Isolation of Acetylcholinesterase from Apoptotic Human Lung Fibroblast Cells by Antibody Affinity Chromatography

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ABSTRACT

Acetylcholinesterase (AChE; EC3.1.1.7) is well known for its role in the hydrolysis of acetylcholine at cholinergic synapses to terminate neurotransmission. In addition to its synaptic presence, AChE has been found to be in non-cholinergic cells such as hematopoietic and osteogenic cells. We have recently reported that AChE is expressed in various cells undergoing apoptosis. To characterize AChE in apoptotic cells and to investigate the role of AChE expression in apoptosis, we devised a method to purify AChE expressed in apoptotic human lung fibroblast cell line HLF. The isolation of this enzyme is mainly based on inhibitor ligand affinity chromatography using immobilized tacrine. However, this method is only effective in isolating active AChE. Here we employed antibody-based chromatography and found that both active and inactive AChE were present in apoptotic HLF cells. Active AChE was predominantly observed in the nuclei of apoptotic cells, while inactive AChE was mainly present in the cytoplasm. Therefore, our method provides an opportunity to investigate further the role of AChE, especially inactive AChE, in apoptosis.

INTRODUCTION

Acetylcholinesterase (AChE; EC3.1.1.7) is responsible for the hydrolysis of acetylcholine at cholinergic synapses in the central and peripheral nervous systems. The enzyme exists in multiple molecular forms distinguished by their subunit associations. There are several AChE catalytic subunits, including a T-type, an H-type, and an R-type, that differ at their C-terminals because of alternative splicing of a single gene in vertebrates (20,21,23,24). AChE can be divided into homomeric and heteromeric forms, derived from catalytic subunits associated with either a lipid-linked subunit (14) or a collagen-like subunit (5). Homomeric forms include a globular G1 monomer and G2 dimer as well as a glycopospholipid-linked dimer. Heteromers consist of the asymmetric forms A4, A8, or A12, which is associated with a collagen-like subunit, and amphiphilic tetramers G4-linked to a 20-kDa hydrophobic anchor.

One interesting feature of AChE is that its expression is not restricted to cholinergic tissues. The enzyme is expressed in non-cholinergic cells such as specific neuronal populations (2,12,17), hematopoietic (25,26,34), and osteogenic cells (11). In these

cells, AChE possesses functions distinct from its well-known catalytic activity (18,32). Recent studies suggest that AChE may function in regulating apoptosis. AChE expression is increased in apoptotic cells due to butyrylcholinesterase (BChE) suppression in retinospheroids using BChE antisense transfection (27). AChE may also participate directly in the regulation of bone marrow cell development by reducing the proliferative and expansive capacity of pluripotent stem cells by promoting apoptosis in their progenies (19,31). Deletion of the AChE gene is associated with both myelodysplasia syndromes and acute myeloid leukemia, suggesting that AChE may in fact act as a tumor suppressor gene (10,33). AChE is expressed in a number of cell lines upon induction of apoptosis by various stimuli (38). When SK-N-SH cells, which normally express AChE, were induced to undergo apoptosis by long-term culture, the expression of AChE increased (37). These data indicate that AChE may function in apoptosis, although detailed mechanisms have yet to be defined.

One critical step in defining the function of AChE is to obtain AChE from various resources. Although AChE could be readily purified by inhibitor ligand affinity chromatography using immobilized N-methylacridinium (9), edrophonium (13), and tacrine (4), inactive AChE binds poorly to these inhibitor ligands (7). To isolate, characterize, and quantify total AChE from apoptotic cells, we developed an affinity chromatography with specific antibodies and were able to purify both active and inactive AChE.

MATERIALS AND METHODS

Inducing Apoptosis by Long-Term Culture and Detecting AChE Activity

HLF cells were grown as a monolayer in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS and 4 mM glutamine. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. To induce apoptosis, HLF cells were maintained in culture without changing the medium for 3–4 days (35). AChE activity was detected by the direct staining with thiocholine (15). The nuclei were stained with 10 µM fluorochrome Hoechst 33258 for 10 min at 37°C.

AChE Cytochemical Staining and TUNEL

AChE activity was detected according to the method of Karnovsky and Roots (15). HLF cells were centrifuged onto slides by Cytospin, subjected to a permeabilization solution (0.1% Triton® X-100, 0.1% sodium citrate) for 2 min on ice, and then incubated with 50 mL TUNEL reaction mixture (Roche Applied Science, Indianapolis, IN, USA) for 60 min at 37°C in the dark. The labeled cells were then washed, transferred onto glass slides, and observed under a fluorescence microscope.

AChE Immunofluorescence Staining and TUNEL

HLF cells were centrifuged onto slides, fixed in 3% paraformaldehyde for 40 min at 4°C, and then treated with the permeabilization solution for 4 min. After blocking in 3% BSA for 1 h at room temperature, the slides were incubated with primary AChE-antibody (BD Biosciences, San Jose, CA, USA) at 1:100 dilution for 2 h and then incubated with the secondary mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution for 1 h at 37°C. After washing three times with PBS, apoptosis was detected using TUNEL as described above.

Preparation of Polyclonal Anti-AChE Antibody

To obtain a large amount of anti-AChE antibody for affinity chromatography, three rabbits were immunized with *Electrophorus electricus* (eel) AChE (Sigma, St. Louis, MO, USA). Four inoculations at 20-day intervals were made: the first injection using complete Freund's adjuvant and three additional inoculations with incomplete Freund's adjuvant. Approximately 1 mg eel AChE was used for each injection. Crude IgG fractions were prepared by ammonium sulfate precipitation. IgG fractions were diluted with 5 mL PBS and dialyzed against PBS to remove ammonia ions. The antibody was then quantitated and lyophilized.

Preparation of Antibody Affinity Column

The purified antibody was coupled to CNBr-Sepharose® (Amersham Biosciences, Piscataway, NJ, USA) at a ratio of approximately 10 mg protein/mL final bed volume, according to the manufacturer's instructions. In brief, the CNBr-Sepharose was hydrated in 1 mM HCl, pH 3.0, and washed three times by sand filtration with 1 mM HCl. The CNBr-Sepharose was then mixed with the antibody at 10 mg/mL in 0.1 M carborate, 0.5 M NaCl, pH 8.3. The reaction was carried out overnight at 4°C with constant rotation. Uncoupled sites were blocked with 0.1 M ethandamine for 2 h at ambient temperature and subjected to three wash cycles alternating between 0.1 M acetate buffer, 0.5 M NaCl, pH 4.0, and 0.1 M Tris-HCl buffer, 0.5 M NaCl, pH 8.0. The column was packed and equilibrated in 0.05 M Tris, 0.15 M NaCl, and 0.02% NaN₃, pH 8.0.

AChE Purification from Apoptotic HLF Cells

Unless stated otherwise, all steps of purification were performed at 4°C.

Preparation of cell lysate. The apoptotic cells were collected and washed with PBS, pH 7.0. A buffer (50 mM Tris-HCl, 150

mM NaCl, 1 µg/mL Aprotinin, 1% Triton X-100, pH 8.0) was added to cell pellets while vortex mixing. The cell lysate was incubated on ice for 30 min and then treated with supersonic wave for 2 min. Afterwards, the lysate was centrifuged at 1700×g for 4 min at 4°C. The supernatant was dialyzed against PBS, pH 7.0, and concentrated to 13 mg/mL by lyophilization.

Protein binding to antibody affinity gel. Approximately 20 mL cell lysate (230 mg protein) was mixed with an antibody affinity gel, which had been equilibrated with 0.05 M PBS, pH 7.5, and interacted for 20 h at 4°C with constant rotation. To remove unbound proteins, the gel was washed by sand filtration with PBS, pH 7.5, and loaded to the column. The column was continually washed until no protein was detected in the filtrate.

AChE elution. The bound AChE was eluted with 0.1 M Glycine-HCl, pH 2.5. All elute was pooled, concentrated by lyophilization, and dialyzed to remove saline. The purified AChE was quantitated and stored in aliquots at -70°C.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to the method of Laemmli et al. (16). Proteins were stained with Coomassie® Brilliant Blue. After electrophoresis, the proteins were transferred onto nitrocellulose membrane electrophoretically. High

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molecular weight markers (Pierce Chemical, Rockford, IL, USA) were included for molecular weight calibration. A constant electric current of 0.065 mA/cm² film was applied for 2 h. Immediately after transfer, the membrane was soaked for 1 h at 37°C in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween® 20, pH 7.2) containing 5% fat-free dried milk. Thereafter, the blot was incubated for 1 h at room temperature with AChE monoclonal antibody diluted to 1/2500 in TBST containing 5% fat-free dried milk. After three washings with TBST for 10 min each, the blot membrane was incubated with HRP-conjugated anti-mouse IgG diluted to 1/2000 for 1 h and then washed three times, each for 10 min, with TBST. HRP activity was detected using ECL® (Santa Cruz Biotechnology).

Determining AChE Activity

AChE activity was determined spectrophotometrically in a 96-well microplate using a modified Ellman's assay as described (10a). The assay was performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer (pH 8.0), 0.1 mg/mL BSA, and 0.3 mM 5',5'-dithiobis-(2-nitrobenzoic acid) at 27°C. The sample's absorbance value at 405 nm was analyzed using a spectrophotometer (model EL311; Bio-Tek, Winooski, VT, USA).

RESULTS AND DISCUSSION

Induction of Apoptosis and Detection of AChE Activity

HLF is a fibroblast cell line derived from human lung that attaches to plastic culture ware in vitro. Although HLF does not require specific growth factors, prolonged culture in vitro could lead to apoptosis. In such aged cultures, apoptotic HLF cells detach from plastic and can be harvested from suspension. In the

suspended cells, we have found that AChE activity appeared in the nuclei of the majority of apoptotic cells and in the cytoplasm of some apoptotic cells upon cytochemical staining with thiocholine (Figure 1A). The cells were also stained with fluoro-chrome Hoechst 33258 to show chromatin condensation and apoptotic bodies (Figure 1B). AChE activity was not present in living HLF cells, as compared to the strong AChE activity found in the apoptotic cells, which could be inhibited by AChE-specific inhibitors BW284c51 and eserine (Figure 1C). In addition to prolonged culture, apoptosis in HLF cells was also induced by treatment with G418, ceramide, or UV-B. Upon receiving these treatments, HLF cells also undergo apoptosis and reveal AChE activity in a similar fashion as that of prolonged culture (data not shown). Because of its simplicity, we have elected prolonged culture as a primary method to induce apoptosis.

Presence of Active and Inactive AChE in Apoptotic HLF Cells

To determine the consistency of the presence of AChE protein and its activity, we detected the enzymatic activity of AChE and total AChE protein with antibodies. Karnovsky and Roots (15) devised a classical method to detect the AChE activity with thiocholine. With this protocol, only active AChE was detected by thiocholine. The presence of active and inactive AChE was also detected by immunofluorescence with AChE-specific polyclonal antibodies. We found that active AChE was mainly detected in the nuclei and apoptotic bodies as indicated by the TUNEL assay. Immunocytochemistry showed that AChE existed in the either nuclei or cytoplasm of apoptotic cells (Figure 2). However, neither AChE protein nor its activity was found in TUNEL-negative HLF cells. (Figure 3).

Figure 1. Existence of AChE in apoptotic cells. HLF cells in a aged culture were stained by (A) AChE cytochemical staining and (B) Hoechst 33258. The arrows indicate negative AChE staining in cells with a normal nucleus. (C) AChE activity was determined spectrophotometrically in a 96-well microplate using a modified Ellman's assay.

Figure 2. Active and inactive AChE is present in apoptotic cells. Both attached and detached apoptotic HLF cells were collected and stained for AChE by cytochemical staining or immunofluorescence to show co-localization of AChE and DNA fragmentation by TUNEL assay in apoptotic cells. (A) Brown stains indicate AChE-positive cells with cytochemical staining. (B) TUNEL-positive cells indicated genomic DNA fragmentation. (C) The cells were observed under a light field microscope. (D) Immunofluorescence indicated that AChE existed in both the nuclei and cytoplasm of apoptotic cells. Some non-apoptotic cells were also positive, which indicated that AChE was expressed at the early stages of apoptosis. (E) A TUNEL assay showed the presence of apoptotic cells.

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Purification of AChE

We have generated a rabbit anti-eel AChE polyclonal antibody that reacts with human AChE expressed by apoptotic HLF. We found that the isolated rabbit anti-eel AChE polyclonal antibody was able to recognize both eel and human AChE expressed by apoptotic HLF cells (Figure 4) on Western analysis. The main band at 80 kDa in lane 1 represents eel AChE, while the subsequent bands at 60, 70, and 30 kDa indicate degraded AChE. Lane 2 contains the apoptotic HLF cell lysate at 68 kDa. The purified protein was again tested for its reactivity to AChE-specific antibodies by Western analysis. As shown in Figure 5, the purified protein strongly react to anti-AChE and is the same molecular weight as AChE in apoptotic cells.

The rabbit anti-eel AChE polyclonal antibody was coupled to Sepharose beads and was used for chromatography to isolate human AChE from apoptotic HLF cells. The concentration of purified AChE was determined by UV absorbance. Approximately 1 mg AChE was isolated from 10^9 apoptotic HLF cells. The purified AChE appears as a 68-kDa band on Western analysis. Therefore, the inactive and active AChE forms have similar immunological reactivity and molecular weights, as others have previously reported (6,8). It has been reported that purified AChE does not always possess enzymatic activity. The difficulty in detecting the activity of AChE may have been

attributed to the following reasons. First, the steps taken in the purification process may have affected AChE activity. Second, a large portion of the isolated AChE may be of the inactive type.

Previous research has shown that inactive AChE existed in increased amounts in the cytoplasm. A stable pool of inactive AChE, 70%–80% of total enzyme, was found in chick brain and muscles (6). Choi et al. (8) found that calcitonin gene-related peptide increased the expression of AChE mRNA and inactive AChE protein in cultured chick myotubes. The proportion of inactive versus active AChE varied significantly during development in quail brain (1). These results indicate that inactive AChE may have a similar role to active AChE. Because inactive and active AChE were present in apoptotic cells at the same time and have similar immunological properties and molecular weights, it is highly possible that the active and inactive forms are inter-convertible. Invertebrate cholinesterases, catalytic subunits with different glycosylation patterns, have been isolated from different tissues (3,22,28,36). In the brain of Alzheimer's disease patients, which correlates with abnormal AChE activity, the level and distribution of AChE isoforms are profoundly altered (29,30). Inactive AChE may have different modifications from active AChE and therefore may have different functions. Another explanation may be that newly synthesized polypeptides fold into different conformations, and the probability of its orientation towards the active or inactive state is regulated by a nonclassical function. These uncertainties may be resolved by further research on the modifications, functions, and properties of inactive and active AChE in apoptotic cells.

Figure 3. Both active and inactive AChE existed in the same apoptotic HLF cell. (A) AChE cytochemical staining exhibits active AChE, as detected by the method of Karnovsky and Roots (15). (B) Immunofluorescence shows that inactive AChE exists in cytoplasm. The negative appearance in the nucleus was due to the brown precipitate obstructing the UV absorbance and fluorescence emission. (C) Hoechst 33258 was used to stain the condensed nucleus.

Figure 4. The purified protein is homogeneous. Western analysis indicates the specificity of the isolated antibody to eel AChE (lane 1) and apoptotic HLF lysate (lane 2).

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Figure 5. The purified protein reacts to antibodies to AChE. AChE purified from apoptotic cells was identified by (A) Coomassie Brilliant Blue staining. Only one band appeared between 50 and 79 kDa. (B) Western analysis with monoclonal antibody to AChE confirmed that the protein is AChE. Lane 1, purified AChE; lane 2, live HLF cells; and lane 3, apoptotic cells.

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