Biological significance of tissue plasminogen activator content in brain tumors

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Fresh brain-tumor samples were obtained at operation and analyzed for their content of tissue type plasminogen activator (tPA) using an activity assay (gel chromatography zymogram) and an enzyme-linked immunospecific assay. The specimens were taken from 23 glioblastomas, 35 metastatic tumors, 42 meningiomas, 16 low-grade gliomas, and seven acoustic neuromas; seven specimens were from normal brain.

A strong correlation was found between the results of the two assays ($r = 0.77$, $p < 0.0001$). There was a threefold difference in the tPA content of the benign tumors as compared to malignant tumors ($p = 0.0006$), the latter having less tPA. Histologically benign meningiomas contained higher tPA than malignant meningiomas ($p = 0.01$); however, the difference between low-grade gliomas and high-grade gliomas was less evident. Overall, regression analysis data have shown an inverse relationship between the tissue content of tPA and the presence and degree of tumor necrosis and peritumoral brain edema ($p = 0.004$ and $p = 0.0004$, respectively).

This finding was most consistent in the glioblastoma group where the correlation coefficient values were $r = 0.53$ and $r = -0.55$, respectively. There was no significant correlation between the tissue tPA content and the age and sex, steroid use, or plasma tPA of the patients or the duration of symptoms.

In summary, this is the first demonstration of tPA in a large series of human brain tumors and in normal brain. The differences observed have clear biological significance and, although the source of tPA in tumor tissue is still unknown, a relative reduction in tPA in tumor tissue may play an integral role in the development of tissue necrosis and tissue edema. The lack of tPA in tumor necrosis was not due to tissue destruction and cell death since urokinase was readily detectable in that tissue.

KEY WORDS: brain neoplasm - tissue plasminogen activator - tumor necrosis - plasminogen activator

Plasminogen activators (PA's) are a group of serine proteolytic enzymes that specifically convert plasminogen to the active proteinase plasmin. The two major types of PA, tissue PA (tPA) and urokinase PA (uPA), differ with respect to their tissue distribution and catalytic, molecular, and immunological properties. The uPA is a 54-kD enzyme composed of two disulfide-linked 30- and 24-kD subunits. The native tPA, in contrast, is a single 70-kD polypeptide chain. Plasminogen activators have been implicated in a wide variety of processes that occur during normal physiological events as well as during neoplastic transformation, growth, and invasiveness. This is well exemplified during mammary gland involution and cancer invasion where the PA system may be partly responsible for the basement membrane destruction. In man, uPA is produced and secreted by several extracranial neoplasms such as adenocarcinomas of the gastrointestinal tract, lung, stomach, and prostate. Although tPA has been associated mainly with vascular fibrinolysis, it has also been shown by several investigators to be produced by cultured rat glioma and human brain-tumor cells. The respective roles played by uPA and tPA in tumors, however, are still under investigation.

Because of the biological importance of PA's and their demonstrated association with human brain tumors, we previously carried out a comparative analysis of the molecular weight forms and the total fibrinolytic activity of several common types of brain tumors. From the results of that study, it was noted that lytic bands in the tPA molecular weight range were lacking in metastatic brain tumors and that the lysis activity associated with the PA's of human brain sarcoma, was unexpectedly prominent. This raised the possibility...
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that the immunological identity of the PA present in benign brain tumors may be in part different from the PA found in malignant brain tumors. Therefore, in this study we have analyzed the tPA content of a large sample of brain tumors using activity and immunological assays, and have correlated the results with the histological nature of the tissue as well as with specific biological parameters.

Materials and Methods

Tissue Processing

Fresh human brain-tumor tissue and normal brain tissue samples were collected in the operating room from patients undergoing craniotomy. A total of 130 specimens included 23 glioblastomas, 35 metastatic brain tumors, 42 meningiomas, 16 low-grade gliomas, seven neurinomas, and seven samples of normal brain.

The samples were immediately placed on dry ice, transferred to a freezer, and stored at −80°C. Specimens were subsequently thawed, weighed, minced, and homogenized at a ratio of 50 mg of tissue to 1 ml of 37.5 mM Tris-HCl, 0.75 mM ethylenediaminetetra-acetic acid, 75 mM NaCl, and 15 mM lysine to a final pH of 9.5.

Methods of Analysis

The protein content of the samples was analyzed using the method of Lowry, et al. The enzymatic activity and molecular weight of electrophoretically separated forms of PA in tumor extracts were determined by our modification of the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) procedure of Laemmli as described in detail previously. Briefly, the SDS-gel contains acrylamide to which purified plasminogen and fibrinogen were added as substrates prior to polymerization. After polymerization and subsequent SDS-PAGE of the brain tissue extracts, the various types of PA (along with other proteins) were separated due to differences in molecular weight. The gel was then treated with a nonionic detergent to remove the SDS, thus renaturing the PA enzymes, and then incubated at 37°C to allow for activation of the plasminogen to plasmin. The active plasmin molecule then hydrolyzed the fibrinogen to soluble products of lower molecular weight which were subsequently eluted from the gel matrix. Upon staining and destaining, the final gel had a uniform blue background except in those regions to which PA had migrated and activated the plasminogen to plasmin. This method is a semiquantitative, direct (two-stage) analysis of PA activity permitting simultaneous correlation of molecular weight and biological activity.

The tPA antigen was measured immunologically using IMUBIND-5, an enzyme-linked immunospecific assay (ELISA) technique. The ELISA for tPA follows the principle of the double-antibody sandwich technique. The wells of a flat-bottom microtiter plate were filled with 200 μl of a coating solution containing goat anti-human tPA immunoglobulin G (IgG) in 0.1 mol/liter NaHCO₃. After incubation for 3 hours at 37°C, the plates were washed three times with 0.15 mol/liter NaCl containing 0.5 gm/liter Tween 20.

A highly purified preparation of tPA from melanoma cell cultures was used as a standard. The dilutions were performed with phosphate-buffered saline (PBS)-Tween buffer (0.2 mol/liter sodium phosphate buffer, pH 7.4, with 0.15 mol/liter NaCl and 0.5 gm/liter Tween 20) containing 1 mg/ml bovine serum albumin; 20 μl of these standard solutions were then added to the wells containing 180 μl PBS-Tween buffer.

Acidified plasma samples were first neutralized by the addition of an equal volume of 0.1 mol/liter Na₂HPO₄, then further diluted 2.5-to-25-fold with PBS-Tween buffer in the wells. The final volume in the wells was kept constant at 200 μl.

After incubation for 18 hours at 25°C in a humid chamber, the wells were emptied and washed as described above. The wells were filled with 200 μl conjugate (anti-tPA IgG), diluted 1:2000 with PBS-Tween, and the plate was incubated at 25°C for 3 hours. Subsequently, 200 μl sodium phosphate buffer (0.1 mol/liter, adjusted to pH 5.0 with 0.1 mol/liter citric acid) containing 0.4 mg/ml o-phenylenediamine and 0.01% H₂O₂ was added and the plate was incubated at 25°C for 30 minutes in the dark. The enzymatic reaction was then stopped by adding 50 μl 4.5 mol/liter H₂SO₄. The absorbance was read at 492 nm.

Determination of Brain Edema

Computerized tomography (CT) scans were obtained for each patient and analyzed according to a previously described procedure. The degree of brain edema was graded on a scale of 0 to 5, with 0 representing total lack of visible edema and 5 severe edema.

Determination of Necrosis

Necrosis on CT scans was defined as an area of low attenuation within an enhancing tumor mass. Surgical correlation was sought in cases where necrosis and tumor cysts could not be differentiated on CT scans. The degree of necrosis was graded on a scale of 0 to 3, with 0 representing the lack of visible necrosis and 3 representing a necrosis area that was greater than 50% of the tumor area.

Histological Subgroups

In addition to the six categories of tissue used in the study, the tumor samples were further subgrouped into benign and malignant categories. The latter subgroup included all glioblastoma and metastatic samples as well as six malignant meningioma samples and one malignant schwannoma. The benign category included the remaining meningiomas and schwannomas as well as all low-grade gliomas.
**Statistical Analysis**

Comparison between the various groups was computed using the analysis of variance method, and correlation coefficients were obtained by linear regression using standard computer software. Statistical significance was set at p ≤ 0.05.

**Results**

The tPA content of all the tissue samples was assayed using the zymographic gel assay as well as ELISA. The results showed a strong correlation between the two methods of analysis (Fig. 1). The correlation coefficient was $r = 0.77$ (p < 0.0001) before adjustment for the protein content of the sample and $r = 0.81$ (p < 0.0001) after adjustment for the protein content of the tissue.

**Protein and tPA Content by Histological Group**

Table 1 demonstrates the mean values (± standard error of the mean) for the protein content, tPA activity, and tPA antigen of the various histological groups. Protein content was lowest in normal brain tissue samples. Benign brain tumors showed slightly higher protein content than normal brain, and the metastatic tumor and glioblastoma samples had a significantly higher (p < 0.003) protein content than normal brain or acoustic neurinoma samples (Table 1).

The mean tPA content as determined by either assay was significantly higher in the acoustic neurinoma group compared to that of the glioma, metastatic tumor, or glioblastoma groups. The mean tPA activity of normal brain was approximately two to three times that of the metastatic tumor and glioblastoma groups. However, this difference was statistically insignificant (Table 1).

To better appreciate the difference in tPA content of the tumor tissue, the samples were separated into two groups. A benign group comprised the benign meningioma, acoustic neurinoma, and low-grade glioma samples. A malignant group included the malignant meningioma, malignant schwannoma, metastatic, and glioblastoma samples. The tPA activity and antigen content of the benign samples were significantly greater (p < 0.0006 and p < 0.001, respectively) than those of the malignant samples.

The difference in tPA content was not related to the difference in origin of the benign tumors with high tPA content (extra-axial tumors) since, within the meningioma group, a statistically significant decrease in the tPA content was noted in the malignant tumors as compared to the benign tumors (Table 2). As shown in Fig. 2, the tPA was approximately three times greater in the benign group than in the malignant group.

Figure 3 illustrates the dense lytic activity in the tPA zone found in several benign meningioma samples obtained from six different patients. This tPA activity can be contrasted with that seen on the zymogram of three glioblastoma samples (Fig. 4); it is apparent from this figure that the necrotic portion of a glioblastoma totally lacked tPA lytic activity and that the tPA activity of even the "viable" portion of the glioblastoma was

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**TABLE 1**

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>No. of Specimens</th>
<th>Protein Content (mg/ml)</th>
<th>tPA Activity (U/mg protein)</th>
<th>tPA Antigen (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal brain</td>
<td>7</td>
<td>2.8 ± 0.3</td>
<td>17.0 ± 1.9</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>acoustic neurinoma</td>
<td>7</td>
<td>3.0 ± 0.5</td>
<td>23.4 ± 10.6$</td>
<td>6.6 ± 1.28</td>
</tr>
<tr>
<td>meningioma</td>
<td>42</td>
<td>3.8 ± 0.2</td>
<td>15.8 ± 3.0</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>glioma</td>
<td>16</td>
<td>3.7 ± 0.3</td>
<td>9.2 ± 1.4</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>metastatic tumor</td>
<td>35</td>
<td>4.3 ± 0.2$</td>
<td>7.4 ± 2.5</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>23</td>
<td>4.4 ± 0.2$</td>
<td>6.3 ± 2.7</td>
<td>2.5 ± 1.0</td>
</tr>
</tbody>
</table>

*Values are given as means ± standard error of the mean. tPA = tissue plasminogen activator. Significance of difference: $**= p < 0.003$ compared to normal brain or acoustic neurinoma; $*$ = p < 0.001 compared to glioma, metastatic tumor, or glioblastoma; and $\dagger$ = p < 0.03 compared to glioma, metastatic tumor, or glioblastoma.

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**TABLE 2**

Presence of tPA in meningioma tissue according to histological grouping and assay used*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Benign Group</th>
<th>Malignant Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Specimens</td>
<td>Mean Value</td>
<td>No. of Specimens</td>
</tr>
<tr>
<td>tPA activity (U/mg)</td>
<td>25</td>
<td>17.93 ± 3.56</td>
<td>6</td>
</tr>
<tr>
<td>tPA antigen (ng/mg)</td>
<td>35</td>
<td>5.54 ± 0.98</td>
<td>7</td>
</tr>
</tbody>
</table>

*Mean values are expressed ± standard error of the mean. tPA = tissue plasminogen activator.
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substantially weaker than that of the meningioma samples (Fig. 3). Of equal importance was the presence of high uPA activity in all the tissue samples assayed and particularly in the samples of necrotic tissue.

Correlation Between tPA and Other Tumor Parameters

Table 3 shows the results of the regression analysis of both tPA activity and antigen versus tumor size, peritumoral brain edema, and tumor necrosis. There was a significant inverse relationship between tPA activity and antigen content and the presence and degree of peritumoral brain edema and tumor necrosis (p < 0.0004 and p < 0.004, respectively). This finding was most consistent in the glioblastoma group (r = 0.53 and r = −0.55, respectively). There was also a reverse correlation between the tissue tPA level and the size of the tumor.

No significant correlation was found between the tPA content of the samples and the age and sex, steroid use, or the plasma tPA of the patients or the duration of symptoms (data not shown).

Discussion

To better understand the role played by PA's in the biology of brain tumors, it has become essential to determine the types of PA produced by the various brain tumors. This study has clearly shown that tPA, whether assessed by an activity assay or measured immunologically, is present in much higher concentrations in benign than in malignant brain tumors.

This study helps to clarify the differences in PA molecular weights described in a previous publication. It is now evident that lytic bands in the 70-kD (± 5 kD) zone represent tPA as documented by a strong correlation between this lysis area and the antigen (ELISA) assay, and also by immunoprecipitation experiments using anti-tPA antibodies (data not shown). The total PA activity of a brain-tumor sample, as generally reported, reflects the presence of both tPA and uPA and therefore may not correlate accurately with the biological function or the histological nature of the neoplasm.

Historical Perspectives

Todd first localized tPA activity histochemically to the vascular endothelium and Takashima, et al.,24 later demonstrated it in the endothelium of normal brain vessels. The distribution of tPA in brain tumors is still unclear. Franks and Ellis22 recently published an immunohistochemical study of the localization of tPA in human brain tumors. Their study material consisted of 38 human brain tumors including astroglial tumors, meningiomas, and metastatic tumors: tPA was localized mainly in endothelial cells of high-grade astrocytomas but the authors could not find any consistent relationship between the pattern of staining and the tumor grade.

Fig. 2. Tissue plasminogen activator (tPA) activity (dark block) and antigen content (light block) of benign and malignant brain tumors. Both parameters were significantly higher in benign brain tumors (p < 0.0006 and p < 0.001, respectively).

Fig. 3. Plasminogen activator (PA) activity as detected on a zymogram. A through F represent six different meningioma samples all showing distinct tissue PA (t-PA) activity. Urokinase (Uk) PA and t-PA controls are shown on the left. Uk-HMW = high-molecular-weight urokinase; Uk-LMW = low-molecular-weight urokinase.

Fig. 4. The zymographic appearance of three different glioblastoma samples (A, B, and C) compared to three samples of tumor necrosis obtained from the same tumors (a, b, and c). Urokinase plasminogen (Uk) activator and tissue plasminogen activator (t-PA) controls are shown on the right. Uk-HMW = high-molecular-weight urokinase; Uk-LMW = low-molecular-weight urokinase.
Tissue Plasminogen Activators vs. Malignancy

Increased PA activity has previously been considered to correlate with an increase in malignancy.\(^{12,31}\) Franks and Ellis\(^{2}\) proposed in their immunohistochemical work that the vascularity of brain tumors might play a significant role as the source of tPA activity and that the recorded activity merely reflects the degree of vascularity of the tumor. The present study does not support this theory, since increasing malignancy is associated with increasing (and pathological) vascularity. Based on this reasoning alone, the tPA activity should be higher in malignant tumors than in their benign counterparts. This was clearly not the case; on the contrary, malignant tumors had significantly less tPA than benign tumors. This is not to say that the only source of tPA is non-endothelial cells. It is likely, however, that both tumor cells, as well as endothelial cells, contribute to the production of tPA. The current study, as designed, cannot address this problem, and further experiments will be necessary to elucidate this question.

The reduced tPA content observed in malignant tumors in this study is not unique to brain tumors. Recent studies of gastrointestinal and breast neoplasms have shown similar results\(^{13,19,29}\), leading some authors to propose the use of tPA measurements as a prognostic indicator for breast cancer.\(^{29}\) Based on these and other studies, it is becoming increasingly recognized that uPA is more likely than tPA to be directly associated with the malignant phenotype of malignant tumors (that is, invasiveness and metastasis).

Benign vs. Malignant Tumors

The difference in tPA production between benign and malignant brain tumors is intriguing, particularly since the literature does not provide an explanation for it. The difference in tPA content is not related to the difference in origin of the benign tumors with high tPA content (that is, extra-axial tumors) since, with the meningioma group, a statistically significant decrease in tPA content was noted in the malignant tumors as compared to the benign ones (Table 2).

Theoretically, changes in the expression of PA's can result from changes in the genetic make-up of tumor cells,\(^{20}\) due to the fact that PA's are gene products. Another hypothesis relates to the regulatory mechanisms that control extracellular proteolysis. In this regard, special consideration must be given to plasminogen activator inhibitor (PAI-1), the most important inhibitor of tPA in vivo.\(^{11,13}\) PAI-1 is produced by glioblastoma cells in culture,\(^{21}\) and some tumor cells simultaneously express PA and PAI-1.\(^{5}\)

Tumor Necrosis Factor

The production of tumor necrosis factor, a mediator of acute-phase response by activated macrophages, has led to an increase in PAI-1 synthesis in humans and in rats.\(^{26}\) The tumor necrosis factor content in various brain tumors is not known and therefore the role of this cytokine in the present findings is difficult to estimate. The tumor necrosis factor is, however, capable of suppressing the deoxyribonucleic acid synthesis of cultured endothelial cells from gerbil brain,\(^{32}\) and has also been shown to stimulate gene transcription of PAI-1 and PAI-2 and simultaneously suppress constitutive gene expression of tPA in human fibrosarcoma cells.\(^{18}\)

In theory, the tumor necrosis factor could be responsible for the decreased tPA of the tumor tissue and subsequent tissue necrosis.

Necrosis and Edema

The tumor tissue content in tPA did not correlate with the patients' age, sex, plasma tPA, or duration of symptoms, nor did it correlate with the tumor protein content. However, a significant and inverse correlation was found between tPA and the presence and degree of tumor necrosis and peritumoral edema. This inverse relationship was most significant for the glioblastoma group. This is a noteworthy observation since, unlike meningiomas and acoustic neurinomas where necrosis is rarely a factor, and unlike metastatic tumors which are almost always associated with a moderate-to-severe degree of peritumoral edema, glioblastomas present with an unpredictable and quite variable CT appearance in regard to these two biological parameters.

A relative reduction in the content of free tPA in neoplastic tissue may play an integral role in the develop-
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development of tissue necrosis and tissue edema. Tissue plasminogen activator is the most important agent in the maintenance of intravascular fibrinolysis and patency. If the regulation of fibrinolysis fails, impaired circulation due to microthrombosis leads to tissue edema and finally to tissue necrosis. As already mentioned, the decrease in tPA activity may be due to the increased production of PAI-1 or decreased secretion of tPA, or both. In addition, the formation of microthrombosis in the tumor, which is probably more common in malignant tumors, leads to a consumption of tPA and an eventual decrease in fibrinolytic activity. The extravasation and activation of plasma-clotting factors finally result in fibrin formation. The deposited fibrin, which is ordinarily cleared via the release of tPA, is allowed to build up because of the overall decrease in intravascular fibrinolytic activity. The end result of this cascade is the failure of microcirculation in the tumor tissue and the ensuing tissue necrosis. Finally, the lack of tPA in the necrotic tumor samples does not appear to be due to a nonspecific tissue destruction and cell death since urokinase was readily detectable in the same tissue, as shown in Fig. 4. The presence of uPA in the necrosis samples is incapable of providing sufficient intravascular fibrinolysis because of the lack of specificity of uPA to fibrin and also due to its localization in the extracellular spaces and on cell surface receptors.

Conclusions

The present study demonstrates for the first time the expression of tPA in a large series of human brain tumors and in normal brain. Although the exact sources of tPA remain unknown, these data show that a high tPA content is associated with benign brain tumors, whereas malignant neoplasms have less tPA than normal brain tissue. In addition, tumor necrosis and peri-tumoral edema correlate inversely with the tPA content of the tumor. In view of the importance of tPA in the maintenance of the microvascular patency and tissue oxygenation, it is anticipated that the pharmacological manipulation of this enzyme is likely to affect the growth and therapy of certain types of brain tumors.

References

26. Tryggvason K, Häyhty M, Salo T: Proteolytic degrada-

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