L-α-Alanine Inhibition of Pyruvate Kinase from Tumors of the Human Central Nervous System

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ABSTRACT

Pyruvate kinase isozyme distribution was studied in 75 intracranial tumors. Well-differentiated gliomas, i.e., astrocytomas, oligodendrogliomas, and ependymomas, showed a relatively high residual pyruvate kinase activity in the presence of 4 mm alanine. The reverse was found for poorly differentiated gliomas. Alanine inhibition of pyruvate kinase correlates well with degree of differentiation of these tumors.

There is also a correlation between alanine inhibition of pyruvate kinase and “one year survival” after “total or subtotal” resection of gliomas in adults. In the tumors of 24 patients who died of a recurrence within one year after the operation, a residual pyruvate kinase activity of less than 15% was found. On the contrary, in the tumors of all patients who survived the operation for more than one year, a residual activity of 15% and higher was found.

With the exception of a medulloblastoma, all poorly differentiated gliomas were characterized by the presence of type K and the hybrid K₂M. In well-differentiated gliomas, besides K₄ and K₃M, M₄ was also present. Alanine inhibition was in agreement with the electrophoretic pattern.

In children (ages 1 and 12 years), gliomas showed no correlation between the distribution of pyruvate kinase isozymes and the histological classification and grading.

Of the nonneuroepithelial tumors studied, relatively high residual activities were found for pyruvate kinase in hemangioblastoma, chromophobe adenoma, craniopharyngioma, and arteriovenous malformation. Other nonneuroepithelial tumors showed much less residual activity. These include benign tumors, neurilemmoma, cavernoma, and malignant metastatic tumors as well as a fibrosarcoma.

Residual activity of pyruvate kinase in the presence of alanine was decreased in the biopsy of the center of intracerebral neoplasms (n = 64) compared with normal brain tissue. Relatively high residual pyruvate kinase activity was found in the relatively well-differentiated gliomas. When the residual activity is determined not in the center of gliomas but more towards the periphery, much higher residual activity is found. It is proposed that a residual activity of less than 70% is indicative for the presence of tumor in a brain specimen.

Because the alanine inhibition can be performed within 10 min, this test can be used during surgery of intracerebral tumors.

The method cannot be used in children because of the presence of both K₄ and M₄ in children’s brain tissue.

INTRODUCTION

There is strong evidence that a massive alteration in isozyme composition exists inside cancer tissue as compared to its pattern in normal tissue. In particular, alterations in carbohydrate metabolism have been reported (11, 14, 15). One of the enzymes involved is pyruvate kinase (EC 2.7.1.40), which catalyzes the conversion of phosphoenolpyruvate to pyruvate with regeneration of ATP and which is a key enzyme in glycolysis. There are at least 3 mammalian isozymes known, according to the organ in which they are found to be predominant: L (liver), M (muscle), and K (kidney) types. Each of these isozymes is composed of 4 identical or nearly identical subunits (3–8). It seems likely (8) that 3 different genes are involved in the synthesis of L, M, and K subunits. Recently, we could identify in white and grey matter of human fetal brain 2 isozymes, M and K types together with 3 hybrids composed of M and K subunits (13). These isozymes and their hybrids are, according to their subunit composition, designated as M₄, K₄M₂, K₃M₃, and K₄.

In white and grey matter of a newborn, we detected mainly K₄ and M₄, whereas in adult human brain, M₄ was found to be predominant with only little of K₄M₃ and K₄. It seems likely that during development of the human brain the synthesis of M subunits is favored as compared to K subunits (13).

In Grade IV astrocytomas, we could detect only K₄ and K₃M, and we concluded that in these highly malignant gliomas a profound shift exists in the synthesis from M to K types of pyruvate kinase as compared to normal brain (13).

Imamura and Tanaka (9) found that the K type of pyruvate kinase was strongly predominant in young rat fetuses and identified it as the fetal form of pyruvate kinase. Imamura and Tanaka (9) also demonstrated that the K type was strongly inhibited by alanine. Farron et al. (7) found this isozyme to be predominant in various experimental tumors of the rat. In hepatomas, Farina et al. (6) and Farron et al. (7) observed a shift of M to K types related to loss of histological differentiation. Tolle et al. (12) in studies on isolated neurons and glial cells of the rat and cultures of neuroblastomas and glioblastomas concluded that these tumors, like those from other tissues, appear to revert their isozyme content to the fetal K type, and in general, the greater the growth rate of the tumor, the greater the ratio of K to M types that is found in the cells.

In this paper, we continue our report (13) about the results of a quantitative detection of K-type pyruvate kinase in intracranial tumors by determination of pyruvate kinase activity in the presence of the inhibitor alanine. These findings are compared with the results obtained with electrophoresis of the enzyme.

The potential value of the alanine inhibition test during sur-
surgery in intracerebral space-occupying lesions is evaluated in respect to prognosis and presence or absence of tumor as observed in the histological examination.

MATERIALS AND METHODS

Materials. NADH (disodium salt), ADP (disodium salt), phosphoenolpyruvate (trisocyclohexylammonium salt), fructose 1,6-diphosphate (trisodium salt), lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and hexokinase were obtained from Boehringer Mannheim, Mannheim G. F. R. Cellulose acetate (gelatinized) was obtained from Chemetron, Milan, Italy. Phenazine methosulfate and thiazolyn blue tetrzählum were obtained from Sigma Chemical Co. (St. Louis, Mo.). L-α-Alanine was purchased from BDH (Poole, England). All other reagents used were of the highest purity available.

Enzymatic Assay and Alanine Inhibition. Pyruvate kinase activity was measured in the coupled lactate dehydrogenase assay as described by Bücher and Pfleiderer (2). The reaction was followed at 340 nm in a Perkin-Elmer 124 split-beam recording spectrophotometer. One unit of pyruvate kinase activity is defined as the amount of enzyme converting 1 μmol of phosphoenolpyruvate per min⁻¹ at 37°. The protein content was determined by the method of Lowry et al. (10) with crystalline bovine serum albumin as standard. Activities are expressed as units/mg protein. For further conditions, see Ref. 13.

For the alanine inhibition test, the assay medium contained the following in a final volume of 3 ml: 0.2 M Tris-HCl (pH 8.0), 65 mM KCl, 20 mM MgSO₄, 0.09 mM NADH, 27 units lactate dehydrogenase, 1 mM phosphoenolpyruvate, 4 mM alanine, and enzyme. The control contained no alanine. The mixture was incubated for 3 min at 37°. The reaction was started by the addition of ADP at a final concentration of 0.2 mM. The 100% value is the activity in the absence of alanine.

Electrophoresis. Electrophoresis on cellulose acetate strips was carried out as previously described. After electrophoresis, bands of pyruvate kinase activity were visualized as described in Ref. 13.

Biopsies and Preparation of Extracts. Biopsies were taken during surgery either from tissue that was estimated to be in or nearest to the center of the tumors or as was done in space-occupying lesions of other origin from the most suspect site. An immediately adjacent specimen was taken for histology; the total volume of "twin" specimen ranged from 0.5 to 2 cu cm. In a number of cases, more than one specimen couple was selected for histological and enzymological comparative evaluation of possible cancer. More, in individual specimens, a comparative evaluation of the histological diagnosis (tumor or not tumor) and the enzymological findings was performed. The histological classification and grading were done according to the World Health Organization Histological Classification of Tumors (16, 17).

Extraction. One part of the tissue was mixed with 3 parts extraction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM sucrose. The mixture was minced by pottering during 1 min. After centrifugation at 20,000 × g for 10 min, the clear supernatant was used for experiments. In case a rapid diagnosis was required, centrifugation was performed for 2 min at 10,000 × g. When the enzyme preparation was stored (at −70°), sucrose was added up to a final concentration of 0.5 M (storage showed no influence on electrophoretic pattern and only little or no loss of enzyme activity).

Tumor Resection. The resection of the tumor was considered "total" if at macroscopic examination no tumor was left after resection.

The resection was considered "subtotal" if after resection of the bulk of the tumor small pieces of the tumor were suspected to be left in place at macroscopic inspection.

The ages of the patients ranged from 1 to 67 years. Tumors of patients younger than 12 years are separately discussed in "Gliomas in Children."

RESULTS

Correlation of Cancer and Degree of Inhibition of Pyruvate Kinase by Alanine

Astrocytomas and Glioblastomas. The possible correlation between histological grading of gliomas and pyruvate kinase activity in the presence of alanine is summarized in Chart 1. The chart shows the mean of residual activity in the different groups of gliomas. The inhibition (expressed as residual activity) of pyruvate kinase by alanine was investigated in 6 astrocytomas according to their histological classification. Well-differentiated tumors are characterized by relatively high residual activity. The reverse is found for the anaplastic astrocytoma and also for the poorly differentiated glioblastomas.

Electrophoresis of pyruvate kinase from the tumors of the different groups is in agreement with the alanine inhibition. Fig. 1 shows the electrophoretic pattern of examples of tumors representative for the respective histological classification. From this figure, it can be concluded that the presence of both M₄ and K₄ and the hybrid K₃M is a specific property of well-differentiated astrocytomas. On the contrary, in highly malignant glioblastomas, the synthesis of K subunits is favored, and therefore, more K₄ and K₃M are observed, in agreement with the alanine inhibition.

Oligodendroglialomas. The degree of inhibition of pyruvate kinase by alanine was investigated in well-differentiated (Grade II) and anaplastic (Grade III) oligodendroglialomas. Also in this variety of gliomas, high residual activity of the enzyme corresponds with low grading of cancer.

Relatively low residual activity is seen in tumors that were classified as more malignant. The electrophoretic pattern of pyruvate kinase from 2 of these tumors is shown in Fig. 2. For these cases, the residual activity of pyruvate kinase in the presence of 4 mM alanine was 23 and 5%, respectively. In these tumors, the same electrophoretic pattern is obtained as compared to the astrocytomas and glioblastomas. M₄, K₄, and the hybrid K₃M are synthesized according to their cancer. The isozyme composition in which relatively more M subunits are present is found in well-differentiated tumors. In less-differen-
Mixed Oligoastrocytoma. For these well-differentiated tumors, histologically corresponding to Grade II, the same correlation as mentioned in the other gliomas is valid. M₄, K₄, and the hybrid K₃M are synthesized in agreement with alanine inhibition of pyruvate kinase.

Other Gliomas. Of 2 ependymomas, the lowest residual activity (3%) was found in the one classified as anaplastic. The other one classified as well differentiated (Grade I) had a residual activity of 29%. The electropherogram of pyruvate kinase of these tumors shows an identical pattern as in other gliomas. In the medulloblastoma, a residual activity of 23% was found. The electrophoretic pattern of pyruvate kinase from this tumor was different compared with the pattern obtained in all other gliomas. In this tumor, the hybrid K₃M is predominant and not K₄; furthermore, K₂M₂ is also present. It can be concluded that the well-differentiated tumors are characterized by higher residual activity than are the poorly differentiated tumors.

Gliomas in Children. The degree of inhibition of pyruvate kinase from gliomas of children (ages 1 to 11 years; n = 8) was investigated. In 2 cases of well-differentiated pilocytic astrocytoma, a similar residual activity of pyruvate kinase is found as in less-differentiated gliomas. The highest residual activity is seen in a medulloblastoma. No correlation between degree of differentiation and pyruvate kinase activity in the presence of alanine is found.

Nonneuroepithelial Tumors. These include haemangioioblastoma (n = 1), chromophobe adenoma (n = 3), craniopharyngioma (n = 1), neurilemmoma (n = 5), fibrosarcoma (n = 1),...
metastatic tumors (n = 4), and 2 vascular malformations [cavernoma (n = 1) and arteriovenous malformation (n = 1)]. The residual activity of pyruvate kinase from these tumors is given in Table 1. Relatively high residual activities were found for pyruvate kinase from a hemangioblastoma, a craniopharyngioma, and chromophobe adenomas (n = 2). Pyruvate kinase from the other tumors within this group as well as the cavernoma shows much less residual activity (see Table 1). A good correlation between the alanine inhibition and electrophoretic pattern was found. However, no correlation could be detected between degree of differentiation and residual pyruvate kinase activity.

**Correlation between Alanine Inhibition and One Year Survival after Total or Subtotal Resection of Gliomas**

Chart 2 shows that of 41 patients investigated, 24 died within one year after surgery because of a recurrence of the tumor. The tumors of these 24 patients showed a residual pyruvate kinase activity of less than 15%. On the other hand, all tumors of 17 patients who had survived the operation for more than one year showed 15% residual activity or more.

**Correlation between Alanine Inhibition and Presence or Absence of Tumor at Histological Examination**

The residual activity of pyruvate kinase in the presence of 4 mM alanine was measured in biopsies taken from the central area of 64 intracerebral lesions that were diagnosed to be of neoplastic origin before surgery. The histological diagnosis is listed in Table 2. The residual activity of all these intracerebral tumors was less than 67%. As was already concluded, this residual activity correlates well with the degree of cancer in case of gliomas. Normal adult brain tissue shows a residual activity of more than 80%.

In our first report (13), we demonstrated maximal alanine inhibition of pyruvate kinase in the center of a poorly differentiated glioma and much less alanine inhibition towards the periphery of the tumor. Therefore, in biopsies away from the center of such tumors, one may expect a correlation which is quite different from which was already reported. For 24 gliomas (16 poorly and 8 well differentiated, according to their histological classification), biopsies were taken from different parts of the tumor and investigated with respect to enzymology as well as pathology. Chart 3 shows the comparison of observed residual activity of pyruvate kinase in the presence of alanine and the diagnosis for the presence of tumor. Chart 3A presents the results for the well-differentiated lesions, and Chart 3B presents the results for the poorly differentiated tumors. For the well-differentiated tumors, all specimens with more than 69% residual activity were diagnosed as normal. For the poorly differentiated tumors, there appeared to be more overlap. Between 53 and 74% residual activity, 7 of 19 of the specimens

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**Table 1**

Residual activity of nonneuroepithelial tumors, metastatic tumors, and vascular malformation

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Mean residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemangioblastoma</td>
<td>44 (1)*</td>
</tr>
<tr>
<td>Chromophobe adenoma</td>
<td>47 (2)</td>
</tr>
<tr>
<td>Craniopharyngioma</td>
<td>37 (1)</td>
</tr>
<tr>
<td>Neurilemmoma</td>
<td>8 (5)</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>8 (1)</td>
</tr>
<tr>
<td>Metastatic tumors</td>
<td>8 (4)</td>
</tr>
<tr>
<td>Cavernoma</td>
<td>11 (1)</td>
</tr>
<tr>
<td>Arteriovenous malformation</td>
<td>77 (1)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of tumors.

**Table 2**

Histological diagnosis of 61 intracerebral neoplasms

Subjects range in age from 11 to 71 years.

<table>
<thead>
<tr>
<th>Intracerebral neoplasms</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>21</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>7</td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma</td>
<td>8</td>
</tr>
<tr>
<td>Mixed oligoastrocytoma</td>
<td>7</td>
</tr>
<tr>
<td>Pilocytic astrocytoma</td>
<td>2</td>
</tr>
<tr>
<td>Astrocytoma Grade II</td>
<td>3</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>1</td>
</tr>
<tr>
<td>Metastatic tumors</td>
<td>4</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>1</td>
</tr>
<tr>
<td>Craniopharyngioma</td>
<td>1</td>
</tr>
<tr>
<td>Ependymoma</td>
<td>2</td>
</tr>
<tr>
<td>Anaplastic ependymoma</td>
<td>1</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>1</td>
</tr>
<tr>
<td>Cavernoma</td>
<td>1</td>
</tr>
<tr>
<td>Hemangioblastoma</td>
<td>1</td>
</tr>
</tbody>
</table>
DISCUSSION

A possible explanation for the disagreement between the diagnosis by the pathologist and the enzyme determination may lie in the biopsy technique. Therefore, we refined the technique of twin biopsies. Each twin specimen was sized to a total of about 20 cu mm instead of 0.5 to 2 cu cm as was normally performed. Chart 4 shows the results for an anaplastic oligodendroglioma. Samples from 12 different locations were taken. All but one (Specimen 11) were diagnosed by pathological examination as tumor. In the same chart, the electrophoretic pattern of pyruvate kinase from the 12 different specimens is given. It was already concluded (13) that the residual activity of pyruvate kinase correlates closely with the electrophoretic pattern, as is shown for this particular case in Chart 4. At the time of surgery, only Specimens 1 to 6 were considered suspect for tumor growth, whereas at microscopic examination, 5 other specimens appeared to contain tumor as well. The ratio of M to K types in the specimens is different from the normal pattern. The results of comparative microscopic and enzymological examination strongly indicate that the overlap already mentioned is due to the biopsy technique.

In an earlier report (13), we concluded that pyruvate kinase from astrocytoma Grade IV (Kernohan's classification) is characterized by the absence of type M, whereas type K and the hybrid K3M are predominant.

In the same study, we examined meningiomas and found that isozyme composition of pyruvate kinase in well-differentiated meningiomas and the poorly differentiated astrocytoma Grade IV were identical. This finding was in contrast with the results of a study by Tolle et al. (12) on neuroblastomas and glioblastomas. They concluded that "tumors of neural origin like those from other tissues appear to revert their isozyme content and in general the greater the growth rate of the tumor, the greater the ratio of types K to M is found." Farron et al. (7) and Farina et al. (6) found in experimental hepatomas that less-differentiated tumors produced only type K but that well-differentiated tumors retained a limited capacity to produce the other types as well.

In human gliomas, a predominance of the K-type pyruvate kinase was found by Bennett et al. (1), but no further correlation with degree of differentiation could be established in their study. This was probably due to the way in which the samples were obtained, after surgery. In the present study, immediate adjacent (twin) specimens for simultaneous enzymological and histological examination were taken during surgery from what was estimated to be the center of the tumor. Tumors were only included in this study if the histological diagnosis of the twin biopsy was also representative for the histological classification and grading of the tumor. In the absence of histochemical procedures, this method seems to be the most appropriate in a simultaneous histological and enzymological study of tumors.

The present report shows that in gliomas a high degree of correlation can be found between the histological grading and the isozyme pattern of pyruvate kinase, as expressed in electrophoretic pattern and alanine inhibition (see Chart 1). Therefore, we conclude that less-differentiated tumors show a higher degree of inhibition of pyruvate kinase activity than do well-differentiated tumors. In gliomas, the range of alanine inhibition is found, dependent on several parameters, e.g., individual biological deviation, the error when taking the specimen, and the error in the determination of pyruvate kinase.

In view of the predominance of the K-type in less-differentiated gliomas, the age of the patient may influence the correlation between enzymological and histological findings. In fetal brain as well as in brain of a newborn (13), considerable amounts of type K are present, and therefore, increased alanine inhibition is found. However, we are not informed about the amount of type K present in normal brain of children. Consequently, we cannot make conclusions with respect to gliomas of children.

Nonneuroepithelial tumors are all mainly characterized by the predominance of K type and the hybrid K3M. In agreement with the observed electrophoretic pattern is the alanine inhibition (see Table 1). All tumors investigated in this study, with the exception of a medulloblastoma, show the presence of mainly type K and the hybrid K3M as was reported by Tolle et al. in their studies. It is, however, quite possible that the cells from which a tumor originates are characterized by the presence of the K type. Therefore, it is not surprising that the assumption that the ratio of types K to M is proportional to growth rate appears in this study to be valid for astrocytomas, oligodendrogliomas, and ependymomas, but not for nonneuroepithelial tumors. The number of cases investigated to date is, however, too low to make definite correlations.

Chart 3. A, residual pyruvate kinase in the presence of 4 mM alanine of well-differentiated cerebral tumors (n = 7). Biopsy taken at different parts of the tumor. O, diagnosed as normal; •, diagnosed as tumor. B, residual pyruvate kinase in the presence of 4 mM alanine of poorly differentiated gliomas (n = 15). Biopsy taken at different parts of the tumors. O, diagnosed as normal; •, diagnosed as tumor.
In gliomas, there exists, apart from a correlation between alanine inhibition of pyruvate kinase and cancer in histological grading, a relationship between alanine inhibition and 1-year survival, as shown in Chart 2. Patients who had a residual activity of less than 15% died from their tumor within 1 year, irrespective of the histological classification and grading of the glioma, whereas those who survived the first year after operation had a residual activity of 15% or more. In the scope of this study, we have included patients of 12 years and older but have not further taken into account the age of the patients, the localization of the tumor, or the error when taking the biopsy specimens. Only 4 patients had postoperative radiotherapy or chemotherapy. It is quite difficult to say if chemo- or radiotherapy has had any influence on the 1-year survival except for the case of the medulloblastoma. It is well known that medulloblastomas are general highly radiosensitive.

In white and gray matter of normal adult brain tissue, we found more than 80% residual pyruvate kinase activity in the presence of alanine. In the present report, we compare pathological findings with residual pyruvate kinase activities from biopsies of gliomas as well as neoplastic lesions of other origin. When the biopsy is taken from the center of the tumor, a decreased residual pyruvate kinase activity is found in all tumors (n = 64) investigated. In surgery of intracerebral neoplastic lesions, a residual activity of less than 80% reflects abnormal distribution of pyruvate kinase isozymes. However, it must be mentioned that the presence of relatively high residual activity may be due to: (a) the presence of a less malignant tumor which contains less K type, even for centrally located tumor tissue; or (b) variation in biopsy technique. Chart 3 clearly shows that in different parts of the tumor different residual activities are found. The results presented in Chart 3 are somewhat conflicting with regard to the periphery of the tumor especially in the region in which 53 to 78% residual activity is found. However, Chart 4 strongly indicates that the biopsy technique is most important when comparing residual
activity and pathological findings. In this particular case, the residual activity and pathological diagnosis correlated well. In 2 cases not described in this study and suspected as space-occupying cerebral lesions, a normal residual activity (more than 80%) was found at surgery. In one case posttraumatic scarring and in the other an inflammatory process was diagnosed at microscopic examination. Moreover, we reported earlier (13) a case of necrosis and scarring caused by thrombosis in an arteriovenous malformation causing a space-occupying lesion in which no alanine inhibition was found despite the presence of normal pyruvate kinase activity.

A residual activity of higher than 70% does not exclude the presence of a tumor but may also denote non-tumor-specific changes. However, based on the results obtained in our comparative histological and enzymological evaluation, we propose that a residual activity below 70% indicates a strong probability of the presence of tumor in an intracerebral biopsy specimen.

The alanine inhibition test can be performed within 10 min after the biopsy is taken and fits well within the scope of a surgical procedure. It is a standardized quantitative test which requires no great experience as compared to histological examination of frozen biopsy material. It can be concluded that alanine inhibition of pyruvate kinase not only can be a valuable help in the determination of cancer and postoperative prognosis of gliomas but also serves as a tool in surgery for detection and demarcation of intracerebral neoplasms.

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