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ORIGINAL ARTICLE

L-Leucine induces growth arrest and persistent ERK activation in glioma cells

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Abstract Glioma is the most common type of brain tumor, and has the worst prognosis in human malignancy. Experimental evidence suggests that the use of high concentrations of various amino acids may perturb neoplastic cell growth. Thus, the aim of this study was to investigate whether essential amino acids can alter the growth and proliferation of glioma cells. Studies were performed using C6 rat glioma cell lines. High concentration of L-leucine induced growth arrest of glioma cell lines. Terminal transferase uridyl nick end labeling assay and cell cycle analysis showed that the effect of L-leucine on glioma cells growth was not cytotoxic, but rather cytostatic. Additionally, the extracellular signal-regulated protein kinase was activated in L-leucine-treated glioma cells, and inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEK) enhanced the effect of L-leucine on glioma cell growth. These data suggest that high concentration L-leucine combined with inhibition of MEK is a potential strategy for glioma cell growth arrest.

Keywords Essential amino acid · L-Leucine · Glioma · Extracellular signal-regulated protein kinase · L-type amino acid transporter 1

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Introduction

Glioma is the most common type of brain tumor, and has the worst prognosis in human malignancy. Despite advances in therapy, malignant gliomas essentially remain fatal (Scott et al. 1998; Stupp et al. 2005). The cause of death in glioma patients is failure of local control of the tumor rather than distant metastasis. Experimental studies suggest that amino acid supply is required to maintain a high degree of protein synthesis in tumor cells. Transporters for essential amino acids are indispensable for protein synthesis (Christensen 1990). The L-type amino acid transporter 1 (LAT1) is a Na^+-independent neutral amino acid transporter essential for transport of large neutral amino acids through the plasma membrane (Kanai et al. 1998; Mastroberardino et al. 1998). LAT1 has also been implicated in the growth and proliferation of tumor cells, as well as in cells during tissue development (Kanai et al. 1998; Sang et al. 1995; Wolf et al. 1996). We previously reported that high expression of LAT1 in malignant gliomas was a significant predictor of outcome (Nawashiro et al. 2006). Furthermore, a specific LAT1 inhibitor, 2-aminobicyclo-2 (2,2,1)-heptane-2-carboxylic acid (BCH), inhibited the growth of glioma cells both in vitro and in vivo (Nawashiro et al. 2006).

LAT1 exhibits high affinity for several nutritionally essential amino acids including leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine, tryptophan, and methionine. There is increasing evidence that these amino acids may compete for LAT1 in the plasma membrane, and that use of high concentrations of various amino acids may perturb neoplastic cell growth (Lind 2004; Nishio et al. 1986; Souba 1993; Wakshlag et al. 2006). For example, the effect of arginine on cell growth has been widely examined (Lind 2004), while other amino acids have been used to

reduce neoplastic cell growth with mixed results (Nishio et al. 1986; Souba 1993; Wakshlag et al. 2006). In the present study, we investigated the effect of essential amino acids on the growth and proliferation of glioma cells.

Methods

Cell line and culture condition

The C6 rat glioma cell line was purchased from Dainippon Pharmaceutical Company (Osaka, Japan). Cells were maintained in a CO_2 incubator at 37°C by in vitro passage at 3- to 4-day intervals in Ham's F10 medium with 15% horse serum and 2.5% fetal bovine serum (Dainippon Pharmaceutical Company). After the cells reached subconfluence, a single-cell suspension was obtained by trypsinization, and cell numbers were counted with a particle counter (Model PC-607; Erma, Tokyo, Japan). Cell suspensions of desired concentrations were prepared and used for the following experiments.

Experiment 1

We compared the effects of 10 mM alanine (Ala), Leu, Val, or Ile on cell growth using the MTS assay. Next, we assessed the ability of several concentrations of Leu (1, 10, and 60 mM) and 20 mM BCH on cell growth using the MTS assay [see section "Analysis of cell viability (MTS assay)"].

Experiment 2

We investigated the mechanism of glioma growth inhibition by Leu (60 mM) using BrdU staining, terminal transferase uridyl nick end labeling (TUNEL) assay, and cell cycle analysis.

Experiment 3

We investigated the relationships among Leu, mitogen-activated protein kinases, and cyclin D1 using Western blot analysis. Furthermore, we determined the effects of 60 mM Leu in combination with the inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 (50, 100, and 150 μM PD98059) (Merck Biosciences) on glioma cells using Western blot analysis, morphological analysis, and MTS assay.

Analysis of cell viability (MTS assay)

Cell viability was determined by using CellTiter 96™ AQueous nonradioactive cell proliferation assay (Promega, Madison, WI, USA), referred to as the MTS assay according to the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] and an electron coupling reagent [phenazine methosulfate (PMS)]. Cells at a desired concentration were plated in 96-well flat-bottomed tissue culture trays (Corning Inc., Corning, NY, USA) until they completely adhered to the bottom. The culture medium was then replaced with media supplemented with various compounds. At the end of the assay, MTS/PMS solution was added to each well and cells were incubated at 37°C for 2 h. The absorbance at 490 nm was measured on an ELISA plate reader (Benchmark microplate reader; Bio-Rad Laboratories, Hercules, CA, USA). Results were obtained with triplicate samples from a single experiment.

BrdU incorporation

Cells were plated at a concentration of 2,000 cells/well onto a chamber slide (Iwaki Glass, Funabashi, Japan), followed by treatment with 10 mM or 60 mM Leu for 2 days. Cells were incubated with BrdU 10 μM (Sigma, St. Louis, MO, USA) for 2 h, followed by removal of labeling medium from cells and gentle washing with PBS. The cells were then fixed in cold 70% ethanol for 25 min. BrdU staining was performed using the Zymed BrdU staining kit (Zymed/Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Results were obtained with four different samples.

Apoptosis assay

The TUNEL assay was performed to assess apoptosis. Cells were plated at a concentration of 1.5×10^5 cells/well in a six-well plate. After 24 h growth, the cells were treated with 20 mM BCH or 60 mM Leu. The assay was performed using an in situ apoptosis detection kit (Wako Pure Chemical, Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. For quantitative evaluation of the apoptotic index, the percentage of TUNEL-positive cells to the total number of cells was calculated for each sample in five random fields. Results were obtained with four different samples.

Cell cycle analysis

Nuclear DNA was detected using the Cycle TEST Plus DNA Reagent Kit (Becton Dickinson (BD), San Jose, CA, USA). Cells were harvested by trypsinization at defined times. For DNA measurements, cells were centrifuged for 5 min at 300*g*, and the pellet was suspended in buffer solution. Nuclei were prepared, stained with propidium iodide, and analyzed by a FACSCalibur (BD) with a gate

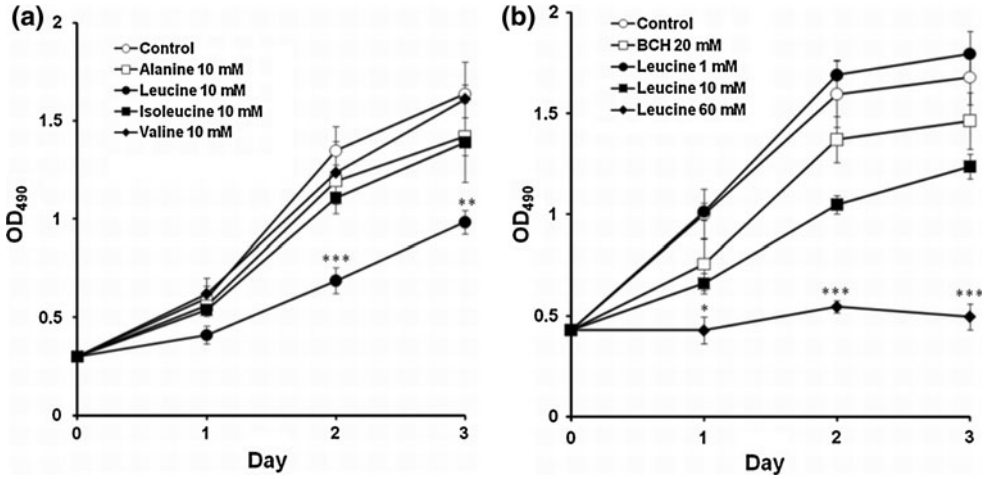

Fig. 1 Effects of amino acids on C6 rat glioma cell growth using MTS assay. **a** Effects of alanine, leucine (Leu), valine (Val), and isoleucine (Ile) (10 mM) on the survival of glioma cells. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus 10 mM Ile. $^{***}P < 0.001$ versus 10 mM Ile. **b** Effects of 20 mM BCH or Leu (1, 10, or 60 mM) on the survival of glioma cells. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{*}P < 0.05$ versus 10 mM Leu. $^{***}P < 0.001$ versus 10 mM Leu. Each data point represents the mean ± standard deviation of triplicate samples. For details see the text and the "Methods" section

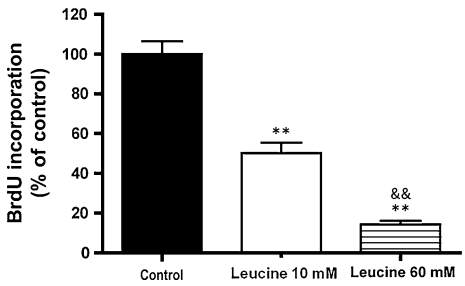

Fig. 2 Effects of L-leucine on C6 rat glioma cell proliferation. Percentage of BrdU-positive cells. BrdU staining was performed after exposure to Leu (10 or 60 mM) for 2 days. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. $^{\&\&}P < 0.01$ versus 10 mM Leu. Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

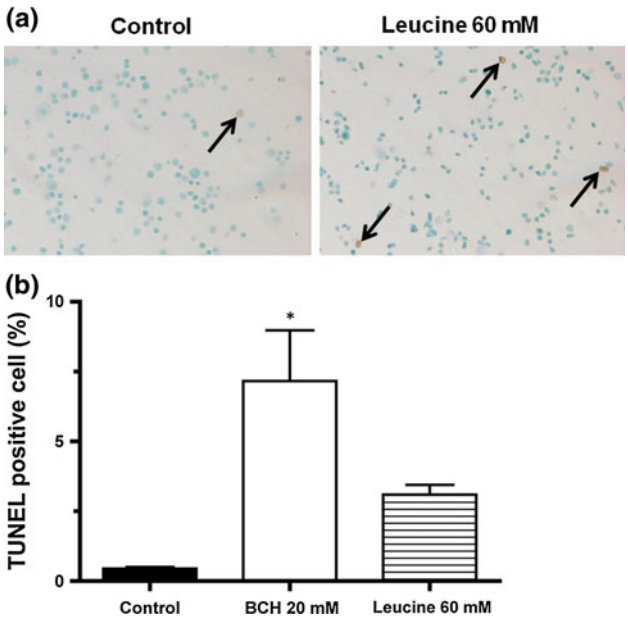

Fig. 3 Effects of L-leucine on C6 rat glioma cell apoptosis. **a** TUNEL staining after exposure to 60 mM Leu for 2 days (*arrows*, TUNEL-positive cells). **b** Percentage of TUNEL-positive cells after exposure to 20 mM BCH or 60 mM Leu for 2 days. The percent ratios of TUNEL-positive cells to the total number of cells were calculated for each sample in five random fields. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

that selects single nuclei within a normal size range. The cell cycle parameters from 10,000 gated nuclei were determined by CELLQuest software (BD). Results were obtained from three or four experiments.

Western blot analysis

Cells were lysed in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 1% Triton X-100, and 0.01% Protease Inhibitor Cocktail Set III (Calbiochem, San Diego, CA, USA). Protein was quantified by the BCA method (Pierce, Rockford, IL, USA). Cell lysates were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. Proteins were transferred onto PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated with primary polyclonal antibodies against phospho-ERK (p-ERK), total-ERK, phospho-stress-activated protein kinase/Jun amino-terminal kinase (p-JNK), phospho-p38 (p-p38),

or cyclin (1:1,000 dilution for all; cell signaling, Beverly, MA, USA). The membranes were then incubated with horseradish-peroxidase conjugated anti-rabbit secondary antibodies (cell signaling). Immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). To quantify Western blot data, densitometric analysis was performed using the NIH ImageJ software.

Morphological studies

The cells were incubated for 4 or 72 h in an atmosphere of 5% CO_2 in the absence or presence of 0.1% dimethylsulfoxide (DMSO) and 60 mM Leu with or without PD (50 or 150 μM). Cells were fixed and photographed using an Olympus camera (Olympus Co. Ltd., Tokyo, Japan).

Statistical methods

All the data are presented as mean ± standard deviation. Comparisons between groups were analyzed by ANOVA followed by Bonferroni/Dunn post hoc analysis. A value of $P < 0.05$ was considered statistically significant. Graphpad Prism 4.0 (GraphPad, San Diego, CA, USA) was used for all statistical analyses.

Results

Experiment 1

High concentration of L-leucine strongly inhibits cell growth

MTS assay revealed that Leu (10 mM) was more potent than Ala, Val, or Ile (10 mM) in inhibiting growth of glioma cells (on day 3, $P < 0.01$ vs. Ile) (Fig. 1a). Furthermore, 60 mM Leu inhibited cell growth more strongly than 1 and 10 mM Leu and BCH (on day 3, $P < 0.001$ vs. 10 mM Leu) (Fig. 1b); the reduction in cell growth at 3 days after Leu treatment compared with the control was 70% for 60 mM Leu, 26% for 10 mM Leu, 13% for BCH, and 7% for 1 mM Leu. MTS assay also revealed that 60 mM Leu was more potent than other amino acids in

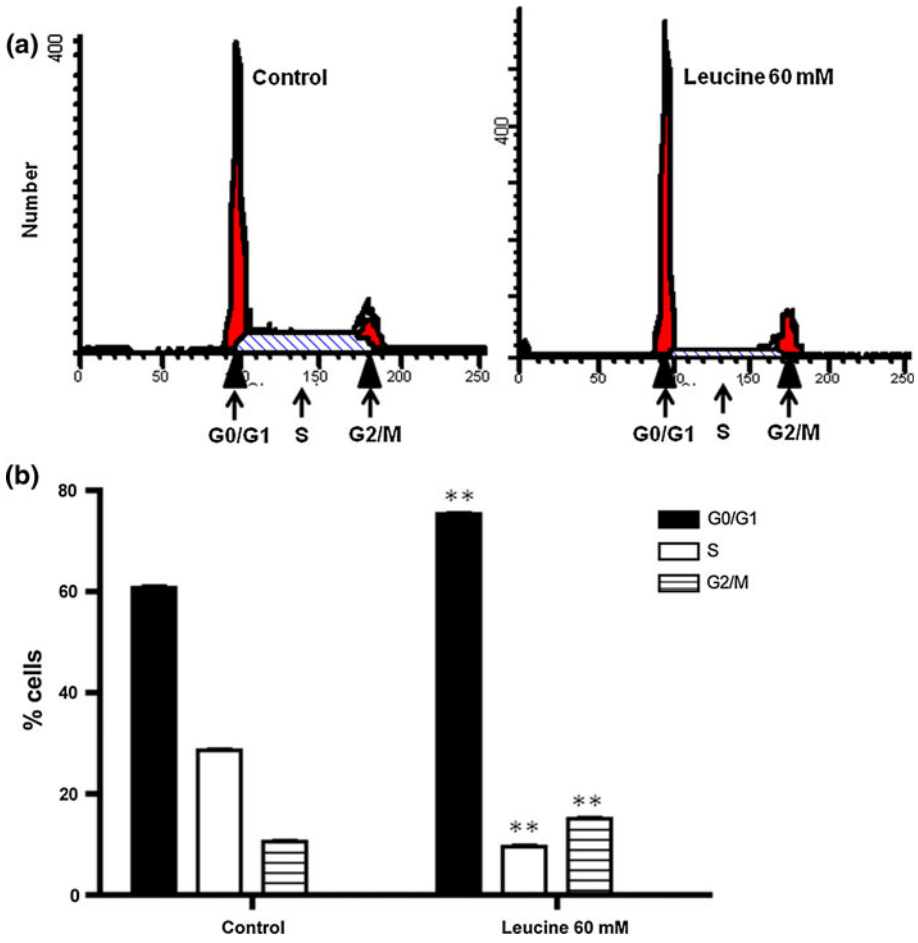

Fig. 4 Effects of L-leucine on C6 rat glioma cell cycle analyzed by flow cytometry. **a** Analysis of the cell cycle by flow cytometry. Analyses were performed after exposure to 60 mM Leu for 2 days. **b** Percentage of cells in each phase. Results are expressed as percentage of cells found in the different cell cycle phases. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. Each data point represents the mean ± standard deviation of at least three experiments. For details see the text and the "Methods" section

inhibiting growth of F6B3 mouse gliosarcoma cells, T98G human glioblastoma cells, and HT1080 human fibrosarcoma cells (data not shown).

Experiment 2

High concentration of L-leucine does not induce apoptosis

The percentage of BrdU incorporation in 10 and 60 mM Leu-treated cells was 50 ($P < 0.01$ vs. control) and 14% ($P < 0.01$ vs. control and 10 mM Leu), respectively (Fig. 2), suggesting a dose-dependent action of Leu on reducing cellular proliferation. The number of TUNEL-positive cells was significantly increased in BCH-treated cells [7.2%, $P < 0.01$ vs. control (0.4%)], but not in 60 mM Leu-treated cells (3.1%) (Fig. 3).

High concentration L-leucine reduces the proportion of cells in S phase

Analysis of the cell cycle by flow cytometry showed that 60 mM Leu significantly reduced the proportion of cells in S phase; control cells accumulated in 61% G0/G1, 29% S, and 11% G2/M phase, while 60 mM Leu-treated cells accumulated in 75% G0/G1, 10% S, and 15% G2/M phase ($P < 0.01$ in each phase) (Fig. 4).

Experiment 3

High concentration of L-leucine activates ERK

Western blot analysis showed that p-ERK expression was significantly higher in both 60 mM Leu and BCH-treated cells as compared with control or 1 mM Leu-treated cells ($P < 0.01$), and that p-ERK expression was increased strongly at day 2, and massively at day 3, in the treatment with 60 mM Leu (Fig. 5). However, total-ERK, p-p38, and p-JNK immunoreactivity did not change over the time course (Figs. 6, 7). Western blot analysis also showed that cyclin D1 expression was significantly reduced in 60 mM Leu-treated cells on day 1 ($P < 0.01$) and day 2 ($P < 0.05$) compared with controls, and was significantly reduced in BCH-treated cells on day 1 ($P < 0.05$) (Fig. 8). Expression of p-ERK was also increased in T98G human glioblastoma cells (data not shown).

PD98059 enhances the effects of L-leucine on cell growth arrest

Western blot analysis showed that expression of p-ERK significantly was decreased in 60 mM Leu plus 150 μM PD98059-treated cells ($P < 0.01$) when compared with 60 mM Leu alone (Fig. 9). Both 100 μM PD98059 alone and 60 mM Leu alone inhibited cell growth compared with the control group ($P < 0.001$), while 60 mM Leu plus 100 μM PD98059 inhibited cell growth more strongly ($P < 0.001$ vs. 100 μM PD98059, $P < 0.05$ vs. 60 mM Leu) (Fig. 10). The treatment with 60 mM Leu plus 150 μM PD98059 was the most potent combination for inducing cell growth inhibition ($P < 0.05$ vs. 60 mM Leu plus 50 μM PD98059) (Figs. 9c, 10); 60 mM Leu plus 150 μM PD98059 inhibited cell growth more strongly as compared with 60 mM Leu plus 100 μM PD98059 on day 1 ($P < 0.01$) (Fig. 10).

Discussion

In the present study, we found that high concentration of Leu could strongly induce growth arrest in glioma cells and lead to persistent activation of p-ERK. Furthermore, these effects could be enhanced by PD. Amino acids can stimulate a number of cell growth pathways. Of these, the mammalian target of rapamycin (mTOR) is a key component of a signaling pathway that integrates inputs from nutrients and growth factors to regulate cell growth (Goberdhan 2010). Essential amino acids, especially Leu,

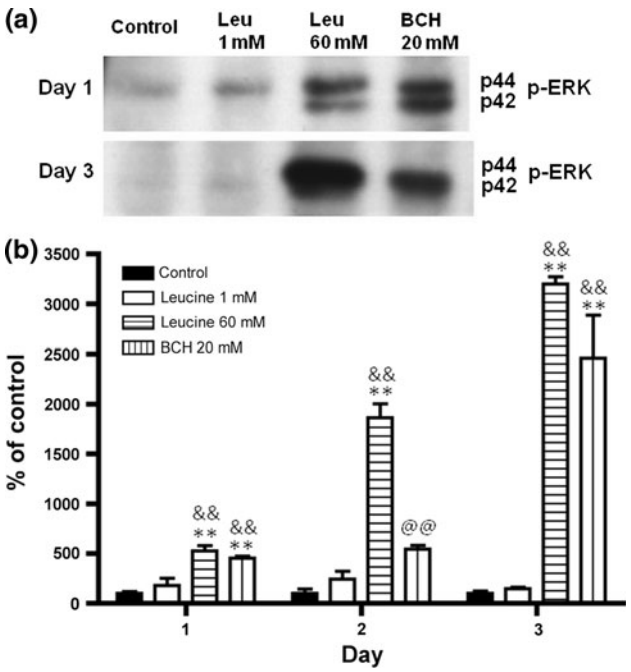

Fig. 5 Expression of p-ERK in C6 rat glioma cells. **a** Western blot analysis of p-ERK expression from cells exposed to Leu (1 or 60 mM) or 20 mM BCH for 1 or 3 days. **b** Quantitative analysis of p-ERK immunoreactivity from the experiment shown in (**a**). Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. $^{\&\&}P < 0.01$ versus 1 mM Leu. $^{@@}P < 0.01$ versus 60 mM Leu. Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

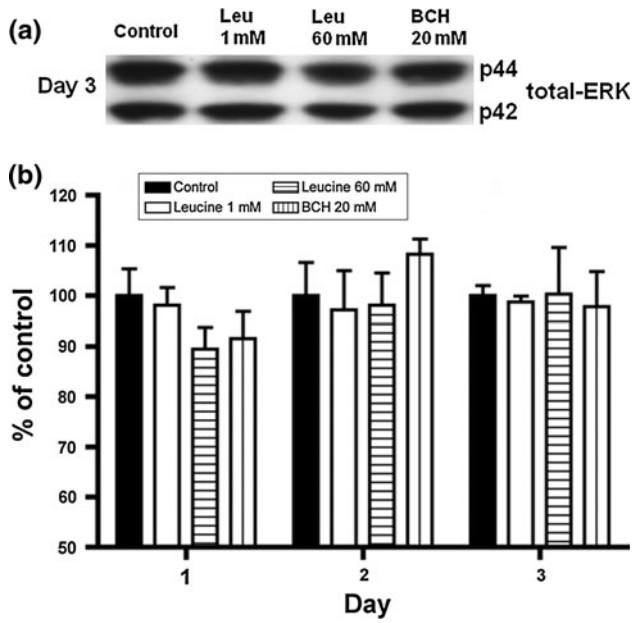

Fig. 6 Expression of total-ERK in C6 rat glioma cells. **a** Western blot analysis of total-ERK expression in cells exposed to Leu (1 or 60 mM) or 20 mM BCH for 1–3 days. **b** Quantitative analysis of total-ERK immunoreactivity from the experiment shown in (**a**). Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

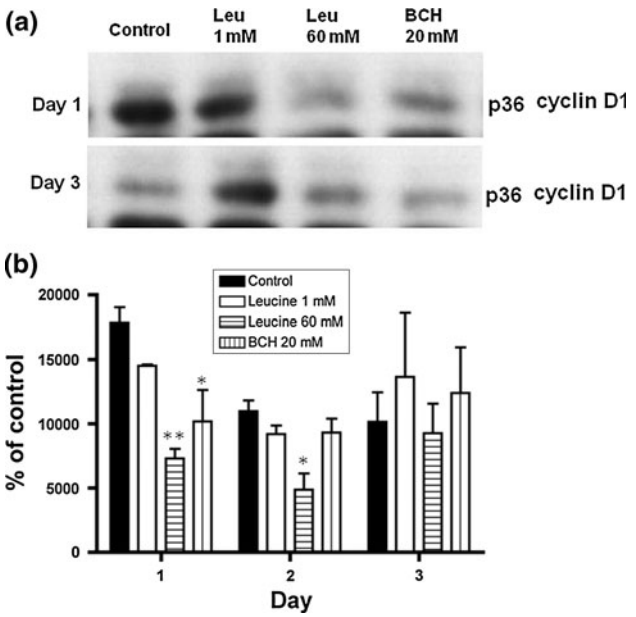

Fig. 8 Effects of L-leucine and BCH on the expression of cyclin D1 in C6 rat glioma cells. **a** Western blot analysis of cyclin D1 expression in cells exposed to Leu or BCH for 1–3 days. **b** Quantitative analysis of cyclin D1 immunoreactivity from the experiment shown in (**a**). Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $*P < 0.05$ versus control. $**P < 0.01$ versus control. Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

are indispensable for mTOR signaling (Avruch et al. 2009; Cohen and Hall 2009; Hara et al. 1998; Hidayat et al. 2003; Ishizuka et al. 2008; Lee et al. 2008; Nicklin et al. 2009; Peyrollier et al. 2000; Sheen et al. 2011), providing a potential mechanism by which deprivation of amino acids may lead to cell growth arrest (Leung-Pineda et al. 2004).

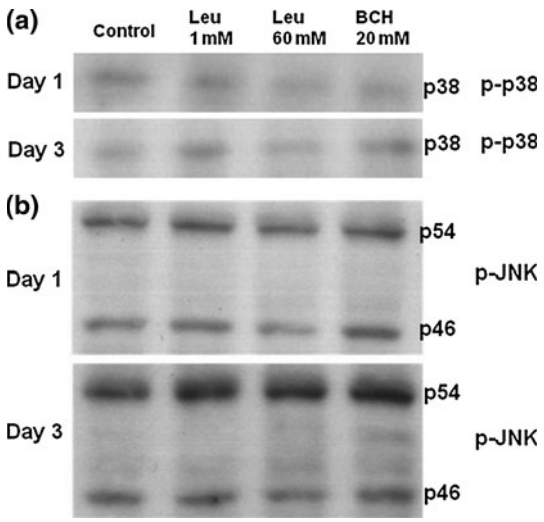

Fig. 7 Expression of p-p38 and p-JNK in C6 rat glioma cells. **a** Western blot analysis of p-p38 expression. **b** Western blot analysis of p-JNK expression. Each data point represents the mean ± standard deviation of four samples. For details see the text and the "Methods" section

Although the mechanism of these paradoxical effects is unclear, we speculate that amino acid imbalance may be important, as the mTOR complex can respond to changes in local amino acid levels (Goberdhan 2010). Accumulating experimental data suggest that high concentrations of various amino acids can also perturb growth of other cell lines (Lind 2004; Nishio et al. 1986; Souba 1993; Wakshlag et al. 2006). Furthermore, LAT1 has been shown to regulate mTOR (Ishizuka et al. 2008; Nicklin et al. 2009), which is a potential mechanism by which amino acid imbalance may lead to LAT1 dysfunction.

We previously reported that the specific LAT1 inhibitor, BCH, inhibited the growth of glioma cells both in vitro and in vivo (Nawashiro et al. 2006). In the present study, the inhibitory effect of 60 mM Leu on cell growth was stronger than for BCH, suggesting that high concentration of Leu may be a potential therapeutic tool for glioma treatment. We also found that BCH treatment induced apoptosis, while 60 mM Leu did not. Furthermore, 60 mM Leu reduced the percentage of cells in the S phase and increased the number of cells in the 0/G1 and G2/M phase. Thus, these data suggest that the action of Leu on glioma cells is cytostatic, not cytotoxic. In contrast, Wakshlag et al. (2006) reported that high concentration of Leu caused apoptosis, increased the percentage of cells in S phase, and decreased those in G2/M phase in canine osteosarcoma cell

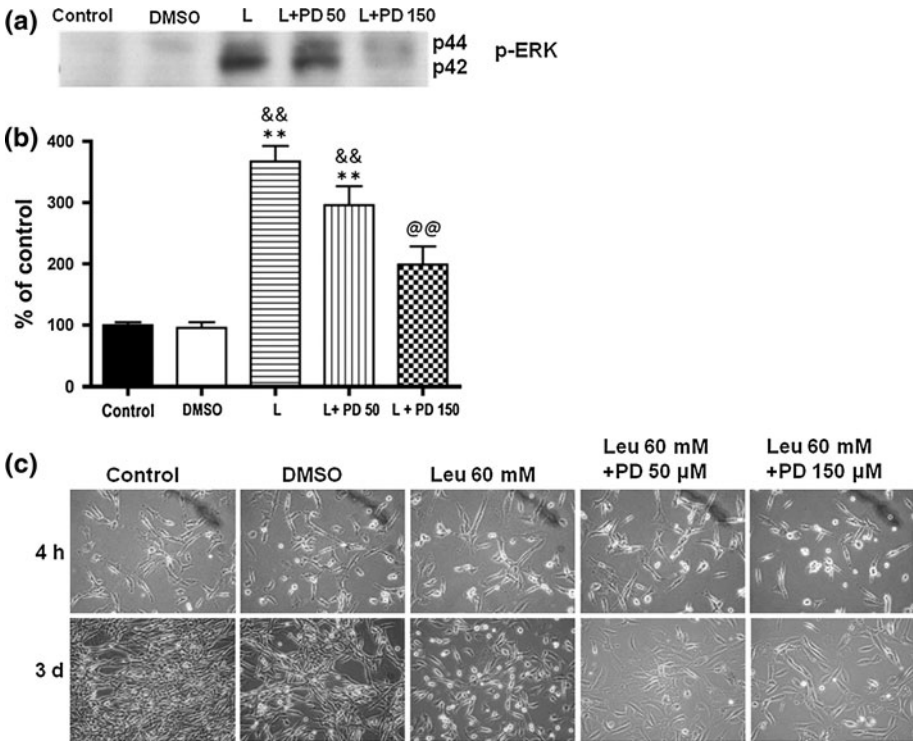

Fig. 9 Effect of 60 mM L-leucine with or without PD98059 on expression of p-ERK and morphology. **a** Western blot analysis of p-ERK expression from cells exposed to medium only, medium with 0.1% DMSO, 60 mM Leu only, 60 mM Leu plus 50 µM PD98059, or 60 mM Leu plus 150 µM PD98059 (both in medium with 0.1% DMSO) for 3 days. **b** Quantitative analysis of p-ERK immunoreactivity on day 3. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. $^{\&\&}P < 0.01$ versus DMSO. $^{@@}P < 0.01$ versus 60 mM Leu. Each data point represents the mean ± standard deviation of four samples. **c** Photomicrographs of cells from the experiment shown in (**a**). For details see the text and the "Methods" section

lines. It is possible that the different cell lines used may have caused these differences.

We found that cyclin D1 expression was weaker in Leu-treated cells than in controls. This may be attributed to the reduction of cells in the S phase as cyclin D1 is an important regulator of G1 to S phase progression in numerous cell types (Alao 2007). Leu treatment also induced a persistent increase in expression of p-ERK, but not total-ERK, indicating ERK activation. This phenomenon may result from adaptation of glioma cells to stress, as the ERK pathway plays an important role in survival, proliferation, and differentiation of cells, and is activated by various stressors (Seger and Krebs 1995). PD98059 can inhibit the ERK pathway, and may suppress the adaptational behavior of glioma cells to Leu exposure, providing a mechanism by which combination treatment with Leu and PD98059 caused an additive effect on glioma cell growth arrest.

Plasma Leu levels greater than 400 µM may cause impaired function of the central nervous system (Mitsubuchi et al. 2005). A 400 µM Leu in plasma is roughly equivalent to 40–80 µM in cerebrospinal fluid, as the cerebrospinal fluid/plasma ratio in Leu was reported at approximately 0.1–0.2 (Scholl-Bürgi et al. 2008). Therefore, the 60 mM Leu used in the present study may cause

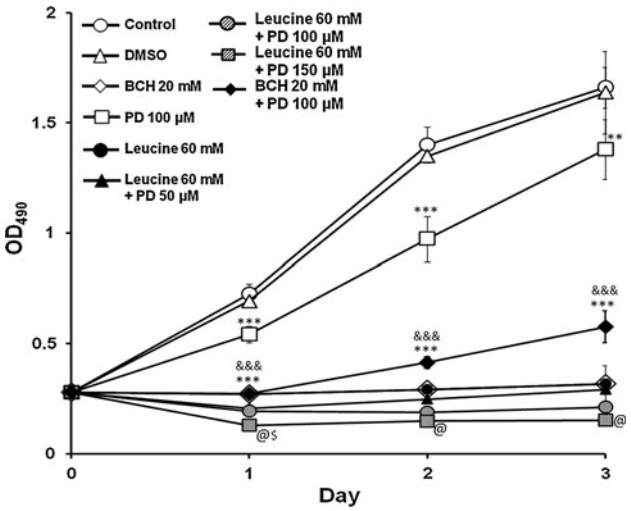

Fig. 10 Effects of LAT1 inhibitor BCH, L-leucine, and PD98059 on glioma cell growth using MTS assay. Data were analyzed by ANOVA followed by Bonferroni/Dunn test. $^{**}P < 0.01$ versus control. $^{***}P < 0.001$ versus control. $^{\&\&\&}P < 0.001$ versus 100 µM PD98059. $^{@}P < 0.05$ versus 60 mM Leu plus 50 µM PD98059. $^{\$}P < 0.01$ versus 60 mM Leu plus 100 µM PD98059. Each data point represents the mean ± standard deviation of triplicate samples. For details see the text and the "Methods" section

dysfunction of normal tissues, likely via an effect on transport of other amino acids, glucose, or other nutrients (Cohen and Hall 2009; Goberdhan 2010; Ishizuka et al. 2008; Nawashiro et al. 2006; Peyrollier et al. 2000). Further studies are required to examine the effects of high concentration of Leu on transport of other substances.

In summary, in the present study, we demonstrated that Leu induces growth arrest and persistent ERK activation in glioma cells. Furthermore, combination treatment with Leu and PD98059 (MEK inhibitor) caused an additive effect of glioma cell growth arrest, which may be a potential therapeutic strategy for the treatment of glioma.

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Conflict of interest The authors declare that they have no conflict of interest.

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