Influence of Glutamine on the Growth of Human Glioma and Medulloblastoma in Culture

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ABSTRACT

Cellular supply of glutamine, an essential substrate for growth, is derived from extracellular fluid and de novo synthesis. We investigated the relative importance of these sources to the growth of six human anaplastic glioma- and one human medulloblastoma-derived permanent cell lines. Exogenous glutamine was limiting for the proliferation of glioma-derived lines D-54 MG, U-118 MG, and U-251 MG. In contrast, medulloblastoma-derived line TE-671 and glioma-derived lines U-373 MG, D-245 MG, and D-259 MG grew in the absence of supplemental glutamine. Two cell lines with contrasting glutamine requirements, D-54 MG and TE-671, were used to explore the pharmacological interference with glutamine metabolism. DL-α-Aminoadipic acid, a reported glutamic acid analogue with gliotoxic properties, significantly inhibited the growth of both lines. These effects were reversed by increasing glutamine, suggesting that the major action of DL-α-aminoadipic acid is as a glutamine antagonist. In contrast, the glutamine synthetase inhibitor β-hydroxylysine demonstrated activity only against TE-671. Acivicin and 6-diazo-5-oxo-L-norleucine, glutamine analogues available for clinical use, reduced the utilization of glutamine are potentially exploitable targets for the chemotherapy of some human gliomas and medulloblastomas.

INTRODUCTION

Eagle’s pioneering efforts to define the nutritional requirements of normal and neoplastic cells in culture led to the identification of glutamine as a critical substrate (10). This amino acid’s multiple contributions to cellular growth include participation in protein, purine, and pyrimidine biosynthesis (22) and energy metabolism (39). The concentration of the substrate in extracellular fluid and rate of de novo synthesis determine its availability for these essential functions. Conversion of α-ketoglutarate, an intermediate in the citric acid cycle, to glutamic acid via aspartate aminotransferase (5) or glutamate dehydrogenase (34) and the subsequent reaction of glutamic acid with ammonia via glutamine synthetase (22) define the pathway for glutamine production. The presence and activities of the enzymes are rate limiting.

In the central nervous system, glutamine not only participates in intermediary metabolism but also detoxifies ammonia and helps to regulate glutamic acid and γ-aminobutyric acid neurotransmission (17). Compartmentation of glutamine metabolism into anatomically and functionally distinct pools (1) underscores the complexity in these activities. Disturbances of the network induce marked alterations in central nervous system physiology. Inborn errors of glutamine and glutamic acid metabolism produce cognitive and motor deficits (28). High doses of glutamic acid cause retinal necrosis in the mouse (18) and precipitate the Chinese restaurant syndrome in humans (32). Structural analogues of glutamic acid selectively damage neurons or glia when injected into newborn mice (24). The treatment of visceral and hematopoietic tumors in patients with glutaminase induces reversible encephalopathy (36).

Glutamine’s contributions to both growth and normal brain function suggest that this amino acid may also be important to the metabolism of primary central nervous system neoplasms. We report here the glutamine requirements of six permanent cell lines derived from anaplastic human gliomas and one line derived from a human medulloblastoma. We have used as probes DL-α-aminoadipic acid, a reported glutamic acid analogue with gliotoxic properties (24), methionine sulfoximine and β-hydroxylysine, glutamine synthetase inhibitors, and acivicin and DON,4 glutamine antagonists, to identify the synthesis and utilization of glutamine as potentially exploitable targets for the chemotherapy of these neoplasms.

MATERIALS AND METHODS

Cell Lines. Cultured cell lines derived from human glioma, medulloblastoma, and osteogenic sarcoma were used. D-54 MG is the Duke University subline A-172 established by G. Todaro (13) from a human glioblastoma multiforme. U-118 MG, U-251 MG, and U-373 MG are lines derived from anaplastic human gliomas, and 2T is a line derived from an osteogenic sarcoma. These lines were established in Uppsala, Sweden, by B. Westermark and J. Pontén. The morphological, karyotypic, and selected biochemical characteristics of these lines have been reported (2). D-245 MG and D-259 MG are lines derived from anaplastic gliomas at Duke (4). TE-671 is a line established by McAllister (19) from a human medulloblastoma and further characterized by Friedman et al. (12).

All cell lines were grown in monolayer culture in Eagle’s glutamine and glutamic acid-free minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer, and 4 mM glutamine in a humidified 5% CO2 atmosphere at 37°C. At confluence, cells were mechanically harvested with a Pasteur pipet following digestion with 0.125% trypsin:0.02% EDTA. All cell lines have been tested to ensure the absence of HeLa cell, inter-, or intra-cell line contamination and Mycoplasma infection.

Chemicals. L-Glutamine, L-glutamic acid, DL-α-aminoadipic acid, β-

1 This work was supported by NIH Grants PO1 NSCA 200023-01 and CA 11888, Duke Comprehensive Cancer Center Developments Funds (CA 14236), and the W. W. Smith charitable trust.
2 Recipient of an Association for Brain Tumor Research Fellowship in memory of Bryce Davis and an American Cancer Society Junior Clinical Faculty Fellowship.
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Received 10/12/84; revised 4/3/85; accepted 5/22/85.

CANCER RESEARCH VOL. 45 SEPTEMBER 1985
4077
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hydroxylysine (mixed dl- and dl-allo-), and L-methionine sulfoximine were obtained from Sigma Chemical Co., St. Louis, MO. Adcin and DON were provided generously by the Developmental Therapeutics Program, National Cancer Institute.

Population Growth Curves. For D-54 MG and TE-671, 10-day growth experiments, in varying concentrations of glutamine and glutamic acid, were performed. Two x 10^6 cells were plated in 35-mm^2 wells containing 2 ml of glutamine- and glutamic acid-free minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid buffer (glutamine- and glutamic acid-free minimal essential medium:10% fetal calf serum), and either glutamine (0 to 4 mM) or glutamic acid (0 to 5 mM). The fetal calf serum was not dialyzed but stored at 4°C for 2 wk prior to use. It contained less than 0.6 mM glutamine and 1.0 mM glutamic acid determined with the Beckman Model 6300 high-performance amino acid analyzer; Beckman Instruments, Inc., Berkeley, CA). Since the serum is diluted 10-fold in the experimental solutions, it contributes to the total less than 0.06 mM glutamine and 0.1 mM glutamic acid. For the purposes of this paper, we regard these levels as insignificant and do not include them in the reported concentrations of the substrates. The osmolality in the dishes ranged between 277 and 296 mOsmol (determined with an Advanced osmometer; Advanced Instruments, Newton Highlands, MA). Medium was replaced on alternate days. Viable cell counts, as assessed by trypan blue exclusion, were determined every 2 days using a hemacytometer. One to two drops of 5% trypan blue were added to 1 or 2 ml of medium, and the coverslips were counted, depending upon growth. Each point was run in triplicate. Mean values are expressed. For the remaining cell lines, this procedure was followed except that viable cell counts were determined only on Day 6 in the presence of 0 mM glutamine and glutamic acid, 0.5 mM glutamine, and 0.5 mM glutamic acid. D-259 MG grew half in monolayer and half in suspension culture; only the former cells were counted.

Glutamine Synthetase Expression. Cells were grown to 80% confluence on 9 x 9-mm coverslips, fixed with -20°C acetone, and rehydrated with 10% normal goat serum in phosphate-buffered saline for 10 min at room temperature. The coverslips were stained with rabbit anti-glutamine synthetase via the avidin:biotin:peroxidase technique previously reported (20). Results were expressed as negative (-), slightly positive (+), moderately positive (++), and markedly positive (+++) for the presence of glutamine synthetase. The primary antibody was prepared by immunizing an adult rabbit with four weekly s.c. injections of 0.1 mg of glutamine synthetase (purified from sheep brain, Sigma G-6632), dissolved in Hanks' balanced salt solution (Grand Island Biological Company, Grand Island, NY), and mixed with an equal volume of Freund's complete adjuvant. A final injection of 0.1 mg of glutamine synthetase in Hanks' balanced salt solution alone was injected 2 wk later, and the first bleeding 12 days later was used as the antibody source. The rabbit antiserum gave a single precipitin line against sheep glutamine synthetase and rat brain homogenate in Ouchterlony immunodiffusion. With avidin:biotin:peroxidase immunohistochemistry against frozen sections of rat brain, astrocyte-specific binding was observed. The antiserum was used at dilutions of 1:400 and 1:800, with normal rabbit serum at the same dilutions serving as control.

Mechanism Studies. Two x 10^6 cells were plated in 35-mm^2 wells containing 2 ml of glutamine- and glutamic acid-free minimal essential medium:10% fetal calf serum supplemented with either dl-α-aminoadipic acid (0 to 10 mM) or l-hydroxylysine (0 to 10 mM) and glutamine (0 to 2 mM). Medium was replaced every other day. Cell viability was measured on Day 6. Each point was run in triplicate.

Efficacy Studies. The procedure was the same as for the mechanism studies except that the glutamine concentration was constant at 0.5 mM with varying levels of DON, acivicin, and methionine sulfoximine.

RESULTS

Population Growth Curves. Chart 1 illustrates the influence of varying concentrations of glutamine and glutamic acid on the growth of the human glioma-derived cell line D-54 MG over a 10-day period. In the absence of exogenous glutamine, the cells were unable to grow. When glutamine was provided, there was a dose-response relationship between growth and glutamine concentration which plateaued at 2 mM. Glutamic acid up to 0.5 mM failed to stimulate the growth of D-54 MG. Levels of 5.0 mM glutamic acid did induce a small response in growth.

Parallel experiments were done with the human medulloblastoma-derived cell line TE-671 (Chart 2). In contrast to D-54 MG, this cell line was able to grow in the absence of glutamine, achieving almost the same saturation density as with 2 mM glutamine. Increasing concentrations of glutamine up to 2 mM did, however, increase the growth rate (differences on Day 6, P < 0.005 by Student's t test). Supplementation with glutamic acid also accelerated the growth of the cell line in a similar fashion.

Glutamine Requirements. Since TE-671 and D-54 MG demonstrated marked differences in their dependence upon exogenous glutamine and glutamic acid for growth, we investigated...
these parameters in the remaining cell lines to better define the spectrum of requirements. Viable cell counts were determined on Day 6 in varying concentrations of glutamine and glutamic acid (Table 1). This time was selected because the full population growth curves with TE-671 and D-54 MG showed that, by this point, viable cell counts were sensitive to different levels of the substrates. In addition to TE-671, three glioma-derived lines U-373 MG, D-245 MG, and D-259 MG were able to proliferate in the absence of supplemental glutamine. In contrast, glioma-derived lines U-118 MG and U-251 MG and osteogenic sarcoma-derived line 2T required 0.5 mM glutamic acid for proliferation. For growth curves with TE-671 and D-54 MG showed that, by this point, viable cell counts were sensitive to different levels of the substrates. In addition to TE-671, three glioma-derived lines U-373 MG, D-245 MG, and D-259 MG were able to proliferate in the absence of supplemental glutamine. In contrast, glioma-derived lines U-118 MG and U-251 MG and osteogenic sarcoma-derived line 2T required 0.5 mM glutamic acid for proliferation. For these lines, 0.5 mM glutamic acid failed to stimulate growth.

Since the ability to grow without glutamine is an indirect measure of functional glutamine synthetase activity, we extended these findings with a direct assay for the presence of this enzyme. Glutamine synthetase expression was detected immunohistochemically only in the four cell lines which grew in the absence of glutamine: TE-671; U-373 MG; D-245 MG; and D-259 MG (Table 1).

**Mechanism Studies.** Having characterized the glutamine nutritional requirements of a panel of human cell lines derived from anaplastic gliomas and medulloblastoma, we next investigated the effects of the gliotoxic compound DL-α-aminoadipic acid on the growth of D-54 MG and TE-671. These two cell lines were chosen for detailed study because of their contrasting origin and glutamine requirements. TE-671, derived from a medulloblastoma, grew without glutamine, while D-54 MG, derived from a glioma, required glutamine for growth. DL-α-Aminoadipic acid (0 to 10 mM) produced dose-dependent growth inhibition against both lines (Chart 3). Increasing glutamine levels reversed the inhibitory effects, especially as the concentration of DL-α-aminoadipic acid was reduced.

Chart 4 shows the effects of δ-hydroxylysine, a glutamine synthetase inhibitor (30), on the growth of TE-671 and D-54 MG. Hydroxylysine only produced inhibition against TE-671, a line with high glutamine synthetase activity. When no supplemental glutamine was provided for TE-671, the inhibitor completely arrested proliferation. Increasing concentrations of glutamine reversed the activity of hydroxylysine, particularly at lower doses of the inhibitor.

**Efficacy Studies.** Since interference with the synthesis and utilization of glutamine caused growth inhibition, it was of interest to test agents with similar mechanisms of action used in the treatment of neoplastic disorders. The activity profile of DON, a glutamine antagonist (7), against TE-671 and D-54 MG was studied (Chart 5). The glutamine concentration was 0.5 mM, the level in cerebrospinal fluid (27). DON produced dose-dependent growth inhibition against both cell lines, with ID₅₀ values of 0.025 μg/ml in TE-671 and 0.04 μg/ml in D-54 MG. The effects of acivicin, a more recently discovered glutamine analogue (15), are also shown in Chart 5. Acivicin produced dose-dependent growth inhibition against both cell lines, with ID₅₀ values of 0.055 μg/ml in TE-671 and 0.015 μg/ml in D-54 MG. Metionine sulfoximine, a glutamine synthetase inhibitor (23) administered to cancer patients in the early 1960s, produced marked growth inhibition only against TE-671, with an ID₅₀ of 3.6 μg/ml.

**DISCUSSION**

Our experiments establish that glutamine is a limiting factor for the growth of human cell lines derived from anaplastic gliomas and medulloblastoma in culture. The cell lines demonstrated heterogeneity in their dependence upon de novo synthesis versus extracellular glutamine. The glioma-derived cell lines D-54 MG, U-118 MG, and U-251 MG failed to proliferate unless supplied with exogenous glutamine. In contrast, the medulloblastoma-derived cell line TE-671 and glioma-derived cell lines U-373 MG, D-245 MG, and D-259 MG grew without glutamine. Differences in glutamine synthetase activity probably account for this variation, since a productive de novo pathway requires high levels of enzyme activity. This spectrum of glutamine dependence among cell lines derived from anaplastic gliomas and medulloblastoma extends the previously reported heterogeneity in gliomas regarding other biochemical, morphological, karyotypic, and antigenic parameters (2, 33, 38). The presence of glutamine synthetase in some of our cell lines complements the previous demonstration of the enzyme in biopsy specimens from patients (26).

Our growth inhibitor studies with DL-α-aminoadipic acid indicate that cultured cell lines derived from primary central nervous system tumors are sensitive to the pharmacological interference with glutamine metabolism. These findings are consistent with those of Campbell et al. (6), who have shown similar effects for DL-α-aminoadipic acid using quantitation of protein content, uptake of radiolabeled lysine, and clonogenicity to measure growth inhibition. Activity against both TE-671, a cell line possessing glutamine synthetase, and D-54 MG, a cell line lacking this enzyme, and reversal by glutamine of these effects together suggest that the major action of α-aminoadipic acid is as a glutamine antagonist. These findings may have relevance for the pathogenesis of α-ketoadipic aciduria (29) and glutaric aciduria (14), two inborn errors of metabolism associated with psychomotor retardation and characterized by increased concentrations of α-aminoadipic acid in the blood and urine. The precise locus of inhibition remains unclear; possibilities include any of the amidotransferase reactions and membrane transport. Other investigations indicate that α-aminoadipic acid decreases the uptake of glutamic acid in cerebral cortical slices (8) and is a weak inhibitor of glutamine synthetase (22). We cannot exclude minor roles for these mechanisms in our tumor lines. The activity profile and glutamine reversal experiments of δ-hydroxylysine, a known

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**Table 1**

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Viable cell counts (x10⁶)</th>
<th>Avidin:biotin: peroxidase reactivity with anti-glutamine synthetase antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-118 MG</td>
<td>2.53 ± 0.06</td>
<td>-</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>3.50 ± 0.79</td>
<td>+, slightly positive</td>
</tr>
<tr>
<td>U-373 MG</td>
<td>9.83 ± 1.20</td>
<td>++, moderately positive</td>
</tr>
<tr>
<td>D-245 MG</td>
<td>5.93 ± 1.17</td>
<td>+++</td>
</tr>
<tr>
<td>D-259 MG</td>
<td>7.23 ± 0.78</td>
<td>++</td>
</tr>
<tr>
<td>2T</td>
<td>2.50 ± 0.30</td>
<td>-</td>
</tr>
<tr>
<td>TE-671</td>
<td>13.40 ± 1.22</td>
<td>+</td>
</tr>
<tr>
<td>D-54 MG</td>
<td>2.07 ± 0.40</td>
<td>-</td>
</tr>
</tbody>
</table>

* Negative; +, slightly positive; ++, moderately positive; ++++, markedly positive.

* GLN, glutamine; GLU, glutamic acid.

* Mean ± SD for triplicate samples on Day 5 following plating 2 x 10⁶ cells on Day 0.
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Chart 3. Influence of glutamine on the growth-inhibitory effects of DL-a-aminoadipic acid. The data for TE-671 are a composite from two experiments. Mean values for maximal growth on Day 6 were 3.16 ± 0.23 x 10^6 and 4.64 ± 0.34 x 10^6 viable cells. For D-54 MG, the mean maximal viable cell count on Day 6 was 1.68 ± 0.10 x 10^6.

Chart 4. Influence of glutamine on the growth-inhibitory effects of D-hydroxylysine. For TE-671, the mean viable cell count for maximal growth on Day 6 was 3.01 ± 0.10 x 10^6. The corresponding value for D-54 MG was 1.21 ± 0.06 x 10^6.

Chart 5. Effects of DON and acivicin on the growth of TE-671 and D-54 MG. Glutamine concentration was 0.5 mM. Data for each curve are a composite from two experiments. For TE-671, mean values for control growth (no drug) on Day 6 ranged from 2.43 to 2.98 x 10^6 viable cells. The corresponding values for D-54 MG ranged from 0.97 to 1.3 x 10^6 viable cells.

Glutamine synthetase inhibitor (30), however, are different from those of α-aminoadipic acid.

Since glutamine antagonists are available for treating patients, our analysis of glutamine metabolism in primary central nervous system tumors can be explored clinically. The antitumor antibiotic DON has shown minor therapeutic activity against a wide variety of visceral and hematopoietic neoplasms (7), but its efficacy against brain tumors has not been explored, perhaps because it failed to increase survival in the ependymoblastoma model (25). Our studies establish that DON has significant growth-inhibitory effects in vitro against the human medulloblastoma-derived cell line TE-671 and the human glioma-derived cell line D-54 MG, with ID50 values of 0.025 and 0.040 μg/ml, respectively, at physiological glutamine concentrations. These ID50 values compare favorably with the tolerable steady-state plasma levels (6.7 to 10.5 μg/ml) achieved in patients during a 24-h infusion of DON (31).

Acivicin, a more recently discovered antitumor antibiotic with the properties of a glutamine analogue (15), also failed to demonstrate activity against murine ependymoblastoma (9). Note worthy in its Phase I trials, however, was dose-limiting central nervous system toxicity ranging from somnolence to psychosis (11, 37). Pharmacokinetic studies in rhesus monkeys revealed important cerebrospinal fluid penetrance (21). Our data indicate that acivicin reduces the proliferation of TE-671 and D-54 MG in vitro, with ID50 values of 0.055 and 0.015 μg/ml, respectively, at physiological glutamine concentrations. Achievable plasma levels in patients during a 24-h infusion of acivicin reach 4.6 μg/ml (37) and 0.89 μg/ml during a 72-h infusion (11).

The glutamine synthetase inhibitor methionine sulfoximine (23) was tested in cancer patients in the early 1960s (16). While no responses were produced in several visceral tumors, prominent dose-dependent central nervous system toxicity was noted. This compound had significant effects against TE-671, a line with high glutamine synthetase activity. The relevant pharmacokinetic parameters in humans have not yet been determined.

The heterogeneity of primary central nervous system tumors implies the need for combination therapy (3). An antimetabolite regimen based on glutamine represents a new approach to the chemotherapy of brain neoplasms that may complement the sensitivity, kinetic, and resistance profiles of the current alkylating
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agents. Since these analogues are not lipid soluble, they also offer the opportunity to better assess the importance of the blood-brain barrier in treatment failure (35). Studies with these agents against human glioma and medulloblastoma tumor lines in athymic mice currently are under way prior to consideration of clinical trials.

REFERENCES


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