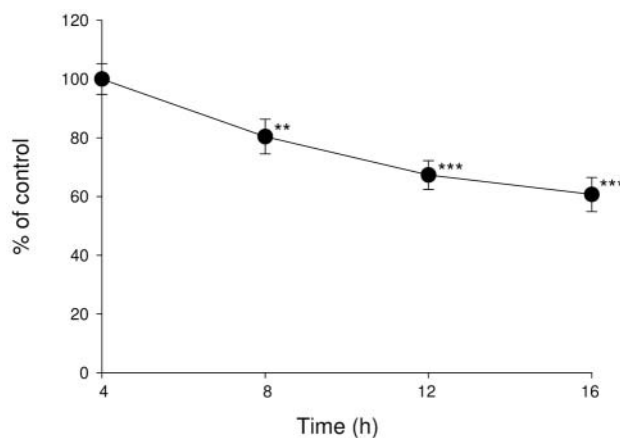d. From 4 to 16 h of 60 pM LAT1 siRNA transfection, the KB cells growth inhibition was dependent on the LAT1 siRNA treatment time (Figure 6). After 12 h there was statistically significant cell growth inhibition in the LAT1 siRNA-treated group (Figure 6).

Discussion

Oral cancer is the sixth most common cancer worldwide (15). In East and South Asian countries, the incidence is much higher, where oral cancers constitute up to 25% of all malignancies (15). The system L amino acid transporters play an important role in growth and proliferation of normal and cancer cells (4, 5, 9, 10). However, the expressions and functional properties of amino acid transporters, including the system L amino acid transporters, in relation to supplying organic nutrition to normal and cancerous oral cells have not been entirely clarified. In the present study, the effects on cell growth of silencing LAT1 expression with siRNA was investigated in KB human oral squamous cell carcinoma.

LAT1 siRNA was introduced into the KB cells and then the expression of LAT1 mRNA was subsequently analyzed using RT-PCR analysis. The expression of LAT1 mRNA was inhibited. The inhibition of LAT1 protein expression was also observed in Western blot analysis. These results are similar to a previous report that the expression of Hsp27 mRNA, related to cancer cell transition, was inhibited by RNAi in the MDA-MB-231 human breast cancer cell line (21). The expression of LAT1 in KB cells was inhibited by
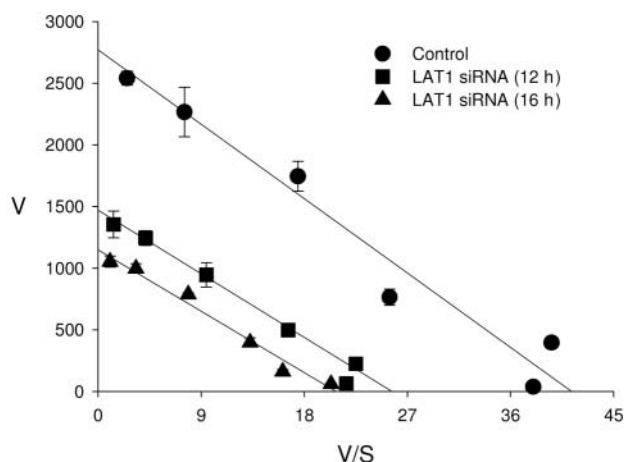
Figure 5. *The Eadie-Hofstee plot of L-leucine uptake in KB cells. The uptake of [^{14}C]L-leucine by KB cells was measured for 1 min and plotted against the L-leucine concentration in the control cells and the LAT1 siRNA-60-pM-treated cells. The Km and Vmax values for [^{14}C]L-leucine uptake in the control KB cells were 65.9±7.1 µM and 2725.8±187.7 pmol/mg protein/min, respectively. The K_m and V_{max} values for [^{14}C]L-leucine uptake in the LAT1 siRNA-treated cells (12 h) were 59.5±5.8 µM and 1473.0±121.8 pmol/mg protein/min, respectively. The K_m and V_{max} values for [^{14}C]L-leucine uptake in the LAT1 siRNA-treated cells (16 h) were 56.3±6.4 µM and 1158.2±118.4 pmol/mg protein/min, respectively. V and S refer to [^{14}C]L-leucine uptake (pmol/mg protein/min) and the [^{14}C]L-leucine concentration, respectively.*

Figure 6. *The time-dependent inhibition of cell viability by LAT1 siRNA in KB cells. The KB cells were treated with LAT1 siRNA 60 pM or scrambled siRNA 60 pM for 4-16 h. Cell viability was determined by the MTT assay. The percentage of cell viability was calculated as a ratio of the LAT1 siRNA-treated cells and scrambled siRNA-treated control cells. Each data-point represents the mean±SEM for three experiments. *p<0.05 vs. control (the control cells measured in the presence of scrambled siRNA 60 pM) and **p<0.01 vs. control.*

LAT1 RNAi fragments. To confirm whether the transport of neutral amino acids was inhibited with LAT1 expression inhibition by RNAi in the KB cells, neutral amino acid L-leucine uptake experiments were performed.

L-leucine transport was significantly inhibited by LAT1 siRNA. The LAT1 siRNA decreased the V_{max} value for L-leucine uptake without affecting the K_m value in the KB cells, indicating that the siRNA decreased the amount of active LAT1 in the KB cells without affecting the affinity of the transporter. These results indicate that LAT1 siRNA induced the LAT1-mediated inhibition of neutral amino acid transport.

With the effect of RNAi, the expression of LAT1 mRNA and protein, as well as the transport of neutral amino acids, were inhibited. The MTT assay was conducted to confirm how such intracellular changes affected cell growth. According to the results of the MTT assay, LAT1 siRNA inhibited KB cell growth in a time-dependent manner. This result corresponds with those of several compounds ([6]-paradol, norcantharidin and baccatin) (20, 22, 23) with antitumor effects, suppressing cancer cell growth in time- and concentration-dependent manners. LAT1 is up-regulated in tumor cells to support continuous growth and proliferation (4, 5, 9, 10). When LAT1 was overexpressed in the KB cells, neutral amino acids were transported for cell growth and proliferation (19). When
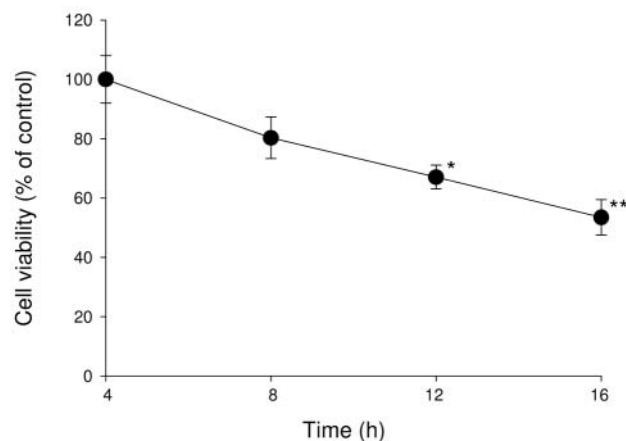
LAT1 siRNA was introduced into the KB cells, however, the LAT1 expression was inhibited and the intracellular depletion of neutral amino acids was induced. We suggest that the inhibition of KB cell growth was thereby induced.

In conclusion, it was considered that the inhibition of amino acid transporter LAT1 expression led to the inhibition of KB cell growth by inducing an intracellular depletion of neutral amino acids such as L-leucine, necessary for cell growth; this study suggests the utility of KB cells in LAT1 studies and provides an insight into the growth inhibition of oral cancer cells *via* the LAT1 inhibitor, LAT1 siRNA. Moreover, LAT1 could be a new target for the suppression of tumor cell growth, including that of oral cancer cells.

Acknowledgements

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