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A glia-derived neurite-promoting factor with protease inhibitory activity

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Brain cells and glioma cells in culture release a protein which induces neurite outgrowth in neuroblastoma cells. This neurite-promoting factor (NPF), which has been purified from serum-free glioma conditioned medium, has an apparent mol. wt. of 43 000. NPF inhibits urokinase as well as plasminogen activator-dependent caseinolysis or fibrinolysis. NPF and urokinase form an SDS-resistant complex. The fact that this glia-derived NPF is a potent protease inhibitor indicates that glial cells modulate the proteolytic activity associated with neuronal cells and suggests that this phenomenon is one of the biochemical events involved in the regulation of neurite growth.

Key words: glia/neuroblastoma/neurite outgrowth/inhibition of plasminogen activation

Introduction

The development of the nervous system requires a multiplicity of cellular interactions. The characterization of the molecules involved in these interactions is necessary to approach the regulation of these developmental events at the biochemical level. Cultured non-neuronal brain cells and glioma cells release macromolecules which influence survival and/or neurite outgrowth of neuronal cells (Monard *et al.*, 1973; Barde *et al.*, 1978; Lindsay, 1979; Sensenbrenner *et al.*, 1980; Tanaka and Obata, 1982; Unsicker *et al.*, 1984).

The glioma-derived neurite-promoting factor (NPF) which induces morphological differentiation in neuroblastoma cells (Monard *et al.*, 1973) is also found in primary cultures of brain tissue established at developmental stages concomitant with or subsequent to the burst of glial cell proliferation (Schuerch-Rathgeb and Monard, 1978). Here we provide evidence that this NPF is a 43 000-dalton protein.

Earlier results indicated that inhibition of neuroblastoma cell-associated proteolytic activity can lead to neurite outgrowth (Monard *et al.*, 1983). The present report shows that the NPF purified from serum-free glioma-conditioned medium is also an inhibitor of urokinase- and plasminogen activator-dependent caseinolysis or fibrinolysis. NPF forms an SDS-resistant complex with urokinase.

Results

Using the neuroblastoma cells as detection system, the NPF present in the serum-free glioma-conditioned medium can be purified in two steps using heparin-Sepharose and Affi-Gel Blue chromatography (see Materials and methods). The inhibition of urokinase-dependent fibrinolysis or caseinolysis and the neurite-promoting activity co-purify. Silver-stained SDS-PAGE illustrates the purity of the protein isolated by this two-step purification procedure (Figure 1). When enough material was loaded on the gel, the purified protein appeared as a doublet at 43 kd. The procedure chosen for the elution from the heparin-Sepharose strongly influences the purity of the material obtained from Affi-Gel Blue chromatography. The elution with 0.8 M NaCl chosen here leads to a poorer recovery than elution with 1.5 M NaCl. It has nevertheless been maintained because elution with the higher salt concentration gives rise to the appearance of two additional protein bands in the active fractions eluted from the subsequent Affi-Gel Blue chromatography. The two-step procedure described leads to 260 μg of pure NPF out of 16 l of serum-free glioma-conditioned medium. The doublet at 43 000 daltons shows a strong reaction upon periodic acid-silver staining, revealing its glycoprotein nature (results not shown). We can estimate that the purified protein promotes neurite outgrowth in 50% of the neuroblastoma cells at 7 ng/ml (6×10^{-11} M). 23 ng are necessary for a 50% inhibition of 0.1 mU urokinase in the 100 μl caseinolysis test (6×10^{-9} M). To demonstrate that both neurite-promoting activity and urokinase inhibitory activity are due to the same protein located at 43 kd, the active material eluted from Affi-Gel Blue was submitted to reversed phase h.p.l.c. followed

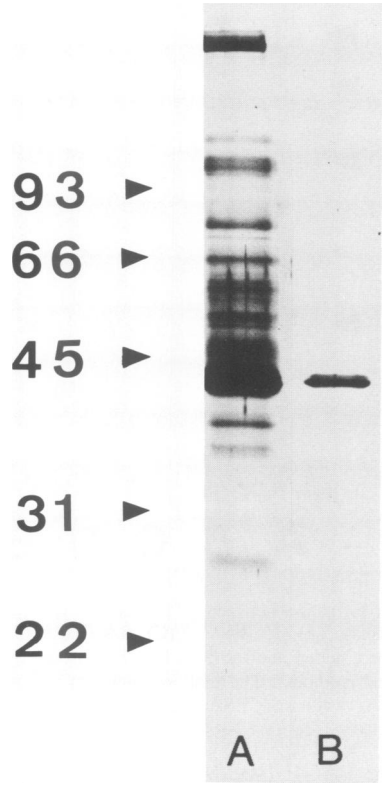

Fig. 1. Silver-stained SDS-gel electrophoresis. Heparin-Sepharose pool *(A)* and Affi-Gel Blue pool **(B)** have been analysed as described in Materials and methods. The mol. wt. markers indicated in kd are: phosphorylase b, BSA, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor.

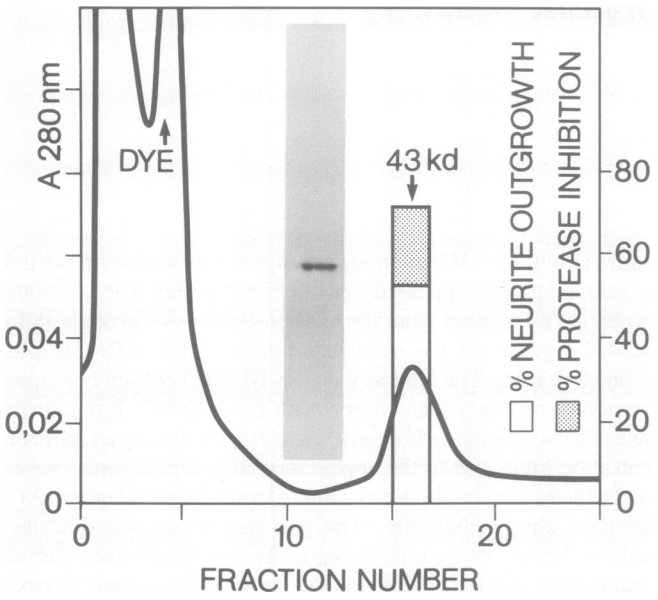

Fig. 2. Preparative SDS-PAGE. Pools of two fractions were made. The assay for neurite outgrowth and protease inhibition were performed after SDS removal. For the neurite outgrowth assay 10 μl of the renatured pools were tested, the background of this biological assay was 23% and the inactive pools gave values of ~25%. The caseinolysis assay was performed using 5 μl of the samples and 0.1 mU of urokinase. The background values were ~5%. Both activities could only be recovered within the protein peak. The insert shows a silver-stained analytical 10% SDS-PAGE of the pool from the fractions 16 and 17.

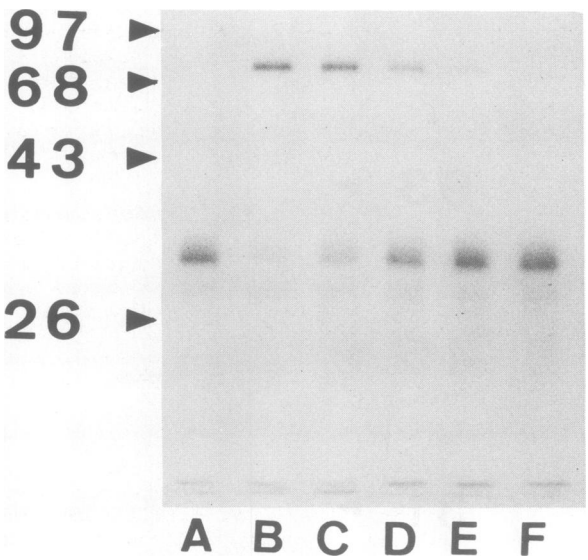

Fig. 3. Complex formation between [^{125}I]urokinase and pure NPF. To 1.5 ng [^{125}I]urokinase **(A)**, 15 **(B)**, 1.5 **(C)**, 0.5 **(D)**, 0.15 **(E)**, 0.05 ng of pure NPF **(F)** were added and the SDS-PAGE analysed by autoradiography (see Materials and methods). The mol. wt. markers indicated in kd are α-chymotrypsinogen, ovalbumin, BSA and phosphorylase B.

by preparative PAGE (see Materials and methods). Figure 2 illustrates that a single protein peak was eluted. When different fractions were tested after renaturation (see Materials and methods) only those located in this protein peak revealed both neurite-promoting activity and urokinase inhibitory activity concomitantly. Each active fraction revealed only the same 43-kd doublet as the Affi-Gel Blue-purified protein when analyzed on silver-stained SDS-PAGE. Figure 3 demonstrates that the purified NPF forms an SDS-resistant complex with urokinase. Preliminary results (not shown) indicate that the 43-kd glia-derived NPF also inhibits thrombin and trypsin, but not chymotrypsin.

Discussion

The material purified by heparin-Sepharose and Affi-Gel Blue chromatography contains both neurite-promoting activity and urokinase inhibitory activity and reveals only the 43-kd doublet upon analysis with silver stained SDS-PAGE. The preparative SDS-PAGE demonstrates that both NPF and urokinase inhibitory activity are due to the same protein located at 43 kd. When the protein purified by heparin-Sepharose and Affi-Gel Blue chromatography is further analyzed by f.p.l.c., two adjacent peaks are resolved. Each of them reveals both activities concomitantly and the 43-kd doublet by SDS-PAGE analysis (unpublished data). This f.p.l.c. behaviour might indicate a microheterogeneity which is presently under investigation.

The fact that the NPF purified from glioma-conditioned medium is also an inhibitor of urokinase and plasminogen activator suggests that one should consider its biological relevance under a new concept. The increase in cell-associated proteolytic activity detected when cerebellar granule cells migrate suggested that immature neuroblasts require such a plasminogen activator-like activity for adequate migration (Krystosek and Seeds, 1981a). This idea is supported by the fact that high concentrations of synthetic inhibitors of serine protease activity interfere with the migration of granule cells when added to cultured paraflocculi of the cerebellum (Moonen *et al.*, 1982). The release of glia-derived neurite-promoting activity in primary cultures of rat brain coincides with a critical developmental stage (3–5 days post-natal) at which most neuronal migrations cease and the burst of glial cell multiplication takes place (Schuerch-Rathgeb and Monard, 1978). The present results suggest that inhibitors similar to the protein we have purified are released by the glial cells and regulate the proteolytic activity associated with the neuronal cells. Through the production of such inhibitors, glial cells would be able to modulate the extent of neuronal migration and cause, at the same time, modifications compatible with the early, target-independent outgrowth of neurites. One has also to envisage that the release of the protease inhibitory proteins could be a tissue culture phenomenon and that, *in vivo*, such proteins would remain associated with the glial cells.

Plasminogen activator-like activity has been found at the neuronal growth cone suggesting that this localisation allows the progression of nerve fibers through the tissue matrix of the developing nervous system (Krystosek and Seeds, 1981b). The fact that the glia-derived protease inhibitor promotes neurite outgrowth in neuroblastoma cells does not seem to be consistent with this hypothesis. The neuroblastoma cells used here as a model are however growing neurites in an *in vitro* environment where the extracellular matrix and its function are certainly far from reproducing the *in vivo* situation and where the adhesion could be more important than the growth cone-associated proteolytic activity. The neuroblastoma cells are, as tumor cells, expected to have an increased cell-associated proteolytic activity which would prevent, or be incompatible with, neurite outgrowth. The glia-derived protease inhibitor purified here could promote neurite outgrowth by reducing this excess of proteolytic activity. This argument is further supported by the promotion of neurite outgrowth by some other protease inhibitors (Monard *et al.*, 1983).

At least six different serine proteases can be detected in neuro-

blastoma cell membrane preparations (Murato and Monard, in preparation). These proteases could affect the turnover of specific membraneous proteins needed for neurite outgrowth; they could also promote the degradation of extracellular matrix elements which influence either the adhesion required for neuritic development or the extent of cellular migration. Each of those proteases could be differentially located on the cell body or at the growth cone. It is not yet known with which of these proteases NPF can interact but the possibility of a selective and localized inhibition has to be considered.

Despite these unknowns, the glia-derived protease inhibitor characterized here can be considered as a modulator which, according to the specific local conditions (amount and nature of the proteolytic activity, concentration of the inhibitor), could promote or even inhibit neurite outgrowth. In other words, a fine localized balance between neuronal proteolytic activity and the glia-derived inhibitor would sustain neurite elongation. Precise dosage and localization of the glia-derived protease inhibitor at different developmental stages will therefore be necessary in considering the biological relevance of such biochemical events.

An inhibitor of urokinase and of plasminogen activator has been purified from human placenta (J.Wun, personal communication). Similar inhibitory proteins, able to complex with urokinase and plasminogen activator, have been detected in the medium conditioned by macrophages, endothelial or hepatoma cells (Vassalli *et al.*, 1984; Loskutoff *et al.*, 1983; Gelehrter *et al.*, 1983). Proteins called protease nexins, able to form selective and stable complexes with serine proteases, are secreted by a variety of cultured cell types (for review, see Knauer *et al.*, 1983). These protease nexins are thought to react with serine proteases in the extracellular environment and thus mediate their cellular binding, internalisation and degradation. One of them has recently been purified (Scott and Baker, 1983).

Brain extracts (Barde *et al.*, 1982; Kligman, 1982; Carri and Ebendal, 1983), medium conditioned by primary glial cell cultures (Lindsay, 1979; Sensenbrenner *et al.*, 1980), by C6 glioma cells (Monard *et al.*, 1973; Barde *et al.*, 1978; Tanaka and Obata, 1982; Unsicker *et al.*, 1984) or by other various cell types (Lander *et al.*, 1982; for review, see Varon and Adler, 1981) contain neurite-promoting activities affecting the morphology of different types of neuronal cells. So far only one of these factors has been purified to homogeneity (Barde *et al.*, 1982) and can be clearly distinguished from the protein purified here.

These neurite-promoting factors, protease inhibitors, protease nexins, as well as the glia-derived neurite-promoting protease inhibitor described here will have to be further characterized by biochemical and immunological means to clearly assign their similarity and their specificity.

Materials and methods

All reagents were analytical grade. MEMα powder medium was from Gibco and fetal calf serum (FCS) from North American Biological Incorporation. Cytodex 1 beads and heparin-Sepharose CL-6B were from Pharmacia and Affi-Gel Blue from Bio-Rad. Spectra/Por 2 MWCO 12 000 – 14 000 dialysis membranes were from Spectrum Medical Industries Inc. The human urokinase routinely used for inhibition assays was from Leo Pharmaeuticals (10 000 Ploug units per ampoule). Plasminogen was prepared from fresh human serum as described by Robbins and Summaria (1976). The pure [^{125}I]urokinase used for complex formation studies was a gift from Dr J.D.Vassalli, Geneva.

Production of serum-free glioma-conditioned medium

MEMα medium supplemented with insulin (2 μg/ml), transferrin (15 μg/ml), penicillin G (150 U/mol) and streptomycin (30 μg/ml) [IT-MEMα] was used to grow C6 rat glioma cells (Benda *et al.*, 1968) on Cytodex microcarrier beads: 10^8 cells were added to 2 g of sterile Cytodex 1 microcarrier beads suspended in 250 ml of IT-MEMα supplemented with 10% FCS. Cell attachment was promoted by stirring the suspension in a 500 ml flask at 37°C on a MCS stirring system (Techne, Cambridge, UK) at 20 r.p.m. After 1 h, 250 ml of IT-MEMα supplemented with 10% FCS were added and the incubation continued for 48 h under the same conditions. The medium was then changed and the microcarrier beads further incubated in IT-MEMα supplemented with 5% FCS for 24 h. Serum-free IT-MEMα was used to transfer the beads into a 1.5 l flask where they were washed, twice for 1 h, with stirring in 500 ml of serum-free IT-MEMα. One litre of serum-free MEM was then conditioned during a 24 h incubation. This 24 h conditioning was repeated for as long as the cells remained attached to the microcarrier beads (up to 10 days). The collected conditioned medium was centrifuged for 15 min at 1000 *g* at 4°C to remove floating cells and kept for up to 1 week at 4°C in order to make up a sufficient amount to start the purification procedure.

Purification procedure

Heparin-Sepharose chromatography. 20 g of heparin-Sepharose CL-6B washed with an excess of 50 mM Tris-HCl buffer pH 8.0 (Tris buffer), was added to 16.5 l of serum-free conditioned medium. This suspension was stirred overnight at 4°C on a rotary shaker at a speed (~120 r.p.m.) required to avoid sedimentation of the heparin-Sepharose. The beads were then collected on a sintered glass filter, washed with Tris buffer and packed into a column. The heparin-Sepharose column (2.6 x 15 cm) was rinsed extensively with ~400 ml 0.4 M NaCl in Tris buffer and glial factor activity eluted with 0.8 M NaCl in the same buffer. The active fractions were pooled. The pool was diluted 2-fold with Tris buffer before it was dialysed against 1 l of Tris buffer with three buffer changes at 2 h intervals. The light floculation produced upon dialysis was spun down at 27 000 *g* for 20 min at 4°C.

Affi-Gel Blue chromatography. The dialysed heparin-Sepharose pool was loaded at a flowrate of 6 ml/h on an Affi-Gel Blue column (1.2 x 2.5 cm). The column was rinsed with 50 ml 0.4 M NaCl in Tris buffer, 25 ml of 1 mM ATP, 1 mM NADH in Tris buffer, 60 ml of 0.6 M NaCl in the same buffer. The following procedure was used to elute NPF: enough 1 M NaCl in Tris buffer was pumped to assure its equilibration with the Affi-Gel Blue. The flow was then stopped for 1 h to promote the best possible desorbtion. Elution was then resumed at 6 ml/h. The collected fractions (33 drops/fraction) containing NPF were pooled. This Affi-Gel Blue pool was dialysed against 1 l of Tris buffer with three changes at 4 h intervals.

Reversed phase h.p.l.c.

This was performed with a wide pore C_8 column 0.4 x 25 cm (Bakerbond, J.T.Baker, RP 7105-0). NPF purified on heparin-Sepharose and Affi-Gel Blue was dialysed against 0.1% trifluoroacetic acid (TFA) and concentrated to 1 ml with a Savant Speed Vac Concentrator and injected. The gradient was 80 – 100% buffer B in 30 min, it was commenced immediately after sample injection (buffer A: 0.1% TFA; buffer B: aqueous 50% acetonitrile containing 0.1% TFA).

SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) in a slab gel (140 x 85 x 1.5 mm) with a 10 – 20% linear gradient in polyacrylamide. The gel was overlayed with 10 mm of stacking gel and run with 20 mA until the bromophenol blue reached the bottom. At this time, the current was kept constant for an additional 40 min to remove the material migrating behind the front and interfering with the silver staining, which was performed as described by Eschenbruch and Buerk (1982). The Bio-Rad low mol. wt. markers were used as standards. In certain cases, the periodic acid silver staining of Dubray and Bezard (1982) was used to reveal glycoproteins.

Preparative SDS-PAGE

NPF purified and concentrated by heparin-Sepharose, Affi-Gel Blue and h.p.l.c. (~1.9 mg) was lyophilized in the Savant Speed Vac Concentrator. 200 μl were neutralized with 1 M Tris-base, 100 μl 3-fold concentrated Laemmli-buffer were added. The electrophoresis was performed in the BRL-Preparative Gel-Electrophoresis system in 10% polyacrylamide 1 cm wide, 4 cm long with a 1 cm stacking gel. The electrophoresis buffer was the same as for the analytical electrophoresis. To the cathode buffer 0.1 mM sodium thioglycolate was added. The elution buffer was 6 g Tris; 28.8 g glycine, 1 g SDS, 154 mg dithiothreitol (DTT)/litre and the flow-rate 6 ml/h. The electrophoresis was run with a 6 mA at room temperature and 1 ml fractions were collected.

Renaturation procedure

Pools of two fractions from the preparative electrophoresis were dialysed against 75 mM NaCl in 50 mM Tris-HCl pH 8.0. 30 μl samples were taken for SDS-PAGE analysis, then 500 μg bovine serum albumin (BSA) were added and SDS removed following the procedure described by Hager and Burgess (1980). The precipitated proteins were dissolved in 20 μl 6 M guanidine-HCl in 50 mM Tris pH 8.0, 20% glycerol, 0.15 M NaCl, 1 mM DTT. It was slowly diluted with 1 ml 75 mM NaCl in 50 mM Tris pH 8.0 and dialysed against this buffer.

Complex formation between urokinase and pure NPF

5 μl 50 mM Tris-HCl pH 8.0 containing 0.1% Triton X-100 and 1.5 ng human [^{125}I]urokinase (33 000 dalton form; sp. act. 3 x 10^6 c.p.m./μg, Vassalli *et al.*, 1984) were incubated for 1 h on ice with 10 μl samples (15; 1.5; 0.5; 0.15; 0.05 ng) of purified NPF in Tris pH 8.0 containing 0.1 mg BSA/ml. These incubation mixtures were supplemented with 15 μl double-concentrated Laemmli buffer (non-reducing conditions) and analyzed without prior boiling on a 10% SDS-PAGE as described above. The pre-stained mol. wt. markers from Bethesda Research Laboratories were used as standards. The fixed and dried gel was overlayed with Kodak XAR-5 film for 20 h.

Inhibition of plasminogen activation

The amount of proteolytic inhibitory activity was estimated in a caseinolysis assay performed in microtiter plates with 96 wells. Each well contained 5 μl of 8% (w/v) milk powder suspension in phosphate-buffered saline, 5 μl of 0.5% Triton X-100 in 50 mM Tris-HCl buffer pH 8.0 containing 0.02 or 0.1 mU human urokinase, the fraction to be tested, Tris buffer to make up to 95 μl and 5 μl Tris buffer containing 1 μg of purified human plasminogen. The interaction between the inhibitor and the urokinase was promoted by a 20 min incubation at 4°C. The reaction was started by the addition of the plasminogen and the reaction mixture incubated at 37°C. Caseinolysis was monitored by measuring, at 1 h intervals, the decrease in turbidity at 405 nm using a Titertek Multiskan MC photometer. In some cases, the plasminogen-dependent degradation of [^{125}I]fibrin (Unkeless *et al.*, 1973) was used to monitor the presence of the inhibitor.

Neurite outgrowth

The neurite-promoting activity was assayed as described earlier by Schuerch-Rathgeb and Monard (1978).

Protein determination

The protein concentration was determined by measuring the extinction at 280 nm. The relationship 1 A_{20} unit = 0.8 mg protein was used to estimate the protein concentration.

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