Acetylcholinesterase Plays a Pivotal Role in Apoptosome Formation

Sang Eun Park,1,2 Nam Deuk Kim,2 and Young Hyun Yoo1

1Department of Anatomy and Cell Biology (BK21 program), Dong-A University College of Medicine and Medical Science Research Center, Seo Gu, Busan, South Korea, and 2Department of Pharmacy, College of Pharmacy, Pusan National University, Geumjeong-gu, Busan, South Korea

Abstract

Although a recent study (Zhang et al. Cell Death Differ 2002; 9:790–800) presented that acetylcholinesterase (AChE) might be an important common component in leading to various types of apoptosis, the molecular mechanism, by which AChE functions, had remained elusive before that study. We explored the role of AChE in apoptosis by silencing the AChE gene. Silencing of the AChE gene abolished the expression of AChE and prevented caspase-9 activation, decrease of cell viability, nuclear condensation and poly(adenosine diphosphate-ribose) polymerase cleavage but not mitochondrial events. Importantly, silencing of the AChE gene blocked the interaction between apoptotic protease-activating factor-1 and cytochrome c. Here we propose that AChE plays a pivotal role in the formation of apoptosome.

Introduction

Acetylcholinesterase (AChE) plays a key role in terminating neurotransmission at cholinergic synapses. However, recent studies have shown that AChE is also expressed in tissues devoid of cholinergic responses (1) and in several types of hematopoietic cells including erythrocytes (2) and megakaryocytes (3), indicating potential functions beyond neurotransmission. A recent study presented the induction of AChE expression in apoptosis in various cell types (4). The study showed that AChE activity was observed in cells showing the positive terminal deoxynucleotidyl transferase-mediated nick end labeling reaction and caspase-3 activation. Because all of the apoptotic cells tested by the study were AChE positive, induction of AChE activity was suggested to be a common phenomenon in cells undergoing apoptosis. The study also revealed that AChE, during apoptosis, was first synthesized in the cytosol and then accumulated in the nucleus, and that apoptosis-associated AChE activity was due to activation of the AChE gene. Furthermore, AChE-specific inhibitors BW284c51 and eserine and antisense AChE inhibited apoptosis.

Materials and Methods

Reagents. The following reagents were obtained commercially: rabbit polyclonal antihorse cytochrome c and caspase-9 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse polyclonal antihuman poly(ADP-ribose) polymerase antibodies were from Oncogene (Cambridge, MA); FITC-conjugated goat antirabbit was from Vector (Burlingame, CA); horseradish peroxidase-conjugated donkey antirabbit was from Amersham Pharmacia Biotech (Piscataway, NJ), 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetrathylbenzimidazolcarbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR). siPORT Amine was from Ambion (Austin, TX), RPMI 1640 and Opti-MEM media, and fetal bovine serum were from Life Technologies, Inc. (Gaithersburg, MD). DMSO, Hoechst 33342, RNase A, protease K, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and protein A agarose beads were from Sigma (St. Louis, MO); SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, IL).

Cell Culture. The human colon adenocarcinoma cell line HT-29 (KCLB 30038) was maintained at 37°C with 5% CO2 in air atmosphere in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated fetal bovine serum.

Etoposide Treatment. Twenty-four h after HT-29 cells were subcultured, etoposide from a stock solution was added to the medium. Stock solutions of etoposide (20 mg/ml) were kept at room temperature. Cells treated with various concentrations of etoposide were harvested, stained with trypan blue, and then counted using a hemacytometer. Because the dose required for half-maximal inhibition of viability at 40 h after treatment was about 40 μg/ml (not shown in “Results” section), this single concentration was used for further assessment of apoptosis.

siRNA Transfection. Twenty-one-nucleotide RNA with 3′-dTdT overhangs was synthesized by Dharmacon Research (Lafayette, CO) in the “ready-to-use” option. The AA-N19 mRNA targets were AChE target sequence (5′-AACGUGCGGAUAGGCUAGAC-3′). Transfection of small interfering RNA (siRNA) was performed by using siPORT Amine and Opti-MEM media according to manufacturers’ recommendations. Cells grown to a confluency of 40–50% in 6-well plates were transfected with 1 nm final siRNA concentration per well.

Morphological Assessment of Apoptosis. Cytocentrifuged cells were stained in 4 μg/ml Hoechst 33342.

Western Blot Analysis. Briefly, 2 × 106 HT-29 cells treated with etoposide were washed twice with ice-cold PBS, were resuspended in 200 μl of ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 μM aprotinin, and 2 μM leupeptin], and were incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA) and equivalent amounts were loaded onto 7.5–15% SDS/PAGE. The gels were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with LAS-3000PLUS (Fuji Photo Film Company, Kanagawa, Japan). Equivalent protein loading was confirmed by Ponceau S staining.

Immunofluorescent Staining. Cytocentrifuged cells were fixed for 10 min in 4% paraformaldehyde, were incubated with antibody to cytochrome c for 1 h, were washed three times each for 5 min, and then were incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS.

Photomicrography and Cell Counting. Cells were observed and photographed under Zeiss Axioskop microscope (Göttingen, Germany). Total cell number, at least 300 cells from each experiment, was counted under DIC optics; and the cell showing condensed or fragmented nuclei in Hoechst staining and cytochrome c release in immunocytochemical staining was counted as an apoptotic cell death.

Received 2/25/04; accepted 3/20/04.

Grant support: Supported by the Brain Korea 21 Project in 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Young Hyun Yoo, Department of Anatomy and Cell Biology (BK21 program), Dong-A University College of Medicine and Medical Science Research Center, 3-1 Dongdaesin Dong, Seo Gu, Busan, South Korea 602-714. Phone: 82-51-240-2926; Fax: 82-51-241-3767; E-mail: yhyoo@daunet.dongac.kr.

2652
**Assay of Mitochondrial Membrane Potential.** JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS, and cells were resuspended in 10 g/ml methanol and incubated at 37°C for 30 min. Flow cytometry to measure mitochondrial membrane potential was performed on an Epics XL (Beckman Coulter, Miami, FL). Data were acquired and analyzed using EXPO32 ADC XL 4 color software. The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris.

**Immunoprecipitation.** Cell extracts were incubated with anti-Apaf-1 antibody in extraction buffer at 4°C overnight. The immunocomplexes were precipitated with protein A-agarose beads for 2 h and were washed five times with extraction buffer prior to boiling in SDS sample buffer. Immunoprecipitated proteins or aliquots containing 40 μg of protein were separated on SDS-polyacrylamide gels, and Western blot analysis with anti-Apaf-1 or anti-cytochrome c was performed as described above.

**Statistical Analysis.** Statistical significance was tested by the nonparametric Kruskal-Wallis test. Four independent experiments were carried out. Statistical results were expressed as the mean ± the SD of the means obtained from triplicates of each independent experiment. In all cases, a P estimated at <0.05 was considered significant.

**Results**

Induction of the expression of AChE was demonstrated by cytochemical staining specific for AChE (Fig. 1A) and by Western blot analysis (Fig. 1B). To specifically decrease the expression of AchE, we introduced 21-mer RNA oligonucleotides into cells by transfection...
siRNA, directed against AchE, efficiently silenced the AchE gene and reduced almost completely the level of AchE (Fig. 1). siRNA, directed against AchE, significantly prevented the etosipside-induced decrease of viability, nuclear condensation, poly (adenosine diphosphate-ribose) polymerase cleavage, and caspase-9 activation (Fig. 2, A–D). However, silencing of the AchE gene blocked neither cytochrome c release from mitochondria into cytosol (Fig. 2E) nor reduction of mitochondrial membrane potential (Fig. 2F). These data suggested that AchE might have influence on a certain apoptotic machinery functioning midway between the release of mitochondrial factors and the activation of caspase-9. Thus, it was natural that we paid attention to Apaf-1 because the involvement of Apaf-1 is indispensable in most cases of stress-induced apoptosis. Western blot analysis showed that there was no prominent change in the level of Apaf-1 even after silencing of the AchE gene (Fig. 3A). Importantly, immunoprecipitation showed that siRNA directed against AchE prevented the physical interaction between Apaf-1 and cytochrome c (Fig. 3B).

Discussion

Previous studies (5–7) showed abnormality of the AchE gene in several tumors. These data suggest that AchE could be a tumor suppressor gene. Furthermore, agricultural use of AchE inhibitors is known to induce several types of tumors (8). In addition, a recent study proved that AchE expression was induced during apoptosis in various cell types and that pharmacological inhibitors or antisense AchE inhibited apoptosis (4). Those data strongly suggest the contribution of AchE to the apoptotic process. We here elucidated that the silencing of the AchE gene inhibited the interaction between Apaf-1 and cytochrome c. Therefore, we concluded that AchE plays an important role in apoptosome formation. To strengthen our claim, we tested the effect of antisense AchE oligonucleotide on etosipside-induced apoptosis in HT-29 cells and found that antisense AchE oligonucleotide showed a similar effect in the silencing of the AchE gene. In addition, in HT-29 cells, AchE was shown to play the same role in apoptosis induced by different apoptotic stimuli (data not shown).

Execution of cellular demolition in apoptosis is carried out by caspases (9, 10). The initially activated caspases launch the proteolytic cascade. These engines of caspase activation are called the apoptosome. The apoptosome is a multiprotein complex composed of the apical protease caspase-9, its cofactor Apaf-1, and the facilitator protein cytochrome c (11, 12). Apaf-1 possesses a long COOH-terminal extension containing 13 repeats of the glycine-histidine and tryptophan-aspartic acid motif, which often mediates protein–protein association. The glycine-histidine and tryptophan-aspartic acid region must negatively regulate Apaf-1, because its deletion generates a constitutively active form (13, 14). Apaf-1 activity is unleashed by binding to cytochrome c, derived from damaged mitochondria. Cytochrome c displaces the head, allowing the compact structure to stretch out into a more linear molecule, and subsequent binding of dATP or ATP to the caspase recruitment domain then allows Apaf-1 multimerization and association of procaspase-9. Seven Apaf-1:cytochrome c heterodimers oligomerize to form a symmetrical “wheel.” As well as containing COOH-terminal glycine-histidine and tryptophan-aspartic acid repeats and a nucleotide binding domain, Apaf-1 has a caspase recruitment domain. The caspase recruitment domain of caspase-9 binds to the Apaf-1 caspase recruitment domain via homophilic and hydrophilic interaction. Inactive procaspase-9 monomers, concentrated on the hub of the apoptosome, recruit additional procaspase monomers to form the active dimer. The caspase-9 zymogen binds to Apaf-1, is activated, and cleaves a neighboring procaspase-9, which then rearranges to form the active site. Thus, the Apaf-1–caspase-9 apoptosome acts as a holoenzyme (15).

The biochemical function of the apoptosome is to activate an initiator caspase, caspase-9.Binding of procaspase-9 to Apaf-1 increases the intrinsic catalytic activity of the caspase-9 protease, leading to the autolytic cleavage of procaspase-9 at Asp115 to yield a large (p35) and a small (p12) subunit (13, 15, 16). Once activated in the apoptosome, caspase-9 relays the death signal, directly activating the executioner caspase-3, -6, and -7 by specific and limited proteolysis (13, 17). Commonly, activated caspase-9 cleaves procaspase-3 at Asp175 and activates the execution caspase, caspase-3.

Taking the current teaching and the data of the present study together, we suggest that AchE plays a pivotal role in apoptosome formation (Fig. 4). In the future, the more detailed molecular mechanism explaining the role of AchE in apoptosis should be elucidated through structure/function analysis.

References

Corrections

p53 and BCNU Resistance in Astrocytes

In the article on p53 and BCNU Resistance in Astrocytes in the June 15, 1996 issue of Cancer Research (1), the title was incorrect. The title should have read “Wild-Type p53 Renders Mouse Astrocytes Resistant to 1,3-Bis(2-chloroethyl)-1-nitrosourea Despite the Absence of a p53-dependent Cell Cycle Arrest.”


AChE in Apoptosis

In the article on AChE in Apoptosis in the April 15, 2004, issue of Cancer Research (1), there is an error on page 2652, in the section under “Materials and Methods” on “siRNA Transfection”. The AChE target sequence should have read 5’-AAGAGUGUCUCUAC-CAAUAU-3’.


Depletion of Methionine Aminopeptidase 2

In the article on Depletion of Methionine Aminopeptidase 2 in the May 1, 2004, issue of Cancer Research (1), there is an error on page 2984, in the section under “Materials and Methods” on “Cell and Enzyme Assays”. The text near the end of the section should have read the following: “The targeting sequence was AAUGCCGUGACACAAACAGUA (Dharmacon Research). The control mismatch sequence was AAUGCCGCGCUACACACAGUA.”


NIS Gene Therapy of Hepatocarcinoma

In the article on NIS Gene Therapy of Hepatocarcinoma in the November 1, 2004, issue of Cancer Research (1), a note should have been included indicating that J. Faivre and J. Clerc contributed equally to the study.


Novel Functions of BRAK

In the article on Novel Functions of BRAK in the November 15, 2004, issue of Cancer Research (1), the following grant support information should have appeared:

This work was supported in part by the University of Texas M.D. Anderson Cancer Center SPORE in Head and Neck Cancer NIH-NCI P50 CA097007 (G. Clayman and M. Frederick), NIH R01 DE013954 (G. Clayman), Cancer Center Support Grant NIH P30 CA016672, Alan J. Ballantyne Distinguished Chair in Head and Neck Surgery Award (G. Clayman), Michael A. O’Bannon Endowment for Cancer Research (G. Clayman), Betty Berry Cancer Research Fund (G. Clayman), and NIH INRS Award T32 CA060374 (G. Clayman).

Acetylcholinesterase Plays a Pivotal Role in Apoptosome Formation

Sang Eun Park, Nam Deuk Kim and Young Hyun Yoo

Cancer Res 2004;64:2652-2655.