The Effects of Branched-Chain Amino Acids on Canine Neoplastic Cell Proliferation and Death¹⁻³

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EXPANDED ABSTRACT

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The use of high concentrations of various amino acids in the diet may perturb neoplastic cell growth. To date, the most effective amino acid for this purpose in cell culture and in vivo has been arginine (1–3). Other amino acids have also been used to diminish neoplastic cell growth with mixed results (4–6). To date, in veterinary medicine, only one clinical study examining supplemental arginine showed diminished neoplastic growth in canines with lymphoma, but unfortunately, the benefit of arginine in the diet remains controversial because the dietary intervention contained multiple variables (7).

Certain neoplastic conditions are often accompanied by changes in metabolism resulting in cachexia. Studies have suggested that branched-chain amino acids might help to preserve lean body mass in models of muscle atrophy (8–10). More recent studies suggest that leucine has profound effects upregulating signaling events (i.e., mTOR activation), leading to protein synthesis in skeletal muscle (11). The exact mechanisms for this upregulation remain elusive, but treatment with branched-chain amino acids (BCAA) remains attractive as a therapeutic modality for ameliorating lean body mass in cachectic patients.

If BCAAs are beneficial during cachexia, determining their effects on neoplastic cell growth is important. Neoplastic cell lines (canine osteosarcoma, canine bronchoalveolar carcinoma, and Madine-Darby canine kidney cells) under the influence of high concentrations of BCAAs (leucine, isoleucine, valine) were examined for their ability to augment or diminish cell growth. Experiments using supplemental arginine were also performed because arginine is the most intensively studied amino acid in cell culture models of neoplasia. Standard growth curves, cell-cycle kinetics, and apoptotic assays were performed to examine the effects of these amino acids.

MATERIALS AND METHODS

Cell culture

Cell lines were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma) at 37°C and 5% CO₂, Madine-Darby canine kidney (MDCK; American Tissue Culture Collection), osteosarcoma (gift from the Cornell comparative oncology program), and bronchoepithelial carcinoma (BACA) (primary cell culture) cells were used.

Cell proliferation

Various amino acids were solubilized to reach final concentrations of 5 mM, 10 mM, 50 mM, and 100 mM as compared with complete media alone. Cells were plated at 40,000 per well in 24-well plates and allowed to attach before treating with amino acids. The 48 h growth curves were done as previously described by Wang et al. (12). Each experiment was performed in triplicate, and a Student’s t test was performed. Bonferroni’s correction was used because of the multiple comparisons, resulting in a α = 0.00625.

Apoptosis assays

Aliquots of 1 × 10⁵ cells were added to tissue culture–treated polystyrene dual-chamber slides (Nalgene) and were allowed to attach and grow for 24 h before being treated with various concentrations of amino acids or no amino acids. After 24 or 48 h of treatment, the medium was removed, and the wells were washed once with PBS and then stained with Hoechst 3222 and propidium iodide using the Vybrant apoptosis kit (Molecular Probes Inc.) The cells were then manually counted and examined for signs of apoptosis (i.e., condensation and nuclear fragmentation). Each experiment was performed in triplicate, and a 1-way ANOVA (α = 0.05) with Tukey’s post hoc comparison was performed.
Flow cytometry

Aliquots of $1 \times 10^6$ cells were plated and allowed to grow for 48 h with the various supplemental amino acids ranging from 10 to 100 mmol/L to examine shifts in cell cycle as previously described (13). Flow cytometry was performed using the Becton Dickenson FACScan flow cytometer. The DNA Star software (DNAStar Inc.) was used to obtain the percentages of cells at different phases of the cell cycle.

Western blot analysis

Osteosarcoma cells were plated to achieve 50% confluency in 100-mm tissue culture–treated polystyrene dishes. The cells were lysed, protein concentrations were determined, and SDS-PAGE and Western blotting were performed as previously described by Antonyak et al. (14). The primary antibodies used were antiactin polyclonal rabbit (Sigma biochemicals), antiphosphotyrosine-cdc2 antibody mouse monoclonal, antiactivated caspase 3 polyclonal rabbit, antiactivated caspase 8 polyclonal rabbit antibody (Cell Signaling), anticaspase 2 rabbit polyclonal antibody (Calbiochem), diluted 1:1000 in tris-buffered saline with 0.5% tween (TTBS) overnight at 4°C. Appropriate horseradish peroxidase–labeled secondary antibodies (Amersham-Pharmacia Inc.) were diluted in TTBS at 1:5000. The immunoblots were then exposed to chemiluminescent reagent and visualized on radiograph film.

RESULTS

Cell proliferation

All amino acids displayed a marked antiproliferative effect that was statistically significant at 100 mmol/L for all three cell lines ($P < 0.00625$). Only arginine and leucine showed a significant antiproliferative effect at 50 and 10 mmol/L in all three cell lines ($P < 0.00625$; Fig. 1).

Apoptosis

Hoechst 3222 staining revealed a marked apoptotic response to arginine and leucine. Leucine treatment (100 mmol/L) induced apoptosis within 36–48 h, whereas arginine treatment (100 mmol/L) caused significant apoptosis by 24 h. The osteosarcoma cell lines demonstrated increased apoptotic rates with arginine and leucine, whereas the MDCK cell line showed a modest increase in apoptosis with arginine treatment (Fig. 2).

Flow cytometry

With analysis of the cell cycle, treatment of the MDCK or BACA cell lines revealed no appreciable differences from control cells after 48 h of treatment with any of the amino acids. When the osteosarcoma cells were treated with 50 mmol/L of arginine, isoleucine, or valine there was little deviation from the average cell cycle analysis ratios of 60:16:24%. Treatment with 50 and 10 mmol/L leucine exhibited a trend toward elimination of the G2M phase, which was most pronounced at 50 mmol/L leucine (Fig. 3), with a G1/S/G2M ratio of 58:39:3%.

Western blot analysis

Because of the profound apoptotic effects of arginine and leucine on osteosarcoma cells, lysates were collected at various time points after treatment with either 100 mmol/L arginine or 100 mmol/L leucine to examine the apoptotic response. The lysates were immunoblotted for active caspase 8, active caspase 2, and active caspase 3, as well as for tyrosine 15 phospho-epitope of cdc2, a potent G2M regulator (Fig. 4). The immunoblots showed that caspases 3 and 2 became activated, whereas caspase 8 does not, suggesting that mitochondrial dysfunction mediates the apoptotic response.

DISCUSSION

The antiproliferative effects of certain amino acids, particularly arginine, have been studied both in vivo and in vitro (1–3). Arginine has been used in critical care medicine for some time, and special formulations of arginine-enriched diets are presently being used in human and veterinary medicine (7,14). The use of branched-chain amino acids as a therapeutic option for the preservation of lean body mass is becoming increasingly popular (15,16). Physicians sometimes advocate their use in cancer-related cachexia; thus, it is increasingly important to understand how high concentrations of these amino acids will affect proliferating neoplastic cells. Regarding cell proliferation, our experiments showed that all amino acids tested had antiproliferative capacities at the highest concentrations (100 mmol/L) but that arginine and leucine were far more suppressive at lower concentrations (50 and 10 mmol/L) than valine or isoleucine.

The antiproliferative effects of all amino acids tested were seen only at extremely high levels. We speculate that this may be because of amino acid imbalance in the medium, considering that all BCAAs compete for the same transporters in the
plasma membrane. This amino acid imbalance may cause problems with protein synthesis leading to suppression of growth. The potency of arginine and leucine at inducing cell death suggests that other mechanisms may be playing a role. Thus, we examined whether this was an apoptotic event. Hoechst staining and Western blotting for caspase activity showed caspase-mediated apoptotic events with both arginine and leucine, whereas valine and isoleucine, although adequate at diminishing proliferation, could not induce apoptosis.

In an attempt to understand this cytotoxic response, cell cycle analysis was performed with flow cytometry, which showed a profound difference between arginine and leucine treatment in the osteosarcoma cell line. Leucine-treated cells showed a loss of the G2M phase of the cell cycle with more cells halted in the S phase (Fig. 3). This phenomenon was not seen during the apoptotic events associated with arginine.

To further elucidate the mechanisms underlying the cytotoxic response, a time course analysis was performed in the osteosarcoma cells with arginine and leucine treatments. The events of apoptosis are often characterized by various caspase cascades being activated, all of which converge onto caspase 3, the major caspase activated during induction of apoptosis. The mitochondrial pathways of apoptosis are complex but often result in the activation of caspase 2, 9, or 7 and eventually activation of caspase 3. Caspase 3 then amplifies caspase activation and irreversibly induces apoptosis (17). Other growth factor–mediated signaling events can lead to caspase 3 activation, which is often mediated through activation of caspase 8 or 10 (18).

We used activated caspase 3 as a universal indication of apoptosis, which was activated in the osteosarcoma with both arginine and leucine. The activation occurred within 24 h in arginine-treated cells and within 36 h in the leucine-treated cells. To help determine whether this was a mitochondrial event or through extracellular stimulation, immunoblotting for caspase 2 and caspase 8 was performed. Results showed that neither arginine nor leucine activate caspase 9 and that caspase 2 activation preceded caspase 3 activation; thus, apoptosis caused by mitochondrial dysfunction is likely.

To further identify how leucine was involved in S phase arrest, the inhibitory phosphorylation site on cdc2 (tyrosine 15), which prevents progression through the G2M by preventing cdc2/cyclinB complex from initiating mitosis, was examined through immunoblotting (19). Cdc2, when bound to cyclin B2, serves as a complex that regulates entry into the G2M phase and changes its phosphorylation state during S phase arrest (19). As expected, more phosphorylation of tyrosine 15 on cdc2 in the leucine-treated cells was observed, but it was no different from arginine treatment, which also showed excessive phosphorylation. In fact, it appears as if both arginine and leucine activate this mechanism to slow cell proliferation.

Although, ultimately, both amino acid insults result in mitochondrial compromise leading to apoptosis at high concentrations, the differences observed in cell cycle analysis imply that there may be different cytotoxic mechanisms. Further investigation is needed to examine both normal cells and other amino acid treatments on cell proliferation, but our data suggest that BCAA treatment does not directly potentiate neoplastic cell growth and may actually diminish neoplastic cell proliferation at supraphysiological concentrations.

**FIGURE 2** Osteosarcoma and MDCK cell apoptosis. Percentage of cells undergoing apoptosis after either 24 or 48 h of treatment with 100 mmol/L of various amino acids. Significant increase in the number of apoptotic cells compared with control, valine, and isoleucine, *P < 0.05.

**FIGURE 3** Cell cycle analysis of osteosarcoma cells. Control cells 60% G1, 16% S, and 25% G2M (A). After 50 mmol/L arginine treatment for 24 h: 62% G1, 18% S, 20% G2M (B). After 50 mmol/L leucine treatment for 48 h: 58% G1, 39% S, 3% G2M, depicting nearly complete loss of G2M phase (C). a = apoptotic debris; G1 = growth phase 1; S = DNA synthesis phase; G2M = growth phase 2/mitosis.

**FIGURE 4** Western blotting for various proteins involved in caspase-mediated apoptosis (activated caspase 8, activated caspase 2, activated caspase 3) and cell cycle regulator cdc-2 phosphoepitope tyrosine 15. Cells were lysed before and after 100 mmol/L arginine or lysine at 0, 12, 36, and 48 h post-treatment. Actin immunoblotting was performed as a marker of equal protein loading. Time course analysis depicts an activation of caspase 2 preceding activation of caspase 3 and no observed activation of caspase 8. Marked increase in phosphorylated tyrosine 15-cdc2 suggests diminished cell proliferation before apoptosis with both arginine and leucine treatment.

**LITERATURE CITED**


