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INVASION OF BRAIN TISSUE BY PRIMARY GLIOMA: EVIDENCE FOR THE INVOLVEMENT OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR AS AN ACTIVATOR OF TYPE IV COLLAGENASE[1]

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The immunocharacterization of a metalloproteinase isolated from rat glioma cell conditioned medium is described and confirms that the enzyme is identical to type IV collagenase. Free, active plasminogen activator (PA) and PA-PAI complexes were identified as being secreted by the same cells. Using affinity-purified metalloproteinase we demonstrate that the enzyme can be partially activated by u-PA but not by plasmin in vitro. On the basis of these findings and previous published work we propose a scheme for the proteolytic degradation of normal brain tissue during tumour invasion.

The invasion of many primary brain tumours is accompanied by remodelling of the vasculature and the destruction of normal brain tissue. This replacement of degraded elements by tumour cells results in a poor prognosis for glioma patients. The arrest of tumour invasion in these cases is particularly important as these tumours do not metastasize and can be regarded as a localised disease without the concomitant concern of secondary tumours arising at remote sites. We have previously identified a proteolytic enzyme secreted by rat glioma cell lines as a metalloproteinase [1] and have shown that it has the ability to degrade normal brain tissue in a model invasion system [2]. However the precise nature of this metalloproteinase has not been reported previously and as it is secreted in a latent form the natural activator in biological systems has not been identified. In this paper we demonstrate that the glioma secreted metalloproteinase is immunologically identical to type IV collagenase, a protein previously reported to be secreted by many tumours possessing a

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Abbreviations:
u-PA, urokinase-type plasminogen activator; AHA, amino hexanoic acid; APMA, aminophenylmercuricacetate; t-PA, tissue-type plasminogen activator; PA, plasminogen activator; PAI, plasminogen activator inhibitor.

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high metastatic potential [3]. We also show that the brain tumour cells capable of tissue destruction also secrete PAs in vitro, and demonstrate that u-PA is capable of activating latent metalloproteinase secreted by rat glioma.

MATERIALS AND METHODS

Cell culture conditions

BT5C is a continuous cell line obtained from fetal rat brain cells which underwent in vitro transformation following in vivo exposure to N-ethyl-N-nitrosourea [4]. These rat glioblastoma cells were cultured as monolayers in 80 cm^2 tissue culture flasks (Nunc, Life Technologies, Paisley, Scotland). The cells were maintained within a standard tissue culture incubator with an atmosphere of 37°C, 100% humidity, 5% CO_2 and 95% air. The BT5C cells were cultured in Dulbecco's modified Eagles medium supplemented with 10% heat inactivated newborn calf serum, four times the prescribed concentration of non-essential amino acids, 2% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (ICN Biomedicals Ltd, Bucks, UK). Serum free media was prepared as above replacing the serum with chemically defined additives [5].

Isolation and immunoblotting of metalloproteinase

The metalloproteinase was affinity-isolated on Gelatin-Agarose from BT5C serum free conditioned medium as described previously [1]. The protease was subjected to SDS polyacrylamide gel electrophoresis according to the method of Laemmli [6] and the protein transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) at 400mA for 3h, thereafter the paper was incubated with 5% milk powder to block non-specific binding sites. The proteases was immunostained with rabbit anti-type IV collagenase. This anti-serum was raised against the native 72kD form of the enzyme isolated from A2058 human melanoma conditioned medium (a gift from Dr.W.G.Stetler-Stevenson, N.C.I., Bethesda, U.S.A.). The antibody is specific for type IV collagenase and also has the ability to inhibit the gelatinolytic activity of the enzyme in vitro (Dr. W.Stetler-Stevenson, personal communication).

In parallel with the immunoidentification of the protease, zymogram analyses were undertaken according to the method of Heussen and Dowdle [7] to establish the presence of electrophoretically separated protein bands which contained proteolytic activity. The molecular weights of the latent and active forms of the purified protease were determined by comparison with rainbow marker molecular weight standards (Amersham, U.K).

Identification of plasminogen activators and inhibitors of fibrinolysis

BT5C cell conditioned media was analysed for PAs by SDS-PAGE [6] using stacking and separating gels of 3% and 10% respectively. SDS was removed from the gel by thoroughly washing in 2.5% Triton X-100 followed by distilled water. The washed gel was placed on a zymographic detector plate composed of 1% Type I agarose (Sigma Chemical Co Ltd, Poole, Dorset, U.K.) in 75mM Tris pH 7.8, 22mM NaCl containing fibrinogen (2 mg/ml, KabiVitrum, Bucks, U.K.), plasminogen (5 μg/ml, Fluorochem Ltd, Derbyshire, U.K.) and thrombin (0.06 U/ml, Leo Laboratories, Bucks, U.K.). [8]. Detector plates were incubated at 37°C overnight. Plasminogen activators diffuse from the separating gel into the zymographic detector plate where they activate plasminogen to plasmin which produces a clear band of lysis against an opaque background. Bands of PA activity were identified by incorporating antibodies to tissue-type plasminogen activator (t-PA) at a final concentration of 20 μg/ml (Biopool, Sweden). Standards of u-PA, a mixture of 33kD and 55kD u-PA, (50 Ploug units/ml, Leo Laboratories, U.K.) and t-PA of 68kD (57 IU/ml, Boehringer Mannheim Ltd, Sussex, U.K.) were used for comparison.

Inhibitors of fibrinolysis were detected using a fibrin detector plate as above which additionally contained u-PA (0.03 Ploug units/ml, Leo Laboratories, U.K.) [8]. In this type of zymogram opaque bands of fibrin resistant to lysis indicated the presence of inhibitors of fibrinolysis.

Activation of metalloproteinase

Preincubation of the purified metalloproteinase with u-PA was performed at 37°C for 1h before assay. At the end of the preincubation stage the u-PA-metalloproteinase mixture was incubated with 10mM amino hexanoic acid to inhibit the u-PA and any contaminating plasmin. Metalloproteinase activity was measured using the release of ^{3}H-gelatin fragments from glutaraldehyde immobilized gelatin on microtitre plates as previously described [9]. These activities were compared to the maximal potential activity of the metalloproteinase when activated by 1mM APMA at 37°C for 1h prior to assay.

RESULTS AND DISCUSSION

Metalloproteinase characterization

The rat glioma cell line BT5C exhibits a degradative mode of invasion both in vivo and in vitro as described previously [10]. The metalloproteinase activity secreted by this cell line, demonstrated by the zymogram technique, showed a single major band of activity following activation of the latent enzyme by preincubation with APMA (Fig.1). Comparison of this band of activity with the molecular weight standards showed that the active form of the metalloproteinase has a Mr of 67kD and the latent form of the enzyme has an Mr of 72kD as demonstrated previously [1]. The presence of the latent and active forms of the metallproteinase in the medium and following affinity isolation is not surprising due to the presence of urokinase type plasminogen activator in the cell conditioned media which we demonstrate has the ability to activate the metalloenzyme (see later). Immunostaining of the metalloproteinase transferred to Immobilon P paper was performed using a polyclonal antibody raised against type IV collagenase and showed that the enzyme was recognised by this antibody. The major band of immunostained protein was coincident with the major band of activity on the zymogram, identified as the active form of the metalloproteinase (Fig.1). This evidence, and the properties of the enzyme described previously including metal chelator inhibition, relative molecular mass studies, activation properties and inhibition by recombinant tissue inhibitor of metalloproteinases (TIMP-1) [1], confirm that the metalloproteinase secreted by the rat glioma cell line BT5C is homologous with type IV collagenase, an enzyme which has been characterized previously [11,12]. The extent of the capacity of tumour cells to secrete this metalloproteinase has been correlated with metastatic potential [3]. It is perhaps surprising that this enzyme is secreted by glioma cells which are unable to metastasize and form secondary tumours outwith the central nervous system

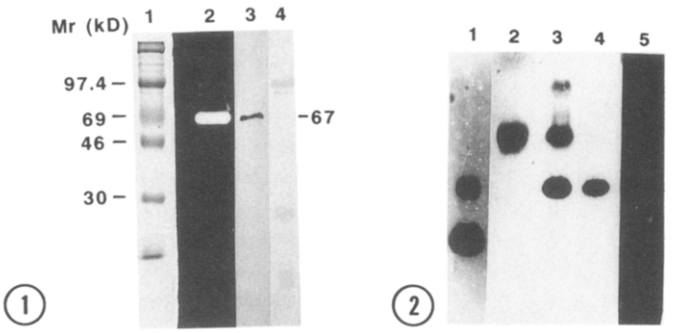

Figure 1. Electrophoresis and immunoblotting of affinity purified glioma cell metalloproteinase. Lane 1; Standard proteins Mr as indicated. Lane 2; Zymogram analysis of APMA-activated metalloproteinase. Lane 3; Immunoblot of electrophoretically separated APMA-activated metalloproteinase immunolocalised with anti-type IV collagenase. Lane 4; Rainbow markers (Amersham) standard proteins transferred to Immobilon P electroblotting paper.

Figure 2. Zymographic analysis for PA activity (Lanes 1 - 4) and for inhibitors of fibrinolysis (Lane 5). Lane 1; u-PA standard (50 Ploug units/ml). Lane 2; t-PA standard (57 IU/ml). Lane 3; BT5C cell conditioned media. Lane 4; as Lane 3 but incorporating 20 μg/ml anti-t-PA IgG into the detector gel. Lane 5; BT5C cell conditioned media.

although we have reported previously that this metalloproteinase is capable of destroying normal brain tissue in a model invasion system [2]. It is possible, for glioma cells, that tissue destruction may also be the principal effect of this enzyme in vivo. A rat glioma cell line BT4Cn, which fails to cause appreciable tissue destruction either in vivo or in vitro [10], does not appear to secrete the metalloproteinase activity in culture as analyed by zymography (results not shown). It may be that, rather than a correlation of type IV collagenase activity with metastatic potential for glioma cells, the secretion of the metalloproteinase indicates a degradative phenotype in vivo and in vitro.

Function of activators and inhibitors of fibrinolysis in glioma media

PA activity was detected in BT5C cell conditioned medium using zymography (Fig.2) and bands of u-PA, t-PA and PA-PAI complex were identified. An excess of PA over PAI was further demonstrated by the lack of PAI activity (PA inhibition) on the zymogram (Fig.2). Thus, PA secreted by the BT5C cells was abundant and present in an active form. PA activity has been described in a variety of human tumour cell lines [13,14]. The secretion of PAs by tumour cells, in particular u-PA, has been associated with tissue damage during tumour invasion and, as for type IV collagenase, with an increased potential for metastatic behaviour [15]. PA activity has also been described in other glioma cell conditioned media

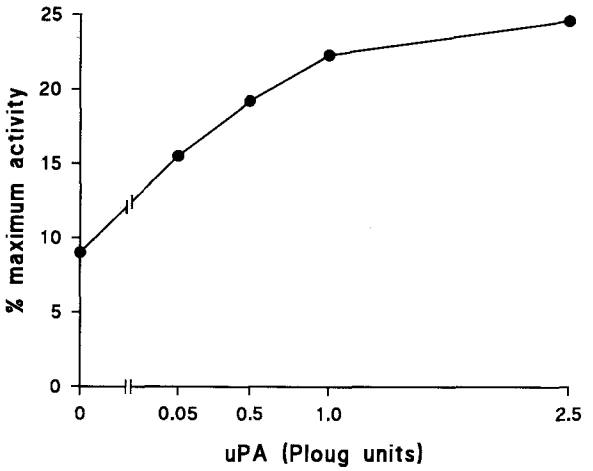

Figure 3. Activation of affinity purified metalloproteinase by incubation of the metalloproteinase with u-PA. The results are expressed as the percentage of the maximal activity obtained by activation of the enzyme by incubation with 1mM APMA at 37°C for 1h using the release of ^{3}H-geletin from coated microtitre plates as described in the methods section.

[16,17] but, as stated earlier, gliomas do not metastasize. This prompted us to investigate whether PA, in particular u-PA, had the potential to activate the metalloproteinase (type IV collagenase) since both are secreted by this rat glioma cell line.

Using the radiolabel release assay of proteolytic activity the results in Figure 3 show that preincubation of the metalloproteinase with u-PA activated the enzyme from 9% to 25% of its potential theoretical maximal activity as estimated by the complete activation of the

Table 1. Attempted activation of affinity purified glioma cell metalloproteinase by plasmin using radiolabel-release assay

	Substrate released (μg gelatin/h)
Non-activated metalloproteinase	0.32
Metalloproteinase + 1mM APMA	2.41
Plasmin (2.5μg)	6.82
Plasmin (2.5μg) + 10mM AHA	0.02
(Metalloproteinase + Plasmin (2.5μg) + AHA	0.36
(Metalloproteinase + Plasmin (2.5μg)) + AHA + 1mM APMA	2.51

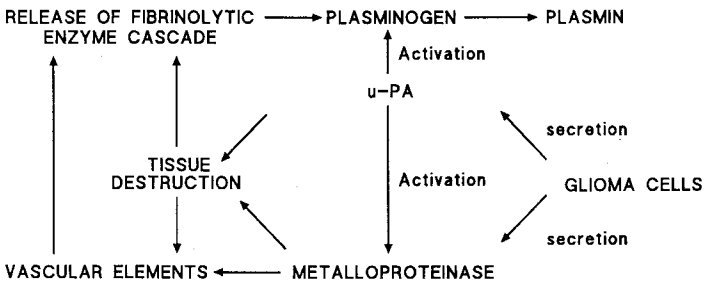

Figure 4. Proposed scheme indicating mechanisms of enzyme activation and tissue damage during glioma invasion.

metalloproteinase by APMA. Between the concentrations of u-PA used in these experiments the activation appeared to be dose dependent. In the presence of the PAs, u-PA and t-PA, plasminogen is converted to the biologically active plasmin. It is of interest that while u-PA activates latent glioma metalloproteinase in the radiolabel release assay, plasmin did not cause any activation of the metalloproteinase (Table 1). Previous studies have reported activation of type IV collagenase by plasmin directly [18] or via the activation of plasminogen to plasmin in human breast carcinoma [19]. Our observations suggest a direct rather than indirect activation of latent glioma type IV collagenase by u-PA in vitro. Upon the basis of our findings in this study and previously [1,2] we propose a mechanism of brain tissue destruction in vivo via the pathways outlined in Figure 4 in which u-PA can contribute to tissue damage through type IV collagenase activation, plasminogen activation to plasmin and by its direct action on tissue. The release of plasminogen following damage of blood vessels [20] and disruption of the blood brain barrier during invasion [21] will further enhance the cycle of normal tissue destruction which occurs during the invasion of tumours with a degradative phenotype.

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