Plasminogen Activator and Inhibitor Activity in Human Glioma Cells and Modulation by Sodium Butyrate

Janet L. Gross, Davette L. Behrens, Debora E. Mullins, Paul L. Kornblith, and Daniel L. Dexter

ABSTRACT

The activity of the serine protease plasminogen activator (PA), which correlates with tumorigenicity and metastatic capacity, was examined using the 125I-Labeled fibrin plate assay in cell extracts from four human glioma lines as a function of growth in vitro. Cell-associated inhibitory activity to plasmin and urokinase-type PA was also measured concurrently. The relative PA activities differed markedly among the lines, whereas inhibitory activities did not. Two lines, SNB-19 and SNB-75, exhibited maximal PA activities (1-6 Ploug units/μg protein) as cultures approached confluence, whereas two other lines, SNB-56 and SNB-78, expressed low PA activity at all times (<0.2 m Ploug units/μg protein). The PA of SNB-19 cell extracts was predominantly urokinase-type PA. In addition to having the highest PA levels, SNB-19 and SNB-75 were the most clonogenic in soft agar and tumorigenic in nude mice. In contrast, SNB-56 and SNB-78 were poorly clonogenic in soft agar and were not tumorigenic in nude mice. Measured directly, inhibitory activities to plasmin and urokinase-type PA were detected in SNB-19 (high PA) and SNB-56 (low PA) cell extracts. However, there were no detectable quantitative differences in inhibitor effects between SNB-19 and SNB-75 suggesting that the differences in PA activity between these lines resulted from changes in PA activity and were not due to differential plasminogen activator inhibitory effects. The ability of the differentiating agent sodium butyrate (NaB) to modulate total PA activity was also examined. Peak SNB-19 cell PA activity was decreased in a concentration (K, 0.75 mM) and time-dependent manner by the addition of nontoxic amounts of NaB. The dose-dependent decrease in PA activity induced by NaB was most likely due to an effect on PA activity, since an earlier addition of NaB resulted in an increased binding in NaB. These results suggest that net cellular PA activity in glioma cells is a balance between relative PA activity and inhibitor(s) effects and that this balance can be modulated by sodium butyrate.

INTRODUCTION

PAs, in addition to their roles in fibrinolysis and other physiological processes (1), have been implicated in several aspects of the malignant phenotype including tumorigenicity (2, 3) and increased invasive and metastatic potential (1, 4). This correlation is further supported by the findings of enhanced PA activity in virally and chemically transformed fibroblasts in vitro (5), elevated PA activities in human tumors in contrast to normal tissues (3, 6), greater metastatic potential of rodent tumor lines with higher PA activity (4), as well as the ability of antibodies to PA to block metastasis (7). High PA activity of high-grade brain tumors correlates well with their invasive characteristics (8, 9). In addition, thromboembolic complications are often associated with intracranial neoplasms (10, 11), further suggesting a role of fibrinolysis in brain neoplasia. Indeed, high levels of PA as well as inhibitors of fibrinolysis have been reported from glioma cells in culture (12-15) as well as from brain neoplasms (8, 9, 16). The local invasiveness of brain neoplasms is particularly important to the lethal progression of this tumor type, since lethality by gliomas is caused by local extension rather than by dissemination. Thus, it is of importance to understand the role of PA in this process.

Differentiating agents are chemical compounds of diverse structures that induce a more benign phenotype in a variety of cultured leukemia and solid tumor cells (for review, see Ref. 17). Differentiating agents have been demonstrated to modulate PA expression as one response in the spectrum of alterations these agents induce in tumor cells (18, 19). Since PA activity is important in the invasiveness of highly malignant Grade III and IV gliomas, the modulation of this enzyme or its inhibitors by differentiating agents may limit the progression of these neoplasms. However, there has been little work done using this approach with cells from intracranial primary cancers. Therefore, we have studied four cell lines established from human brain tumors to address two questions. (a) Since the net cellular expression of PA activity is the sum of constantly changing levels of both PA and PA inhibitors (PAI) (1, 20), what are the relative levels of PA and PAI in these cell lines? (b) Can maturational agents cause a decrease in net PA expression concomitantly with the induction of a more benign phenotype in glioma cells? This report describes the results of our investigation; portions of this work have been presented in preliminary form (21).

MATERIALS AND METHODS

Chemicals. Dimethylformamide was from Aldrich Chemical Co., Milwaukee, WI. NaB and phorbol 12-myristate 13-acetate were from Sigma Chemical Co., St. Louis, MO.

Cell Lines. Glioma lines were derived from primary human neoplasms, each histologically confirmed as intracranial astrocytoma or glioblastoma multiforme. SNB-19 was established in 1980 from the left parieto-occipital glioblastoma from a 47-yr-old male. SNB-56 was derived in 1981 from a 39-yr-old man who underwent a left parieto-occipital craniotomy for glioblastoma. SNB-75 was from a Grade IV glioblastoma obtained in 1980 from a 78-yr-old female. SNB-78 was established in 1984 from a glioblastoma in a 71-yr-old male who underwent craniotomy. Cells were maintained in a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eiger's medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% (vol/vol) heat-inactivated (56°C, 30 min) fetal calf serum (HyClone Laboratories, Logan, UT). The cells were maintained in a humidified atmosphere of 95% air-5% CO2 at 37°C. Chromosomal analysis was provided by A. Parmiter, Department of Dermatology, University of Pennsylvania.

In Vitro Growth Parameters. Cell doubling times and saturation densities were determined as previously described (22) except that initial cell-plating density was 1.6 × 10⁴ cells/cm². The concentration of compounds required to inhibit the number of cell doublings by 50% in...
SODIUM BUTYRATE MODULATION OF GLIOMA CELL PLASMINOGEN ACTIVATOR

Sodium butyrate was used to study the modulation of plasminogen activator (PA) expression in glioma cells. The effects on cell growth, plasminogen activator (u-PA and t-PA) expression, and the kinetics of substrate hydrolysis were analyzed. The results showed that sodium butyrate inhibited the expression of both u-PA and t-PA, and decreased the rate of substrate hydrolysis.

**RESULTS**

**Cell Culture Studies.** Four human glioma lines were compared in vitro with respect to growth parameters and chromosomal analysis; a summary of their properties is presented in Table 1. All four lines reached plateau growth approximately 1 week postplating (data not shown); the saturation densities were slightly different (Table 1). The doubling times of three lines were similar (50–58 h), whereas SNB-56 had a doubling time of 33 h. All four lines were aneuploid. SNB-19 demonstrated a bimodal chromosome range.

<table>
<thead>
<tr>
<th>Line</th>
<th>Doubling time (h)</th>
<th>Saturation density (cells/cm²)</th>
<th>Chromosome Range</th>
<th>Modal no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNB-19</td>
<td>52 ± 4</td>
<td>7.8 × 10⁶</td>
<td>65–99</td>
<td>79</td>
</tr>
<tr>
<td>SNB-75</td>
<td>58 ± 2</td>
<td>8.8 × 10⁶</td>
<td>150–300</td>
<td>60</td>
</tr>
<tr>
<td>SNB-56</td>
<td>33 ± 3</td>
<td>1.5 × 10⁷</td>
<td>64–209</td>
<td>80</td>
</tr>
<tr>
<td>SNB-78</td>
<td>50 ± 2</td>
<td>6.7 × 10⁷</td>
<td>58–166</td>
<td>77</td>
</tr>
</tbody>
</table>
ences in cloning efficiencies among the lines, as shown in Table 2. SNB-19 was the most clonogenic in soft agar, with a cloning efficiency of 41.5%. Interestingly, the tumor from which this line was derived was extremely aggressive in the patient. SNB-75 was moderately clonogenic in semisolid medium (cloning efficiency, 8.9%) whereas SNB-56 and SNB-78 were the least clonogenic. Thus, although there were only slight differences in anchorage-dependent growth characteristics (Table 1), there were marked differences in anchorage-independent growth among the lines.

Tumorigenicity in Nude Mice. The relative tumorigenicity of each glioma line in nude mice was examined; the results are presented in Table 2. Two lines, SNB-56 and SNB-78, not clonogenic in soft agar, were also not tumorigenic in mice up to 110 days postinjection of 10^5 cells per s.c. site. SNB-75 cells, modestly clonogenic in soft agar (Table 2), produced tumors which developed slowly and which were first measurable 80 days postinjection. SNB-19 cells, the most clonogenic in soft agar, were also the most tumorigenic. SNB-19 tumors appeared 30–35 days postinjection and animals were sacrificed at 97 days postinjection due to excessive tumor burden (average tumor weight, 6.8 g). Thus, there was a correlation between anchorage-independent growth in vitro and tumorigenicity in nude mice for each glioma line.

Plasminogen Activator Activity. The total PA activity was measured using the [125I]-labeled fibrin plate assay in detergent extracts from each glioma line as a function of growth in culture. The time course of PA activity differed significantly among the lines, as illustrated in Fig. 1. Two lines exhibited maximal cellassociated PA activity as cultures approached confluence, in agreement with that observed with other cells at confluence (25, 32). Although the absolute PA activity varied slightly among experiments, presumably due to culture conditions and the passage level of each line (25), consistent differences in PA activity among the lines were apparent. SNB-19 had the highest PA activity, which peaked 3–4 days after cell plating (1–6 m Plough units/μg protein; 3.2 m Plough units average). SNB-75 maintained moderate PA activity 3 days postseeding and thereafter (0.8–3.2 m Plough units/μg protein; 2.0 m Plough units average). Two lines, SNB-56 and SNB-78, exhibited low PA activity at all times in culture (Fig. 1). A summary of the PA activity of each line on Day 3 in culture is presented in Table 1. The relative PA activities of the four human glioma lines correlated with the malignant parameters of anchorage-independent growth and tumorigenicity in nude mice (Table 2). The PA of detergent-extracted SNB-19 cells was predominantly u-PA, since hydrolysis of the synthetic substrate S2251 by SNB-19 cell extracts was independent of fibrin in the assay mixture (data not shown) (27). This is in agreement with Dans et al. (13), who demonstrated urokinase immunohistocytochemically in a human glioma line in vitro.

Effects of Plasminogen Activator Inhibitors. The differences in net cellular PA activity among the four glioma lines as a function of growth in culture could be the result of changes in PA activities and/or PAI effects. Therefore, two lines with contrasting PA activity were analyzed for changes in PAI as a function of time in culture. Direct inhibition of urokinase was examined in extracts from SNB-19 (high PA) and SNB-56 cultures (low PA) grown for up to 8 days in culture. Plasmin inhibition in the same extracts was also examined, since plasmin inhibition by brain homogenates has been reported previously (16). Both extracts contained inhibitory activities to urokinase and to plasmin. The pattern of urokinase inhibition by both glioma lines was similar; urokinase inhibition was high (50–75% inhibition of 250 m Plough units) 1 day after cell plating and disappeared by 3–4 days postplating (Fig. 2). The pattern of plasmin inhibition by both lines was also similar, but differed from those of urokinase inhibition. Inhibition of plasmin by SNB-19 and SNB-56 cell extracts remained relatively constant (25–40% inhibition of 2.5 μg of plasmin) with time (Fig. 2). Thus, there were no absolute differences in urokinase or plasmin inhibition between SNB-19 and SNB-56 cells as a function of time in culture.

Glioma extracts were next examined for direct inhibition of t-PA. Two lines with contrasting PA activities, Day 7 SNB-19
cultures (2 m Plough units/µg protein; Fig. 1), and Day 2 SNB-56 cultures (0.1 m Plough units/µg protein; Fig. 1) were compared for their ability to inhibit t-PA (80 Plough units) as a function of cell protein. Direct inhibition of t-PA by both SNB-19 and SNB-56 cell extracts was observed (Fig. 3). The inhibition of t-PA was dependent on the concentration of added cell protein; the degree of inhibition was similar for both lines (Fig. 3). Taken together, these results suggest that the differences between the PA activities in two glioma lines were not due to variations in cellular PAI effects.

Modulation of Glioma PA Activity by Differentiating Agents. Since SNB-19 cells had high PA activity, it was of interest to examine the effects of known maturational agents on SNB-19 cell-associated PA activity. Differentiating agents of three structural classes were added to SNB-19 cultures when PA activity was at its peak (Day 3). Thirty h after the addition of nontoxic concentrations of the potent phorbol diester phorbol 12-myristate 13-acetate (5 x 10^-8 M), the polar solvent dimethylformamide (1.25% w/v), or NaB (1 mM), cell-associated PA activity was measured. As shown in Table 3, 1 mM NaB reduced SNB-19 PA activity approximately 3-fold, whereas the other agents were ineffective.

Since SNB-19 activity was reduced significantly by exposure to NaB, it was expected that NaB treatment could modulate another biochemical parameter correlated with tumorigenicity (1, 2). The clonogenicity of SNB-19 cells grown in soft agar with or without NaB was assessed. As shown in Table 4, SNB-19 clonogenicity in soft agar was reduced by 92 and 98% when the cells were cultured in agar containing 0.25 mM and 0.5 mM NaB, respectively. The growth of SNB-19 cells in monolayer culture with 0.25 and 0.5 mM NaB was not significantly affected compared to untreated cells (data not shown).

The effect of NaB on peak SNB-19 PA activity was examined for concentration and time dependency. Varying concentrations of NaB were added to Day 3 SNB-19 cultures and 30 h later

![Fig. 3. Direct inhibition of t-PA by SNB-19 and SNB-56 extracts. Varying amounts of cell extracts (C, SNB-19; O, SNB-56) were incubated for 40 min at room temperature with 80 units of t-PA. The inhibition of absorbance at 405 nm was measured as described in "Materials and Methods."](image)

![Table 3. Effect of known differentiating agents on SNB-19 PA activity](image)

<table>
<thead>
<tr>
<th>Agent</th>
<th>PA activity (m Plough units/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.09</td>
</tr>
<tr>
<td>PMA (5 x 10^-9 M)</td>
<td>2.43</td>
</tr>
<tr>
<td>DMF (1.25%)</td>
<td>2.33</td>
</tr>
<tr>
<td>NaB (1.0 mM)</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of sodium butyrate concentration on SNB-19 PA activity. SNB-19 cells were plated on Day 0 and fresh growth medium was added daily. Three days postplating, varying concentrations of sodium butyrate were added to the cells. Cells from triplicate cultures were harvested 30 h later and extracted with Triton X-100. Cell extracts (1 µg) were assayed for PA activity (5, 25).

![Fig. 5. Time course of modulation of SNB-19 PA activity with sodium butyrate. SNB-19 cells were plated on Day 0 and fresh growth medium was added daily. Three days postplating, varying concentrations of sodium butyrate were added. At various times after sodium butyrate addition, cells from triplicate cultures were harvested, pelleted, and detergent extracted. Cell-associated PA activity was measured in extracts containing 1 µg cell protein (5, 25).](image)

Table 4. Effect of NaB on SNB-19 clonogenicity in soft agar

<table>
<thead>
<tr>
<th>Addition</th>
<th>Clonogenicity in soft agar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>49.0</td>
</tr>
<tr>
<td>0.25 mM NaB</td>
<td>4.0</td>
</tr>
<tr>
<td>0.5 mM NaB</td>
<td>0.9</td>
</tr>
</tbody>
</table>

SNB-19 cells were cloned in soft agar containing 0, 0.25 mM, or 0.5 mM NaB and were fed weekly. Colony formation was assessed after 4 wk (22). Cells in parallel were cultured at equivalent plating densities in monolayer culture in medium containing 0, 0.25 mM, or 0.5 mM NaB.

Fig. 4. Effect of sodium butyrate concentration on SNB-19 PA activity. SNB-19 cells were plated on Day 0 and fresh growth medium was added daily. Three days postplating, varying concentrations of sodium butyrate were added to the cells. Cells from triplicate cultures were harvested 30 h later and extracted with Triton X-100. Cell extracts (1 µg) were assayed for PA activity (5, 25).

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SODIUM BUTYRATE MODULATION OF GLIOMA CELL PLASMINOGEN ACTIVATOR
Changes in inhibitor levels with butyrate treatment. The decrease in PA activity of NaB-treated SNB-19 cultures was due to reduced PA activity and direct inhibitory activity to urokinase. As shown in Table 5, untreated Day 4 SNB-19 extracts exhibited high PA activity (1.9 m Plough units/µg) and low inhibitory activity to urokinase (15% inhibition; see also Fig. 2). The exposure of SNB-19 cultures to increasing concentrations of NaB resulted in a dose-dependent decrease in total PA activity with no reduction of that exhibited by control, untreated cultures.

Since glioma cell PA is predominantly u-PA (13), the inhibition of urokinase by untreated and NaB-treated SNB-19 cell extracts was measured to estimate whether the net lowered PA activity of NaB-treated cultures was due to reduced PA activity or enhanced PAI effects. If the NaB concentration-dependent, reduced PA activity was due to an increased inhibition by PAI, then the PAI effect should be dependent on the concentration of sodium butyrate added to SNB-19 cultures. To test this, Day 3 SNB-19 cultures were exposed for 30 h to varying concentrations of NaB and cell extracts were examined for total PA activity and direct inhibitory activity to urokinase. As shown in Table 5, untreated Day 4 SNB-19 extracts exhibited high PA activity (1.9 m Plough units/µg) and low inhibitory activity to urokinase (15% inhibition; see also Fig. 2). The exposure of SNB-19 cultures to increasing concentrations of NaB resulted in a dose-dependent decrease in total PA activity with no elevation in urokinase inhibitory activity, suggesting that the reduced PA activity was directly due to reduced PA activity and not to an enhanced PAI effect.

DISCUSSION

In order to study the role of PA in brain tumors, we examined PA activity in four human glioblastoma lines. Interestingly, although all four lines were derived from high-grade brain neoplasms, two lines had significantly high PA activity whereas two lines expressed low PA activity. Those lines which had high PA activity were also clonogenic in soft agar in vitro and in addition were tumorigenic in nude mice, thus extending to human gliomas the correlation of high PA activity and tumor necrosis observed for other tumors (6). This correlation suggests that the modulation of glioma PA may be a means of controlling the progression of these highly invasive tumors. The modulation of human glioma PA and clonogenicity in soft agar was achieved with the maturation agent sodium butyrate. PA activity of SNB-19 and SNB-75 human glioma cells is reduced significantly by sodium butyrate. The inhibition of PA activity is most likely caused by a change in PA synthesis, since direct measurement of urokinase inhibition showed no changes in inhibitor levels with butyrate treatment. The decreased PA activity correlates with greatly reduced clonogenicity in agar when the glioma cells were grown in the presence of NaB. Ossowski and Belin (18) reported that human malignant carcinoma HEp-3 cells treated with another differentiating agent (DMSO) have decreased PA activity and morphological changes similar to nonmalignant HEp-3 cells. The decreased HEp-3 PA activity was not due to increased PAIs but rather to decreased messenger RNA for u-PA (18). Frager et al. (19) have reported that human renal carcinoma cell PA secretion is also reduced after exposure to DMSO, sodium butyrate, or retinoic acid; this decrease was also apparently due to inhibition of PA synthesis.

The spectrum of inhibitory activities to serine proteases of these human glioma lines is interesting; the simultaneous inhibition of u-PA, t-PA, and plasmin has not been previously defined for this tumor type. Plasmin inhibition has been demonstrated from homogenates of gliomas and other brain tumors (9, 16). Glooor et al. (14) reported that cultured rat glioma cells release an M, 43,000 protein which promotes neuroblastoma cell growth, inhibits urokinase, t-PA, thrombin, and trypsin activities, and which is a member of a family of protease nexins (33). Dans et al. (15) have evidence from a human glioblastoma line of an inhibitor to u-PA which is distinct from protease nexin but immunologically similar to PAI-1, an inhibitor derived from vascular endothelial cells (34). Both PAI-1 and PAI-2 (35), two biochemically and immunologically distinct inhibitors, inhibit u-PA and t-PA (but not plasmin) and are members of the Serpin gene family (36). The type(s) of PAI synthesized by the human glioma lines reported in this paper remains to be characterized.

Our study has demonstrated that in vitro at least one glioma line, SNB-19, expressed high PA activity simultaneously with inhibitory activity to u-PA, t-PA, and plasmin. Since net PA activity is the result of interaction between PA and PAI (1, 20), the data suggest that PA activity most likely could not be completely inhibited by PAI in SNB-19 cells. SNB-56 cells also contained inhibitor(s) to u-PA, t-PA, and plasmin, but expressed negligible PA activity; these results likewise suggest that the PA activity in SNB-56 cells was completely inhibited by the PAI in these cells. These results emphasize the importance of the balance between PA and PAI in the regulation of PA expression in tumor cells. The mechanism of the modulation of this balance by NaB and other agents is currently under investigation in our laboratory.

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