Purine Metabolism of Human Glioblastoma in Vivo

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ABSTRACT

The aim of this study was to identify targets for rational chemotherapy of glioblastoma. In order to elucidate differences in the biochemistry of tumor and normal human brain, in vivo pool sizes of purine nucleotides, nucleosides, and nucleobases and of purine metabolizing enzymes in biopsy material from 14 grade IV astrocytomas and 4 normal temporal lobe samples were analyzed. Specimens were collected during surgery using the freeze-clamp sampling technique and analyzed by high pressure liquid chromatography. Total purine nucleotides, adenylates, and guanylates in the tumors were 2186, 1865, and 310 nmol/g (wet weight), respectively, which corresponds to 61, 60, and 71% of normal brain tissue concentrations. Relative to normal brain the tumors had significantly lower ATP and GTP levels, essentially normal pool sizes of purine nucleosides and bases, unchanged activities of the salvage enzymes hypoxanthine-guanine phosphoribosyltransferase, adenosine phosphoribosyltransferase, and adenine kinase (659, 456, and 98 nmol/h/mg protein, respectively) and 4-fold higher activities of IMP dehydrogenase (11.6 nmol/h/mg protein); the latter is the rate limiting enzyme for guanylate de novo synthesis. IMP pools in the tumors were 60% of values obtained from 2 additional patients revealed that significant amounts of the active metabolite thiazole-4-carboxamide appear to be a rational therapeutic approach. Preliminary in vivo experiments with normal and malignant tissue specimens from 2 additional patients showed that significant amounts of the active metabolite thiazole-4-carboxamide adenine dinucleotide are formed from tiazofurin. At a concentration of 200 μM this drug was able to deplete guanylate pools in the tumors to a median of 54% of phosphate buffered saline treated controls. Flux studies with [14C]formate showed that tiazofurin strongly inhibited de novo synthesis of guanylates in glioblastoma to an average of 10% of controls. This effect was more pronounced in the tumors as compared to normal brain. No inhibition of salvage of [14C]guanine by tiazofurin could be observed in normal and malignant tissues. Supportive measures have to be considered to inhibit the highly active salvage enzyme hypoxanthine-guanine phosphoribosyltransferase that can partly antagonize a tiazofurin to be considered to inhibit the highly active salvage enzyme hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, and adenosine kinase (659, 456, and 98 nmol/h/mg protein, respectively) and 4-fold higher activities of IMP dehydrogenase (11.6 nmol/h/mg protein); the latter is the rate limiting enzyme for guanylate de novo synthesis. IMP pools in the tumors were 60% of values obtained from 2 additional patients revealed that significant amounts of the active metabolite thiazole-4-carboxamide appear to be a rational therapeutic approach. Preliminary in vivo experiments with normal and malignant tissue specimens from 2 additional patients showed that significant amounts of the active metabolite thiazole-4-carboxamide adenine dinucleotide are formed from tiazofurin. 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(3, 4) have performed in vitro biochemical studies with brain tumor cell lines and advocated chemotherapeutic treatment with anticytosine agents. The purpose of our study with human brain tumors in vivo was to elucidate differences in purine metabolism in grade IV astrocytomas and relevant normal tissue as a basis for better design of chemotherapy. With the aid of the molecular correlation concept Weber (5) during the past years has identified sensitive targets in purine and pyrimidine metabolism for chemotherapeutic treatment of malignant tumors. To our knowledge no data on nucleic acid precursor metabolism are available either on human brain tumors, other solid human tumors, or mouse and rat brain tumors. Brain tumors are representative for studying purine metabolism. In vivo pool sizes of nucleotides, nucleosides, and nucleobases can be measured in biopsies before surgical removal of tissue and without the perturbing effects of ischemia caused by ligation of blood vessels. Based on knowledge about salvage and key enzyme activities, rational treatment strategies with tumor selective agents can be developed. Enzyme directed chemotherapy has already yielded promising clinical results in the treatment of hematological malignancies in humans (6, 7). Better insight into strategic differences between metabolism of glioblastomas and normal tissue should also aid in improving current chemotherapeutic results for patients suffering from these malignancies.

INTRODUCTION

The prognosis for patients with anaplastic gliomas remains poor despite efforts to combine radiotherapy and chemotherapy with surgical intervention. The median survival of adults with these tumors increased from 14 to 36 weeks when radiation therapy was administered following surgical resection. Among the few active agents crossing the blood-brain barrier the nitrosoureas, especially carmustine, have brought therapeutic improvement. Inclusion of carmustine into treatment regimens has increased the proportion of survivors after 18 months from 4 to 19% (1). However, the benefit conferred by the addition of chemotherapy was slight, leading this group to conclude that "it is best to use radiotherapy in the post surgical treatment of malignant glioma and to continue the search for an effective chemotherapeutic regimen" (1). Among the other drugs thought to be effective are antimetabolites such as methotrexate, 1-β-D-arabinofuranosylcytosine, 5-fluorouracil, and the alkylating agents cisplatin and teniposide. However, no mono- or combination therapy has been clearly shown to be superior to carmustine (1). Recently Drono et al. (3, 4) have performed in vitro biochemical studies with brain tumor cell lines and advocated chemotherapeutic treatment with anticytosine agents. The purpose of our study with human brain tumors in vivo was to elucidate differences in purine metabolism in grade IV astrocytomas and relevant normal tissue as a basis for better design of chemotherapy. With the aid of the molecular correlation concept Weber (5) during the past years has identified sensitive targets in purine and pyrimidine metabolism for chemotherapeutic treatment of malignant tumors. To our knowledge no data on nucleic acid precursor metabolism are available either on human brain tumors, other solid human tumors, or mouse and rat brain tumors. Brain tumors are representative for studying purine metabolism. In vivo pool sizes of nucleotides, nucleosides, and nucleobases can be measured in biopsies before surgical removal of tissue and without the perturbing effects of ischemia caused by ligation of blood vessels. Based on knowledge about salvage and key enzyme activities, rational treatment strategies with tumor selective agents can be developed. Enzyme directed chemotherapy has already yielded promising clinical results in the treatment of hematological malignancies in humans (6, 7). Better insight into strategic differences between metabolism of glioblastomas and normal tissue should also aid in improving current chemotherapeutic results for patients suffering from these malignancies.

MATERIALS AND METHODS

Patient Material. Glioblastoma tissue (9 males, 5 females; median age, 57 ± 17 (SD) years) was obtained during surgery at the University of Innsbruck. In 4 cases the whole temporal lobe had to be removed during brain tumor resection due to tumor infiltration, and macroscopically and histologically normal portions of this region served as control. Additionally glioblastoma and normal temporal lobe brain tissue was obtained from 2 females (patients 15 and 16, ages 53 and 62 years, respectively) for ex vivo evaluation of biochemical response to treatment with an IMP dehydrogenase inhibitor.

Materials. Tiazofurin (2-β-D-ribofuranosylthiazolo-4-carboxamide), NSC 286193, was obtained from Dr. H. N. Jayaram, Laboratory for Experimental Oncology, Indianapolis, IN. [8-14C]Tiazofurin (specific radioactivity, 27.0 mCi/mmol) was provided by Dr. Ven Narayanan, National Cancer Institute, Bethesda, MD. [14C]Formic acid (specific activity, 58 mCi/mmol) and [8-14C]guanine (specific activity, 52.6 mCi/mmol) were purchased from New England Nuclear, Vienna, Austria. All other chemicals were of the highest purity available.

Sampling Technique. Biopsy specimens (0.1 to 2.0 g) were obtained by using a rapid freeze-clamping procedure, ground in a mortar, and stored in liquid nitrogen. This method proved to be necessary to measure accurate in vivo concentrations of nucleotides, nucleosides, and bases. For in vitro biochemical studies 5 g of each normal and malignant brain tissue were obtained during surgery and transferred immediately into tissue culture flasks containing 20 ml of RPMI 1640...
supplemented with glutamine (2 mm) and 10% fetal calf serum.

Ribonucleotides, Nucleosides, and Nucleobases. Frozen tissue powder was weighed and immediately extracted with ice cold 10% trichloroacetic acid (1:4, w/v). After centrifugation at 4°C and at 12,000 g for 2 min the acid soluble extracts were neutralized immediately with a mixture of tri-n-octylamine in Freon (1:2, v/v), according to the method of Khyrm (8). Aliquots of the neutralized extracts were analyzed on a Beckman System Gold high pressure liquid chromatography unit using techniques described earlier (9–11).

Enzyme Assays. Radiochemical assays were performed in 100,000 × g supernatants of frozen tissue dissolved in extraction buffer, according to published methods (12–15). Hypoxanthine was used as a substrate for measuring hypoxanthine-guanine phosphoribosyltransferase activity. All assay conditions were optimized for glioblastoma and normal brain tissue with respect to pH, concentrations of the substrates, cofactors, and the amount of enzyme protein according to published methods. Assays were performed in duplicate and SDs were less than 5%. Enzyme activities were linear over 30 min. Protein concentration was measured with the Bio-Rad assay kit according to the method of Bradford (16).

Measurement of IMP Dehydrogenase. Activity was measured according to the method of Holmes et al. (14). Briefly, the incubation mixture contained 50 mM potassium phosphate buffer (pH 7.4), 100 mM potassium chloride, 1 mM EDTA, 0.3 mM NAD* (Bohringer Mannheim, Mannheim, Federal Republic of Germany), 5 mM NaF, [8-3H]IMP (56 Ci/mmol, 50 μCi/ml; Amersham International, Amersham, United Kingdom) at a final concentration of 119 μM and 0.15–0.20 mg of protein in a total volume of 60 μl. The reaction was terminated by spotting 2-μl aliquots onto polyethyleneimine cellulose plates (Merck, Darmstadt, Federal Republic of Germany) and IMP substrate and XMP product were separated by 10% KH2PO4 (w/v) in water with IMP and XMP as standards. Spots were visualized under UV light (254 nm) and cut out, and radioactivity was desorbed from plates by 1 N HCl:methanol (1:1, v/v) for 30 min and counted in a Packard CA 2000 liquid scintillation counter.

In Vitro Studies. Surgical specimens were immediately transferred to the laboratory, weighed, dissected into 250-mg portions, and finely minced with scissors in Petri dishes containing 10 ml of RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf serum. Tissue slices were then incubated for 30 min in a humidified atmosphere of 95% air-5% CO2 at 37°C. Subsequently samples were incubated further with unlabeled or radiolabeled [2-3H]thiazofurin (200 μM, 5.4 μCi/ml) for 2 h. To examine the flux of precursors through the purine biosynthetic pathway, 5 μCi of [14C]formate or [8-3H]guanine at a final concentration of 10 μM were added for the last 30 min of the incubation period. Tissue pieces were then quickly centrifuged, washed twice with cold PBS, extracted with cold 10% trichloroacetic acid, and quickly neutralized with tri-n-octylamine/Freon as described above. Nucleotides and thiazofurin metabolites were analyzed by high pressure liquid chromatography methods as described above (11).

Statistical Analysis. Data were subjected to statistical analysis with the two tailed t-test for small samples. Normal distribution and variance of data were established by the Pearson correlation coefficient and F test to assure applicability of the t-test analysis. Data yielded probability values lower than 0.05 were considered as significantly different.

RESULTS

Patient Material. Histological grading of the 16 tumor specimens determined that they were grade IV astrocytomas.

Purine Nucleotides, Nucleosides, and Nucleobases. In preliminary time course experiments with ischemic rat brain (definitive lesion) sampling conditions during surgery were simulated and the freeze-clamp sampling procedure was established to measure correct in vivo amounts of ribonucleotides, nucleosides, and nucleobases in brain tissue (data not shown). Concentrations of total purine nucleotides were significantly lower in human glioblastoma (2186 ± 119 nmol/g, 61%) when compared to normal brain tissue (3580.4 ± 279 nmol/g). Concentrations of adenylates and guanylates were both significantly lower in the tumors (60 and 71%, respectively) as compared to normal brain tissues (Table 1). Pool sizes of ADP and ATP in glioblastomas were 67 and 53% of normal brain (528 ± 48 and 1105 ± 78 nmol/g, respectively). AMP pools were not significantly different (241 ± 54 nmol/g in the tumor). Concentrations of guanine nucleoside diphosphates and nucleoside triphosphates were also lower in tumors (74 ± 13 and 226 ± 22 nmol/g, respectively); GMP pools were not significantly different (22 ± 6 nmol/g in the tumor). Purine nucleoside and base concentrations in tumors and normal tissue were in the low micromolar range. No significant differences between pool sizes were seen between tumor and normal tissue (Table 1). Concentrations of deoxyadenosine, deoxyguanosine, deoxyinosine, and xanthosine were below the limit of detection (100 pmol/g).

Purine Enzymes. Among the salvage enzyme activities in the tumors hypoxanthine-guanine phosphoribosyltransferase was 659 ± 359 nmol/h/mg protein (102% of normal brain activity) and adenosine phosphoribosyltransferase was 456 ± 39 nmol/h/mg protein (94% of normal brain activity). Adenosine kinase activity in the tumors was 98 ± 15 nmol/h/mg protein (118% of normal brain activity). However, IMP dehydrogenase activity in the tumor was significantly higher than in normal brain tissue (11.6 ± 1.9 nmol/h/mg protein, 414% of normal) (Table 2).

Table 1 Purine nucleotides, nucleosides, and bases in normal human brain and human glioblastomas

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normal brain</th>
<th>Glioblastoma</th>
<th>% of normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>242.3 ± 19.8</td>
<td>241.2 ± 53.6</td>
<td>99</td>
</tr>
<tr>
<td>ADP</td>
<td>788.4 ± 41.0</td>
<td>527.7 ± 48.5</td>
<td>66</td>
</tr>
<tr>
<td>ATP</td>
<td>2099.8 ± 118.8</td>
<td>1105.3 ± 77.7</td>
<td>53*</td>
</tr>
<tr>
<td>Total adenylates</td>
<td>3120.5 ± 245.2</td>
<td>1865.4 ± 95.4</td>
<td>60*</td>
</tr>
<tr>
<td>GMP</td>
<td>25.9 ± 2.8</td>
<td>21.9 ± 6.1</td>
<td>85</td>
</tr>
<tr>
<td>GDP</td>
<td>1690.0 ± 8.0</td>
<td>74.1 ± 12.7</td>
<td>44*</td>
</tr>
<tr>
<td>GTP</td>
<td>299.9 ± 16.6</td>
<td>225.7 ± 21.7</td>
<td>75*</td>
</tr>
<tr>
<td>Total guanylates</td>
<td>4358.5 ± 32.7</td>
<td>310.4 ± 35.6</td>
<td>71*</td>
</tr>
<tr>
<td>IMP</td>
<td>17.2 ± 2.4</td>
<td>11.1 ± 2.8</td>
<td>64*</td>
</tr>
<tr>
<td>Adenine</td>
<td>4.5 ± 1.8</td>
<td>5.0 ± 0.6</td>
<td>111</td>
</tr>
<tr>
<td>Adenosine</td>
<td>23.3 ± 3.0</td>
<td>26.3 ± 5.4</td>
<td>113</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>150</td>
</tr>
<tr>
<td>Guanosine</td>
<td>5.3 ± 0.2</td>
<td>6.2 ± 1.4</td>
<td>117</td>
</tr>
<tr>
<td>Inosine</td>
<td>27.5 ± 5.4</td>
<td>30.9 ± 7.4</td>
<td>112</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>32.6 ± 6.5</td>
<td>30.4 ± 7.4</td>
<td>93</td>
</tr>
<tr>
<td>Xanthine</td>
<td>34.2 ± 4.7</td>
<td>32.6 ± 6.9</td>
<td>95</td>
</tr>
</tbody>
</table>

* Significantly different from normal brain (P < 0.05).

Table 2 Purine enzymes in normal human brain and human glioblastoma

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal brain</th>
<th>Glioblastoma</th>
<th>% of normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine-guanine</td>
<td>648 ± 124</td>
<td>659 ± 359</td>
<td>13 102</td>
</tr>
<tr>
<td>phosphoribosyltransferase</td>
<td>485 ± 28</td>
<td>456 ± 39</td>
<td>7 94</td>
</tr>
<tr>
<td>Adenine phosphoribosyl</td>
<td>83 ± 9</td>
<td>98 ± 15</td>
<td>7 94</td>
</tr>
<tr>
<td>transferase</td>
<td>2.8 ± 1.3</td>
<td>11.6 ± 1.9</td>
<td>4 414*</td>
</tr>
</tbody>
</table>

* Significantly different from normal brain (P < 0.05).
toma tissue from 2 patients was incubated for 2 h with tiazofurin (100 μM) but no significant depletion of guanylate pools could be observed at this concentration compared to PBS treated tumor control tissue. Therefore normal and malignant ex vivo biopsy material of patients 15 and 16 with glioblastoma infiltrating the temporal lobe was incubated for 2 h with cold or [2-14C]tiazofurin at 200 μM and the effects on nucleotide pools, [14C]formate incorporation (de novo pathway), [8-14C]guanine incorporation (salvage pathway), and formation of the active metabolite of the drug TAD were examined. ATP/AMP ratios were 23.4 and 17.8 (mean values) in tumor and normal brain tissue slices, respectively. Tumors formed 2641 and 1696 pmol/g tissue of TAD compared to 496 and 470 pmol/g in normal brain (mean values of duplicate samples from patients 15 and 16, respectively). Guanylate pools in the tumors were significantly depleted to 45 and 63% of PBS treated controls compared to 73 and 91% in normal brain tissue. After tiazofurin treatment, de novo synthesis of guanylates (incorporation of radiolabeled formate) was curtailed significantly to 11.0 and 16.2% in glioblastomas and to 27.0 and 41.2% in normal brain compared to PBS treated controls. However, no expansion of the IMP pool size or increased incorporation of radioactivity from [14C]formate into IMP could be detected after inhibition of IMP dehydrogenase with tiazofurin. Salvage of [8-14C]guanine could not be inhibited by tiazofurin in both tissues.

DISCUSSION

The elucidation of purine and pyrimidine metabolic pathways in human brain tumors as well as differences between normal and neoplastic brain tissue might represent a first step in identifying potential targets for an enzyme directed chemotherapy. Biochemical differences between tumor and normal brain tissue should help in identifying strategic targets and might thus provide the basis for rational tumor chemotherapy. The only system in which sufficient information about differences between normal and neoplastic tissue is presently available is the rat hepatoma-normal rat liver model (5). The pattern of purine enzyme activities and tissue concentrations of their substrates in glioblastoma and normal human brain shows similarities with those of rapidly growing hepatoma 3924A and normal liver in the rat model (5, 9, 17). In glioblastoma total adenylate and guanylate pools are lower than in normal human brain. The same relationship has also been found between hepatoma 3924A and normal rat liver (17). Similarly GTP pools in leukemic cells from bone marrow aspirates of leukemic patients were significantly different from normal bone marrow cells of healthy volunteers (18). IMP dehydrogenase activity in glioblastoma was 4-fold higher than in normal brain tissue. The activities of purine salvage enzymes hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase were similar in glioblastoma and normal brain tissue. Salvage enzyme activities in tumors were found to be 57- and 39-fold higher than IMP dehydrogenase activity. Also in rat liver, in rat hepatomas of different growth rates, and in primary colon carcinoma in humans, activities of purine salvage enzymes were orders of magnitude higher than those of the rate limiting enzymes of de novo biosynthesis (19-21). Adenosine kinase activities in glioblastomas and normal brain tissues were not significantly different. Pool sizes of nucleosides and bases were essentially identical in both normal and neoplastic tissue, which is in agreement with similar activities of salvage enzymes and adenosine kinase.

Hypoxanthine and guanosine levels in glioblastoma are higher than concentrations measured in rat hepatoma 3924A (9). Efficient salvage of these metabolites might allow one to circumvent cytotoxic effects of purine de novo synthesis inhibitors such as the antiglutamine agents azaserine, 6-diazo-5-oxo-L-norleucine, or acicin by repleting purine nucleotide pools.

Weber et al. (22) first advocated the use of IMP dehydrogenase inhibitors for the treatment of cancer. With the IMP dehydrogenase inhibitor tiazofurin Weber et al. (23) succeeded for the first time in suppressing growth of s.c. transplanted hepatoma 3924A in rats. With this approach a high rate of complete remissions are being achieved in a current phase I/II study in patients suffering from chronic granulocytic leukemia in blast crisis (7).

To elucidate potentially favorable therapeutic effects of tiazofurin and to test the hypothesis that inhibition of IMP dehydrogenase should also have a biochemical impact in glioblastoma we have performed preliminary experiments with normal and malignant ex vivo biopsy material from two patients suffering from this tumor. Following tiazofurin treatment depletion of guanylate pools was more pronounced in glioblastoma than in normal brain. Also de novo guanylate synthesis measured as incorporation of [14C]formate into nucleotides was inhibited to a greater extent in glioblastoma than in normal brain. The same effects, although slightly more pronounced, have been observed in leukemic cells of acute nonlymphocytic leukemia patients and in normal WBC of healthy volunteers treated with tiazofurin in vitro in a previous study (18). Glioblastoma tissue formed less of the active metabolite TAD than normal brain tissue to inhibit guanine salvage and thus improve the one can take advantage of high basal levels of hypoxanthine in glioblastoma and further investigations should reveal whether inhibition of IMP dehydrogenase with tiazofurin could not be inhibited by tiazofurin in both tissues.
therapeutic potential of tiazofurin. In addition the effects of other IMP dehydrogenase inhibitors such as 3-deazaguanine will be investigated.

Our data indicate that inhibition of IMP dehydrogenase in glioblastoma might confer favorable biochemical effects. Results of this study justify further pursuit of this therapeutic approach which is already used successfully in patients with hematological malignancies in order to overcome resistance to chemotherapy in human brain tumors.

REFERENCES

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