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Human glioma U-251 cells contain type 1 plasminogen activator inhibitor in a rapidly releasable form

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Abstract Because recent information suggests that the localized deposition of protease inhibitors is one mechanism by which cells regulate pericellular proteolysis during tissue invasion, the distribution of type 1 plasminogen activator inhibitor (PAI-1) associated with the invasive human glioma cell line U-251 was investigated. Direct and reverse fibrin zymography indicated the presence of urokinase-like plasminogen activator (u-PA) and PAI-1 in U-251 conditioned media and cell lysates. PAI-1 antigen was detected immunologically in cytoplasmic granules present within cellular processes of U-251 cells and these organelles could be isolated on Percoll density gradients in a high density band. In contrast, u-PA activity and another secreted protein, amyloid β-protein precursor, were only present in the low density region of the gradients. Functional analysis of PAI-1 in the granules contained within the high density fractions revealed the presence of active PAI-1. Incubation of U-251 cells with the secretagogue, 8-bromoadenosine 3′:5′-cyclic monophosphate, resulted in a 3-fold increase in the release of PAI-1 in the media conditioned by these cells. These data suggest that the human glioma cell line U-251 contains PAI-1 in a rapidly releasable form, which may provide another mechanism by which these tumors could regulate proteolytic activity in a localized manner.

Key words: Cytoplasmic granule; Glioma; Urokinase; Plasminogen activator; Plasminogen activator inhibitor

1. Introduction

Primary tumors of the central nervous system account for approx. 1.2% of all autopsied deaths and 40–50% of intracranial tumors, with the majority of these tumors being derived not from neurons but from the glia (reviews [1–3]). Malignant gliomas are classified histologically according to their resemblance to normal cells from the central nervous system, with astrocytes and oligodendrocytes being two major groups of glia within the central nervous system [1–3]. The poor prognosis of gliomas is partly mediated by the resistance of these tumors to cytostatics and irradiation, and because a total tumor resection is often difficult due to diffuse infiltrative growth into adjacent brain areas [1–3]. The infiltrative growth pattern of gliomas is usually along preformed nerve pathways (e.g. corpus callosum), around vessels, or the leptomeninges. Because proteolytic enzymes play a key role in modifying the extracellular matrix (ECM) during tumor infiltration within the human brain (reviews [3–6]), detailed information on the mechanisms that regulate protease activities secreted by gliomas is essential for an understanding of their infiltrative growth pattern.

Current information suggests that the plasminogen activation system plays an important role in the degradation of the ECM required for the migration of cells (reviews [3–8]). Plasminogen circulates as a proenzyme at high concentrations in the vasculature and is proteolytically converted into an active enzyme, plasmin, which is not only capable of degrading matrix components (e.g. fibronectin and laminin), but also of activating latent matrix-degrading enzymes (e.g. procollagenases and macrophage elastase). The activation of the zymogen plasminogen is primarily mediated by the activity of two distinct classes of enzymes (i.e. tissue-type plasminogen activator, t-PA; urokinase-like plasminogen activator, u-PA) [3–8]. Bindal et al. [4] noted that high levels of u-PA and low or absent t-PA activity correlated with histologically malignant brain tumors, aggressive infiltrative characteristics, and shorter patient survival. The ability of u-PA to bind to and be localized to a receptor at the leading edge of migrating cells has been proposed as one mechanism that cells utilize to direct the extracellular proteolysis to the cell-cell and cell-substratum contacts [8]. In situ analysis has revealed an enhanced expression for the u-PA receptor at the leading edge of human gliomas [9], and antibodies to the u-PA receptor effectively block the invasion of four glioblastoma cell lines (e.g. U-251 cells) in an in vitro matrigel assay [10]. Immunohistochemical localization studies for u-PA antigen have revealed that all anaplastic astrocytomas and glioblastomas contain a preponderance of neoplastic cells immunoreactive for u-PA [11,12].

A series of serine protease inhibitors (serpins) appears to be utilized by cells to control the activation of plasminogen, with prevailing data suggesting that type 1 plasminogen activator inhibitor (PAI-1) is the primary regulator of both t-PA and u-PA with dissociation rate constants greater than 10^{-7} M^{-1} s^{-1} (review [13]). The detection of PAI-1 mRNA and antigen associated with human gliomas both in vivo [11,14–18] and in vitro [19–23] has led to the concept that this inhibitor may play a role in regulating excessive degradation of the ECM in the brain (review [15]). Research in our laboratory and in others (reviews [13,24,25]) has revealed that the localized distribution of PAI-1 is one mechanism which cells utilize to further control the activation of plasminogen and the degradation of matrix proteins. More specifically, PAI-1 is able to bind to vitronectin present in the ECM, which results in the localization and stabilization of PAI-1 in the active form [13,24,25]. Our group has observed that not only is the secretion of PAI-1 elevated in cultured endothelial cells following their treatment with cytokines but an enhanced deposition of active PAI-1 both into the ECM and onto the luminal cell surface is also observed [26]. Furthermore, PAI-1 is deposited

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into the storage granules of platelets (i.e. platelet α-granules) in a rapidly releasable form that provides one mechanism to control the fibrinolytic system within a local environment in the peripheral vasculature (review [27]). Transfection experiments with expression constructs encoding full length PAI-1 and a mouse pituitary cell line (i.e. AtT-20 cells) indicated that this inhibitor contains a functional domain capable of directing it into the regulated secretory pathway [28]. Thus, PAI-1 contains a number of domains that can regulate its intracellular and extracellular distribution. Although immunohistochemical and in situ hybridization analyses of brain tissues reveals prominent staining for PAI-1 both in anaplastic cells and in areas of necrosis [11,14,16,17], little information currently exists on the distribution of PAI-1 associated directly with the gliomas. This study was initiated to investigate this issue by utilizing cultured human glioma U-251 cells, an invasive cell line [10,21] previously observed to contain high levels of PAI-1 mRNA [21,29] but no PAI-1 activity in its conditioned media [29]. We report that U-251 cells contain active PAI-1 stored within intracellular granules in a rapidly secretable form.

2. Materials and methods

2.1. Analysis of PA and PAI activities associated with U-251 cells by direct and reverse fibrin zymography

The human glioma U-251 cell line was obtained from Dr. Darrel Bigner (Duke University Medical Center, Durham, NC) and cultured as described [21]. Media conditioned by U-251 cells for 24 h and cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously utilizing a 4% stacking gel and a 9% separating gel under non-reducing conditions [26,28]. The electrophoresed gel was soaked for 1.5 h in 2.5% Triton X-100 and subsequently analyzed by either direct or reverse fibrin autography as described previously [26,28]. Markers for the gels included Rainbow Coloured Protein Molecular Weight Markers (Amersham Life Sciences, Arlington Heights, IL), purified urokinase (product no. 124, 80000 IU/mg, mixture of 50 and 33 kDa forms; American Diagnostica, Greenwich, CT), purified t-PA (product no. 111, 700000 IU/mg, 66 kDa, American Diagnostica), and purified PAI-1 [28]. Immunodepletion experiments were performed using a modification of previously described procedures [30,31]. Briefly, non-immune rabbit serum or antiserum to either urokinase [32], t-PA [32], PAI-1 [33] (20 μl, respectively) were added to protein A-coated Sepharose CL-4B beads (80 μg; Pharmacia, Uppsala, Sweden) that had been rehydrated and washed according to the manufacturer's instructions. U-251 conditioned media or cell lysates (100 μl corresponding to 10^5 cells) were incubated (1 h, 23°C) with the beads. The beads were removed by centrifugation and the supernatant fluid analyzed by SDS-PAGE and direct or reverse fibrin autography.

2.2. Immunofluorescence analysis of PAI-1

Human U-251 cells (10^4 cells/cm^2) were seeded onto fibronectin-coated (10 μg/cm^2; Sigma Chemical Co.) glass coverslips (12 mm diameter; Fisher Scientific Co., Pittsburgh, PA) in serum-free medium. The next day, the cells were fixed with 4% paraformaldehyde (20 min, 4°C), and PAI-1 antigen localized immunofluorescently utilizing previously described protocols [28].

2.3. Detection of PAI-1 and amyloid β-protein precursor (APP) in subcellular fractions of U-251 cells

U-251 cells (10^8 cells) were homogenized, and the membrane/organelle-rich fraction was isolated by differential centrifugation and subsequently fractionated on 20% Percoll gradients as described previously [28]. PAI-1 activity was quantitated as previously described [26,31] by using immobilized t-PA (American Diagnostica) to bind active PAI-1 in a sample and the bound PAI-1 immunologically detected by incubation with affinity-purified rabbit anti-PAI-1 (10 μg/ml [33]) followed by ^{125}I-goat anti-rabbit IgG (50000 cpm/well; Amersham). PAI-1 antigen was quantitated using a two-site immunoradiometric assay (IRMA) [31] that employed immobilized monoclonal antibody against human PAI-1 (clone 2D2; Gibco, Glasgow, UK) to bind PAI-1 antigen in a sample and bound PAI-1 detected as described above. The distribution of APP in the fractions from the Percoll gradient were determined by dot blotting using a modification of previously described protocols [28] that employed monoclonal antibody 22C11 anti-APP (1 μg/ml; Boehringer Mannheim, Indianapolis, IN) as the primary antibody.

2.4. Secretagogue treatment of U-251

U-251 cells (10^6 cells/dish) were plated in 60 mm diameter tissue culture dishes and the cells were allowed to equilibrate by incubation (37°C, 24 h) in growth media. For experimental analysis, the cells were incubated with two changes of serum-free media over a 2 h period, and subsequently incubated for 1 h in serum-free media supplemented either with or without the secretagogue, 5 mM 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP) [28,34,35]. Conditioned media was collected during each 1 h period.

3. Results and discussion

3.1. Fibrinolytic profile of U-251 cells

Fractionation of the conditioned media of U-251 cells on non-reducing SDS-PAGE followed by fibrin autography revealed the presence of a single zone of fibrinolysis at 50 kDa (Fig. 1A, lane 3). The enzyme responsible for this fibrinolytic zone co-migrated with the 50 kDa lytic zone observed utilizing a commercially available urokinase preparation (Fig. 1A, lane 1) and as observed by Sitrin and co-workers [29] using casein-zymographic analysis, the enzyme secreted by U-251 cells that was responsible for the 50 kDa lytic zone was dependent upon the presence of plasminogen (data not shown). A similar 50 kDa zone of fibrinolysis was detected using U-251 cell lysates (Fig. 1A, lane 4). Fig. 1B demonstrates the parallel analysis of the U-251 samples for PAI activity by SDS-PAGE and reverse fibrin autography. A single lysis-resistant zone was pres-

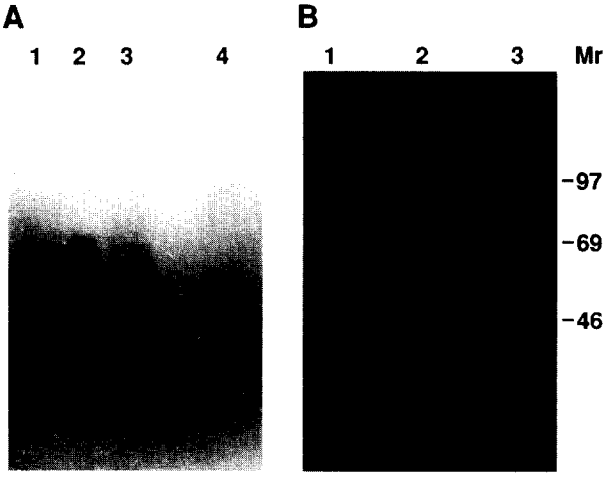

Fig. 1. Fibrinolytic profile of PAs and PAIs expressed by U-251 cells. (A) Samples were subjected to SDS-PAGE and analyzed by fibrin zymography. Lanes: 1, purified urokinase (10 ng); 2, purified t-PA standard (10 ng); 3, conditioned medium from U-251 cell culture after 24 h incubation under serum-free conditions (100 μl corresponding to the media conditioned by 10^5 cells); 4, U-251 cell lysate (10^5 cells/lane). (B) Samples were subjected to SDS-PAGE and analyzed by reverse fibrin zymography. Lanes: 1, purified PAI-1 (50 ng); 2, conditioned medium from U-251 cell culture after 24 h incubation under serum-free conditions (100 μl corresponding to the media conditioned by 10^5 cells)); 3, U-251 cell lysate (10^5 cells/lane). Positions of molecular weight standards are indicated.

ent in the media conditioned by the U-251 cells (Fig. 1B, lane 2), as well as associated with the U-251 cell lysates (Fig. 1B, lane 3), and the lysis-resistant zone co-migrated with the activity of purified PAI-1 (Fig. 1B, lane 1). The inhibitor responsible for the lysis resistant zone could be immunodepleted from both U-251 conditioned media and cell lysates using Sepharose anti-PAI-1, whereas the enzyme responsible for the 50 kDa lytic zone could be immunodepleted using Sepharose anti-urokinase but not with Sepharose anti-t-PA (data not shown). It should be noted that active PAI-1 released into solution is rapidly converted at physiological temperatures ($T_{1/2}$, 1 h) into a latent form whose activity can be detected following treatment with denaturants (e.g. SDS-PAGE) [13] and thus, the presence of primarily latent PAI-1 coupled with high levels of u-PA in the conditioned media would account for the inability of Sitrin and co-workers [29] to detect PAI-1 in the U-251 conditioned media.

3.2. Subcellular distribution of PAI-1 in U-251 cells

Immunofluorescence analysis was initially utilized to investigate the cellular distribution of this inhibitor. Fig. 2 indicates that intense staining for PAI-1 was observed in two distinct regions: (i) in the perinuclear region (Fig. 2A) and (ii) in cellular processes (Fig. 2C). Examination of these latter fields by phase contrast microscopy suggested that the punctate staining of PAI-1 within the cellular processes was localized to membrane-bound organelles (data not shown). No specific staining was observed utilizing non-immune IgG as the primary antibody (Fig. 2B,D). A similar pattern of staining was observed using a commercially available monoclonal antibody (no. 380, American Diagnostica) against PAI-1 as the primary antibody (data not shown).

Because the punctate PAI-1 staining pattern in U-251 cells was reminiscent of the distribution of PAI-1 in the dense core storage granules present within the cellular processes of PAI-1-transfected AtT-20 cells [28], we utilized density gradient fractionation to further characterize the PAI-1-containing subcellular organelles of U-251 cells. For this purpose, U-251 cells were homogenized, the subcellular organelles were

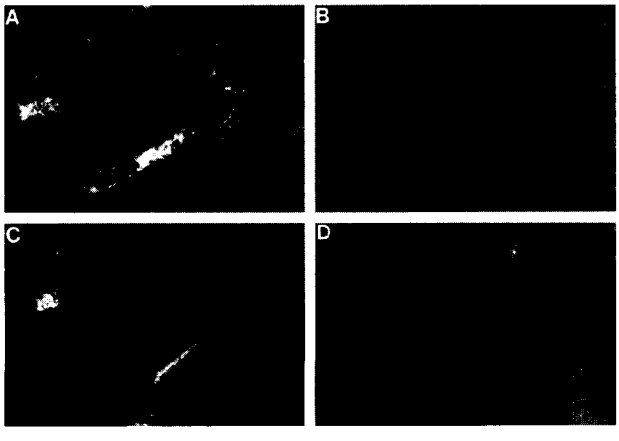

Fig. 2. Immunofluorescence staining of PAI-1 in human U-251 cells. Subconfluent cultures of human U-251 cells were grown overnight in serum-free medium, fixed with paraformaldehyde, permeabilized with 1% Triton X-100 and incubated with affinity purified rabbit anti-PAI-1 (A,C [28]) or with nonimmune rabbit IgG (B,D). Bound antibodies were detected by incubation with fluorescein-labelled goat anti-rabbit IgG. The samples were examined using an Olympus BH-RFL microscope equipped with a DN-100 dichroic mirror, an O-515 barrier filter, and a mercury lamp for excitation. Samples were photographed using a Kodak 3200 ASA film and a 10 s exposure.

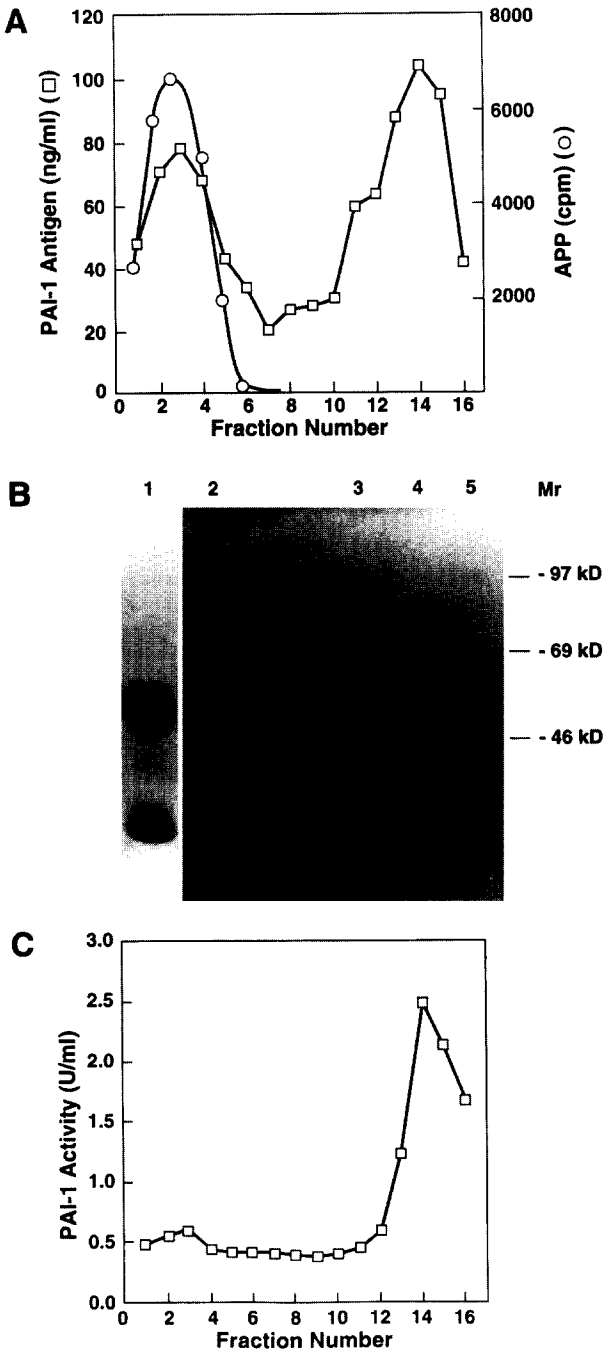

Fig. 3. Subcellular fractionation of U-251 cells. U-251 cells (10^8 cells) were homogenized and their cellular organelles were centrifuged on a 20% Percoll density gradient. Fractions (800 μl) were collected starting from the top of the gradient. (A) Fractions were analyzed for PAI-1 antigen in a two-site IRMA. APP antigen was analyzed by dot blotting using anti-APP clone 22C11 as the primary antibody followed by ^{125}I-goat anti-mouse IgG (10^5 cpm/ml) and the radioactivity associated with each dot determined in a gamma-counter. (B) Zymographic analysis of u-PA in Percoll gradient subcellular fractions of U-251 cells: urokinase (lane 1, 10 ng); t-PA (lane 2, 10 ng); fractions 3 (lane 3, 100 μl); fraction 9 (lane 4, 100 μl); fraction 14 (lane 5, 100 μl). (C) Analysis of PAI-1 activity in U-251 subcellular fractions utilizing a functional immunoassay.

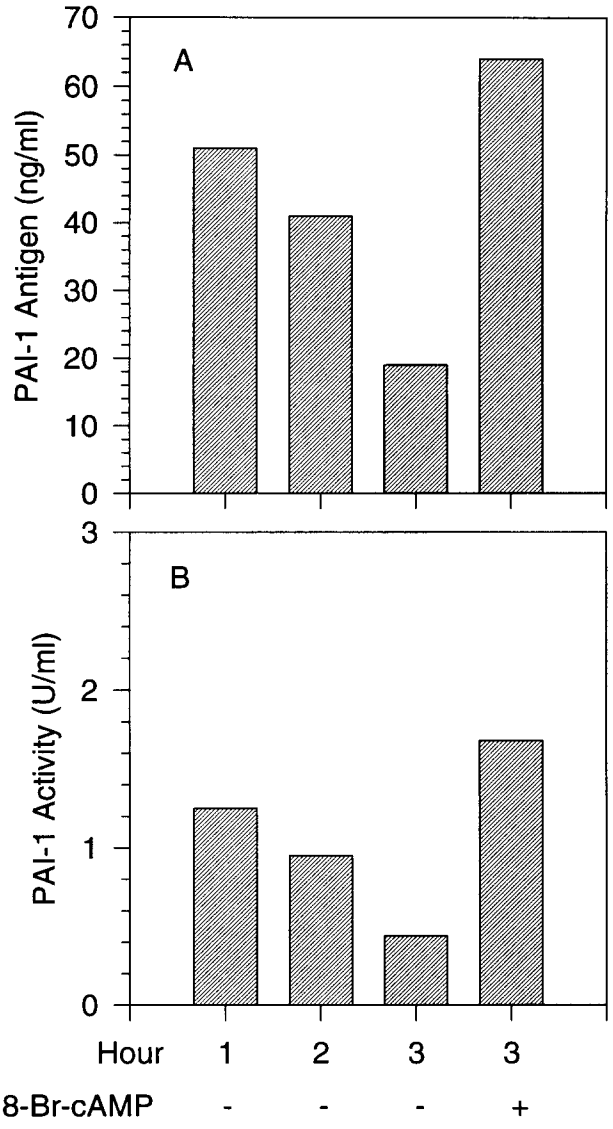

Fig. 4. Secretagogue-induced secretion of PAI-1 from U-251 cells. U-251 cells were incubated for two consecutive 1 h time periods under serum-free conditions and the conditioned media harvested to define basal PAI-1 secretion. The cells were subsequently incubated for 1 h in serum-free media in the absence or presence of 5 mM 8-Br-cAMP. The harvested conditioned media were analyzed for PAI-1 antigen in a two-site IRMA (A) and for PAI-1 activity in a functional immunoassay (B).

pelleted by centrifugation, and subjected to ultracentrifugation on 20% Percoll. Analysis of PAI-1 in the isolated fractions revealed that PAI-1 was present in two distinct areas of the gradient (Fig. 3A): (i) a low density band that has been observed to correspond to rough endoplasmic reticulum, Golgi apparatus, cellular membranes [34,35] and (ii) a high density, storage granule-containing region [34,35]. To demonstrate specificity for this fractionation protocol, APP was selected because of its distribution within diverse cell subfractions. For example, APP has been observed (i) to be stored in conjunction with other proteins (e.g. PAI-1) within platelet α-granules, (ii) to undergo fast axonal transport in neurons, and (iii) to be endocytosed and proteolytically processed in lysosomes; however, this protein is constitutively secreted in all other cell types examined so far including a classical model cell system (i.e. AtT-20 cells) for investigating the packaging of proteins (e.g. PAI-1 [28]) into the regulated secretory pathway [36]. Thus, data on the distribution of APP in the subfactions would provide an insight into the specificity of this cell's regulated secretory pathway for PAI-1. Dot blotting analysis of APP using a monoclonal antibody against the extracellular domain of APP revealed that this protein was present only in the low density region (Fig. 3A).

Because complexes between u-PA and PAI-1 have been observed to be internalized via the lipoprotein-receptor related protein into lysosomes [8], we investigated the possibility that we were immunologically detecting PAI-1 antigen associated with u-PA in endosomes or lysosomes contaminating the high density region of the gradient. Firstly, fibrin zymography of isolated fractions revealed that u-PA activity could only be detected in the low density region (Fig. 3B, lane 3) and was absent in the high density region (Fig. 3B, lane 5). Secondly, analysis of the activity for PAI-1 in a functional immunologic assay revealed that the high density region contained the majority of active PAI-1 (Fig. 3C).

3.3. *Secretagogue-induced secretion of PAI-1 from U-251 cells*

The presence of active PAI-1 in the high density regions of the Percoll gradient, which have been observed to contain dense core secretory granules of AtT-20 cells [34,35], raised the possibility that PAI-1 may be present in a rapidly releasable form in U-251 cells. To examine this issue, U-251 cells were washed repeatedly over a 2 h period and subsequently incubated for 1 h in the presence and absence of 8-Br-cAMP, a secretagogue of the regulated secretory pathway [34,35]. Fig. 4 indicates that the presence of 8-Br-cAMP resulted in a 3-fold increase in the PAI-1 antigen (panel A) and activity (panel B) of the media conditioned by U-251 cells in comparison to the media conditioned in the absence of this secretagogue.

Taken together, our data indicate that U-251 cells contain active PAI-1 stored in a rapidly releasable state. The role of this form of PAI-1 may be related to the wide-spread distribution of u-PA detected in gliomas in vivo [11,12]. Although cell-associated u-PA appears to be required for the migration of numerous types of cells [8] including U-251 cells [10], our observations coupled with the report of Sitrin and co-workers [29] indicate that the secretion of high levels of u-PA from U-251 cells results in a net plasminogen activation activity in the milieu surrounding these cells. Because u-PA is able to directly activate latent collagenase [37], Reith and Rucklidge [37] have proposed a cyclic model in which these two enzymes could initiate cellular damage, disruption of blood brain barrier, and subsequent release of plasminogen from the damaged vessel. Thus, high levels of rapidly releasable active PAI-1 might be important as a local control mechanism to prevent proteases, which are secreted by the tumor cells themselves, from excessively degrading the surrounding ECM and causing necrosis of the tumors. In light of the ability of neuronal cells to package and secrete a wide variety of proteins and other molecules [38], the derivation of gliomas from neuroectodermal cellular lineage [2] raises the possibility that a regulated secretory pathway may exist in other types of gliomas and hence, the packaging of PAI-1 and its role in regulating excessive protease activity within the brain may be more complex than previously envisioned.

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