At the beginning of this century, the fibrinolytic properties of neoplastic tissue were noted by Carrel and Burrows 5 and by Fischer 12 through observations of plasma clot dissolution by cancerous cells. Several hypotheses were subsequently presented to explain this phenomenon. However, it was not until a decade ago that Ossowski and coworkers 3,16 demonstrated that the transformation of cultured cells by oncogenic viruses consistently induced an increase in extracellular proteolytic activity due mainly to the release of plasminogen activators (PA’s) from the transformed cells. Plasminogen activators constitute a group of serine proteases that generate fibrinolytic activity by limited proteolysis of the inactive zymogen plasminogen, to form the active proteolytic enzyme plasmin.

More recently, PA’s have been implicated in a wide variety of processes associated with neoplastic transformation, including tumor invasiveness and metastasis, cell migration and decreased adhesiveness, tumor hemorrhage, tumor-host interactions in the form of coagulopathies and thromboembolic events, regulation of local proteolytic activity, increased microvascular permeability, and immunosuppression. 3,13,16,27,31,32,35,42 Because of their association with neoplasia, PA’s have also been proposed as potential biological markers for tumors in man. 20,33

The association of brain tumors and PA function has not received wide attention. Lysis of fibrin by extracts of human brain tumors was demonstrated in the early 1970’s by Böck, et al. 2 and Tovi, et al. 38 using the fibrin plate assay. Other investigators have studied PA production by cultured human and rat glioblastoma cell lines, 8,15,18,19,22,28,37,39,44-47 and in some instances conflicting results were reported concerning the molecular weight and the immunological identity of the specific type of PA present in human brain tumors. 4,28,39,44

Because of the important biological role played by PA’s and their demonstrated association with human brain tumors, we have comparatively analyzed the molecular weight forms and the overall fibrinolytic activity of five common types of brain tumors and of normal brain. The study utilized a newly developed zymographic technique which provides a greater sensitivity than the overlay techniques used previously. This method has facilitated the identification and comparison of the multiple molecular weight forms of PA present in fresh human brain-tumor samples.

Materials and Methods

Tissue Processing

Fresh human tumor and brain tissue was collected in the operating room from patients undergoing surgery, placed immediately on dry ice, and transferred to a -80°C freezer. The tissue was subsequently thawed, weighed, minced, and homogenized at a ratio of 50 mg to 1 ml of 37.5 mM Tris HCl, 0.75 mM ethylenedi-
aminetetra-acetic acid, 75 mM NaCl, and 15 mM lysine at a final pH of 9.5. The protein content of each sample was analyzed according to the method of Lowry, et al.28

Zymographic Analysis

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 (PAGE) procedure of Laemmli.21 The following components were combined and poured into a slab gel apparatus* to form two gels of 9 cm x 14 cm x 1.5 mm, consisting of 17.1 ml of double-distilled water (DDW), 15.0 ml of 1.5 M Tris (pH 8.8), 0.6 ml of 10% SDS, 22.0 ml of 30% acrylamide containing 1.2% bis-acrylamide, 4.0 ml of fibrinogen (10 mg/ml), 1.0 ml of purified human plasminogen (1.1 mg/ml), 0.3 ml of 10% ammonium persulfate, and 0.03 ml TEMED. The resulting 11% acrylamide separating gel was poured into the gel form to a height of 9.3 cm to allow shrinkage to a final height of 9.0 cm upon polymerization. The 3% acrylamide stacking gel consisted of 6.3 ml of DDW, 2.5 ml of 0.5 M Tris (pH 6.8), 0.1 ml of 10% SDS, 1.0 ml of 30% acrylamide containing 1.2% bis-acrylamide, 0.15 ml of 10% ammonium persulfate, and 0.01 ml of TEMED. Plasminogen-free gels were prepared by using purified plasminogen-free fibrinogen following lysine-sepharose chromatography32 and by substituting an equal volume of DDW for plasminogen in the gel mixture.

Sample buffer, consisting of 2.0 ml of 0.5 M Tris (pH 6.8), 3.0 ml of glycerol, 0.2 ml of 0.05% bromophenol blue, and 2.8 ml of 10% SDS, was added in a 1:1 (vol/vol) ratio to the unknowns. Diluted samples were then loaded onto the gels without boiling. The anodal and cathodal running buffers were identical, consisting of 0.5 M Tris, 0.38 M glycine, and 0.1% SDS and adjusted to pH 8.3. Electrophoresis was carried out at 4°C under a constant current of 12.5 mA/slab for 60 minutes followed by 65 mA/slab until the tracking dye reached the bottom of the separating gel. After electrophoresis, the gels were rinsed with DDW and washed twice for 30 minutes each in 1 liter of 2.5% Triton X-100 to remove the SDS. Gel slabs were then incubated overnight (approximately 16 hours) at 37°C in 1 liter of 0.1 M glycine, pH 8.0. The gels were stained in 0.1% amido black in 10% acetic acid and 15% isopropanol for 2 hours and destained in a mixture of 10% acetic acid and 20% methanol. Zones of lysis appeared as transparent clear areas against a uniform background of darkly stained fibrinogen. By using 50 to 75 μg of marker proteins, it was possible to visualize the molecular standards since they stained darker than the fibrinogen in the gel matrix, thus permitting accurate determination of PA molecular weight. When the highly purified tissue type of PA (T-PA), urokinase, or human plasmin is analyzed by our method, the molecular weights and mobilities of each are in agreement with their known molecular weights obtained from sequence data or gel filtration (± 4000 to 5000 daltons). By comparing lysis zones obtained on plasminogen fibrinogen gels with those obtained on plasminogen-free fibrinogen gels, one can specifically distinguish PA activity from plasminolysis or nonspecific proteolysis.

Determination of Lysis Intensity

Each lysis zone within a given sample lane was analyzed by scanning densitometry.† The total lysis per sample, as compared to a background non-lysed zone, was proportional to the integrated surface area outlined by the densitometer. Values were expressed as lysis per microliter of sample per milligram of protein.

Determination of Brain Edema

Computerized tomography (CT) scans were analyzed for each patient according to a previously described procedure.14 The degree of brain edema was graded on a scale of 0 to 5. A value of 0 represented total lack of visible edema while 5 represented severe edema.

Statistical Analyses

The mean protein concentration, lysis, and brain edema for each sample were analyzed by standard statistical procedures. Differences among groups were evaluated by analysis of variance, and correlation coefficients were obtained by linear regression.

Results

A total of 58 samples were analyzed, and the results are reported below by sample group according to histological diagnosis as follows: metastatic, acoustic neurinoma, glioblastoma, meningioma, low-grade glioma, and normal brain. The protein content of the homogenized human tissue was not statistically different among the groups (Table 1). The PA activity, molecular weight patterns, and major characteristics are presented for each group in Figs. 1 to 4.

Metastatic Group

The metastatic group comprised 10 patients. The primary neoplasms originated from the lungs in seven patients, from an unidentified source in two, and from the kidneys in one. This group demonstrated the highest degree of PA activity, which was statistically significantly greater than the other groups with the exception of the acoustic neurinoma group (Table 1).

The molecular weight pattern was highly characteristic and uniform for this group (Fig. 3). In addition to the prominent lytic bands seen at 36, 60, and 94 kD in all the samples, lytic bands were found at the 84-kD

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* Slab gel apparatus, Model 220, manufactured by Bio-Rad Laboratories, Richmond, California.

† Scanning densitometer manufactured by Ephortec-Joyce-Loebl, Newcastle, England.
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### TABLE 1

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>No. of Samples</th>
<th>Protein Content† (mg/ml)</th>
<th>PA Activity (lysis/μl/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>metastatic</td>
<td>10</td>
<td>39.8 ± 8.9</td>
<td>151.4 ± 66.6</td>
</tr>
<tr>
<td>acoustic neurinoma</td>
<td>10</td>
<td>49.2 ± 13.1</td>
<td>150 ± 40.1</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>13</td>
<td>47.5 ± 14.3</td>
<td>105 ± 40.0</td>
</tr>
<tr>
<td>meningioma</td>
<td>16</td>
<td>47.1 ± 17.2</td>
<td>87.5 ± 31.2</td>
</tr>
<tr>
<td>low-grade glioma</td>
<td>6</td>
<td>41.6 ± 5.3</td>
<td>73.7 ± 31.2</td>
</tr>
<tr>
<td>normal brain</td>
<td>3</td>
<td>39.4 ± 2.5</td>
<td>52.3 ± 18.6</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations. PA = plasminogen activator.
† No statistically significant difference.
‡ Statistically different (p < 0.05).

zone in six of the samples and at the 50- to 55-kD zone in three. A “trail” of lytic activity was noted between the 60- and 94-kD bands (Fig. 1). Of particular significance was the lack of prominent lytic bands in the molecular weight range of 60 to 84 kD. Although minor lytic bands may have been present within this zone and could have been masked by the trail, the lack of major lytic zones within this range in all 10 samples clearly separates this group from all of the other primary brain-tumor groups.

**Acoustic Neurinoma Group**

The acoustic neurinoma group consisted of 10 patients. The PA activity of these tumors was surprisingly high, almost identical to that of the metastatic group. The elevation of PA levels was statistically significant when compared to the remaining four groups (Table 1). This group had the greatest number of lytic bands with a characteristic molecular weight pattern (Fig. 3). In addition to the lytic bands found in all samples at 60, 74, and 94 kD, lytic bands were noted at 36 kD in nine samples, at 100 kD in seven, at 84 kD in five, and at 68 kD in four. It is important to note the frequent finding of the band at 36 kD (which is also seen in the highly malignant samples) and the characteristically dense lytic band at 74 kD (Fig. 1).

**Glioblastoma Group**

There were 13 patients in the group with highly anaplastic glioma. The mean PA activity for this group was consistently higher than that obtained for normal brain, low-grade glioma, and meningioma; however, this increased level was not statistically significant (Table 1). Four of the six samples with lytic activity below the mean level for this group were derived from tumors that had been previously irradiated (within 6 months of the surgery). Only one of the seven samples with lytic activity above the mean value for this group was derived from a tumor that was irradiated (3 years previously).

The molecular weight pattern of PA’s in this group of tumors varied greatly and in some instances resembled that observed in the acoustic neurinoma or low-grade glioma groups (Fig. 3). In addition to the 60-kD band found in all samples, the 74-kD band was found in 10 samples, the 36- and 94-kD bands in eight, the 84-kD bands in seven, and the 68-kD bands in six. In three samples the pattern was indistinguishable from that seen with low-grade glioma (which may be a result of sampling during surgery of low-grade tumor tissue contained in this malignant glioma), and in two samples the pattern was similar to that of acoustic neurinoma. The primary differentiating element of the glioblastoma group was either a prominent 84-kD band or an absent 94-kD band.

**Meningioma Group**

The meningioma group was the largest group and was composed of 16 patients. The mean PA activity for this group was consistently, but not significantly, higher than that of the low-grade glioma group. The PA molecular weight pattern was very constant and characteristic. All tumor samples in this group had a lytic band at 60, 68, 74, and 84 kD, with only one sample lacking a detectable lytic band at 84 kD. The lytic band most characteristic for this group was that migrating at 84 kD. While some variation in intensity was evident, this band is important in differentiating the meningioma group from the low-grade glioma group.

**Low-Grade Glioma Group**

Six patients comprised the low-grade glioma group. Three patients had a grade 2 astrocytoma, two patients had an oligodendroglioma, and one patient had a mixed astrocytoma-oligodendroglioma. The mean PA activity for this group was slightly higher but not statistically different from that of the normal brain group (Table 1). The two highest values were obtained in samples from a patient with a recurrent oligodendroglioma and another patient with a mixed glioma. All six tumors
had similar migrating PA forms, with lytic bands at 60, 68, and 74 kD. No other lytic bands were noted for any of the six samples.

**Normal Brain Group**

Cortical brain samples were obtained from three patients: two with hypertensive intracerebral hematoma and one with an anaplastic astrocytoma located in the deep white matter of the frontal lobe. The PA molecular weight distribution was characteristically composed of a distant band at 60 kD. Although two of the samples had faint lytic bands at 68 and 74 kD (Fig. 3), the intensity of these latter bands was consistently less than that observed in the low-grade glioma samples.

**Brain Edema**

Mean brain edema scores (obtained from CT scan data) for the entire group of 55 brain tumors were as follows: metastatic 3.5, glioblastoma 2.1, low-grade glioma 1.2, meningioma 1.1, and acoustic neurinoma 1. The correlation between PA activity and edema score for all the groups was $r = 0.321$ ($p < 0.05$). The

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**Fig. 2.** Densitometric tracings of various tumor extracts and normal brain following zymographic analysis.

**Fig. 3.** Molecular weight heterogeneity of plasminogen activators in human brain tumors. Numbers in parentheses represent the total number of samples per group; molecular weight is expressed in daltons.
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FIG. 4. Plasminogen activator activity in extracts of human brain tumors. Bars represent the mean activity for each tumor type.

correlation coefficients for the meningioma, glioblastoma, and metastatic groups were 0.402, 0.376, and 0.220, respectively. The remaining two groups were too small to evaluate separately.

Discussion

Plasminogen activators in tumors of the central nervous system (CNS) have received very little study despite the intense investigation into the possible role of this enzyme in cancer biology in general. In this study, we have used modifications of the techniques described by Laemmli and by Roche, et al., to comparatively analyze the molecular weight forms of PA's and the overall fibrinolytic capacity of five common types of brain tumor and of normal brain. By incorporating the physiological substrates fibrinogen and plasminogen as co-polymers into SDS polyacrylamide gels, we were able to gain greater sensitivity and visibility of lytic bands with our zymographic method than has been possible with conventional overlay techniques.

The molecular weight pattern for each type of brain tumor was uniquely different, with few exceptions. The lack of appreciable lytic bands at 68 and 74 kD in the metastatic group is of particular interest because of the marked biological differences between primary and metastatic brain tumors. Furthermore, our data suggest that the distinct molecular weight patterns of PA’s in various brain tumors may allow their use as tumor markers.

The finding of multiple molecular weight forms of PA in non-CNS neoplastic tissue has been described previously, with values ranging from 28 to 165 kD. This has raised several unanswered questions. For instance, are the larger PA forms the product of different genes, or are they aggregates of the smaller forms? The precise cellular origin of the various forms of brain-tumor PA’s is not known. Undoubtedly, some cells that were uniformly present in all of our tumor samples account for some of the PA detected. For example, the 60-kD form, present in all tumor extracts, might be produced in part by the endothelial cells that are certainly present in each of the tumor extracts. However, the finding of consistent differences among the groups is more in favor of tumor cell-specific forms of PA. This theory is also supported by the variability in molecular weight patterns seen within the glioblastoma group, a tumor known for its heterogeneous cell population.

Our studies demonstrate a lack of association between a specific molecular weight form of PA and the malignant phenotype since extracts of acoustic neurinoma (a benign tumor) consistently contained lytic bands at 36 and 94 kD, similar to those seen in metastatic tumors in glioblastomas. On the other hand, differences were evident within the glioma group, and low-grade gliomas can in most circumstances be differentiated from highly malignant gliomas because the former contains fewer PA forms and has a lower fibrinolytic capacity (Figs. 3 and 4).

Tumor-associated PA can also be differentiated immunologically. The urokinase type of PA (U-PA) is generally associated with PA forms of 30- and 60-kD molecular weight, while T-PA is associated with PA’s with a molecular weight of 70 kD. Other molecular weight forms are of uncertain types and may be associated with either U-PA or with other unspecified forms of PA. Preliminary data from our laboratory indicate that both PA types are produced by the brain tumors studied and that some PA forms are resistant to antibodies against both PA types. Colombi, et al., have shown that PA forms detected in human breast tumors were different from U-PA and the melanoma T-PA, adding support to our findings in brain tumors; namely, that additional forms of PA are likely to be present in the various tumors studied, offering exciting diagnostic and therapeutic potential. On the other hand, recent studies have shown the potential prognostic significance of identifying the immunological type of PA associated with the neoplastic tissue since T-PA exerted transformation-enhancing activity that was not detected with U-PA. Although the molecular weight patterns of the PA’s present in each tumor type were quite distinct, significant differences in the overall fibrinolytic capacity were only demonstrable for the metastatic and acoustic neurinoma groups.

The markedly increased lysis associated with acoustic
neurinoma was unexpected because of the increasingly recognized role of PA’s in metastasis and malignancy. The following explanations analyze our paradoxical finding of a relatively high PA activity in acoustic neurinoma (a benign, noninvasive, and nonmetastasizing tumor) in comparison to the malignant brain tumors. 1) It is possible that the actual fibrinolytic activity of the malignant tumors studied is higher than that detected by our zymographic assay. The fibrinolytic activity, as measured in this study, represents the average of the activity of all the homogenized tissue material present in the sample. Since metastatic and glioblastoma samples frequently contain necrotic tissue with potentially lower fibrinolytic activity, 2 it is possible that the actual fibrinolytic activity of these two tumors is even higher than that demonstrated in this study. In addition, the PA activity of the glioblastoma group is further reduced by the inclusion of tumor samples recently subjected to radiation therapy. 17 2) Inhibitors of fibrinolysis may be present in acoustic tumors and therefore may prevent these tumors from metastasizing. Malone, et al., 21 have shown that tumor spread correlated directly with tumor activation of fibrinolysis and inversely with inhibition of fibrinolysis. This concept is very relevant to the condition in our experiments since the electrophoretic technique used in our laboratory separated the non-covalently bound inhibitors from the activators on the basis of their molecular weight. 3) Plasminogen activators may represent only one essential step in promoting invasiveness and metastasis. Additional factors that are lacking in acoustic tumors may be required to carry the metastatic process further. 35 4) Finally, the immunological identity of the PA’s present in acoustic neurinomas may be different from that of the PA’s present in malignant brain tumors, since, as discussed previously, certain PA types have greater transformation effect on cells than others. 9

The enhanced fibrinolytic activity of the various forms of brain tumors in comparison to normal brain tissue may have practical clinical significance. For instance, spontaneous hemorrhages have frequently been reported in association with both the benign and malignant forms of brain tumors. 26,43 The most common explanation given relies on a mechanistic theory of vessel rupture secondary to the stretching or invasion of the tumor vessels. 45 Our results suggest that spontaneous tumor hemorrhage in the absence of evident systemic coagulopathy may be due to a locally enhanced fibrinolytic activity which, in turn, may be aggravated by tumor necrosis and vessel rupture. 5,24

Brain edema frequently occurs in association with brain tumors and is most prominent with metastatic brain tumors. 14 It is thought that the enhanced fibrinolytic activity of the tumor may affect the vascular permeability in the region of the tumor. 32 Our own results correlating fibrinolysis and brain edema as detected on CT scanning have shown a definite correlation (p = 0.046), but one that is weak (r = 0.321). The limitations of this evaluation relate to the qualitative measurement of brain edema and to the fact that other enzymatic processes could be involved in the proteolytic degradation of fibrin and in the disruption of the blood-brain barrier, with the resulting vasogenic brain edema. Based on our results, we do not believe that PA’s could solely account for brain edema associated with brain tumors.

Finally, PA inhibition may find a role in the therapy of brain tumors. For instance, we have demonstrated that antifibrinolytic therapy with epsilon-aminocaproic acid (EACA) retarded the growth of a human glioblastoma transplanted subcutaneously in nude mice and prolonged the survival of the treated animals. 36 Recently, Ossowski and Reich 30 have effectively inhibited the metastasis of a human tumor cell line inoculated into chicken embryos by the use of antibodies to urokinase. Further studies identifying and differentiating the role of the various types of PA’s will undoubtedly provide important and critical information concerning the clinical and biological roles of the fibrinolytic enzyme system in brain tumors.

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Address reprint requests to: Raymond Sawaya, M.D., Department of Neurosurgery, University of Cincinnati Medical Center, 231 Bethesda Avenue (M.L. 515), Cincinnati, Ohio 45267.