ISOLATION OF A GLIAL MATURATION FACTOR FROM BEEF BRAIN

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1. Introduction

Soluble brain extracts from chick embryos and from newborn rats stimulate the morphological maturation of chick neuronal and glial cells in mixed cultures [1,2]. Chick brain extracts induce morphological changes in pure astroglial cells in culture derived from brains of newborn rats, with an increase of the level of the glial-specific protein S100 [3]. In these rat glial structures, brain extracts prepared from postnatal rats and adult beef elicit the same morphological and biochemical stimulatory effects [4] as well as enhance glial cell proliferation. Similar changes induced by brain extracts from rats and pigs in different astroglial cultures have been reported [5–7].

It is not known whether the different effects, i.e., morphological changes, enhanced proliferation and biochemical maturation are induced by a single active factor or by several different factors. Therefore, we purified a crude fraction from beef brain extract and could demonstrate that the isolated factor induces the three events simultaneously.

2. Materials and methods

2.1. *Cell cultures*

Cultures of newborn rat astroblasts were prepared and maintained as in [8]. The culture medium consisted of 90% Waymouth nutrient medium (Gibco no. 705/1 MD) and 10% fetal calf serum (Gibco). In experimental cultures, the crude brain extract or purified fractions were added after 5 days cultivation in normal nutrient medium for various times.

2.2. *Crude brain extract preparation*

Beef brains were homogenized in 20 vol. chloroform–methanol (2:1, v/v) at 4°C. The homogenate was maintained at 4°C under agitation for 12 h. The precipitate was then filtered through a glass filter, washed with the chloroform–methanol mixture, and finally acetone-dried. The powder was resuspended in distilled water (3 mg/ml) and the mixture was stirred for 2 h at 4°C and centrifuged at 20 000 × *g* for 30 min. The supernatant, called 'crude brain extract', was kept frozen at −20°C.

2.3. *Purification of the active factor(s)*

2.3.1. Criteria of purification

The effect of the crude extract and of its subsequent fractions was determined by both morphological and biochemical modifications. The morphological effect (i.e., cells becoming very fibrous) was evaluated in the cultures after 24 h or 48 h treatment (i.e., in 6- or 7-day-old cultures). Only those fractions which modified >50% of the cells were considered active.

The effect on the biochemical maturation was estimated after 15 days treatment (i.e., 20 days in culture) by the increase of the level of S100 protein. Such treated cultures were changed every 2nd day. The level of S100 protein was measured by a radioimmunological assay by the method in [9].

2.3.2. Purification procedure

The crude brain extract was first applied on a DEAE–Sephacel column (Pharmacia); each fraction was tested on the cultured cells. The most active fractions of this DEAE–Sephacel eluate were submitted to gel filtration on an Ultrogel column (LKB). The most active fractions of this first gel filtration were identified and fractionated by a further step of gel filtration, on the same column under the same conditions.

2.4. *Gel electrophoresis analysis of the purified factor(s)*

Electrophoresis was done as in [10], with some modifications: electrophoresis under non-dissociating conditions was performed in 4–20% gradient acrylamide slab gels (8 × 8 × 0.4 cm) at 15 V for 16 h. Protein samples (10 μl) were applied to each slab. Under dissociating conditions, the gel and the electrophoresis buffers contained 0.1% SDS; the protein samples contained 2% SDS.

Gels were fixed in 25% isopropanol, 10% acetic acid in water, and then stained with 0.05% Coomassie brilliant blue in 50% methanol, 10% acetic acid in water for 12 h. Slab gels were then destained in 7.5% methanol, 10% acetic acid in water.

3. Results and discussion

3.1. *Effects of the crude brain extract*

Morphological changes of the cells induced by treatment with crude beef brain extract are shown in fig.1. As early as 48 h after addition of the extract, i.e., 7 days in culture, the cells appeared very fibrous

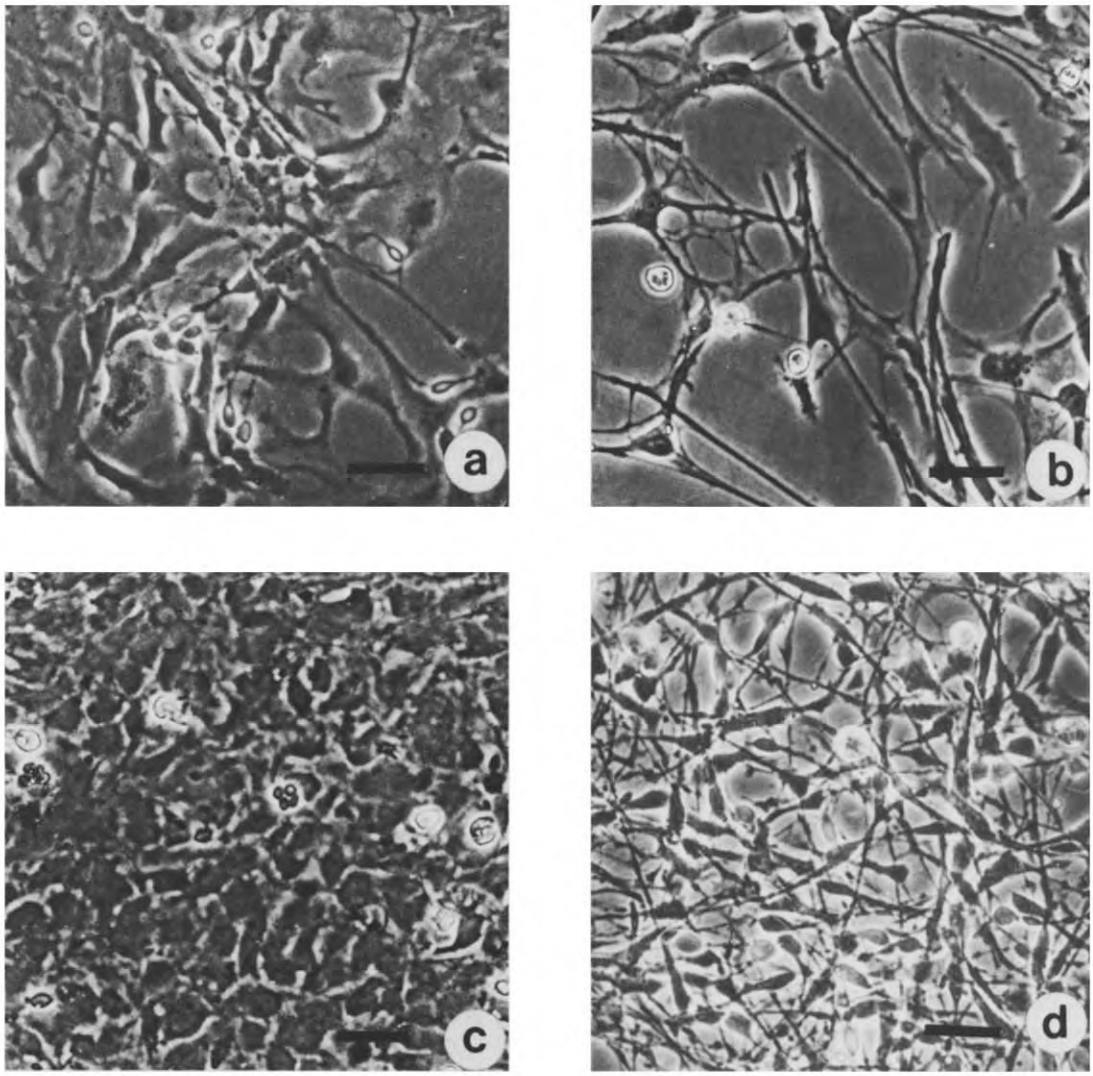

Fig.1. Astroglial cultures from cerebral hemispheres of newborn rat. Cultures were grown in nutrient medium alone, for 7 days (a), and for 20 days (c). Identical cultures were grown in the presence of crude beef brain extract from day 5, for 2 days (b) and for 15 days (d). Scale bar: 50 μm.

(b), whereas untreated cells were flat and polygonal (a). After 15 days of treatment, the cells still retained their fibrous aspect (d) while the cells in control culture remained flat (c). At that time, i.e., 20 days in culture, the level of S100 protein in treated cells was 250% of the level measured in untreated cells. These results were obtained with 60 μg brain protein/ml culture medium.

3.2. *Effects of the fractions obtained by the purification steps*

The various fractions of the DEAE–Sephacel chromatography were tested on the cultured cells (fig.2). The fractions which induced morphological modifications were also able to increase the level of S100 protein (fig.2b). Activities were present mainly in fractions 7 and 8, and to a lesser extent in fraction 6. These fractions correspond to very acidic proteins. Protein at 12 μg/ml culture medium from the fractions 7 or 8 was required to obtain the same morphological and biochemical changes as those elicited by a concentration of 60 μg crude brain extract protein, indicating an ~5-fold purification.

After gel filtration on Ultrogel of the two most active fractions (7,8) from the DEAE–Sephacel eluate (fig.3a,b), both the morphological and biochemical effectivenesses were present in the same fraction. The calibration of the Ultrogel column showed that the active fraction corresponds to $M_r \sim 20\,000$. To obtain the same morphological and biochemical stimulation as with crude brain extract, only 0.5 μg protein/ml culture medium was required. Thus, the fac-

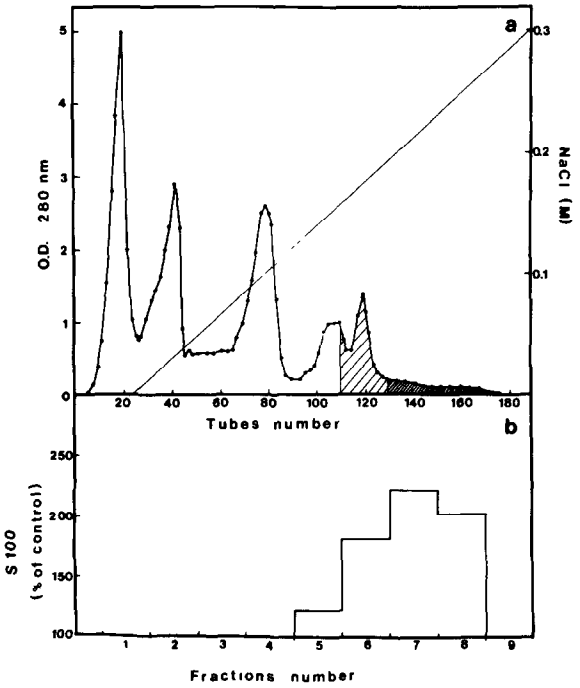

Fig.2. (a) DEAE–Sephacel (Pharmacia) chromatography of crude beef brain extract. Extract (100 ml) dialyzed against 5 mM Tris–HCl (pH 7.4) was applied onto the column (2.8 × 15 cm) pre-equilibrated with the same buffer. The column was washed with 100 ml buffer, then elution was performed with a linear gradient of NaCl (1200 ml). The flowrate was 20 ml/h. The eluate was 7 ml tube. Starting from tube no. 10, each subsequent 20 tubes were pooled to give 9 fractions of 140 ml each. Every fraction was tested for morphological and biochemical activity on the cultures. Morphological alteration of the cells is indicated by hatched areas. (b) Stimulation of S100 protein level, which was 100% in untreated cells.

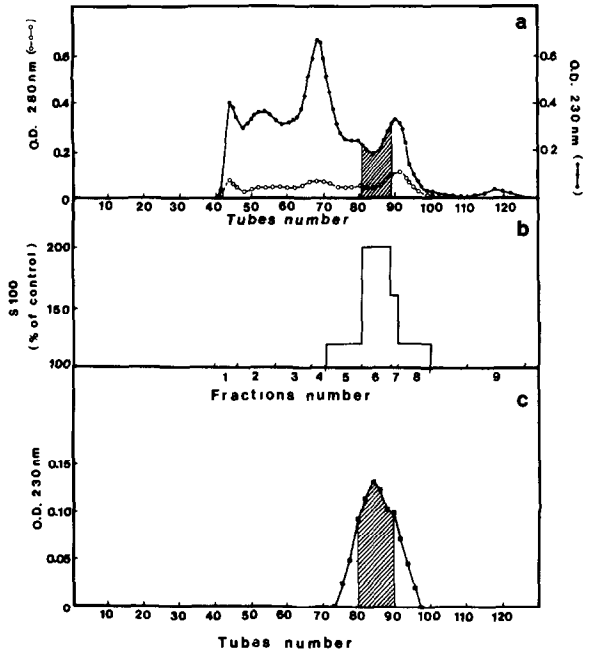

Fig.3. (a) Ultrogel chromatography of fractions 7,8 from DEAE–Sephacelcolumn. The pooled fractions were concentrated and dialyzed against a 10 mM sodium phosphate buffer (pH 7.4) containing 140 mM NaCl. The 2 ml sample containing 20 mg protein was applied on a 1.6 × 80 cm column of Ultrogel ACA 44 (LKB) equilibrated in the same buffer. The volume eluate was 1 ml/tube and the flowrate, 20 ml/h. Every tube from no. 40–100 was assayed for morphological activity. The hatched areas indicate the activity. Tubes were pooled as shown (b) to give 9 fractions which were tested for activities on S100 level. Tubes 81–89 were pooled, concentrated and dialyzed. The sample was applied on the same column (c). Hatched fraction (with morphological activity) was concentrated and dialyzed for subsequent electrophoresis analysis.

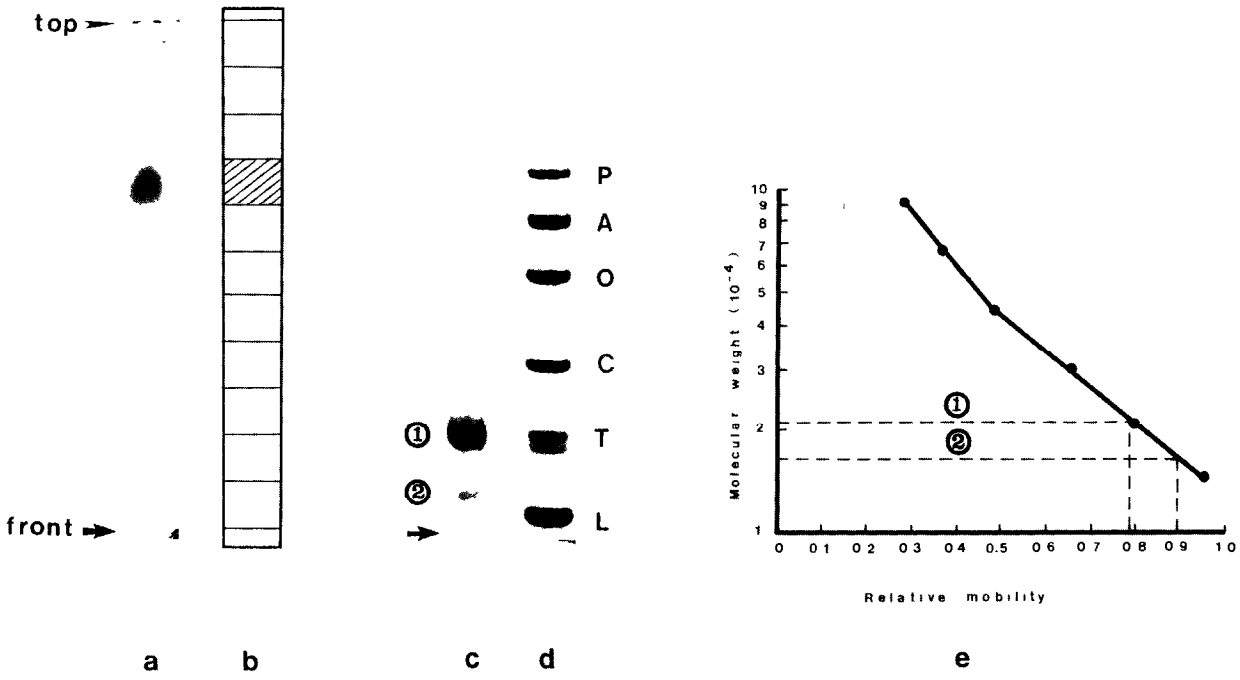

Fig.4. Polyacrylamide gel electrophoresis (arrows indicate the front dye of bromophenol blue): (a) Non-dissociating electrophoresis of the active fraction from fig.3c. Duplicate, unstained gel was cut as shown in (b) and eluates of the fractions were tested for morphological activity on cultured astroblasts. Activity is indicated by hatching; (c) dissociating SDS electrophoresis of the same fraction from fig.3c; (d) various calibration proteins (Pharmacia kit) were submitted to SDS gel electrophoresis: *Abbreviations* (M_r): P, phosphorylase *b* (94 000); A, albumin (67 000); O, ovalbumin (43 000); C, carbonic anhydrase (30 000); T, trypsin inhibitor (20 100); L, α-lactalbumin (14 400); (e) determination of the M_r of the active factor.

tor(s) was purified 120-fold. Considering that the first extraction of the acetone powder corresponds to a 6-fold purification of a brain soluble extract, total enrichment could be considered as 700-fold.

A further gel filtration of the most active fractions of the first gel filtration eluate was performed and provided an homogeneous peak with a slight shoulder (fig.3c). Morphological and biochemical activities of this second eluate were of the same range as that of the active fractions of the first gel filtration.

The central part of the peak was analyzed by gel electrophoresis.

In non-dissociating conditions (fig.4a), one major band and one minor band were detected. Only the fraction corresponding to the major band induced morphological modification (fig.4b). In SDS-gel electrophoresis, two bands were observed (fig.4c). These two proteins were estimated to be M_r 20 000 and 16 000 (fig.4d,e).

The active fraction after Ulrogel filtration has been estimated to $M_r \sim 20\,000$. Therefore, the band which corresponds to M_r 16 000 may be a partially degraded form of the M_r 20 000 protein rather than a subunit of a putative M_r 36 000 protein.

3.3. *Some properties of the factor*

The purified factor was shown to be thermolabile (treated at 70°C for 10 min, the activity was destroyed) but resistant to trypsin. Beside its ability to stimulate the maturation of astroglial cells, the factor is also able to enhance DNA synthesis as evidenced by incorporation of [^{3}H]thymidine. In the treated cultures, the purified factor (0.5 μg/ml culture medium) was added during culture days 5–8. One μCi [^{3}H]thymidine was added in each culture on day 7; after 24 h incubation, the incorporation of [^{3}H]thymidine was 3-times higher in treated than in control cultures, demonstrating the mitogenic activity of the factor.

Other authors [11] have purified active factors from pig brain, to some extent. Two molecular forms of $M_r \sim 40\,000$ and 200 000 were detected, and it

was reported that these factors had morphological and mitogenic activities. The authors showed that both activities were contained in the same fractions, but the activity on biochemical maturation was not studied. Our results demonstrate that a single protein of M_r 20 000, isolated from beef brain will induce morphological and biochemical maturation as well as cell proliferation in glial cell cultures from rat brain.

4. Conclusion

This paper reports the purification of a soluble protein from beef brain which induces the maturation of astroglial cells. The factor was purified 700-fold and was active at 0.5 μg/ml culture medium. It was characterized as an acidic protein of $M_r \sim 20\,000$.

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