Enolase isoenzymes in human gliomas

ERNST M. H. VAN DEN DOEL, M.D., GERT RIJKSEN, PH.D., PAUL J. M. ROHOLL, PH.D.,
CEES W. M. VAN VEELEN, M.D., AND GERARD E. J. STAAL, PH.D.

Department of Clinical Neurophysiology, Neurological Clinic, University Hospital and Department of
Hematology, Division of Medical Enzymology, Institute for Pathology, and Department of
Neurosurgery, University Hospital, Utrecht, The Netherlands

Gamma-enolase (one of the three possible subunits of the dimeric enzyme enolase (EC 4.2.1.11)) has been
reported as a marker for human neurons. Studies investigating the presence of γ-enolase in human gliomas
have given conflicting results, but a definite finding is important for further studies of the biology of these
tumors and the possible use of γ-enolase as a marker for tumors originating in nervous tissue or for neuronal
damage. Using electrophoresis of tumor tissue extracts as well as immunohistochemistry the authors have
demonstrated the presence of γ-enolase in human gliomas. Analysis of the γ-enolase content in the plasma of
patients with brain neoplasms further revealed that, although this enzyme may be present in the tumor itself,
its concentration in blood is not a reliable marker for a tumor of the human central nervous system.

KEY WORDS □9 brain neoplasm □9 enolase □9 immunohistochemistry □9 tumor marker □9 glioma

Enolase (2-phospho-D-glycerate hydrdrolase, EC 4.2.1.11) catalyzes the conversion of 2-phospho-D-
glycerate to phosphoenolpyruvate in the glycolytic pathway. It is a dimeric enzyme of about 90,000-
dalton molecular weight; three subunits have been recognized and designated α, β, and γ. Of the six
possible combinations of two subunits, only αα, ββ, αβ, αγ, and γγ have been found in human tissues; βγ has
not been demonstrated.

In human tissues, the αα-isoenzyme is most frequently encountered; however, as ββ is the predominant
enzyme in muscle, quantitatively this is the most common form. The γ chain of enolase has been reported
to occur exclusively in neurons and cells of the amine precursor uptake and decarboxylation (APUD) system,
where it was originally described as a 14-3-2 protein. Substantial amounts of αγ enolase can be
found in erythrocytes and blood platelets. As most studies of enolase isoenzymes have been conducted
with immunological methods, protein reacting with anti-γ antibody has been designated "neuron-specific
enolase (NSE);" however, cross-reactivity with the αγ isoenzyme is often seen and this is not neuron-specific.
In our immunological studies, we therefore prefer the term "γ-enolase."

Human glial cells have been found to contain principally α-enolase and no γ-enolase; this finding has led
to numerous studies into the value of γ-enolase as a marker for neuronal tissue in tumors of the nervous
and APUD systems. Among the "APUD-omas," γ-enolase activity could be demonstrated in neuroblastomas,
melanomas, retinoblastomas, pheochromocytomas, medullary thyroid carcinomas, oat-cell tumors of
the lung, non-functioning islet-cell carcinomas of the pancreas, and carcinomas of the gut.

Studies, mainly immunohistochemical, of human gliomas have given conflicting results regarding the
presence of γ-enolase in the tumor tissue. Taking histopathological structure and the varying biological
behavior of human gliomas into consideration, γ-enolase could be expected in at least some of these
tumors. In addition, cultured glioma cells have been found to contain γ-enolase.

To clarify these various findings, we studied human gliomas with a combination of electrophoresis (which
gives quantitative results and discriminates between γ- and αγ-enolases) and immunohistochemistry (to avoid
the pitfall of varying specificity and sensitivity of the antibodies used). The results prompted us to evaluate
the significance of the γ-enolase level in plasma as a marker for brain neoplasms, because elevated serum
levels have been reported in patients severely ill with neuroblastomas and several other tumors of the APUD
system. The γ-enolase content of gliomas
being known, this investigation would give further insight into the mechanism of elevated serum γ-enolase levels in tumors of various kinds.

**Materials and Methods**

**Materials and Sources of Tissue**

The auxiliary enzymes, nucleotides, and substrates used in this study were all of analytical grade.* Normal brain tissue was obtained from four autopsies within 12 hours after death, from anamnestically and macroscopically normal brains. Specimens were taken from the pre-rolandic motor area and separated under moderate magnification (× 10) into white and gray matter. Tumor tissue was obtained from the apparent center of the tumor in patients undergoing neurosurgical procedures. In addition, twin biopsies were taken from a limited number of tumors: they consisted of two identical biopsies as near to each other as possible, which were then analyzed separately, one in the enzymology division and the other in the pathology department. All measurements were repeated four times.

**Preparation of Tissue Extracts**

Tumors were dissected free from necrotic areas and hemorrhages. Tissues were homogenized in three volumes of enolase extraction buffer, pH 8.0, containing 50 mM Tris-HCl buffer, 100 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, and 100 mM sucrose. Cell debris and particulate constituents were removed by centrifugation for 10 minutes at 48,000 G. All experiments were performed at 4°C. Protein content was measured according to the method of Lowry, et al.¹⁹

**Plasma Samples**

Blood from tumor patients and from 30 healthy controls was collected in standard 2-ml Vacutainer tubes containing 0.2 ml Na-heparin and was immediately processed by centrifugation for 10 minutes at 10,000 G. The plasma was stored at −30°C until analysis. The plasma was tested because hemolysis might influence γ-enolase levels.² Plasma of all patients referred to the neurosurgery department of the State University Hospital, Utrecht, for operation on a brain tumor during a 6-month period was analyzed. For technical reasons (such as a quick return of the patient to the referring hospital) only a limited number of patients could be examined on the 4th postoperative day. All surgical specimens were analyzed in the pathology department, and Kernohan's tumor staging³ was applied. Plasma γ-enolase measurements were made using a recently introduced radioimmunoassay,** as described by Cooper, et al.⁸ Normal values were 1.6 to 16.0 ng enolase/ml of plasma.

**Enolase Activity**

Enolase activity was measured in 100 mM Tris-HCl buffer, pH 8.0, containing 10 mM MgCl₂, 10 mM KCl, 0.5 mM ethylenediaminetetra-acetic acid (EDTA), 1.5 mM adenosine diphosphate (ADP), 0.2 mM nicotinamide adenine dinucleotide, reduced (NADH), as well as pyruvate kinase 0.5 U/liter, and lactate dehydrogenase 1.0 U/ml. The reaction was started with the substrate glycero-2-phosphate (1 mM) and was measured in a Kontron Uvikon 610 CL spectrophotometer.† One unit of enolase activity is defined as the amount of enzyme that converts 1 μmol of substrate/min at 37°C.

**Pyruvate Kinase Assay and Alanine Inhibition**

Pyruvate kinase activity was measured in the coupled lactate dehydrogenase assay as described by Bücher and Pfeiderer.² The percentage of inhibition of pyruvate kinase by alanine was measured as described earlier.³⁷ ³⁸

The 100% value is the activity in the absence of alanine.

**Cellulose Acetate Electrophoresis**

Enolase isoenzymes were separated on cellulose acetate strips in 40 mM of Na-phosphate buffer, pH 7.0. Samples containing 1.5 to 2 mU of enolase activity were used. Electrophoresis was carried out at room temperature for 45 minutes at 160 V. Enolase activity was detected by incubating the gels in the dark at 37°C in a solution containing 100 mM Tris-HCl buffer, 10 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, 1 mM glycero-2-phosphate, 1.5 mM ADP, 20 mM adenosine monophosphate (AMP), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADP), 1 mM glucose, 0.5 mM 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide, and 0.17 mM phenazine methosulfate, as well as pyruvate kinase 0.5 U/ml, hexokinase 0.14 U/ml, and glucose-6-phosphate dehydrogenase 0.07 U/ml. Controls included incubation of gels in the absence of glycero-2-phosphate and specific inhibition of enolase by potassium fluoride. Isoenzymes were quantified by densitographic analysis of the gels at 550 nm in a Beckman CDS 200 densitometer.‡

**Purification of αα- and γγ-Enolases**

Human αα- and γγ-enolase were purified by a combination of the methods of Fletcher, et al.,¹¹ and Shimizu, et al.,¹³ with some modifications. In brief, frozen human brain tissue obtained within 12 hours postmortem was homogenized in three volumes of 15 mM Na-phosphate buffer, pH 6.9, containing 5 mM MgSO₄ and 0.1 mM EDTA. After centrifugation for 20 minutes at 20,000 G, the supernatant was applied to a CM-

---

* Enzymes, nucleotides, and substrates obtained from Boehringer, Mannheim, Federal Republic of Germany; ion-exchange matrices from Pharmacia, Uppsala, Sweden; and cellulose-acetate gels from Cellogel, Milan, Italy.

** NSE-RIA test kit manufactured by Pharmacia, Uppsala, Sweden.

† Kontron Uvikon 610 CL spectrophotometer manufactured by Kontron AG, Zürich, Switzerland.

‡ Densitometer, Model CDS 200, manufactured by Beckman Instruments Inc., Fullerton, California.

---

E. M. H. van den Doel, et al.

J. Neurosurg. / Volume 65 / September, 1986
Enolase isoenzymes in human gliomas

Sephadex A50 column equilibrated in the same buffer. Enolase activity passed through the column without binding and the sample was fractionated consecutively by (NH₄)SO₄ precipitation (40% to 80% saturation). The precipitate containing enolase activity was dissolved in 15 mM Na-phosphate buffer, pH 7.9, supplemented with 5 mM MgSO₄ and 0.1 mM EDTA (referred to as "buffer 7.9") and extensively dialyzed against the same buffer. This fraction was subjected to ion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A50 in buffer 7.9. The αα-enzyme ran through the column, and αγ- and γγ-enzymes were eluted by applying a linear gradient of 0.1 to 0.7 M NaCl in buffer 7.9. The αγ fraction was dissociated and recombined to form αα- and γγ-isoenzymes by using a chromatofocusing column (PBE 94), I essentially as described by Shimizu, et al.33 The αα and γγ fractions obtained from the DEAE A50 column were combined and dissociated by adjusting the pH to 2.8 with citric acid; recombination to form αα-enolase (25%), αγ-enolase (50%), and γγ-enolase (25%) was performed by readjusting the pH to 7.9 by adding solid Tris. The αγ fraction of this dissociation-reassociation experiment was isolated by DEAE A50 chromatography and was used in the chromatofocusing experiment.

The αα and γγ fractions obtained after the PBE column were dialyzed against 20 mM Tris-HCl buffer, pH 7.9, 5 mM MgSO₄, and 0.1 mM EDTA and were further purified by fast protein liquid chromatography (FPLC) on a Mono Q column equilibrated in the same buffer.* The αα-enzyme was not retained in these conditions, while the γγ-enolase had to be eluted by applying a 0.0- to 1.0-NaCl linear gradient. The purified enzymes appeared to be completely homogeneous upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Preparation of Antibodies

Antibodies against purified αα- and γγ-enolases were raised in sheep: 500 to 1000 µg of antigen in 200 µl of phosphate-buffered saline (PBS) emulsified with 200 µl Freund's complete adjuvant was injected subcutaneously at multiple sites at the back of the animal. After 2 and 4 weeks, a booster injection was given intramuscularly with the same amount of antigen in Freund's incomplete adjuvant. The sheep were bled after 6 weeks. The antisera were specific for the respective antigens as judged by immunodiffusion, immunoelectrophoresis, and immunoprecipitation studies. The anti-γγ antibody reacted with the αγ-enolase as well; however, no cross-reactivity was observed with the anti-αα antibodies and the αγγ-hybrid.

Preparation of Enzyme Standards

Enolase isoenzymes in normal brain (electrophoresis)*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enolase Activity (U/mg protein)</th>
<th>% Total Enolase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αα-Enolase</td>
<td>αγ-Enolase</td>
</tr>
<tr>
<td>gray matter</td>
<td>1.50 ± 0.1</td>
<td>37 ± 1.4</td>
</tr>
<tr>
<td>white matter</td>
<td>1.28 ± 0.6</td>
<td>44 ± 4.3</td>
</tr>
</tbody>
</table>

* Mean values ± standard deviation in four measurements.

The anti-αα and anti-γγ antibodies were purified by precipitation with αα and γγ fractions, respectively, obtained after DEAE A50 chromatography (see above). After centrifugation for 20 minutes at 48,000 G, the antigen-antibody complex was dissociated in glycine-citric acid buffer (0.133 M glycine, 0.104 M citric acid, 0.514 M NaCl) and the components were separated by gel filtration on a S-200 column† in the same buffer. After gel filtration, the immunoglobulin(Ig) G fraction was dialyzed against PBS containing 0.2% NaN₃.

Immunohistochemistry

The avidin-biotin-peroxidase complex method was used for immunohistochemistry, according to the technique of Hsu, et al.34 (described in detail by de Jong, et al.9). After dewaxing, the sections were incubated for 1 hour with 10% (v/v) normal rabbit serum or 10% normal goat serum in 0.01% PBS, pH 7.6, to reduce background staining. Sections were successively incubated with rabbit or sheep anti-γ antibody or with sheep anti-α antibody, diluted 1:1000 to 1:5000 in 10% (v/v) normal rabbit serum overnight at 4°C, with biotinylated rabbit anti-sheep IgG (dilution 1:250 in 1% (v/v) normal goat serum) for 30 minutes at room temperature, and with avidin-biotin-peroxidase complex‡ (dilution 1:125 in 1% (v/v) normal rabbit or goat serum) for 1 hour at room temperature. The incubation steps were followed by washes in PBS. Peroxidase activity was visualized by developing in a freshly made solution of benzidine (0.5 mg/ml), 0.1 M imidazole, and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6) in the dark for 1 hour at room temperature.

The antibodies used were our own purified sheep anti-α and anti-γ enolase as well as the rabbit anti-γ enolase developed by Taylor, et al.,36 and kindly donated by Dr. Taylor.

Results

Electrophoresis

Normal Brain. Table 1 gives the isozymic composition of enolase in normal white and gray matter. Both

---

§ CM-Sephadex A50 manufactured by Pharmacia, Uppsala, Sweden.
† Chromatofocusing column, Model PBE 94, manufactured by Pharmacia, Uppsala, Sweden.
‡ FPLC and Mono Q manufactured by Pharmacia, Uppsala, Sweden.
* Mean values ± standard deviation in four measurements.
† Sephacryl S-200 manufactured by Pharmacia, Uppsala, Sweden.
‡ Dilution with avidin-biotin-peroxidase complex according to the instructions of Vector Laboratories, Burlingame, California.

J. Neurosurg. / Volume 65 / September, 1986 347
TABLE 2

Electrophoresis of enolase in astrocytomas*

<table>
<thead>
<tr>
<th>Tumor Pathology</th>
<th>No. of Cases</th>
<th>Enolase Activity (U/mg protein)</th>
<th>$%$ Total Enolase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>astrocytoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>grade I</td>
<td>1</td>
<td>0.57</td>
<td>50</td>
</tr>
<tr>
<td>grades II-III</td>
<td>3</td>
<td>0.59 ± 0.03 69 ± 5.6</td>
<td>22.3 ± 7.6 10.0 ± 6.2</td>
</tr>
<tr>
<td>grade III</td>
<td>8</td>
<td>0.43 ± 0.11 68.6 ± 6.1</td>
<td>21.6 ± 5.0 11.0 ± 5.8</td>
</tr>
<tr>
<td>grade IV</td>
<td>5</td>
<td>0.38 ± 0.20 65.3 ± 11.3</td>
<td>24.2 ± 9.8 10.2 ± 3.6</td>
</tr>
</tbody>
</table>

* Values are mean ± standard deviation.

\[ \gamma \gamma \text{-enolase and } \alpha \gamma \text{-enolase were present in considerable amounts. This is in accordance with the findings of Kato, et al.,}^{17} \text{ although the } \gamma \gamma \text{-enolase content seemed to be slightly higher than the } \alpha \gamma \text{ fraction in our study. In addition, the total enolase activity tended to be slightly lower in the white matter. This could be due to the fact that myelin makes up a part of white matter. The difference, however, is not significant (} p = 0.25)\text{. The enolase isoenzyme pattern is more or less the same in white and gray matter (Fig. 1).}

\[ \text{Gliomas. Table 2 summarizes the mean enolase pattern and total enolase activity of 17 astrocytomas of varying malignancy. In addition, enolase activity in two oligodendrogliomas, two ependymomas, and three cerebral metastases is given in Table 3. The varying morphology and heterogeneous histological structure of gliomas play an important role in producing large standard deviations, but a tendency toward lower total enolase activity can be seen with increasing malignancy. The most important finding, however, is the presence of considerable amounts of } \gamma \gamma \text{- and/or } \alpha \gamma \text{-enolase in all tumors examined. The } \gamma \gamma \text{ fraction is lower than in normal brain, but the } \alpha \gamma \text{ level is comparable; only in one ependymoma (Case 10, Table 3) was the } \alpha \gamma \text{ fraction elevated. The } \alpha \alpha \text{ fraction in gliomas, with the exception of the same ependymoma (Case 10), was higher than in normal brain; this could be expected theoretically, as these tumors derive from the } \alpha \alpha \text{-enolase containing glia.}

\[ \text{Immunohistochemistry}

\[ \text{Normal Brain. Our findings in immunohistochemical stainings of normal brain were similar to those reported by other authors.}^{21,27,32} \text{ There was positive staining for } \gamma \text{-enolase in neurons (which did not stain for } \alpha \text{-enolase) and positive staining for } \alpha \text{-enolase in astrocytes, oligodendrocytes, and ependymal cells (Fig. 2). In addition, erythrocytes stained positive for } \gamma \text{-enolase.}

\[ \text{Gliomas. Table 3 compares the enzymological and immunohistochemical analyses of 11 individual gliomas in adult patients. The clear presence of } \gamma \gamma \text{-enolase and } \alpha \gamma \text{-enolase was reflected by positive } \gamma \text{-enolase staining in all tumors examined. Theoretically, the presence of } \gamma \text{-enolase in the electrophoresis could be produced by the erythrocytes, which are present in nearly all gliomas, especially the more malignant ones. The immunohistochemical stains proved that this was not the case. Due to the varying histopathological structure of the tumors, cells were not always easy to identify, but generally the staining for } \gamma \text{-enolase was weak in cells that still could be recognized as neurons, and negative in cells that could be classified as normal glial cells. Some cells appeared as reactive astrocytes and stained positive. Most of the positive staining, however, was found in cells that were clearly part of the tumor and were glial in configuration (Fig. 2B). The necrotic parts, which could be observed in the more malignant tumors, also contained material that stained positive.}

\[ \text{Fig. 1. Electrophoresis examples of gray (left) and white (right) matter. From above: } \gamma \gamma, \alpha \gamma, \text{ and } \alpha \alpha \text{ fractions.}
Enolase isoenzymes in human gliomas

TABLE 3
Results of immunohistochemical staining using anti-\(\gamma\)-enolase antibody*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor Pathology</th>
<th>Activity (%)</th>
<th>% Total Enolase Activity on Electrophoresis</th>
<th>Staining</th>
<th>PK Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Activity (U/mg protein)</td>
<td>(\alpha)-Enolase</td>
<td>(\gamma)-Enolase</td>
<td>(\gamma)-Enolase</td>
</tr>
<tr>
<td>gliomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>astrocytoma grade I</td>
<td>0.57</td>
<td>50</td>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>astrocytoma grade II</td>
<td>0.60</td>
<td>68</td>
<td>29</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>astrocytoma grade III</td>
<td>0.39</td>
<td>67</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>astrocytoma grade III</td>
<td>0.46</td>
<td>77</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>astrocytoma grade III</td>
<td>0.44</td>
<td>61</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>astrocytoma grade IV</td>
<td>0.65</td>
<td>59</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>astrocytoma grade IV</td>
<td>0.77</td>
<td>77</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>oligodendroglioma grade II</td>
<td>0.77</td>
<td>75</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>oligodendroglioma grade III</td>
<td>0.12</td>
<td>46</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>ependymoma grade II</td>
<td>0.16</td>
<td>33</td>
<td>51</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>ependymoma with astrocytic differentiation</td>
<td>0.13</td>
<td>70</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>cerebral metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>carcinoma</td>
<td>0.22</td>
<td>69</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>adenocarcinoma</td>
<td>0.22</td>
<td>76</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>B-cell lymphoma</td>
<td>0.46</td>
<td>92</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

* All patients were older than 18 years.

Because consecutive brain tumors were biopsied, we were also able to examine a number of metastases by immunohistochemical staining (Fig. 2C and D). These metastases were nearly all positive, but showed varying intensities of staining (Table 3). This was true of metastases from adenocarcinomas, anaplastic carcinomas, and in one case of a B-cell lymphoma, possibly reflecting in the fact that \(\gamma\)-enolase can be produced in varying quantities by malignant cells. A number of tumors were also examined with the antibody developed by Taylor, et al., the same results were obtained, although the staining was weaker.

Alanine Inhibition of Pyruvate Kinase

The pyruvate kinase isoenzyme present in normal brain tissue was not inhibited by alanine. In gliomas with increasing malignancy, an isoenzyme transformation took place to a form that is inhibited by alanine, so that in the most malignant gliomas only a small percentage of pyruvate kinase remained active in the presence of alanine. This "pyruvate kinase residual activity" has been shown to correlate well with malignancy. As it was of utmost importance that enzymological results of the biopsy studies correlated well with the immunohistochemical findings, this measurement served as an additional control in the twin biopsies. The pyruvate kinase residual activity was expected to diminish with increasing malignancy, with a value generally below 15% in gliomas of grades III and IV. As can be seen in Table 3, the correlation was valid.

Plasma \(\gamma\)-Enolase Level in Patients

Table 4 shows the results of the \(\gamma\)-enolase values in the plasma of patients with cerebral neoplasms as determined 1 day preoperatively and on the 4th day

TABLE 4
Pre- and postoperative \(\gamma\)-enolase levels in plasma of patients with brain neoplasms

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor Pathology</th>
<th>Clinical Course (mos)</th>
<th>(\gamma)-Enolase (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Preop</td>
</tr>
<tr>
<td>1</td>
<td>astrocytoma grade I</td>
<td>&gt; 3</td>
<td>8.8</td>
</tr>
<tr>
<td>2</td>
<td>astrocytoma grade I</td>
<td>&gt; 3</td>
<td>9.8</td>
</tr>
<tr>
<td>3</td>
<td>astrocytoma grade I</td>
<td>&gt; 3</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>astrocytoma grade II</td>
<td>&gt; 3</td>
<td>6.6</td>
</tr>
<tr>
<td>5</td>
<td>oligodendroglioma grade II</td>
<td>&gt; 3</td>
<td>15.0</td>
</tr>
<tr>
<td>6</td>
<td>astrocytoma grade III</td>
<td>1~3</td>
<td>43.0</td>
</tr>
<tr>
<td>7</td>
<td>oligodendroglioma grade III</td>
<td>1~3</td>
<td>8.1</td>
</tr>
<tr>
<td>8</td>
<td>oligodendroglioma grade III</td>
<td>&gt; 3</td>
<td>15.5</td>
</tr>
<tr>
<td>9</td>
<td>astrocytoma grade III</td>
<td>&gt; 3</td>
<td>8.8</td>
</tr>
<tr>
<td>10</td>
<td>astrocytoma grade IV</td>
<td>&lt; 1~3</td>
<td>9.5</td>
</tr>
<tr>
<td>11</td>
<td>astrocytoma grade IV</td>
<td>&lt; 1~3</td>
<td>12.0</td>
</tr>
<tr>
<td>12</td>
<td>astrocytoma grade IV</td>
<td>1~3</td>
<td>9.8</td>
</tr>
<tr>
<td>13</td>
<td>astrocytoma grade IV</td>
<td>1~3</td>
<td>10.2</td>
</tr>
<tr>
<td>14</td>
<td>astrocytoma grade IV</td>
<td>&lt; 1~3</td>
<td>8.1</td>
</tr>
<tr>
<td>15</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>13.5</td>
</tr>
<tr>
<td>16</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>10.2</td>
</tr>
<tr>
<td>17</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>11.7</td>
</tr>
<tr>
<td>18</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>8.8</td>
</tr>
<tr>
<td>19</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>7.5</td>
</tr>
<tr>
<td>20</td>
<td>meningioma</td>
<td>&gt; 3</td>
<td>8.8</td>
</tr>
<tr>
<td>21</td>
<td>neurofibroma</td>
<td>&gt; 3</td>
<td>10.2</td>
</tr>
<tr>
<td>22</td>
<td>acoustic neuroma</td>
<td>&gt; 3</td>
<td>12.0</td>
</tr>
<tr>
<td>23</td>
<td>melanoma</td>
<td>1~3</td>
<td>10.4</td>
</tr>
<tr>
<td>24</td>
<td>carcinoma</td>
<td>&lt; 1~3</td>
<td>10.7</td>
</tr>
<tr>
<td>25</td>
<td>carcinoma</td>
<td>&lt; 1~3</td>
<td>24.0</td>
</tr>
<tr>
<td>26</td>
<td>adenocarcinoma</td>
<td>1~3</td>
<td>6.0</td>
</tr>
<tr>
<td>27</td>
<td>Grawitz tumor</td>
<td>1~3</td>
<td>10.4</td>
</tr>
<tr>
<td>28</td>
<td>carcinoma</td>
<td>1~3</td>
<td>16.0</td>
</tr>
</tbody>
</table>

* ND = not done.
FIG. 2. Examples of immunohistochemical staining for \( \gamma \)-enolase. \( \times \) 340. A: Normal tissue. Neurons can be seen to stain positive. Glial cells do not stain. B: Astrocytoma grade III. The cytoplasm of the tumor cells stains positive. C: B-cell lymphoma. Some neurons appear swollen but stain positive. The cytoplasm of some tumor cells can be seen to stain faintly, but are positive. D: Carcinoma. The cytoplasm of the tumor cells stains positive.

postoperatively. As can be seen, only two patients had clearly elevated levels of \( \gamma \)-enolase (Cases 6 and 25, Table 4), while one patient had a marginally elevated value (Case 8). One of these (Case 6) had an astrocytoma in the right frontal lobe. His symptoms started about 2 months before the operation and rapidly pro-
gressed over the last 3 weeks. At surgery, a tumor with a diameter of about 3 cm was found. In Case 25, a metastasis from a carcinoma was found deep in the left frontal lobe; symptoms began about 4 weeks before the operation. The third patient (Case 8) had an oligodendroglioma in the left frontal lobe; the course progressed
Enolase isoenzymes in human gliomas

slowly, with symptoms existing for 2 years. In all three patients, abnormal values receded to within normal ranges after the operation. Normal values were obtained in all other patients, even in those patients with a large highly malignant tumor showing a rapidly progressive course; for instance, in Case 24 only 14 days elapsed between the first symptoms and the operation, during which a tumor with a diameter of 5 cm was found. All patients with benign tumors had normal plasma γ-enolase levels. On the 4th postoperative day, when it could be assumed that any elevation of γ-enolase activity due to necrosis of cerebral tissue or hemolysis as a result of the operative procedure had disappeared,13 no abnormal values were found.

Discussion

Gliomas consist principally of glial cells with varying degrees of malignancy. These cells demonstrate a heterogeneous morphology, first described by Virchow41 and subsequently confirmed by all authors describing the morphology of gliomas.12,25 Apart from neoplastic cells, elements with varying morphology and reactive and degenerative cells with atypical appearance may be found.29,31 Besides this heterogeneous morphology, studies with monoclonal antibodies showed an antigenic heterogeneity in human gliomas.5 This antigenic heterogeneity in human gliomas seems to vary not only from case to case, but even within one glioma at different times.

In 1940, Scherer40 commented on the methods of human glioma growth. He found that the only glioma to show purely expansive growth is the ependymoma, and stressed the fact that all other gliomas, without exception, have an infiltrative growth pattern. This explains why even in the most malignant gliomas, especially at the periphery of tumors, nests of normal or atypical brain tissue can be found. Due to this morphology, a sudden transition from significant amounts of γ-enolase in normal brain tissue to total absence of γ-enolase in all gliomas, as described by Roys, et al.,27 would seem surprising.

In human gliomas several enzymes show a change in isoenzyme composition. This has been described for lactate dehydrogenase, hexokinase, and notably pyruvate kinase.37,38 This mechanism could theoretically be possible for enolase too. Vinore, et al.,40 using a radioimmunoassay, found specific amounts of γ-enolase in F98 anaplastic glioma cells. They suggested that anaplastic glioma cells may be potential precursors for either glial or neuronal cells, reflected by the presence of γ-enolase. Our findings in metastases suggest that other malignant cells may also possess the capacity to produce γ-enolase, possibly in relation to a changed metabolism, as suggested by Vinore, et al.39 The studies of Roys, et al.,27 have subsequently been disputed by Nakajima, et al.,21 and Vinore, et al.39 Nakajima, et al., using immunohistochemistry, found γ-enolase present in three of 18 astrocytomas, two of four oligodendrogliomas, and two of three ependymomas. Vinore, et al., also using immunohistochemistry, found γ-enolase present in 12 of 14 glioblastomas, 16 of 23 astrocytomas, one of two oligodendrogliomas, and two of three ependymomas. Positive staining was also found in a number of other malignancies, such as fibroadenoma and infiltrating ductal carcinoma. Because the specificity and sensitivity of the antiserum employed may play a role in these varying findings, we employed the double analysis described (that is, combination of electrophoresis of enolase in gliomas and immunohistochemistry). We can confirm the findings of Nakajima, et al., and Vinore, et al.; in our study, however, all gliomas examined showed some γ-enolase. The double analysis provided additional evidence that this finding was not due to a nonspecific binding reaction of the antibody.

Gamma-enolase is increasingly used as a tumor marker in plasma for several malignancies, such as neuroblastoma, small-cell lung cancer, and pancreatic islet-cell carcinoma,1,2,7,8,15,16,43 as well as in histopathological differential diagnosis.10,23 For interpretation of these studies, it is important to understand the mechanisms producing changes in the isoenzyme spectrum of enolase in tumors, or an elevated level of an isoenzyme in plasma. The finding of γ-enolase in a tumor might reflect the involvement of neuronal tissue, but it might also indicate the presence of a malignant cell of other origin, as this study has demonstrated the capacity of cells not deriving from neuronal tissue to produce amounts of γ-enolase. The presence of γ-enolase in a wide range of metastatic brain tumors was reported recently by Staal, et al.,24 who used electrophoresis. Our first conclusion, therefore, is that analysis of the enolase spectrum in a brain tumor provides no clues to its cell of origin.

The fact that γ-enolase seems to be present in all human gliomas and even in some metastases of other malignancies could provide insight into the mechanism of elevated levels of γ-enolase in the serum of patients with extensive “APUD-omas,” which are known to contain considerable amounts of γ-enolase.

Various authors have reported significant increases of γ-enolase levels in the serum of patients with widespread neuroblastoma,20,43 small-cell lung cancer,1,2,7,8 and nonfunctioning pancreatic islet-cell carcinoma.24 In immunohistochemical studies, significant amounts of γ-enolase could be demonstrated in tumors arising from the APUD system, such as islet-cell tumors, pheochromocytomas, and medullary thyroid carcinomas, as well as in neuroblastomas and retinoblastomas.7,15,36,39

In the case of an intracranial tumor, three possible mechanisms suggest themselves for producing an elevated level of γ-enolase in blood: 1) existing neurons are damaged, either by the tumor itself or indirectly (such as by edema or ischemia), thereby releasing γ-enolase which then should pass the blood-brain barrier;
2) the tumor contains elevated levels of γ-enolase and this, either by secretion or as the result of necrosis, produces elevated blood levels; or 3) the tumor contains elevated levels of γ-enolase, but only that located outside the nervous system produces elevated blood levels, and therefore elevated blood levels should reflect widespread disease. So far, only the third proposition seems to be the case, as elevated levels of γ-enolase have been found only in patients with tumors containing large amounts of γ-enolase and lying outside the central nervous system (as with the “APUD-omas,” retinoblastomas, and neuroblastomas). The first mechanism could be tested by studies measuring γ-enolase in the plasma of patients with a large cerebral infarct. This mechanism should be responsible for an elevated γ-enolase level in the case of gliomas and benign intracranial tumors, because gliomas contain less γ-enolase than does normal brain tissue, as demonstrated in our study.

As a marker in blood for the presence of a possible brain tumor or the recurrence of a previously treated brain tumor, the determination of the γ-enolase level in plasma ideally should produce few false negatives. As can be seen from our data, however, this seems not to be the case. Although we found some patients with a clearly elevated plasma level of enolase, who had a large brain tumor, many patients with a large brain tumor did not have an elevated level of γ-enolase in plasma. The γ-enolase level in the cerebrospinal fluid may be more reliable (as suggested by Royds, et al.26,28), but because of advances in preoperative diagnostic techniques, few lumbar punctures are performed nowadays in patients with brain tumors.

Conclusions

We conclude that the determination of the level of γ-enolase in plasma seems to be of little value as screening for brain tumors other than neuroblastomas, retinoblastomas, and “APUD-omas.” Investigation of human gliomas by electrophoresis and immunohistochemistry showed that γ-enolase was present in nearly all tumors examined, as well as in a number of metastases. Determination of the presence of γ-enolase in a cerebral neoplasm therefore provides no clue to its cells of origin.

Acknowledgments

The authors wish to thank Dr. J. J. Jansen for reviewing the manuscript, Dr. B. van Ketel for control of pathological specimens, Mrs. M. Streefkerk and A. M. C. Vlug for technical assistance, and Mrs. E. L. Huisman-Backer Dirks for preparing the manuscript.

References

Enolase isoenzymes in human gliomas


Manuscript received January 4, 1986.
Address reprint requests to: Ernst M. H. van den Doel, M.D., Department of Clinical Neurophysiology, Neurological Clinic, Nicolaas Beetsstraat 2-4, 3511 HV Utrecht, The Netherlands.