In Vitro Plasminogen Activator Activity in Human Brain Tumors


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ABSTRACT

Cell cultures were prepared from nine human brain tumors. Fibrin plate assays showed plasminogen-dependent fibrinolytic activity in lysates and in material released by these neoplastic cells but not in those from normal adult human white matter.

Antibodies against human urokinase caused catalytic inhibition of the urokinase and of the plasminogen activator from WI-38 cells, simian virus 40-transformed WI-38 cells, human prostatic cells, and human ovarian carcinoma cells. However, the anti-urokinase immunoglobulin G did not inhibit the plasminogen activator activity of any of the human brain tumor preparations. These studies indicate that the plasminogen activator produced by human brain tumor cells is antigenically different from the plasminogen activator of other human normal and neoplastic cells.

INTRODUCTION

An increase in the production of plasminogen activator enzymes after transformation by chemical carcinogens or oncogenic viruses has been demonstrated in mammalian and avian cells in culture (25, 27, 36). The significance of this altered proteolytic activity of transformed cells to the behavior of tumor cells in vivo remains uncertain. Although there are exceptions (24, 29, 35), increasing evidence supports the role of plasminogen activator enzymes in the induction and maintenance of the transformed phenotype in vitro.

There is no agreement about the nature of the plasminogen activator enzyme(s) found in human normal tissues or the relationship of the enzyme(s) to UK,4 the plasminogen activator found in urine. It has been suggested that certain human tissue plasminogen activators and UK are identical, at least immunologically (5, 6), but other evidence suggests that they differ (2, 18, 19). However, immunological cross-reactivity has been reported between UK and the plasminogen activator produced by cultured human brain tumor cells of different ovarian carcinomas (3).

Studies have been made of the role of plasminogen activator activity in human tumors. Both fragments of freshly removed tumor tissue and cultured tumor cells have been assayed for fibrinolysins (3, 7, 8, 12, 13, 21, 26, 31, 37, 38), and they have shown increased production of plasminogen activator enzyme(s). Although fragments of brain tumors of several histological types have shown plasminogen activator activity (8) and histochemical studies of normal human brain have shown that the fibrinolytic activity is localized to the meninges and vessels (34), no report is available on the plasminogen activator of cultured normal and neoplastic brain cells. In this study, in order to eliminate any enzymes produced by vessels or related to secondary changes in the tumor tissue, cell cultures were used to study the plasminogen activator activity of human brain tumors.

In this paper we report on the plasminogen activator activity of cultures of several types of brain tumors, normal brain, prostate, ovarian carcinoma cells, WI-38 cells, and SV40-transformed WI-38 cells. We have used an antibody against UK to examine these plasminogen activator substances immunologically.

MATERIALS AND METHODS

Cell Cultures. Fresh human brain tumor tissue, obtained at operation, was explanted and grown, with the use of Eagle's modified minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 15% fetal bovine serum. Subconfluent cultures were examined for fibrinolytic activity. The cultures had undergone fewer than 10 passages in vitro with the exception of glioblastoma cultures. One of these is an established line (VH) and has been used in previous reports (17), while the other (H) had undergone over 20 passages in vitro.

WI-38 human embryo lung cells, and SV40-transformed WI-38 cells were kindly provided by Dr. J. M. Lehman (University of Colorado Medical Center, Denver, Colo.). Human ovarian carcinoma cells in culture (COLO-110) were purchased from Dr. G. Moore (Denver General Hospital, Denver, Colo.). Normal human prostate cells in culture were provided by Dr. M. Webber (University of Colorado, Denver, Colo.).

Brain tumor cells were also stained by the indirect immunoperoxidase method for the presence of S-100 protein (23, 33) to verify their neuroectodermal origin. Dr. B. W. Moore (Washington University, St. Louis, Mo.) kindly provided the rabbit antiserum directed against bovine S-100 protein.

Preparation of Cell Lysates. The method used to prepare lysates of subconfluent cell cultures was similar to that reported by Pollack et al. (27) and by Wigler et al. (39). The total protein concentration of each lysate was determined by the method of Lowry et al. (22) and adjusted to a value of 1.0 mg/ml.

Released Plasminogen Activator. The medium was removed from subconfluent cell cultures, and the cells washed once with PBS. The cells were then incubated for 24 hr in 10 ml of this buffer, which was collected and

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4 The abbreviations used are: UK, urokinase; SV40, simian virus 40; PBS, 0.05 M phosphate-0.15 M NaCl, pH 7.4; DFP, diisopropylfluorophosphate.
clarified by centrifugation at 10,000 × g for 10 min and then concentrated 100 times by pressure dialysis. It was not possible to detect protein, even in the concentrated specimens. Medium with serum was added to the cell cultures after this procedure, and the cells remained viable.

**Preparation of Fibrin Plates.** A modification of the fibrin plate method of Astrup and Müllertz (4) was used. Plates were made by pouring a total volume of 2.0 ml of the material onto a 75 × 25 glass microscope slide. If no plasminogen was added or if the amount were altered, the amount of buffer used was correspondingly modified to keep constant the volume, and hence the thickness, of the fibrin layer. Each plate contained 2.2 NIH units of bovine thrombin [Thrombin, Topical (Bovine Origin); Parke, Davis, and Co., Detroit, Mich.]. Crude bovine fibrinogen, Fraction 1 (Calbiochem, San Diego, Calif.) was purified by the method of Laki (20) and stored in plastic tubes at −20°. Seven mg of this fibrinogen preparation were incorporated in each plate. Dog plasminogen was prepared by lysine-agarose affinity chromatography (10). The amount of dog plasminogen in each plate was 15 μg unless otherwise specified. The thrombin, plasminogen, and finally the fibrinogen were added to aliquots of agarose (Sigma Chemical Co., St. Louis, Mo.) to yield a final concentration of 1% agarose in PBS at 55°; mixed quickly, and poured onto the glass slides.

For assay of a sample, 10 μl of cell lysate or cell supernate were placed in a well in the fibrin plate and incubated at 37° in a moist atmosphere. Periodic observation for 48 hr showed that active preparations produced a concentric zone of lysis about the center well which enlarged with time (Fig. 1). The area of the lytic zones reflects the activity of the material. Slides were fixed in a 1% solution of picric acid in 15% acetic acid at the conclusion of each assay.

**Lysis of ¹²⁵I-Labeled Fibrin.** Iodinated bovine ¹²⁵I-labeled fibrinogen was diluted with nonradioactive fibrinogen to 9.1 × 10⁷ cpm/mg protein. Aliquots were pipetted into the 16-mm-diameter wells of plastic trays and dried to a film as described by Unkeless et al. (35). Each well contained 1.0 × 10⁶ cpm. The ¹²⁵I-labeled fibrinogen film was converted to fibrin by a 15-min exposure to 0.4 NIH unit of bovine thrombin (Parke-Davis) in 1.0 ml of PBS. The thrombin solution was removed, and the ¹²⁵I-labeled fibrin was washed twice with the buffer. Then 0.9 ml of buffer, 0.05 ml of dog plasminogen (0.3 mg/ml), and 0.05 ml of test sample were added to each well; the mixture was incubated at 37°, and 0.05-ml aliquots were removed at selected intervals. The radioactivity released into the solution was measured. Control values were obtained, with the use of identical preparations, except that 0.05 ml of the buffered solvent used to prepare the lysates was added instead of an enzymatic preparation. Control values were subtracted from the values obtained at the same time for the active preparations.

**Effect of DFP.** The tumor plasminogen activator preparations were treated with DFP to assess inhibition (15). A stock solution of 0.5 M DFP in isopropyl alcohol was diluted to 10 mM with 0.1 M Tris-HCl, pH 7.0. One ml of the buffered 10 mM DFP solution was added to 1 ml of the tumor cell lysate, the mixture was incubated for 0.5 hr at room temperature, and the treatment was repeated with another ml of DFP solution. The Tris-HCl buffer, without DFP, was used to treat control tumor preparations similarly. Then the treated and control materials were dialyzed against 1000 volumes of PBS at 4° for 4 hr. The activity of the treated and control material was then tested in parallel fibrin plate assays.

**Preparation and Testing of Anti-UK Antibody.** Rabbit antiserum was prepared against human UK (2128 Ploug units/mg; ICN Pharmaceuticals, Inc., Cleveland, Ohio). A solution of 2.0 mg/ml was prepared in PBS, and 0.5 ml of this solution was mixed with incomplete Freund’s adjuvant and injected i.c. The animals were boosted with 0.08 ml of the UK injected i.v. All tests were carried out with serum obtained at the time of exsanguination.

Because normal serum contains protease inhibitors (28), IgG was prepared from the antiserum by affinity chromatography.

Immunodiffusion tests were carried out in 1% agarose in PBS. Immunelectrophoresis was done with gels of 1% agarose in barbital buffer (ionic strength, 0.02; pH 8.4). Tests for the inhibitory effect of anti-UK IgG on fibrinolytically active material were carried out in standard fibrin plates containing plasminogen as described above, according to a method based on a procedure described by Holm and Möller (16), and modified for plasminogen activator studies by Christian et al. (11). The lytic material was placed in the center well with anti-UK IgG and control rabbit IgG placed in surrounding wells. The plates were then incubated at 4° for 12 hr to allow diffusion and antigen-antibody interaction, and then they were incubated at 37° to activate the fibrinolytic enzymes. Inhibition was evident when the enlarging circle of lysis about the center well was sharply restricted toward the well containing anti-UK IgG.

**RESULTS**

**Brain Tumor Plasminogen Activator.** Cultures of the primary brain tumor cells stained positively for S-100 protein with the indirect immunoperoxidase method, confirming their neuroectodermal origin (23). Fibrinolytic activity was demonstrated by the fibrin plate method in lysates and released material from cultures of all 8 primary brain tumors and from a cerebral secondary melanoma. Cells cultured from normal adult brain showed no detectable fibrinolytic activity. The primary brain tumors cultured included 5 glioblastomas, an ependymoblastoma, and 2 meningiomas. There were quantitative differences in the fibrinolytic activity of lysates from different tumors. A crude approximation of the relative activity of the various tumor lysates, matched in protein concentration (1.0 mg/ml total protein), is given in Table 1. The area of lysis of standard fibrin plates in 1 hr was compared with the lysis produced by a 1.0-mg/ml solution of UK (B grade; Calbiochem). The UK activity was given an arbitrary value of 100 units. Similar quantitative estimates could not be made for the plasminogen activator activity released from the cells because the protein levels were below the limits of detection.

The fibrinolytic activity of the tumor lysates and released material was plasminogen dependent. The fibrin plates without plasminogen showed negligible or no lysis in the time required to produce large lytic zones in the plates.
with plasminogen. The major fibrinolytic action of the tumor material was due to the added plasminogen. Human plasminogen was also activated by the released plasminogen activator and by the lysates of all the brain tumors. There was a linear relationship between the amount of plasminogen and the resultant fibrinolysis in the range of 0 to 50 μg of dog plasminogen per plate; addition of more than 50 μg of plasminogen did not result in larger zones of fibrinolysis.

Treatment of the brain tumor lysates and released material with DFP resulted in complete inhibition of their plasminogen activator activity as compared to control preparations. Thus, the brain tumor cells produce a serine protease (15) that can convert the zymogen, plasminogen, to the active proteolytic enzyme, plasmin, which in turn causes lysis of fibrin (10).

Immunological Studies with Anti-UK Antibody. In an effort to further characterize the brain tumor plasminogen activator(s), an antiserum was prepared in the rabbit against human UK as described above. Experiments showed that nonimmunized control rabbit serum inhibited fibrinolysis by UK and by tumor material, but normal rabbit IgG isolated from this serum did not. Thus, in all inhibition studies, identically prepared rabbit control and anti-UK IgG were used.

Both the anti-UK serum and its IgG gave single precipitin lines in immunodiffusion and immunoelectrophoresis against UK (Fig. 2). Sharp inhibition of fibrinolysis was seen adjacent to the anti-UK IgG well when UK was assayed on fibrin plates surrounded by control and anti-UK IgG solutions (Fig. 3A). Immune precipitin lines were seen with difficulty in the latter type of preparation because of obscuration by fibrin incorporated in the plates, but a precipitin line is visible in Fig. 3B.

For further demonstration of the inhibitory effect of anti-UK IgG on plasminogen activation by UK, the effect on lysis of 125I-labeled fibrin by the enzyme was studied. The rate of release of 125I-labeled fibrin split products was clearly inhibited by a final concentration of 0.1 mg of anti-UK IgG per ml, compared to a control preparation with 0.1 mg of normal rabbit IgG per ml. The effect was enhanced with 1.0 mg of anti-UK IgG per ml, while the more concentrated control IgG solution had no greater effect than the dilute control (Table 2).

In order to demonstrate that the anti-UK serum and its IgG were directed against the plasminogen activator activity and not against the plasmin activity, aliquots of dog plasminogen were activated by incubation at 37° for 1 hr with either UK or Varidase (streptokinase-streptodornase; Ledere Laboratories, Pearl River, N. Y.). The Varidase-activated plasmin was used in immune diffusion against anti-UK serum, and no precipitin line was seen. Both preparations were assayed on fibrin plates that contained no added plasminogen, with anti-UK and control IgG in surrounding wells. In each case there was no inhibitory effect; the resultant zones of lysis were circular with no restriction toward the anti-UK well.

In order to ascertain whether cultured cell lysates could be inhibited with the anti-UK IgG, lysates from a line of human ovarian carcinoma cells (COLO-110) and normal human prostatic epithelial cells were studied. In both the ovarian carcinoma cells and the normal prostatic cells, plasminogen-dependent fibrinolytic activity was demonstrable in cell lysates, the prostate being the more active material. Furthermore, the anti-UK IgG inhibited the lysis of fibrin plates by both human prostatic and ovarian carcinoma cell lysates in a manner similar to that observed with UK. The prostatic lysate gave a single line of identity with the UK line in an immune diffusion test against the anti-UK serum. The ovarian carcinoma lysate did not give a detectable precipitin line with the antiserum.

Lysates were also prepared from WI-38 human embryo lung cells and their SV40-transformed counterparts. Both the WI-38 cells and the SV40-transformed WI-38 cells pro-

Table 1

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Relative plasminogen activator activity</th>
</tr>
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<tbody>
<tr>
<td>UK solution</td>
<td>100</td>
</tr>
<tr>
<td>Normal adult glial lysate</td>
<td>0</td>
</tr>
<tr>
<td>Glioblastoma lysate 1 (VH)</td>
<td>3</td>
</tr>
<tr>
<td>Glioblastoma lysate 2 (H)</td>
<td>6</td>
</tr>
<tr>
<td>Glioblastoma lysate 3</td>
<td>3</td>
</tr>
<tr>
<td>Glioblastoma lysate 4</td>
<td>11</td>
</tr>
<tr>
<td>Glioblastoma lysate 5</td>
<td>3</td>
</tr>
<tr>
<td>Ependymoblastoma lysate</td>
<td>5</td>
</tr>
<tr>
<td>Meningioma lysate 1</td>
<td>9</td>
</tr>
<tr>
<td>Meningioma lysate 2</td>
<td>2</td>
</tr>
<tr>
<td>Melanoma lysate</td>
<td>20</td>
</tr>
</tbody>
</table>

Effect of anti-UK IgG on the release of 125I-labeled fibrin split products by UK

The anti-UK IgG inhibits the release of 125I-labeled fibrin split products by UK, and the inhibitory effect is increased by the use of a more concentrated anti-UK solution. The concentration of UK in each case is 1.25 μg/ml.

<table>
<thead>
<tr>
<th>IgG</th>
<th>Concentration (mg/ml)</th>
<th>30 min (x 10^4)</th>
<th>60 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbit IgG</td>
<td>0.1</td>
<td>5.9</td>
<td>6.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal rabbit IgG</td>
<td>1.0</td>
<td>5.5</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-UK IgG</td>
<td>0.1</td>
<td>3.8</td>
<td>7.1</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Anti-UK IgG</td>
<td>1.0</td>
<td>2.0</td>
<td>4.7</td>
<td>64</td>
<td>34</td>
</tr>
</tbody>
</table>

* Values represent the mean of triplicate determinations.
duced very active lysates that caused plasminogen-dependent lysis of fibrin plates. The SV40-transformed cell lysate was somewhat less active than the untransformed cell material was. However, both lysates were inhibited by the anti-UK IgG. Fig. 2B shows the restriction of WI-38 lysis adjacent to the well of anti-UK IgG. Lysates from the transformed and untransformed WI-38 cultures gave single immune precipitin lines in immunodiffusion tests with the anti-UK serum. The prostate, COLO-110, WI-38, and SV40-transformed WI-38 cell lysates and UK were all inhibited by the DFP treatment used for the brain tumor preparations.

**Brain Tumor Plasminogen Activator and Anti-UK Antibody.** Each of the brain tumor lysates was tested in immune diffusion and immunoelectrophoresis against the anti-UK serum, and no precipitin lines were seen. The brain tumor lysates were also tested on fibrin plates to see whether the anti-UK IgG would restrict lysis. There was no inhibition (Fig. 3C). Similarly, anti-UK IgG had no inhibitory effect on fibrin plate lysis by the plasminogen activator released by brain tumors in vitro.

Thus, the plasminogen activator substance(s) produced by a variety of brain tumor cells in vitro is (are) immunologically distinct from UK, while the plasminogen activator produced by transformed and untransformed WI-38 human embryo lung cells, an ovarian carcinoma, and normal prostate cells in vitro are immunologically similar to UK.

**DISCUSSION**

This study demonstrates that brain tumor cells produce a substance or substances in vitro that can activate the fibrinolytic system by converting the zymogen, plasminogen, to the active protease, plasmin. The plasminogen activator is found in lysates made from the cultured cells and is released by the cells when they are incubated in PBS. The activating substance is inhibited by DFP and is therefore a serine protease.

Assays of lysates of cultured normal human glial cells suggest that they do not produce detectable plasminogen activator in vitro. However, it is possible that the plasminogen activator action is masked by inhibitors such as the plasminogen activator inhibitors reported in serum and tissues by Aoki and Kawano (1) or the trypsin inhibitor found in normal brain by Brecher and Quinn (9). The histochemical work of Takashima et al. (34), reporting that the fibrinolytic activity of human brain appears to be confined to vascular endothelial cells, meninges, and choroid plexus, supports our results. Whether the neoplastic glial cells in vitro produce plasminogen activators not produced by their normal counterparts or produce larger quantities of plasminogen activators that overwhelm available inhibitors, or merely produce less inhibitor, is uncertain. The net effect is that the glial tumor cells are associated in vitro with increased fibrinolytic activity.

Meningioma cells in culture produce plasminogen activators, but it is unknown whether their fibrinolytic activity exceeds that of their normal arachnoidal counterparts. Studies in several species, including humans, suggest that the leptomeninges normally produce high plasminogen activator levels (14, 30, 34), but to our knowledge cultured arachnoidal cells have not been studied for this function.

There is, as outlined earlier, no agreement about the relationship of normal tissue plasminogen activator enzymes to UK. We have found that the plasminogen activator from ovarian carcinoma is immunologically similar to UK, as has been reported by Åstedt and Holmberg (3). The normal prostate cells were selected for study because of the relationship of the prostate to the genitourinary system where UK is normally found and because of reports of high levels of plasminogen activator in the prostate gland of various species. The plasminogen activator from normal prostate epithelial cells is immunologically similar to UK. This study shows that the plasminogen activator(s) produced by cultured brain tumor cells in the present study is (are) immunologically different from UK.

Christman et al. (11) concluded that different hamster transformed cell lines produced different plasminogen activators, although they were unable to determine whether the type of plasminogen activator enzyme produced was determined by the original cell type or by the agent transforming the cells. Rifkin et al. (28) and Laug et al. (21) have described the plasminogen activator activity of WI-38 cells, and Bernik et al. (5) have reported that the released plasminogen activator activity from WI-38 cells can be partially neutralized by an anti-UK serum. We have demonstrated that the plasminogen activator enzyme produced by WI-38 human embryo lung cells before and after transformation is immunologically similar. Whether or not this is a general phenomenon will await the examination of further normal and transformed cell lines.

The present studies reveal that brain tumor cells both produce and release plasminogen activator which is immunologically different from UK. However, further study must be made to determine whether the invasiveness and the perineoplastic edema associated with these tumors are correlated with the amount and type of plasminogen activator produced.

**REFERENCES**

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Fig. 1. Fibrin plate assay of the plasminogen-dependent lytic activity of a cultured glioblastoma cell lysate. The plate was incubated with the lysate for 24 hr at 37°C in a moist atmosphere. Left to right, undiluted lysate (protein concentration, 1.0 mg/ml); a 1:4 dilution; a 1:10 dilution; control buffered solvent (0.5% Triton X-100-0.1 M Tris-HCl, pH 8.1).

Fig. 2. A single immune precipitin line is seen when UK and anti-UK IgG are reacted in immunoelectrophoresis.

Fig. 3. A, inhibition of the zone of fibrinolysis caused by UK (center well) on a fibrin plate is seen by the sharp restriction of the otherwise circular zone toward the well containing anti-UK IgG (right upper). The other wells, containing control rabbit IgG, cause no inhibition of lysis. B, inhibition of fibrinolysis, due to a lysate of WI-38 cells. Center well, WI-38 cell lysate; right upper well, anti-UK IgG; control rabbit IgG is in the other 3 wells. Note the fine precipitin line between the lysate and the anti-UK IgG. C, no inhibition of fibrinolysis by a brain tumor lysate (center well) is seen when anti-UK IgG is placed adjacent to it (right upper well). The other surrounding wells contain normal rabbit IgG.
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