Inhibition of L-Type Amino Acid Transporter Modulates the Expression of Cell Cycle Regulatory Factors in KB Oral Cancer Cells

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The purpose of this study was to examine the effect of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), an inhibitor of L-type amino acid transporters, on the cell growth suppression in KB human oral cancer cells and to study the roles of cell cycle regulatory factors in the BCH-induced growth inhibition. The effect of BCH on cell growth suppression and the influence of BCH to cell cycle regulatory factors in KB cell growth inhibition were examined using cell cycle analysis, immunoblotting and immunoprecipitation. The BCH treatment induced cell cycle arrest at G1 phase in KB cells. The expression of cyclin D3 was remarkably decreased by BCH treatment. The BCH inhibited the expression of cyclin-dependent protein kinase 6 (CDK6) in a time-dependent manner. In addition, the expression of CDK inhibitor p27 was increased by BCH treatment in KB cells, but not CDK inhibitors p21 and p15. These results suggest that, in KB cells, the inhibition of LAT1 by BCH causes cell cycle arrest at G1 phase by inhibiting cyclin D3–CDK6 complex whereas increasing expression of a CDK inhibitor p27.

Key words L-type amino acid transporter; cancer cell; cell cycle regulatory factor; 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid

Amino acid transport system L is a cell membrane protein that transports neutral amino acid and known to be the major route of neutral amino acids for cell proliferation.[1—3] Kanai *et al.* identified the first isoform of amino acid transport system L (L-type amino acid transporter 1, LAT1) from C6 glioma cells.[4] It is predicted to be 12-membrane-spanning proteins, and mediates Na^+-independent amino acid exchange and prefers large neutral amino acids with bulky or branched side chains for its substrates.[4—8] Following the molecular identification of LAT1, the second isoform of amino acid transport system L (L-type amino acid transporter 2, LAT2) has been identified.[9—11] It has the characteristics of transporting small neutral amino acids as well as large neutral amino acids.[10—12] LAT1 is only expressed in restricted organs such as brain, spleen, placenta and testis.[4,13] However, LAT2 is more ubiquitously expressed than LAT1.[10—12] Importantly, LAT1 is highly expressed in malignant tumors presumably to support their continuous growth and proliferation.[4,5,14,15]

Based on the characteristics of LAT1 and LAT2, it is proposed that the manipulation of system L activity, particularly that of LAT1, would have therapeutic implications for cancer treatment. If the activity of LAT1 should be suppressed and thereby the depletion of intracellular neutral amino acids should be induced, this would be helpful for inducing the inhibition of cancer cell growth. However, the mechanism by which inhibition of LAT1 can cause cancer cell growth suppression or cytotoxicity of cancer cells is not entirely clear.

2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) is a model compound for the study of amino acid transporters, as it is a system L selective inhibitor.[4—6,11,16,17] Substrates for amino acid transport system L include several essential amino acids such as leucine, isoleucine, valine, phenylalanine, methionine and histidine.[1—3] Therefore, the suppression of L-type amino transpoter activity using BCH could be effective in the retardation of tumor cell growth by depleting the intracellular essential amino acids. In this study, we attempted to clarify the effect of BCH on the expression of factors regulating the cell cycle to cause cell cycle arrest in KB human oral cancer cells. We report here that the BCH arrests KB cell growth at G1 phase of cell cycle through the inhibition of cyclin D3–cyclin-dependent protein kinase 6 (CDK6) complex expression while increasing the expression of p27, a CDK inhibitor.

MATERIALS AND METHODS

Materials BCH and *N*-methylthiotetrazole (MTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-cyclin D1 antibody, anti-cyclin D3 antibody, anti-CDK4 antibody, anti-CDK6 antibody, anti-p15 antibody, anti-p21 antibody, anti-p27 antibody and anti-β-actin antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Other analytical reagents were purchased based on the analytical grade. KB human oral cancer cells were supplied by American Type Culture Collection (ATCC, Rockville, MD, U.S.A.).

Cell Cultures KB cells were grown in Dulbecco's modi-

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fied Eagle's medium (DMEM) and F-12 media with the ratio of 3 : 1 supplemented with 10% fetal bovine serum (FBS). Cells were maintained as monolayers in plastic culture plate at 37 °C in a humidified atmosphere containing 5% CO_2.

Flow Cytometric Cell Analysis KB cells were cultured in 100-mm tissue-culture dishes at a density of 1×10^6 cells/dish for 24 h and were treated with 20 mM BCH for 24 or 48 h. After treatment, cells were collected and washed with PBS. Flow cytometric analysis was performed using Cycle TEST^{TM} PLUS DNA Reagent Kit (Becton Dickinson, Erembodegem-Aalst, Belgium) and FACScan (Becton Dickinson, Erembodegem-Aalst, Belgium) according to the instructions of the manufacture. Results were analyzed using the CellQuest software.

Co-immunoprecipitation Cells were lysed in a buffer containing 1% Triton X-100, 10 mM Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaCl, 50 mM NaF, supplemented with 1 mg/ml antipain, 1 mg/ml leupeptin, 100 nM phenylmethylsulfonyl fluoride (PMSF) and 100 nM sodium orthovanadate. Approximately 1 mg of the clarified cell lysate was incubated overnight at 4 °C with anti-CDK6 (2 μg) or anti-cyclin D3 (2 μg). Immunoprecipitates were recovered on protein G-Sepharose beads. Proteins were transferred on to polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with anti-CDK6 and anti-cyclin D3.

Immunoblotting To examine the mechanisms by which BCH inhibits the cell growth, the immunoblotting analyses were performed. The immunoblotting (10 μg of protein loaded) was performed according to the previously-described method.[18,19] The primary antibodies used herein include anti-cyclin D1 antibody (1 : 1000), anti-cyclin D3 antibody (1 : 1000), anti-CDK4 antibody (1 : 1000), anti-CDK6 antibody (1 : 1000), anti-p15 antibody (1 : 1000), anti-p21 antibody (1 : 1000), anti-p27 antibody (1 : 1000) and anti-β-actin antibody (1 : 2000).

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

RESULTS

Cell Cycle Analysis of KB Cells Our previous study shown that BCH inhibited KB cell growth in a time and dose dependent manner.[20] Inhibition of KB cell proliferation by BCH led us to predict that BCH would impinge upon KB cell cycle progression. To test this hypothesis, KB cells were treated with or without 20 mM BCH for 24 h or 48 h and the stages of cellcycle were determined by FACScan analysis. As shown in Fig. 1, BCH treatment led to increase the percentage of cells at the G1 cell cycle phase from 53.55 to 60.29% after 24 h treatment. BCH caused a progressive increase in G1 phase cells; 70.82% of cells were at the G1 phase after 48 h treatment. The results suggested that BCH-induced inhibition of cell growth was correlated with an arrest in G1 of the cell cycle.

The Effect of BCH on Cyclin D Expression Progression of cell cycle depends on the coordinated activation/inactivation of a series of cyclins, CDKs and cyclin inhibitors. Thus, we examined the effect of BCH on expression of cyclin D1

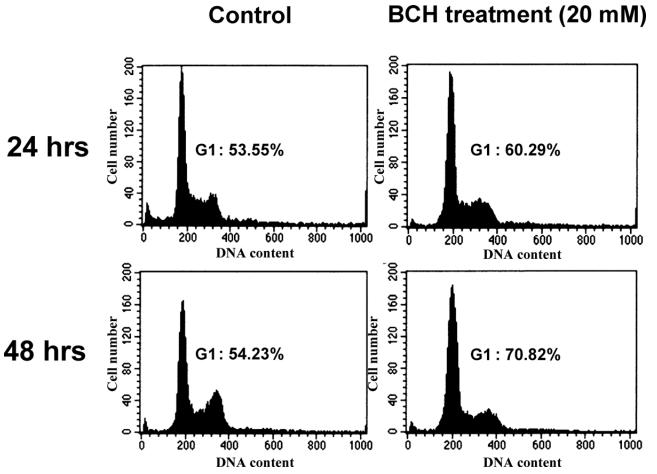

Fig. 1. Accumulation of Cells with G1 DNA Content in BCH-Treated KB Cells

KB cells were treated with BCH 20 mM for −24 and 48 h. Cell distribution according to DNA content was measured by Cycle TEST^{TM} PLUS DNA Reagent Kit and FACScan. This data indicated that the BCH arrested cell cycle at G1 phase in KB cells.

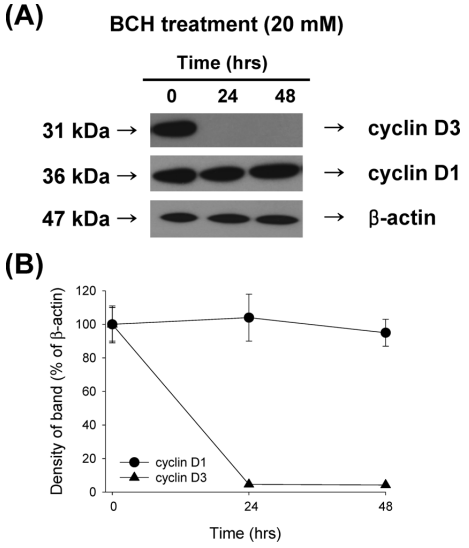

Fig. 2. The Effect of BCH on the Expression Levels of Cyclin D

(A) Expression levels of cyclin D1 and cyclin D3 by BCH were measured in KB cells. The cells were treated with BCH 20 mM for 0, 24 and 48 h. The cell lysate was prepared and analyzed by immunoblotting as described in Materials and Methods. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

and D3 by densiometric analysis of immunoblots. The expression level of cyclin D1 showed no significant difference as compared with the control group following the treatment with 20 mM BCH (Fig. 2). In contrast, the degree of expression of cyclin D3 was dramatically changed upon BCH treatment. The high basal expression of cyclin D3 in KB cells was decreased down to the undetectable level after 2 d of BCH treatment indicating that inhibition of LAT1 activity by BCH induces down regulation of cyclin D3 expression but not cyclin D1 expression (Fig. 2).

Differential Expression of CDKs by BCH Cyclin D binds to and activates CDK4 or CDK6, the activity of which is required for the G1 to S transition. To measure the degree of expression of CDK4 and CDK6, KB cells were treated with 20 mM BCH. As shown in Fig. 3, the expression of

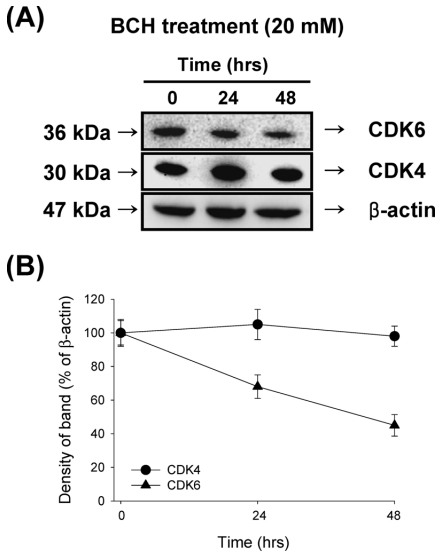

Fig. 3. The Effect of BCH on the Expression Levels of CDK4 and CDK6

(A) Expression levels of CDK4 and CDK6 by BCH were measured in KB cells. The cells were treated with BCH 20 mM for 0, 24 and 48 h. The cell lysate was prepared and analyzed by immunoblotting as described in Materials and Methods. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

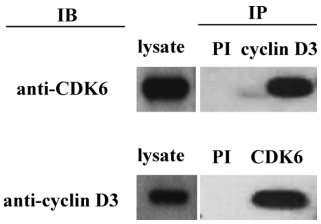

Fig. 4. CDK6 Interacts with Cyclin D3 in KB Cells

Protein was harvested and immunoprecipitated with CDK6 or cyclin D3 antibody. Immune complexes were subjected to Western blot analysis with cyclin D3 or CDK6 antibody after washing with RIPA buffer.

CDK4 showed no significant difference following BCH treatment as compared with the control group. In the control group, the degree of expression of CDK6 was relatively higher. However, the high basal expression level of CDK6 was decreased in proportion to the time of BCH treatment (Fig. 3). Furthermore, we also confirmed whether cyclin D3 binds with CDK6 in KB cells, co-immunoprecipitation was performed. The lysates from KB cells were immunoprecipitated with cyclin D3, CDK6 and pre-immune serum (PI) (as a negative control) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with CDK6 and cyclin D3 antibody, respectively. As shown in Fig. 4, CDK6 interacted with cyclin D3 to form CDK6–cyclin D3 complex in KB cells. Together with inhibition of cyclin D3 expression, this result indicates that one aspect of BCH induced cell cycle arrest is due to the down regulation of cyclin D3–CDK6 complex.

The Effect of BCH on the Expression of CDK Inhibitors The activity of CDK4 and CDK6 is opposed by members of the INK4 family, while the CIP/KIP family binds to and inhibits cyclin/CDK complexes. To measure the degree of expression of CDK inhibitors such as p15 (INK4 family), p21 (CIP1) and p27 (KIP1)[21,23] KB cells were treated with 20 mM BCH for 24 and 48 h. As shown in Fig. 5,

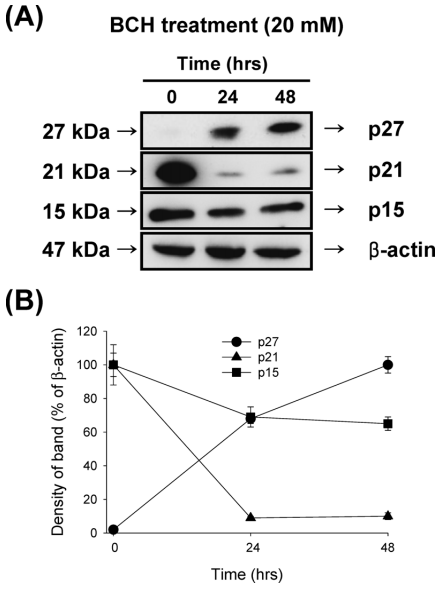

Fig. 5. The Effect of BCH Treatment on the Expression Levels of CDK Inhibitors (p15, p21 and p27)

(A) Expression level of p15, p21 and p27 by BCH were measured in KB cells. The cells were treated with BCH 20 mM for 0, 24 and 48 h. The cell lysate was prepared and analyzed by immunoblotting as described in Materials and Methods. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

p27 expression level was very low in the control group. After BCH treatment, however, its expression was markedly increased in proportion to the time of BCH treatment. In contrast, the relatively higher basal expression of p21 was decreased after 24 h BCH treatment implying BCH inhibition of LAT1 activity modulates the expression of CDK inhibitors, p27 and p21, bifunctional way. Although the expression level of p15 was also decreased upon BCH treatment, the effect was not significantly changed as in p21 (Fig. 5).

DISCUSSION

In this study, we investigated the differential expression of factors regulating the cell cycle on the inhibitory effect on cell growth by BCH, a selective inhibitor of L-type amino acid transporters, in KB human oral cancer cells.

Our previous study, BCH suppressed the growth of KB cells in a dose- and a time-dependent manner.[20] These results were in agreement with various reports that various compounds ([6]-paradol, norcantharidin, baccatin, *etc.*) suppressed the growth of cancer cells in a dose- and a time-dependent manner.[20,24,25] According to our previous studies, of the L-type amino acid transporters, LAT1 and its cofactor 4F2hc were expressed in KB cells, however, LAT2, another subtype of L-type amino acid transporters, was not expressed.[26] We also reported that LAT1 played a key role in transporting neutral amino acids including L-leucine in KB cells for cell growth and proliferation.[19,26] Therefore, intracellular depletion of essential amino acids by BCH mediated specific inhibition of LAT1 activity could be the relying mechanism of cell growth arrest upon BCH treatment.

The induction of cancer cell apoptosis is the most useful strategy of the development of anti-cancer drugs.[27,28] In

association with this, many authors have conducted the studies to induce the apoptosis. In our previous study, we reported that BCH induces apoptosis in KB cells in caspase-3 and caspase-7 dependent manner.[19] In addition to apoptosis, the current study using flow cytometric analysis of live cells shows that the BCH treatment induced arrest of cell cycle at G1 phase in KB cells which is a prior step to activate apoptosis (Fig. 1). Accordingly, we further examined the mechanisms by which BCH regulates the cell cycle, especially G1 phase, and thereby inhibits the growth of KB cells.

Recent studies have shown that such chemicals derived from natural materials as indole-3-carbinol, isothiocyanate and resveratrol have an inhibitory effect on the cell growth in cancer cells.[29] It has been reported that this effect alters various factors associated with the cell cycle and thereby induce the apoptotic cell death.[28] Of CDKs which have been known to be the regulatory factors for cell cycle, CDK4 and CDK6 play a key role in regulating G1 phase and G1–S phase along with cyclin D.[21,29] Of cyclin Ds, cyclin D1 is a proto-oncogene and it has been reported to affect the cell cycle.[21] Recent studies reported the biological functions of cyclin D2, which are similar to those of cyclin D1, and the role of cyclin D3 in human cancer cells.[21,22] In the current study, we found that there is no significant difference in the expression of cyclin D1 between the BCH treatment group and the control group in KB cells. However, a significant reduction in cyclin D3 levels was observed in BCH treated KB cells (Fig. 2). Cyclin D, in response to mitotic signals, binds to existing CDK4 and CDK6 forming active cyclin D–CDK complexes that transits cell cycle from G1 to S phase. BCH dependent inhibition of LAT1 activity selectively down regulated CDK6 expression over CDK4 (Fig. 3). Thus it can be postulated that the lower level of cyclin D3–CDK6 complex renders KB cell cycle arrest at G1 phase in KB cells upon BCH inhibition of LAT1 activity. We also confirmed that cyclin D3 interacted with CDK6 in KB cells (Fig. 4).

The cell cycle progression activity of cyclin D–CDK complexes is counteracted by INK4 and CIP/KIP family members. These factors have been reported to bind to the cyclin–CDK complex and thereby to suppress the phosphorylation activity.[21] We examined the expression levels of three CDK inhibitors, p15 which suppresses the phosphorylation of pRb associated with cyclin D/CDK4 or cyclin D/CDK6 complex,[21] p21 protein binds to cyclin D/CDK4 complex or cyclin D/CDK6 complex and suppresses the phosphorylation activity, and p27 protein suppresses the phosphorylation activity which is dependent on cyclin D or cyclin E during the transition of G1 phase to S phase.[23] Our results showed that the expression of p27 was significantly increased in proportion to the time of BCH treatment (Fig. 5). But the expression of p21 and p15 was decreased. This indicates that BCH increases the expression of p27, one of CDK inhibitors, and thereby further inhibits the activity of cyclin D3–CDK6 complex which was down regulated in BCH treated KB cells. BCH thus can selectively target several components of the cell cycle machinery to arrest the cell cycle at G1 phase.

Although the exact mechanism by which LAT1 inhibition by BCH induces cell cycle arrest of oral cancer cells warrants future research, the mammalian target of rapamycin (mTOR) could be a probable target to mediate the observed growth inhibitory effects. Recently, it has been shown that BCH treatment inhibits mTOR pathway in carcinoma cell lines.[30] Since mTOR integrates various extracellular and intracellular signals especially by sensing intracellular amino acid pools, it is reasonable to postulate that the depletion of amino acids in KB cell by the inhibition of LAT1 activity by BCH suppresses mTOR activity thereby down regulates the expression of cell cycle modulators like cyclins, CDKs and CDK inhibitors to arrest cell cycle at G1 phase.

In conclusion, we found that the inhibition of LAT1 activity which is highly expressed in KB human oral cancer cells contributes to the cell cycle arrest at G1 phase. The observed growth inhibition was regulated by the decreased expression of cyclin D3 and CDK6 and the increased expression of p27, a cyclin D–CDK6 complex inhibitor. Based on our results, it can be inferred that the use of LAT1 specific inhibitors would be another possibility for the suppression of growth of oral cancer cells.

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