Expression of Heterogeneous Profiles of Plasminogen Activators and Plasminogen Activator Inhibitors by Human Glioma Lines

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ABSTRACT

Expression of plasminogen activator (PA) enzyme activity is believed to be one of the mechanisms by which malignant cells cause pericellular proteolysis of stromal structures during implantation and tissue invasion. In this study, four cell lines derived from human gliomas were studied to ascertain which PA enzymes and PA inhibitors determine the level of secreted PA activity. A plasminogen-dependent esterolytic assay was used, and two lines (U251 and U373) were found to secrete high levels of PA activity, while PA activity was undetectable in the conditioned media from the remaining two lines (U138 and LM). The PA produced by U251 and U373 resolved as single bands comigrating with high molecular weight urokinase (M, 54,000) on casein-plasminogen zymography. Northern blot analysis demonstrated high levels of mRNA for urokinase-type PA (uPA) in U251 and U373, as well as a considerably lower level of uPA message in LM. U251 and U373 also contained mRNA for tissue-type PA (tPA), although secreted tPA activity was not demonstrated by zymography. The U138 line contained essentially undetectable levels of mRNA for either uPA or tPA. U138 was also unique in secreting PA inhibitor activity and contained high levels of mRNA for PA inhibitor 2, which was not seen in any other line. All cell lines contained PA inhibitor 1 mRNA, with substantially more expression in the U138 and LM lines than in U251 and U373. None of the lines secreted measurable anti-plasmin activity. We conclude that there is considerable heterogeneity among human glioma cells in expression of PA enzymes and PA inhibitors. The coordinated regulation of these proteins likely determines secreted PA activity and the resultant role of plasminogen activation in tumor implantation and invasion.

INTRODUCTION

There is a wealth of evidence to establish that augmented expression of PA activity is a common, if not characteristic, feature of the malignant phenotype (1). Enhanced expression of PA activity has been demonstrated histochemically in tumors in situ, in tumor extracts, and in a variety of malignant cell lines (1). Viral transformation of nonmalignant cells in vitro and treatment with tumor promoters likewise result in increased PA activity (1). Although high levels of PA activity have been consistently associated with several types of malignant cells, we (2) and others (3, 4) have found significant variability in PA secretion by glioma cells. In our recent studies, we found high levels of PA activity in culture fluids from 12 of 12 human squamous carcinoma and malignant melanoma lines, while culture fluids from only 2 of 6 glioma lines had detectable activity (2). We did not investigate whether the failure to detect PA activity in the glioma culture fluids reflects a lack of PA synthesis or overproduction of PA inhibitors. In general, this question has been pursued in only a fragmentary way. Many studies have only shown the results of functional assays for PA activity which could not distinguish between uPA and tPA (1, 4). Because the two forms of PA are functionally different, the type(s) of PA produced by a malignant cell may be as critically important as the level of activity in determining the role of PA in malignant behavior (1). Additionally, the possible presence of specific PA inhibitors (PAI-1 and PAI-2), as well as nonspecific PA or plasmin inhibitors, was also not adequately addressed in some earlier studies (1, 2, 4). To overcome these limitations, we have in the present study utilized a combination of enzymatic assays, casein-plasminogen zymography, and Northern blot analysis to characterize the PA and PA inhibitor proteins produced by a series of four human glioma lines.

MATERIALS AND METHODS

Reagents. Plasminogen was prepared from human plasma by lysis-Sepharose affinity chromatography (Pharmacia Chemicals, Piscataway, NJ) and quantitated by protein content (5). Cyanogen bromide digest of human fibrinogen was prepared as described previously (6). The human PAI-2 cDNA was provided by Andrew C. Webb, Ph.D. (Wellesley College). This cDNA was the subcloned internal PstI-DraI fragment of the pCD 1214 clone in pGEM-2, inserted between the PstI and Smal sites in the polylinker (7). A full length human urokinase cDNA inserted in the PstI-PvuII site of a pBR322 vector was obtained from the Japanese Center Resources Bank-Gene, National Institute of Health, Tokyo, Japan (8). The human full length tPA clone, inserted in the Psrl site of pBR322 was provided by Sandra J. F. Degen, Ph.D. (Children's Hospital, University of Cincinnati); 9. The cDNA clone of human PAI-1 is a full length segment inserted in the EcoRI site of pUC13, provided by D. Ginsburg, M.D. (Howard Hughes Medical Institute, University of Michigan (10)).

Cell Culture. The four glioma lines used in this study included U373 and U251, previously shown to express high levels of PA activity, and U138 and LM, for which PA expression was undetectable (2). The cell lines were grown in 25-cm² polystyrene flasks (Falcon Plastics, Oxnard, CA) in complete medium consisting of Dulbecco's minimal essential medium (Hazleton, Denver, PA) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), fungizone (2.5 µg/ml), 1-glutamine (2 mM), nonessential amino acids (10 mM) (MEM), and 15% fetal bovine serum (Hyclone, Logan, UT). For evaluation of secreted PA and PA inhibitor activities, the monolayers were washed with MEM and the media replaced with serum-free medium consisting of MEM supplemented with antibiotics and 0.1% (w/v) bovine serum albumin (Sigma Chemical Co.). Cells were subsequently incubated for an additional 48 h. The conditioned media were removed and frozen at −20°C for subsequent analysis. For analysis of mRNA, cells were grown to confluence in serum-supplemented medium and washed twice with MEM. Cells were lifted from the monolayer with 5 mM EDTA (pH 7.4) in phosphate-buffered saline, collected by centrifugation, washed, and flash frozen as a dry pellet at −70°C for RNA extraction.

Esterolytic Assay for PA and PA Inhibitor Activities. Plasminogen activator activity was measured with the esterolytic assay of Coleman and Green (11), with minor adaptations for use in microtiter plates. Test samples (10 µl) were incubated for 60 min, at 37°C, with an optimal amount of plasminogen in 0.67 M glycine/0.17% bovine serum albumin/1.7 µM Tris/0.02% Triton X-100 (50 µl). The plasmin-gener-
ated during this initial incubation was quantified by addition of thiobenzyl benzoylcarboxy-7-l-lysinate (0.2 mM; Calbiochem, LaJolla, CA) and 5,5'-dithio-bis-2-nitrobenzoic acid (2.2 mM; Calbiochem) in 200 μl of 0.2M phosphate/0.2 mM NaCl/1% Triton X-100. After 30–60 min at 37°C, optical absorbance was read at 414 nm with a Titertek Plus multichannel spectrophotometer (Flow Labs, McLean, VA). PA activity was determined from a standard curve generated with commercially prepared urokinase (Calbiochem) and expressed in mPU. This assay reliably detected levels of PA activity as low as 20 mPU/ml. To measure PA inhibitor activity, serial dilutions of the test samples were incubated with 2 mPU of commercial urokinase, and the residual PA activity was measured in standard fashion. PA inhibitor activity was calculated from a regression equation describing the relationship between sample concentration and residual PA activity and expressed as PAI units/ml (1 PAI unit = 1 mPU PA inhibited).

Zymographic Identification of Plasminogen Activators. Plasminogen activator activity was identified by molecular weight using an adaptation of polyacrylamide gel electrophoresis-casein zymography (12). Slab gels (18 x 16 x 0.15 cm) were prepared with 10% acrylamide supplemented with 0.1% sodium dodecyl sulfate, 0.5 mg/ml α-casein, (Sigma) and 5 μg/ml plasminogen. Cynogen bromide-digested fibrinogen (100 μg/ml) was also added to the running gel to optimize detection of tPA activity (6). Conditioned media from confluent monolayers of the glioma lines were subjected to electrophoresis under nonreducing conditions. High molecular weight urokinase (M, 54,000; American Diagnostica, Greenwich, CT) and two-chain tissue plasminogen activator (68,000; American Diagnostica) were run as standards in every gel. The gels were then sequentially washed in 1 and 0.1% Tween-80 and incubated in 0.1% Tween-80 at 25°C for 16 h. Once the gels were stained with Coomassie blue, PA activity was identified as focal zones of caseinolysis.

mRNA Analysis. To extract cellular RNA, glioma cell pellets were sonicated on ice in urea (5 mM), LiCl (3 mM), and heparin (14 units/ml), according to the method of Auffray and Rougeon (13). RNA was precipitated at -20°C and repeatedly extracted with phenol/chloroform. The RNA was size fractionated electrophoretically on 1% agarose gels containing 3.5 μM formaldehyde and 20 μg/ml ethidium bromide (14). Visualization of ribosomal bands under UV light provided internal size markers for each lane and was also used to verify that the RNA content was equal among corresponding lanes. The RNA was transferred to Hybond nylon filters (Amersham, Arlington Heights, IL) (14). The cDNA clone of interest was labeled with [32P]dCTP (Amersham) by random priming (15). The nylon filters were hybridized with 2 x 10⁹ cpm of 32P-cDNA for 3 h at 65°C, followed by serial washes of increasing stringency, with the final wash consisting of 0.1 x standard saline citrate (0.15M NaCl/0.15M Na citrate)/0.1% sodium dodecyl sulfate at 68°C (14). The filters were then developed by autoradiography, using Kodak XAR-5 X-Omat AR film at -70°C.

RESULTS

Growth Characteristics of Glioma Cell Lines. The four glioma cell lines were plated at 10⁵ cells/well in 16-mm wells in MEM with 10% fetal bovine serum. During days 1–8, the growth rates of the U251 and U373 cells were substantially greater than either the U138 or LM lines (Fig. 1). Cell viability at saturation density was >95% for all cell lines.

PA and PA Inhibitor Activities of Glioma Cell Lines. The four glioma lines were grown in parallel and allowed to reach confluence in 25-cm² flasks containing 10 ml of medium. Two-day serum-free culture fluids were generated from each of the four glioma lines and assayed for PA, PA inhibitor, and anti-plasmin activities. Table 1 shows the activities found in culture medium from the four cell lines grown and assayed in parallel. Cell lines U251 and U373 secreted substantial levels of PA activity, expressing 593 and 599 mPU/ml, respectively. By contrast, secreted PA activity was not demonstrable in the U138 and LM lines (<20 mPU/ml). Assaying specifically for PA inhibitor activity revealed that U138 produced significant amounts of PA inhibitor (85 PAI units/ml). LM demonstrated no PA inhibitor activity. To determine whether any of the cell lines secreted appreciable anti-plasmin activity, conditioned media were assayed by a modified form of the colorimetric PA assay.

Table 1: Plasminogen activator and inhibitor activities secreted by glioma lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PA activity (mPU/ml)</th>
<th>PA inhibitor activity (PAI units/ml)</th>
<th>Anti-plasmin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>U251</td>
<td>593 ± 1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>U373</td>
<td>599 ± 44</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>U138</td>
<td>0</td>
<td>85 ± 17</td>
<td>0</td>
</tr>
<tr>
<td>LM</td>
<td>0</td>
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<td>0</td>
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</tbody>
</table>

* Mean values ± SD of duplicate assays. Results are from a representative experiment in which the four cell lines were cultured in parallel. Three independent cultures of each cell line were assayed, yielding virtually identical results.

Fig. 1. Growth of human glioma cells in monolayer culture. Cells were plated at 10⁵ cells/well in a 24-well dish using MEM supplemented with 10% fetal bovine serum. Culture medium was changed at 2-day intervals and duplicate dishes were harvested and counted at various times. Values shown represent averages ± differences between individual values and averages, based on duplicate samples in a single experiment. The experiment was repeated two times with similar results.

Zymographic Identification of Plasminogen Activators. As shown in Fig. 2, casein-plasminogen zymography identified single bands of PA activity in the U373 and U251 lines. In both cases, the PA activity exactly comigrated with the high molecular weight uPA standard (M, 54,000). No areas of PA activity were reliably detected levels of PA activity as low as 20 mPU/ml. To measure PA inhibitor activity, serial dilutions of the test samples were incubated with 2 mPU of commercial urokinase, and the residual PA activity was measured in standard fashion. PA inhibitor activity was calculated from a regression equation describing the relationship between sample concentration and residual PA activity and expressed as PAI units/ml (1 PAI unit = 1 mPU PA inhibited).
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Fig. 2. Casein-plasminogen zymography of glioma-conditioned media. Areas of PA activity are shown as focal zones of caseinolysis. Lane 1, tissue plasminogen activator standard (M, 68,000; 10 ng/lane); lane 2, urokinase-type plasminogen activator standard (350 mPU/lane), showing a lytic band corresponding to high molecular weight (M, 54,000) and a minor band of low molecular weight (M, 32,000) urokinase. Conditioned media from the U373 line (lane 3) and the U251 line (lane 4) demonstrated a single lytic band which comigrated with high molecular weight uPA. Lines U138 (lane 5) and LM (lane 6) did not demonstrate any detectable PA activity by this method.

Fig. 3. Northern blot analysis of plasminogen activators. Top, hybridization with uPA cDNA. U251 and U373 expressed high levels of uPA mRNA (2.4 kilobases). LM expressed substantially lower levels of uPA mRNA, while uPA mRNA was essentially undetectable in U138. Bottom, hybridization with tPA cDNA. tPA mRNA (2.5 kilobases) was clearly demonstrable in the U373 and U251 lines, while little or no tPA message was detectable in U138 and LM (10 µg RNA/lane).

Fig. 4. Northern blot analysis of plasminogen activator inhibitors. Top, hybridization with PAI-2 cDNA. U138 cells contained high levels of PAI-2 mRNA (2.0 kilobases), while none was detectable in any of the remaining three cell lines. Bottom, hybridization with PAI-1 cDNA. All lines contained demonstrable PAI-1 mRNA (3.0 and 2.0 kilobases), most prominently expressed in the U138 and LM lines (10 µg RNA/lane).

PAI-2. U138, which secreted PA inhibitor activity, contained a high steady state level of mRNA for PAI-2 (Fig. 4). PAI-2 mRNA was not detectable in any of the other three lines, even with prolonged exposures on autoradiography. PAI-1 mRNA was detectable in all four cell lines but was substantially more prominent in U138 and LM than in U373 or U251 (Fig. 4).

DISCUSSION

Malignant cells of various types typically synthesize high levels of uPA, tPA, or both (1). Prior studies of gliomas and glioma-derived cell lines have shown that PA activity is highly variable in these cells (2–4). Characterization of glioma PA activity has been limited to a few studies but has consistently shown uPA secreted in vitro in a zymogen form (3, 16, 17). tPA production in gliomas appears to be largely, if not exclusively, limited to the accompanying vascular endothelium (18). Secretion of plasmin inhibitors also has been described (16), but this does not appear to be responsible for the variability among glioma lines in secreted PA activity.

To precisely define the molecular basis of PA heterogeneity in gliomas, it is necessary to profile the production of PA enzymes and PA inhibitors by a series of glioma lines under uniform set of culture conditions. The present study is an attempt to do this. Utilizing four human glioma lines, two PA producers and two that produce undetectable levels of PA activity, we applied a series of approaches to identify PA enzymes and inhibitors produced by these cells. Both of the PA-producer lines (U251 and U373) demonstrated a substantial amount of uPA but no detectable tPA by casein-plasminogen zymography. The presence of detectable uPA was associated with a high level of uPA mRNA. Although tPA was undetectable enzymatically, the PA-producer lines also contained tPA mRNA. We cannot conclude from this study whether these cells secreted amounts of tPA which were insufficient for detection by zymography or, alternatively, the presence of tPA mRNA did not eventuate in the synthesis of functional enzyme.

In addition to producing high levels of uPA, the PA-producing cells failed to exhibit detectable PA inhibitor activity and expressed little mRNA for PAI-1 and none for PAI-2. By contrast, the two PA-nonproducer cell lines expressed greater...
levels of mRNA for PAI-1. One of these, U138, also contained abundant mRNA for PAI-2 and, correspondingly, secreted high levels of PA inhibitor activity. To our knowledge, production of PAI-2 by a human glioma cell line has not been demonstrated previously. We have, however, identified a second glioma line with a high level of PAI-2 mRNA. Although not fully characterized, this second line (UM-6) was also identified as a PA nonproducer in our previous study (2). Thus, the production of PAI-2 does not appear to be unique to the U138 line. It is useful to compare these results to those reported in a survey of PA and PA inhibitor expression by 22 human non-glioma tumor lines (19). Both studies demonstrated that uPA and tPA may be expressed concurrently by tumor cells. However, Quax et al. (19) reported that all cells expressed some sort of PA and that the level of PA expression correlated with that of PA inhibitor, suggesting common regulatory mechanisms. By contrast, we found that the U138 line had no detectable uPA or tPA but expressed very high levels of PA-2 and appreciable PAI-1 mRNA, which is clearly an example of divergent regulation. Likewise, the PA-nonproducer LM line expressed low levels of uPA but appreciable PAI-1 mRNA. While the functional significance of these observations is unknown, it appears that some glioma lines are unique in expressing little or no PA activity, a property which may still permit tissue invasion in brain tissue which contains relatively little connective tissue stroma.

The significance of the differences among glioma lines in PA activity is not fully understood. It has been suggested on the basis of several experimental approaches that PA secretion influences tumor cell invasion at the primary tumor site and implantation of metastases (20). Several components of the extracellular matrix, including laminin, fibronectin, and proteoglycans, as well as fibrin-rich provisional matrix, are susceptible to plasmin-mediated hydrolysis (21–24). While collagen is plasmin resistant, the ability of plasmin to activate latent collagenases could provide a mechanism by which PA leads to destruction of plasmin-resistant matrix constituents as well (25). Perhaps the strongest evidence of the role of uPA in metastasis is the direct demonstration of uPA at sites of murine tumor invasion (26) and the finding by Ossowski and Reich (27) that a neutralizing uPA antibody blocked pulmonary metastasis but did not block a human tumor transplanted into chick chorioallantoic membrane.

At present, it is not known whether gliomas also utilize PA to facilitate invasion or whether gliomas differ in vivo behavior as a function of PA production. We do know, however, that in vitro behavior is influenced by expression of PA activity. For example, we found that PA-producing lines grow more rapidly than the PA nonproducers (Fig. 1), although a causal relationship was not shown. Our previous study (2) showed that attachment and spreading of glioma cells on fibronectin was sensitive to exogenous plasminogen. Both PA-producer and PA-nonproducer cells formed stable attachments and spread on fibronectin-coated plates in the absence of plasminogen, but in the presence of plasminogen, PA-producer cells released from their attachment sites. Exogenous plasminogen had much less effect on PA-nonproducing cells. PA production also affected attachment to fibronectin in the presence of plasminogen. These observations suggest that PA production enables these cells to degrade the provisional fibrin/fibronectin stroma of tumors in a way that facilitates cell detachment and, possibly, invasiveness. It will be necessary for future studies to determine how these in vitro observations relate to tumor invasion and metastasis in vivo.

Finally, it is of interest to note that the PA-producer lines U251 and U373 are also positive for GFAP, while the PA-nonproducers LM and U138 are GFAP negative (28, 29). Although rare, GFAP-positive glial cell lines tend to express the markers closest to those of differentiated gliomas in situ (28, 29). Thus, one could speculate that events in glioma differentiation regulate expression of both PA activity and GFAP. It is also noteworthy that, while both PA-producing and PA-nonproducing glioma lines express extracellular matrix protein, the matrix components are most highly expressed on the cells lacking PA activity (28–31). Elevated expression of matrix proteins may thus result from an absence of plasmin-mediated matrix degradation. Studies with additional cell lines will be necessary to extend these observations.

REFERENCES

2. Varani, J., McKeever, P., Fligiel, S. E. G., and Sitrin, R. G. Plasminogen activator expression by human tumor cell lines and localization of tissue activator activity in breast tumor lines (19). Both studies demonstrated that uPA and tPA may be expressed concurrently by tumor cells. However, Quax et al. (19) reported that all cells expressed some sort of PA and that the level of PA expression correlated with that of PA inhibitor, suggesting common regulatory mechanisms. By contrast, we found that the U138 line had no detectable uPA or tPA but expressed very high levels of PA-2 and appreciable PAI-1 mRNA, which is clearly an example of divergent regulation. Likewise, the PA-nonproducer LM line expressed low levels of uPA but appreciable PAI-1 mRNA. While the functional significance of these observations is unknown, it appears that some glioma lines are unique in expressing little or no PA activity, a property which may still permit tissue invasion in brain tissue which contains relatively little connective tissue stroma.

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