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A Factor That Prevents EDTA-Induced Cell-Growth Inhibition: Purification of Transthyretin from Chick Embryo Brain

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Abstract. We previously reported the inhibition of cell-growth in Neuro-2A cells, mouse neuroblastoma, by Zn^{2+} chelation with EDTA. This paper describes the purification of a factor that prevents EDTA-induced cell-growth inhibition from chick embryo brain. The purified factor has a molecular mass of 16 kDa on SDS-polyacrylamide gel electrophoresis under reducing conditions. This factor prevents the cell-growth inhibition in a dose-dependent manner and also binds thyroxine. Analysis of the N-terminal amino acid sequence revealed that 40 residues coincide with the sequence of chicken liver transthyretin.

Key words: Apoptosis inhibition, Chick embryo brain, Transthyretin

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EDTA induced cell-growth inhibition in Neuro-2A cells by Zn^{2+} chelation was previously reported [1]. The importance of Zn^{2+}, rather than Ca^{2+}, in the apoptosis of chronic lymphatic leukemia cells [2], lymphocytes [3] and thymocytes [4] was suggested. The inhibition of endonucleases for DNA cleavage by Zn^{2+} was also detected *in vitro* [5, 6].

Concerning the physiological function of apoptosis in neural cells, it is important for neural network formation. Neurons require signals from other cells to avoid death and are thought to compete for limited amounts of survival signals secreted by the target cells [7]. Only about half of the neurons receive enough signal to survive, and the others undergo death [8]. In this study, Neuro-2A, a cell line separated from mouse neuroblastoma C1300 and having the potential for neurite outgrowth [9], was used as a neural cell model. Neurotrophins that were isolated from brain, for example BDNF, prevent neuronal cell death [10]. Brain was used as a starting material for purification of a factor that prevents EDTA-induced cell-growth inhibition.

The factor was purified from 10-day chick embryo brain. Forty amino acids at the N-terminal correspond to those of chick liver TTR (transthyretin). This paper is the first report on the purification of TTR from the brain.

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Materials and Methods

Materials

DMEM (Dulbecco's modified essential medium) and F12 nutrient mixtures (Ham F12) were purchased from Gibco (Rockville). FBS (Fetal bovine serum) and HS (horse serum) were obtained from Flow Laboratory (Irvine). BSA (Bovine serum albumin),

MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium), Proteinase K, RNase A, and human plasma TTR were from Sigma (St. Louis). EDTA-2Na was from Dojindo (Kumamoto). Penicillin G and streptomycin were from Wako Pure Chemicals (Osaka). ^{125}I-Thyroxine (T_4) was from NEN™ Life Science Products (USA). All other reagents were of reagent grade.

Cell culture

Neuro-2A cells (a gift from Dr. T. Horiuchi, Gunma University) were cultured in 45% (v/v) DMEM and 45% (v/v) Ham's F-12 supplemented with 5% (v/v) FBS, 5% (v/v) HS, and antibiotics (100 μg/ml each of penicillin G and streptomycin). The cells were cultured at 37°C in an atmosphere of 5% CO_2 at 90% humidity.

Cell growth assay and calculation of inhibitory activity

One hundred microliters of culture medium containing Neuro-2A cells (5.5×10^4 cells/ml) was pipetted into each well of a 96-well culture dish. After incubation for 24 hrs at 37°C, the culture medium was changed to serum-free medium (50% DMEM, 50% F-12), and 50 μl of test sample, or PBS(−) (Dulbecco's phosphate buffered saline) as a control, sterilized by passing through a filter (pore size 0.2 μm), was added to each well. The cells were further incubated for the pre-determined optimal time. Cell-growth was measured by the modified MTT method described by Mosmann [11] and Alley *et al.* [12], measuring the absorbance at $A_{570-620}$ with a plate reader (Toso, Type MPR-A4, Tokyo). Inhibitory activity of EDTA-induced cell-growth inhibition was calculated from the following equation: $20 \times [A_{570-620}$ (sample containing 166 μM EDTA)-$A_{570-620}$ (PBS(−) containing 166 μM EDTA)/$A_{570-620}$ (PBS(−))]. The correlation between MTT absorbance and the number of cells, determined with a TATAI Eosinophil Counter and a microscope, was confirmed.

Purification of the factor from chick embryo brain

Fertilized chicken eggs were obtained from a local chicken farm. Three hundred brains were removed from 10-day chick embryos, rinsed in extraction buffer (14 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM PMSF (phenylmethanesulfonyl fluoride), 1 mM DTT (dithiothreitol), pH 7.6), mixed with 50 ml extraction buffer and boiled rapidly for 5 min. The boiled brains were homogenized in 200 ml of extraction buffer in a Teflon-glass homogenizer. The homogenate was centrifuged at $8,000 \times g$ for 30 min and the supernatant was re-centrifuged at $54,000 \times g$ for 30 min. The supernatant (crude extract) was applied to a DEAE-Toyopearl 650 column (3×30 cm, Toso, Tokyo), equilibrated with buffer A (extraction buffer containing 10% glycerol). Adsorbed proteins were eluted with 600 mM NaCl solution in buffer A at a flow rate of 5.0 ml/min. Eluted fractions were pooled and concentrated to 5 ml through a YM3 membrane (Amicon, Beverly). The concentrated fraction was subjected to gel filtration chromatography on a Toyopearl HW-50 column (2×90 cm, Toso, Tokyo) in buffer A containing 0.1 M NaCl at a flow rate of 0.25 ml/min. Standard proteins (Catalase 232 kDa, BSA 67 kDa, Ribonuclease A 13.7 kDa, Pharmacia Biotech Inc., Tokyo) were used to estimate the apparent molecular mass of the inhibitory factor of EDTA-induced cell-growth inhibition. A portion of each fraction was dialyzed overnight with a PM 1,000 dialyzing membrane against PBS(−) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4) and then incubated with Neuro-2A cells for the MTT assay. Active fractions were pooled and dialyzed overnight against buffer A. The dialysate was applied to an EAH-Sepharose column (1.6×5 cm, Pharmacia Biotech, Tokyo) and the proteins were eluted with a linear gradient from 0 to 400 mM NaCl in buffer A at a flow rate of 1.0 ml/min. The active fractions were pooled, dialyzed overnight against buffer A, and applied to Econo-Pac CHT-2 (5 ml; Bio-Rad, Tokyo). The proteins were eluted with a linear gradient from 0 to 500 mM potassium phosphate buffer (pH 7.6) containing 10% glycerol, 1 mM DTT and 0.1 mM PMSF at a flow rate of 1.0 ml/min. The inhibitory activity was eluted in the flow-through fraction. The concentration of NaCl or potassium phosphate in each fraction was determined by conductivity measurement (M & S Instrument, CD-35M2, Tokyo). All procedures except for heat-treatment were carried out at 4°C.

Binding Assay

^{125}I-T_4 binding activity was measured by the method described by Emmer *et al.* [13]. The 0.2 ml assay mixture contained 31.6 pM ^{125}I-T_4, 5,000–8,000 cpm, the CHT-2 fraction and 0.1 mg/ml BSA as a carrier protein in potassium phosphate buffer (pH 7.6) containing 10% glycerol, 1 mM DTT and 0.1 mM PMSF. The samples were incubated for 10 min on ice, after which 4 vol of cold saturated $(NH_4)_2SO_4$ in PBS(−) was added. Ten minutes later, the precipitates were collected by centrifugation for 30 min at 10,000 × g, dissolved in 1 ml PBS(−), and the radioactivity was measured with a γ-counter (ALOKA, ARC-370M, Tokyo).

SDS-PAGE

Each fraction was mixed with an equal volume of SDS sample solution (14% SDS, 140 mM DTT, 40 mM EDTA, 10% Glycerol, 0.1% Bromophenol Blue, pH 7.5) and the mixture was boiled for 5 min. SDS-PAGE was carried out in 12% acrylamide gels

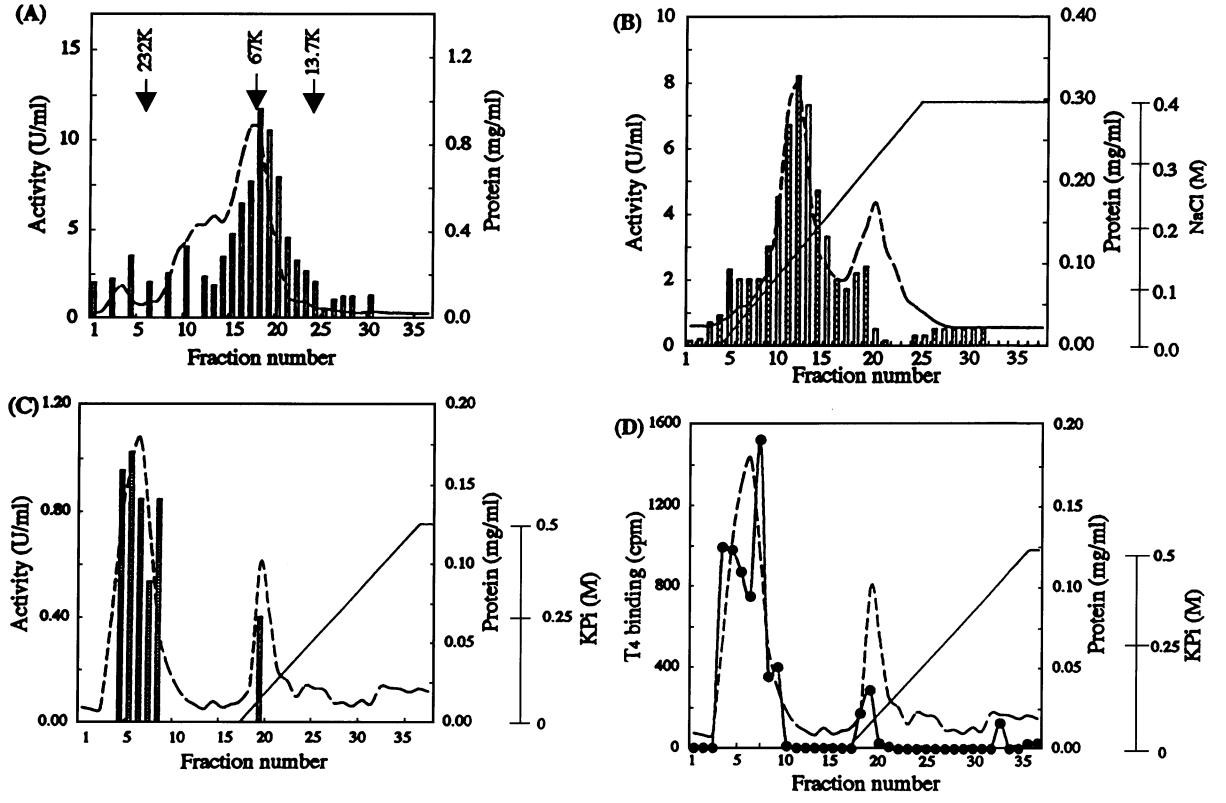

Fig. 1. (A) Elution profile of proteins and inhibitory activity of EDTA-induced cell-growth inhibition by gel filtration column chromatography (TSK-gel HW55F). The protein concentration of each fraction (---) was determined by the Coomassie dye binding assay (Bio-Rad, Tokyo). Vertical bars indicate inhibitory activity measured by the MTT assay. Elution positions of the standard proteins are indicated by arrows. (B) Elution profile of proteins and inhibitory activity of EDTA-induced cell-growth inhibition by EAH-sepharose column chromatography. Adsorbed proteins were eluted with a linear gradient of 0–0.4 M NaCl (—) in Buffer A. The protein concentration of each fraction (---) was determined by the Coomassie dye binding assay (Bio-Rad, Tokyo). Vertical bars indicate inhibitory activity measured by the MTT assay. (C) Elution profile of proteins and inhibitory activity of EDTA-induced cell-growth inhibition by CHT-2 column chromatography. Adsorbed proteins were eluted with a linear gradient of 0–0.5 M potassium phosphate buffer (—). The protein concentration of each fraction (---) was determined by the Coomassie dye binding assay (Bio-Rad, Tokyo). Vertical bars indicate inhibitory activity measured by the MTT assay. (D) Elution profile of proteins and ^{125}I-T_4 binding activity by CHT-2 column chromatography. The same fractions as shown in (C) were used for the measurement of ^{125}I-T_4 binding activity (-●-). Dashed line (---) shows the protein concentration of each fraction.

according to a modification of the method of Kadenbach *et al.* [14] and the separated proteins were visualized by silver staining [15]. Apparent molecular masses were determined relative to the mobilities of protein standards (Pharmacia Biotech LMW Kit E containing phosphorylase b 94 kDa, bovine albumin 67 kDa, ovalbmin 43 kDa, carbonic anhydrase B 30 kDa, trypsin inhibitor 20.1 kDa, and α-lactoalbumin 14.4 kDa). Stained proteins in the gel were scanned with a color image scanner JX-250 (SHARP, Tokyo). The purity of the inhibitory factor was analyzed on a Macintosh computer with an NIH image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI).

N-terminal amino acid sequence analysis

Proteins in the active fraction from the EAH-Sepharose elution were separated by SDS-PAGE under reducing conditions and then electroblotted onto a clear blot membrane-P (Atto, Tokyo) and stained with Coomassie Brillant Blue R-250. The amino-acid sequence at the amino terminal region of the 16 kDa protein was analyzed with a sequencer (Applied Biosystems Inc., Protein Sequencer 473A, Foster City) by Edman degradation.

Determination of protein concentration

The protein concentration was determined by the Bradford method (Coomassie dye binding assay) with bovine serum albumin as the standard protein [16].

Results and Discussion

Purification of a factor that prevents EDTA-induced Neuro-2A cell-growth inhibition was performed as described in MATERIALS AND METHODS. The MTT assay assessed the viability of Neuro-2A cells when 166 μM EDTA plus each brain embryo fraction was added to the cells. Heat treatment (100°C, 5 min) was performed to remove heat-unstable proteins and this step enabled the purification of the factor. At each purification step, the major active fractions were pooled and subjected to the next step. The inhibitory activity of EDTA-induced cell-growth inhibition was co-eluted in the DEAE bound fraction. The elution profile from Toyopearl HW-55 F gel filtration showed one major peak of activity containing a protein with an apparent molecular mass of 58 kDa, estimated from the elution positions of standard proteins (Fig. 1 (A)). The inhibitory activity was eluted in the NaCl concentration range of 0.1 to 0.25 M by EAH-Sepharose chromatography (Fig. 1 (B)). Most of the activity appeared in the flow-through fraction by Econopak CHT-2 chromatography (Bio-Rad, Tokyo) and there was minor activity in the bound fraction (Fig. 1 (C)). The ^{125}I-T_4 binding activity in the flow-through fraction of CHT-2 was confirmed (Fig. 1 (D)). Finally, the factor was purified as a single band with a molecular mass of 16 kDa on SDS-polyacylamide gel

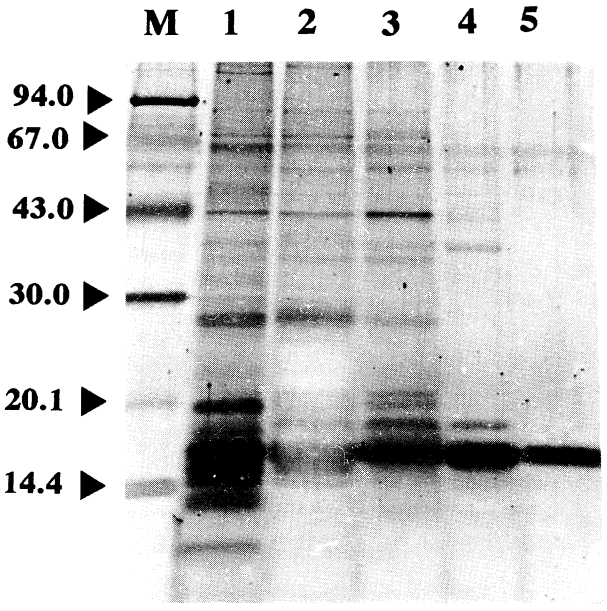

Fig. 2. SDS-PAGE of samples from each purification step. Samples from each purification step were dissolved in SDS sample solution and aliquots were applied to 12% polyacrylamide gel. Lane 1, 1.01 μg protein from the crude extract; lane 2, 1.27 μg protein from the DEAE pool fraction; lane 3, 0.84 μg protein from the TSK-gel pool fraction; lane 4, 0.56 μg protein from the EAH pool fraction; lane 5, 0.17 μg protein from the CHT-2 pool fraction. After electrophoresis, proteins in the gel were stained with a silver staining reagent. Arrowheads to the left indicate the positions of the protein standards.

Table 1. Summary of purification of the factor from 10-day chick embryo brain. Three hundred brains (40 g wet weight) were used as starting material to purify the factor.

Fraction	Protein	Total activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	%
Brain extract	115	313	2.70	1.00	100
DEAE-Toyopearl 650M	41.7	536	12.9	4.80	172
TSK gel-Toyopearl HW 55F	13.8	194	14.0	5.18	62.0
EAH-Sepharose	2.00	51.8	25.9	9.60	16.6
Econo-Pac CHT-2	1.05	42.0	40.0	14.8	13.5

electrophoresis under reducing conditions (Fig. 2). The purity of the purified factor (Fig. 2, lane 5) was 96.3%. The purification procedure is illustrated in Table 1. The total amount of factor from 300 brains (40 g wet weight) was 1.05 mg.

The purified factor prevented EDTA-induced cell-growth inhibition in a dose-dependent manner as measured by the MTT assay (Fig. 3). Half maximal activity was at a concentration of 1 μg/ml.

Proteins in the purified fraction (EAH-Sepharose pool) were separated by SDS-PAGE with electrophoretic transfer to the membrane. The 16 kDa protein band was cut out for analysis of the N-terminal amino acid sequence. The N-terminal amino acid sequence (40 residues) coincided completely with that of mature chicken liver TTR [17] (Fig. 4). TTR is one of the most strongly conserved plasma proteins. The amino acid sequences of chicken TTR has more than 75% similarity to that of several mammalian TTR [17]. These data suggest that the purified factor is identical to TTR. TTR is a homotetramer (55 kDa), with a monomer molecular mass of 13,836 Da [17], close to the band observed on

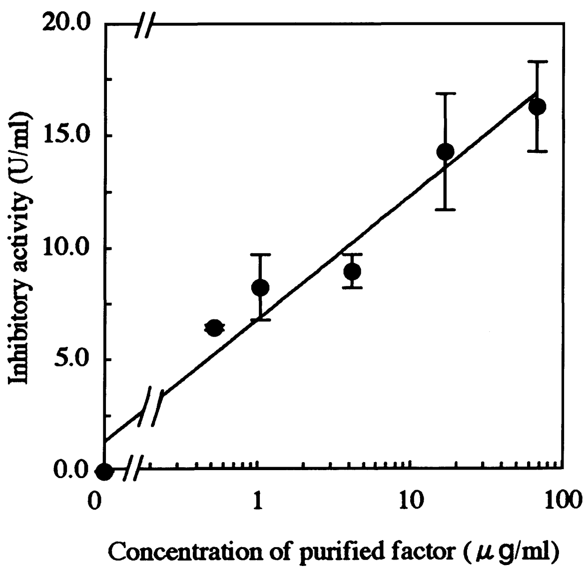

Fig. 3. Dose-effect of the purified factor on EDTA-induced cell-growth inhibition. Neuro-2A cells were cultured for 48 hrs in a mixture of 166 μM EDTA and various concentrations of purified factor. Inhibitory activities were measured by MTT assay. Each value represents the mean$\pm$SD (n=3).

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                     -20          -10          -1 1        10           20
Liver     TTR       MAFHSTLLVFLAGLVFLSEAAPLVSHGSVDSKCPLMVKVL

Purified factor     --------------------APLVSHGSVDSKCPLMVKVL

                                  30           40
Liver     TTR       DAVRGSPAANVAVKVFKKAA

Purified factor     DAVRGSPAANVAVKVFKKAA
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Fig. 4. Correspondence between the N-terminal amino acid sequence of the purified factor and that of the chicken liver TTR. The amino acid sequence at positions -1 to -20 (under-lined) is the signal sequence.

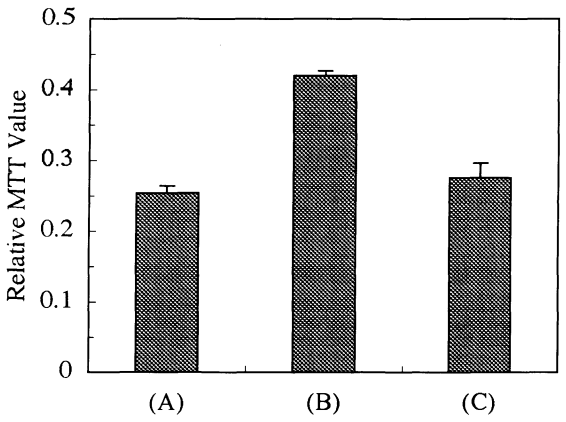

Fig. 5. Competitive experiment with EDTA and T_4 for TTR by MTT assay. Each histogram shows relative MTT value of Neuro-2A cells treated with 166 μM EDTA (A), 166 μM EDTA and 150 μg/ml (protein) DEAE active fraction (B) and 166 μM EDTA, 150 μg/ml (protein) DEAE active fraction and 150 nM T_4 (C). Each value represents the mean$\pm$SD (n=3).

SDS-PAGE (Fig. 2, lane 5). Nevertheless, commercially available TTR from human plasma (Sigma, St. Louis) did not prevent EDTA-induced cell-growth inhibition (data not shown). Since it was isolated by SDS-polyaclyamide gel electrophoresis under non-reducing conditions, this TTR might be denatured by SDS.

There is possibility that the prevention of cell-growth inhibition is due to binding of EDTA to the factor, detected by competitive experiment with EDTA and T_4 (Fig. 5).

The molecular mass of the factor, estimated from the elution position of standard proteins on gel filtration chromatography, was 58 kDa, but the factor has a molecular mass of 16 kDa on SDS-polyacrylamide gel electrophoresis under reducing conditions. These data suggest that TTR in chick brain also exists as a homotetramer.

The brain is an important target organ for thyroid hormones [18], particularly during development [19]. Thyroid hormones are distributed throughout the body by binding to proteins synthesized in the liver. These proteins are thyroxine-binding globulin (TBG), TTR, and albumin [20]. The TTR mRNA is expressed not only in the liver but also in the brain. The TTR mRNA concentration in the rat choroid plexus is 11.3 times higher [21], and in chicken choroid plexus it is 21.4 times higher [17] than the concentration in the liver. The TTR expression was also detected in developing rat brain by an immunohistochemical method [21] and *in situ* hybridization [22].

The brain is separated from the bloodstream by the blood-brain and blood-CSF barriers. These barriers are not freely permeable to amphipathic molecules, such as thyroid hormones. Schreiber's hypothesis [23] was derived to explain the transport mechanism of thyroid hormones from the bloodstream to the brain in participation with transthyretin, which is synthesized in the choroid plexus and secreted into the CSF. The results of our purification suggest that TTR exists in the choroid plexus and CSF for the purpose of transporting thyroid hormone from the bloodstream.

The functions of TTR and thyroid hormones in the brain, especially in development, have not yet been clearly revealed. The next target of investigation will be the function of TTR and thyroid hormones in the developing brain.

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