

Induction of acetylcholinesterase expression during apoptosis in various cell types

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Received 1.10.01; revised 22.1.02; accepted 28.1.02
Edited by DR Green

Abstract

Acetylcholinesterase (AChE) plays a key role in terminating neurotransmission at cholinergic synapses. AChE is also found in tissues devoid of cholinergic responses, indicating potential functions beyond neurotransmission. It has been suggested that AChE may participate in development, differentiation, and pathogenic processes such as Alzheimer's disease and tumorigenesis. We examined AChE expression in a number of cell lines upon induction of apoptosis by various stimuli. AChE is induced in all apoptotic cells examined as determined by cytochemical staining, immunological analysis, affinity chromatography purification, and molecular cloning. The AChE protein was found in the cytoplasm at the initiation of apoptosis and then in the nucleus or apoptotic bodies upon commitment to cell death. Sequence analysis revealed that AChE expressed in apoptotic cells is identical to the synapse type AChE. Pharmacological inhibitors of AChE prevented apoptosis. Furthermore, blocking the expression of AChE with antisense inhibited apoptosis. Therefore, our studies demonstrate that AChE is potentially a marker and a regulator of apoptosis.

Cell Death and Differentiation (2002) 9, 790–800. doi:10.1038/sj.cdd.4401034

Keywords: apoptosis; acetylcholinesterase; noncholinergic

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ACh, acetylcholine; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labeling; CHX, cycloheximide

Introduction

Apoptosis in nucleated metazoan cells is executed through an evolutionarily conserved 'suicide' program,¹ responsible for the elimination of excessive cells during development² and various pathophysiological processes.³ Despite its highly conserved nature, the molecular mechanisms underlying apoptosis have only recently been identified.⁴ Investigations in the last few years have revealed that under many circumstances, apoptosis is initiated by the activation of a cascade of proteases called caspases.^{5,6} The activation of caspases is tightly regulated by cellular signals such as that instigated by ligation of the TNF family receptors and release of mitochondrial cytochrome *c*. It is believed that besides being responsible for the characteristic apoptotic morphological changes, caspases also mediate degradation of the inhibitor of caspase-activated deoxyribonuclease, resulting in the liberation of caspase-activated deoxyribonuclease. This nuclease cleaves genomic DNA at the internucleosomal linker regions leading to condensation of the nuclei and fragmentation of genomic DNA.⁷ However, detailed mechanisms regulating the activity of the components in this general scheme of the apoptotic process remain undefined.

Cholinesterases are members of the serine hydrolase family using a serine residue at their active site.⁸ Other than cleaving acetylcholine (ACh), it has been revealed that this enzyme may also be active in peptide hydrolysis,^{9,10} although some reservations regarding this activity have been raised.¹¹ In addition, these enzymes have also been shown to exhibit aryl acylamidase activity.¹² There are two major types of cholinesterases: acetylcholinesterase (AChE) which preferentially hydrolyzes ACh, and butyrylcholinesterase (BChE) which hydrolyzes butyrylcholine. The function of BChE remains a puzzle. It has no known specific natural substrate, although it is capable of hydrolyzing ACh with a very low efficiency. Mutation in BChE does not result in significant physiological consequences.¹³ On the other hand, besides its pivotal role in neurotransmission, recent studies have revealed that AChE is also expressed in tissues that are not innervated by cholinergic nerves.⁸ It is found in several types of hematopoietic cells including erythrocytes¹⁴ and megakaryocytes.^{15,16} Overexpression of cholinesterases could lead to abnormal megakaryocytopoiesis.^{8,17} Transient expression of AChE has been found in nerve tissues during development.¹⁸ Early work showed that high doses of γ -irradiation induced erythroleukemic K562 cells to elevate AChE-activity accompanied by cessation of cell proliferation.¹⁹ Administration of cortisone to neonatal mice could

increase AChE activity in the cortex of the thymus.²⁰ AChE activity in Graafian follicles was found to gradually increase during follicular maturation and ovulation.^{21,22} Although these data indicate a possible relationship between AChE expression and apoptosis, this premise has never been systemically investigated. Considering the established role of apoptosis in gamma irradiation, cortisone treatment, and ovulation, we hypothesize that the induction of AChE activity is related to apoptosis. We report herein that AChE is expressed during apoptosis induced by various stimuli in a number of cell lines. Inhibition of AChE with pharmacological inhibitors could prevent apoptosis. Furthermore, AChE antisense, which inhibits the expression of AChE, also prevented apoptosis. Thus, we have shown that AChE is likely a novel marker and a regulator of apoptosis.

Results

AChE activity is associated with apoptosis induction in various cell lines

Since AChE has been shown to be associated with tissue remodelling during development^{18,23,24} and decreased in cell proliferation,^{14,25–27} it is possible that AChE expression accompanies apoptosis. To evaluate this association, we determined the expression of AChE in several cell lines by employing a well-established cytochemical staining protocol^{28–32} upon induction of apoptosis with specific stimuli. This detection method was originally described by Karnovsky and Roots²⁸ for the demonstration of acetylcholinesterase (AChE) and modified by Hanker *et al.*³¹ and Kobayashi *et al.*³⁰ It is specific for AChE³² and has been widely used for diagnosis in detecting AChE activity in research and in clinical diagnosis of nerve disorders such as Hirschsprung's disease.^{29,31} This protocol is based on the ability of acetylthiocholine to catalyse the hydrolysis of acetylthiocholine, which contains a sulfur atom substituted for an oxygen in the ester linkage. Enzymatic cleavage of acetylthiocholine by AChE yields acetate and thiocholine, whose sulphhydryl group reduces ferricyanide to ferrocyanide. Copper ferrocyanide, which is colored brown, is formed at the site of enzymatic activity and immediately precipitates. The brownish precipitate thus marks the site of AChE activity. We have performed the cytochemical staining on a variety of cell types. AChE activity was only observed in apoptotic cells. One such example is shown in Figure 1, where only apoptotic HLF human fibroblast cells (Figure 1B) in long term-culture³³ appeared dark brown upon AChE cytochemical staining.

Specificity of the modified Karnovsky and Roots staining

Although the Karnovsky and Roots²⁸ staining for AChE was established more than three decades ago, this is the first time to associate AChE activity with apoptotic cells. To prove the specificity of this methods, we employed AChE specific inhibitors. As shown in Figure 2, the staining of apoptotic cells is inhibited by BW284c51 (Figure 2B). To further investigate that the specific staining is indeed due to presence of AChE protein in apoptotic cells, we incubated cells with

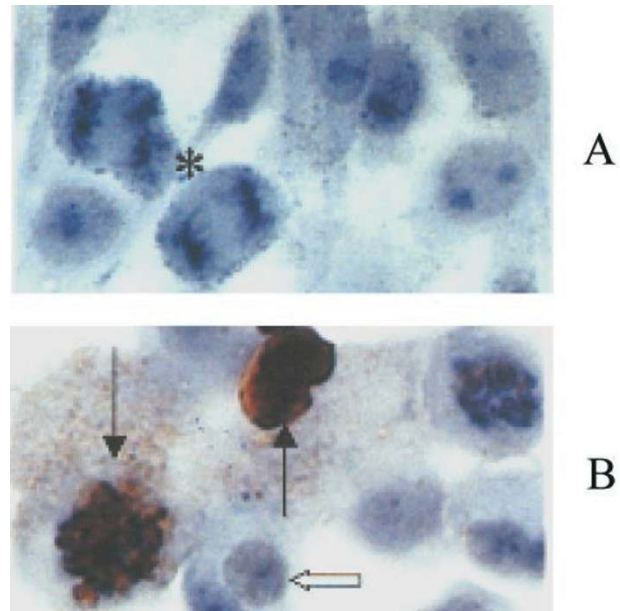


Figure 1 Apoptotic cells are positive for AChE. Human fibroblast cell line HLF either at exponential growth phase (A) or in aging culture as described in Materials and Methods (B) were stained by AChE cytochemical staining and hematoxylin. Note: The asterisk in panel A shows a mitotic figure, indicating that the cells are in a exponential phase. The dark brown staining in panel B demonstrates a positive reaction for AChE. The open arrow in panel B shows that a nearby live cell is negative for AChE staining. The photographs were taken under a light microscope at 600 × magnification

polyclonal rabbit anti-AChE. As shown in Figure 2C, antiserum to AChE also dramatically reduced the appearance of the brownish precipitate upon Karnovsky and Roots staining. The same results were also obtained with other cell lines. This result indicates that the activity of AChE is due to the presence of AChE protein.

Cells dying by apoptosis display specific characteristics. In addition to major morphological changes, the fragmentation of genomic DNA has been used as a diagnostic feature of apoptotic cell. To establish the correlation between DNA fragmentation and AChE expression, GM-CSF-dependent cell line M07e was deprived of GM-CSF and FCS for 18 h. Apoptotic cells were harvested and stained for genomic DNA fragmentation by terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) staining and for the presence of AChE protein by immunocytochemistry. As shown in Figure 3, all apoptotic cells are positive for both AChE and TUNEL staining. However, live cells are negative for both TUNEL and AChE. We also tested the relationship between AChE cytochemical staining and genomic fragmentation. As shown in Figure 4, only cells stained by Karnovsky and Roots staining were positive in the TUNEL assay. We also found that the activation of caspase 3 associated with the presence of AChE activity (data not shown). Therefore, AChE is present in apoptotic cells.

AChE exists in various types of apoptotic cells

To generalize the association of AChE activity with apoptosis, we tested the presence of AChE activity with the modified

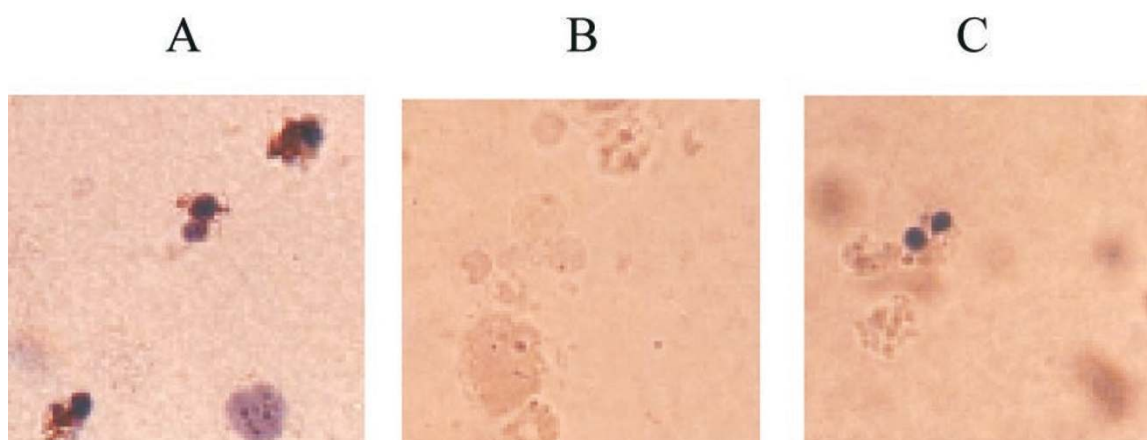


Figure 2 The specificity of AChE cytochemical staining. M07e cells were induced to undergo apoptosis by GM-CSF deprivation for 48 hours. Cells were stained by AChE cytochemical staining and hematoxylin. (A) AChE cytochemical staining plus Hematoxylin staining. (B) Cells were treated with AChE specific inhibitor, BW284c51 at 10 mM and then AChE cytochemical staining. (C) Cells treated with a rabbit antiserum against human AChE (c-16) before AChE cytochemical staining. The photographs were taken under a light microscope at 320 \times magnification

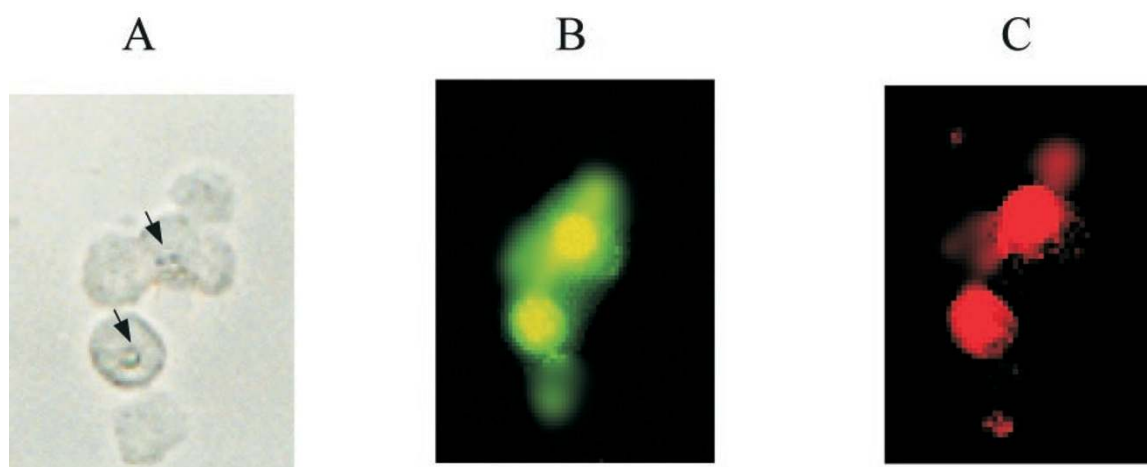


Figure 3 Co-localization of AChE and DNA fragmentation in apoptotic cells. M07e cells were induced to undergo apoptosis by GM-CSF deprivation. Detached apoptotic cells were collected and stained for both AChE and genomic DNA fragmentation. (A) Phase-contrast image under light microscope. (B) TUNEL positive, as detected by biotinylated nucleotides and binding to Streptavidin conjugated with FITC. (C) The cells were stained with anti human AChE antibody (AChE c-16), followed by secondary antibody conjugated with Rhodamine

Karnovsky and Roots staining and a modified ELISA assay using the Ellman's method.^{34,35} As shown in Table 1, no other live cells except mature megakaryocytes^{15,16,36} express AChE. Interestingly, all apoptotic cells tested are AChE positive. Thus, induction of AChE activity in cells undergoing apoptosis is a common phenomenon in various cell types induced by different stimuli.

The AChE activity in all apoptotic cells could be inhibited by the AChE specific inhibitors, BW284c51 and eserine, but not by a BuChE specific inhibitor iso-OMPA (tetra-isopropyl pyrophosphoramidate). Two such examples of detection of AChE activity by the Ellman's ELISA assay are illustrated in Figure 5. We used purified red blood cell AChE as our standard ELISA assay control (Figure 5A), illustrating the sensitivity of the assay and the specificity of the inhibitors. Two cell lines were used as examples:

apoptosis in HLF cells were induced by prolonged culture and in SK-N-SH induced by TNF treatment. Detached apoptotic fibroblast cell line HLF (Figure 5B) and neuroblastoma SH-N-SH cells (Figure 5C) were positive for AChE activity, which could be specifically inhibited by BW284c51 and eserine, but not by iso-OMPA. Only negligible amounts of AChE activity could be detected in live attached cells.

To further investigate the existence of AChE protein in apoptotic cells, we performed Western blot analysis on several cell lines with and without apoptosis induction. As shown in Figure 5D, antibody specific to the carboxyl terminus of human AChE (C-16, Santa Cruz Biotechnology, Inc.) detected a 65 kd band in the cell lysate of apoptotic, but not live cells, in several cell types examined. This molecular weight is equivalent to the size of human

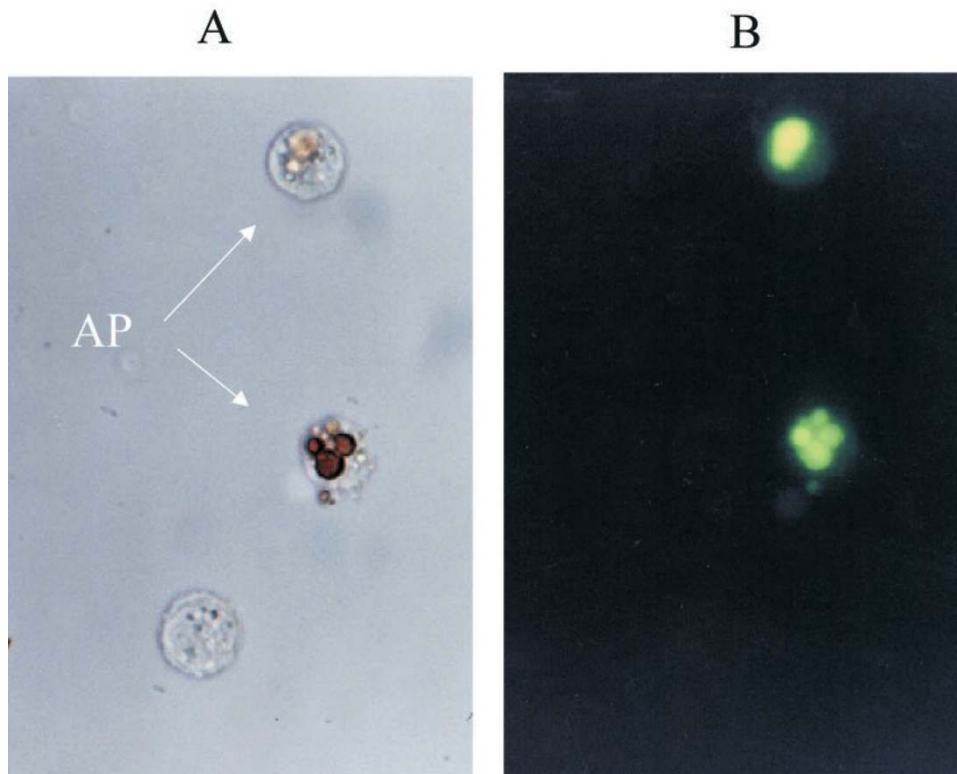


Figure 4 Co-localization of cytochemical staining with TUNEL assay. Detached apoptotic HLF cells were collected and stained for AChE by cytochemical staining and for genomic DNA fragmentation by TUNEL assay. AP stands for apoptotic cells. (A) Brown indicates AChE positive with cytochemical staining. (B) TUNEL positive cells (green) indicate genomic DNA fragmentation

Table 1 Expression of AChE in various cell lines undergoing apoptosis

	Cell type	Apoptosis stimuli	Live cells	Apoptotic cells#	BW284c51	Iso-OMPA	Eserine
<i>Human</i>							
	HLF	Fibroblast	Aging	—	+	—	+
	HFL-1	Fibroblast	Aging	—	+	—	+
	Hela	Cervical Cancer	TNF α +CHX	—	+	—	+
	M07e	Leukemia	Minus GM-CSF	—	+	—	+
	Meg-01	Leukemia	TGF- β	—	+	—	+
	Dami	Leukemia	TGF- β	—	+	—	+
	HL-60	Leukemia	Daunorubicin	—	+	—	+
	SK-N-SH	Neuroblastoma	TNF α +CHX	—	+	—	+
	PC-3	Prostate Cancer	TGF- β	±	++	—	+
	HOS	Osteosarcoma	TGF- β	—	+	—	+
<i>Mouse</i>							
	NIH/3T3	Fibroblast	Detachment	—	+	—	+
	BrE-VimTts	Endothelial line	TGF- α	—	+	—	+
	MK*	Megakaryocyte	ND	+	ND	—	+
<i>Rat</i>							
	BRL	Liver	Detachment	—	+	—	+
	SMC	Smooth muscle	TGF- β	—	+	—	+
	MK*	Megakaryocyte	ND	+	ND	—	+
<i>Swine</i>							
	EC	Prim. Endothelia	Detachment	—	+	—	+
<i>Bovine</i>							
	EC	Prim. Endothelia	TGF- β	—	+	—	+

Induction of apoptosis: Apoptosis in M07e was induced by deprivation of GM-CSF and FCS for 18 h. Apoptosis in Hela, endothelial cell lines, and primary rat aorta SMC was induced by 1 ng/ml recombinant human TNF- α in presence of cycloheximide (10 μ g/ml) or TGF- α (5 ng/ml). Aging-induced apoptosis was carried out by prolonged culture. Daunorubicin (1 μ M) was used to induce apoptosis in HL-60 cells. Eserine and BW284c51 are used at 10 μ M, iso-OMPA at 1.5 mM. #, apoptosis was determined by Trypan blue exclusion and MTT conversion and Ellman's assay. *MK, normal rat and mice Megakaryocytes, AChE positive. +, positive for AChE cytochemical staining. —, negative for AChE cytochemical staining. ND, not determined. CHX, cycloheximide

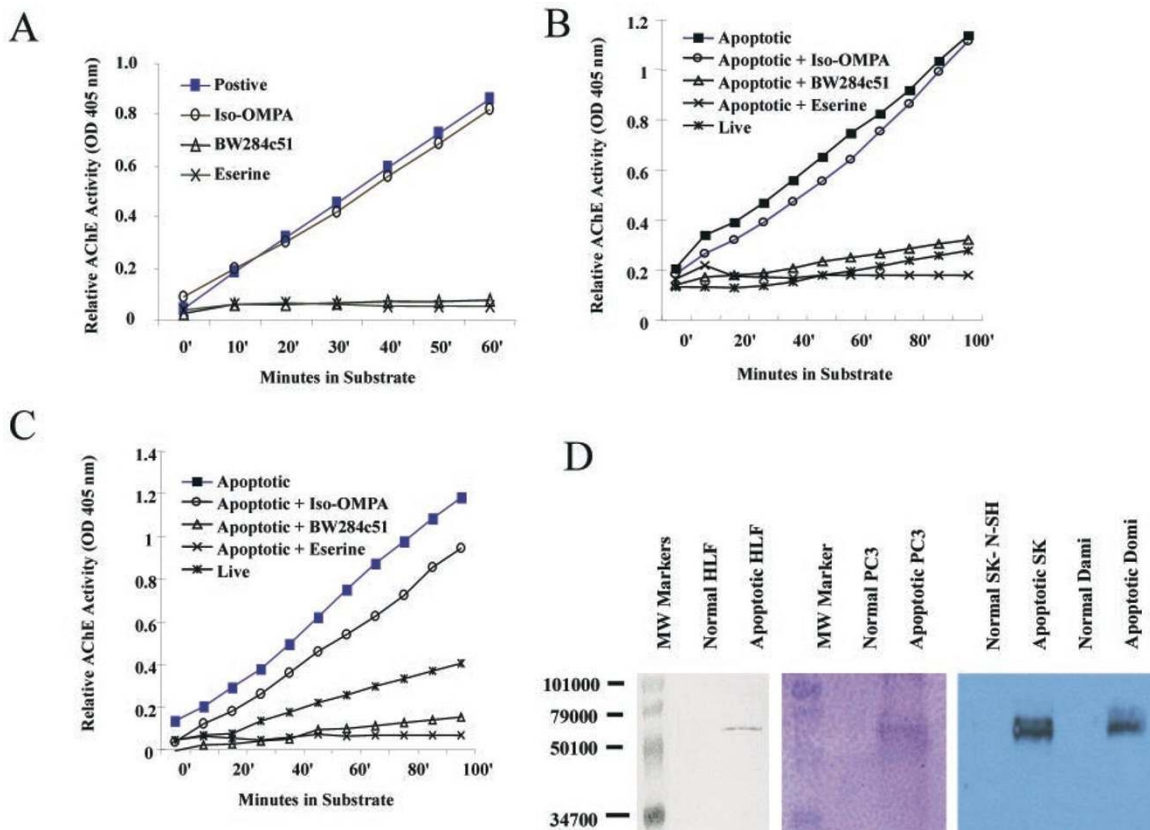


Figure 5 Apoptotic cells express AChE. AChE activity was determined by the Ellman's method. (A) Standard control of human red blood cell AChE. (B) Apoptotic HLF cells induced by prolonged culture. (C) Apoptotic SK-N-SH induced by TNF (1 ng/ml) in the presence of cycloheximide (10 mg/ml). Live cells were employed as controls

synaptic AChE. In addition, when tacrine affinity chromatography³⁷ was performed, the elute of the cell lysate of apoptotic but not live cells also contains a 65 kd protein on SDS-PAGE (Figure 7).

Time course of AChE expression during apoptosis induction

In our cytochemical staining and immunohistochemistry, we found that the presence of AChE is not homogeneous in either cytoplasm or nucleus. To find out whether this heterogeneity is due to the stage of apoptosis induction, we determined the time course of AChE expression in leukemic cell line Meg-01 cells upon treatment with TGF- β . The expression of AChE was detected by cytochemical staining. As shown in Figure 6, AChE initially appears in the cytosol (Figure 6B, 2 h) and then in the nucleus (Figure 6C,D, 4 and 6 h respectively). AChE follows the nuclear morphological changes such as condensation and fragmentation (Figure 6F-H, 8, 10 and 12 h). Eventually, when the cell membrane ruptures, AChE is released (Figure 6I, 18 h). We also examined the expression of AChE by transmission electromicroscopy. At 2 h after TGF- β treatment, AChE was only observed in the cytosol (Figure 6J) and all moved to the nucleus by 4 h. Therefore, during apoptosis induction, AChE is first synthesized in the cytosol and then accumulates in the nucleus.

Apoptosis-associated AChE activity is due to activation of the AChE gene

To further confirm the identity of the cholinesterase induced during apoptosis, we performed RT-PCR with total RNA extracted from both apoptotic (detached) and live (attached) HLF cells with specific primers for each of the alternatively spliced forms of human AChE. Only the mRNA encoding the synaptic type AChE mRNA (482 bp) was detected (Figure 7A). Very low amount of RT-PCR product was amplified from RNA of attached live cells. To verify the identity of the RT-PCR product, we sequenced the 482 bp RT-PCR product and found that the sequence was identical to the synaptic type AChE (GenBank, accession number M55040). Furthermore, the molecular weight of the cholinesterase present in apoptotic cells was validated by its specific binding to tacrine. Detached apoptotic and attached viable HLF cells were lysed and tacrine affinity chromatography was performed.³⁷ The eluate from tacrine affinity chromatography column was also analyzed on SDS-PAGE. As shown in Figure 7B, no protein in viable cells was found to bind to tacrine. However, the elute from apoptotic cells contains a protein as a single band with a molecular weight of approximately 65 kDa on SDS-PAGE, which is identical to that of standard human erythrocyte AChE. In addition, we have also detected the presence of AChE by Western blot analysis with a rabbit polyclonal antibody

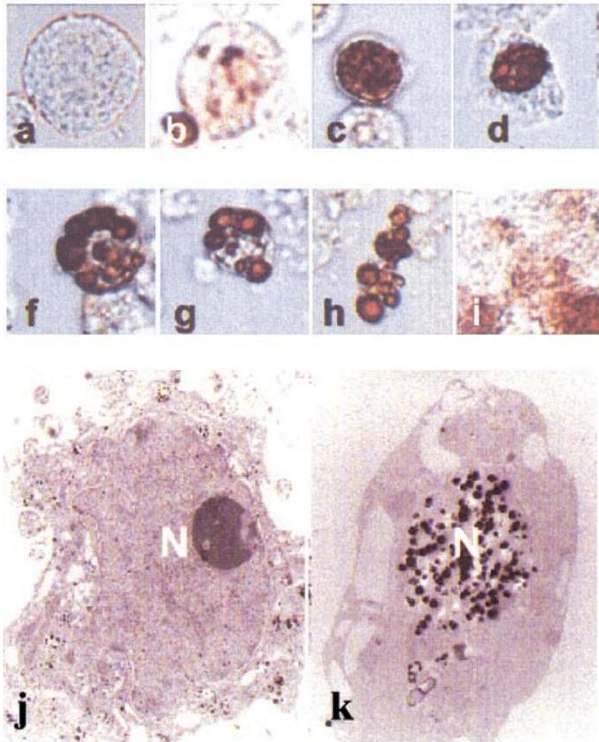


Figure 6 Intracellular localization of AChE in cells undergoing apoptosis. Leukemic cell line Meg-01 cells were treated with TGF- β at 5 ng/ml. The expression of AChE was detected by cytochemical staining at 0 h (A), 2 h (B), 4 h (C), 6 h (D), 8 h (F), 10 h (G), 12 h (H), and 18 h (I) after treatment. The brown color in the micrographs indicates the presence of AChE. The presence of AChE was also detected by transmission electromicroscope at 2 h (J) and 4 h (K) after treatment

specific to human AChE. Similar to tacrine chromatography, Western blot analysis also detected a 65 kd protein in apoptotic, but not in live cells (Figure 7C). Therefore, these studies strongly suggest that the cholinesterase in apoptotic cells is synapse AChE.

Inhibition of AChE blocks apoptosis

To examine whether AChE inhibitors could modulate apoptosis, we tested the effects of tacrine, BW284c51, and eserine. We found that all these inhibitors reduced cell death in several cell lines including Dami, HLF, SK-N-SH, NIH/3T3, and BRL. Figure 8 shows the effect of these inhibitors on TNF induced apoptosis in Dami (Figure 8A) and long-term-culture induced³³apoptosis in HLF cells (Figure 8B). Therefore, the expression of AChE likely participates in the process leading to apoptosis.

It has been reported that antisense oligodeoxynucleotides against AChE messenger RNA could dramatically increase the proliferation of bone marrow cells *in vitro*.²⁵ To further determine whether AChE expression has a role in the execution of apoptosis, we employed two antisense oligodeoxynucleotides against the AChE mRNA (AS-AChE) (one complementary to exon 1 and the other to exon 2). Their corresponding sense (S-AChE) and mismatched oligodeoxynucleotides were used as controls. As shown

in Figure 9, both antisense oligos completely blocked apoptosis in Dami (Figure 9A) and HLF (Figure 9B) cells, while the control oligos did not affect apoptosis. To determine the specificity of the oligodeoxynucleotides, we examined the effect of the oligodeoxynucleotides on the expression of acetylcholinesterase by Western blot analysis. We found that antisense oligo blocked the expression of AChE in HLF cells undergoing apoptosis, while the control oligos did not have any effect (Figure 9C).

Discussion

The primary function of AChE is to hydrolyze ACh and thus terminates cholinergic neurotransmission. However, recent studies have shown that this gene is also expressed in other cells including developing brain,^{24,38} and some hematopoietic cells,³⁹ such as red blood cells and megakaryocytes. In 1988, Bulloch and Lucito²⁰ found that administration of cortisone could lead to an increase in AChE activity in the thymus. The authors proposed that the increase in AChE induced by steroids is due to an activation of quiescent cholinergic nerves and nerve-related structures. Schwenke *et al.*¹⁹ showed that γ -irradiation of erythroleukemic K562 cells caused an increase in AChE-activity accompanied by cell differentiation and cessation of cell proliferation. In addition, it has been shown that gonadotrophin treatment in ovarian follicles also induces nerve like AChE activity.^{21,22} These studies have attributed the expression of AChE to the possible extension of neural ends and proposed the role of nerve extension in the physiology controlled by these treatments. Recently, Soreq *et al.*²⁵ demonstrated that antisense oligodeoxynucleotide mediated inhibition of acetylcholinesterase gene expression induces expansion hematopoietic progenitors. Taking together, although the investigations were not designed to investigate the association between AChE and apoptosis, they did reveal that these treatments, which are now known to induce apoptosis, could affect AChE activity. The results presented in our study demonstrate for the first time that AChE is induced and plays an important role in apoptosis.

Abnormality of the AChE gene is also observed in some tumors.²⁶ Recent genomic studies strongly supported this notion. Yeast artificial chromosome (YAC) mapping has revealed that the AChE gene is located in the 7q21-32 fragment⁴⁰ and was mapped to 7q22.⁴¹ Human genome sequencing has further verified that this gene is at 7q22. Interestingly, loss of heterozygosity (LOH) at 7q22 has been found to occur in ovarian cancer,⁴² breast cancer,⁴³ prostate cancer,^{44,45} and several types of leukemia.⁴⁶⁻⁴⁸ LOH of 7q22 has also been found in uterine leiomyoma⁴⁹ and liver cancer.⁵⁰ These data and our finding of the role of AChE in apoptosis strongly suggest that AChE could be a tumor suppressor gene, a notion suggested by Stephenson *et al.*⁵¹ In addition, epidemiological evidence and animal studies have revealed that many pesticides are carcinogenic. It has long been suggested that agricultural use of organophosphorus insecticides, which are inhibitors of AChE, is linked to non-Hodgkin's lymphoma and leukemia development.⁵² Although the molecular mechanism is not known, it has been shown that prolonged exposure of rats to organophosphorous pesticides could induce mammary

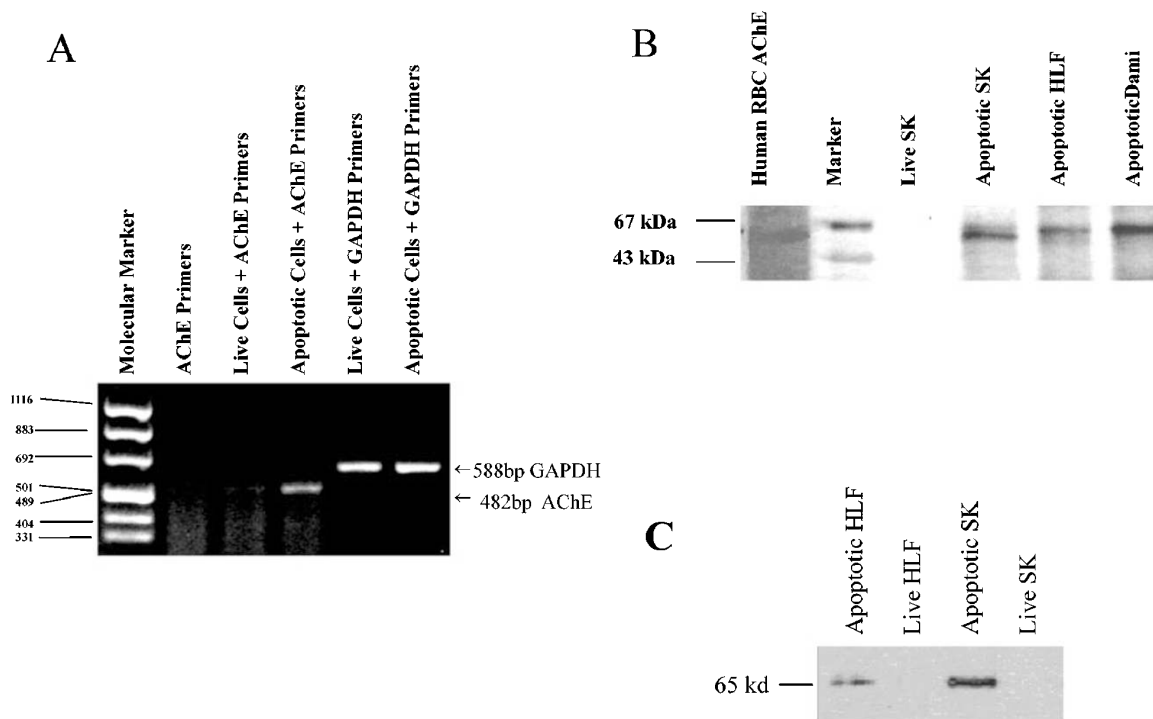


Figure 7 Detection of AChE expression in apoptotic cells. Messenger RNA was detected by RT-PCR and protein was examined by chromatography and Western blot analysis. **(A)** RT-PCR of synaptic specific AChE mRNA. GAPDH was used as a mRNA quantity control. RT-PCR detection of AChE was carried out in HLF cells. Similar result was obtained with PC-3, HeLa, SK-N-SH, and Dami cells. **(B)** SDS-PAGE analysis of the protein isolated from SK-N-SH, HLF and Dami cells by tacrine affinity chromatography. **(C)** Western blot analysis of AChE in HLF and SK-N-SH with a rabbit anti-human AChE polyclonal antibody

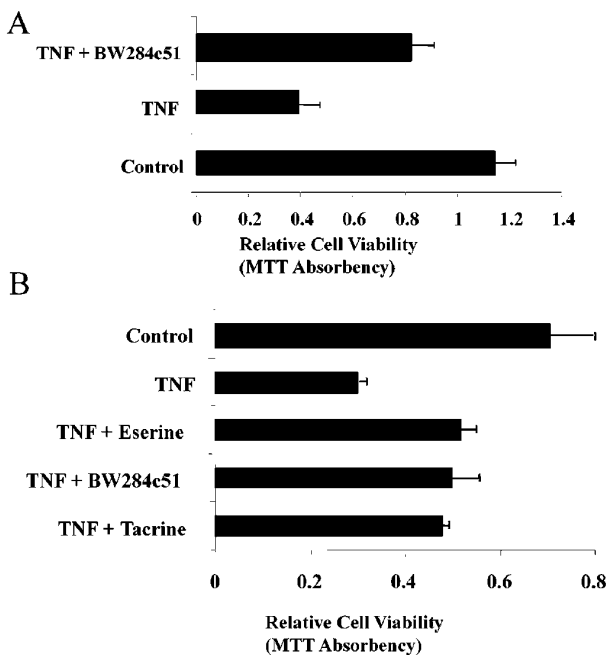


Figure 8 The effects of AChE inhibitors on apoptosis. **(A)** Apoptosis in Dami cells induced by TNF- α and cycloheximide. **(B)** Apoptosis in HLF cells induced by TNF (1 ng/ml) and cycloheximide (10 μ g/ml). Cell viability was assessed by MTT. The inhibitor also inhibited apoptosis in SK-N-SH, NIH/3T3 and BRL cells. Tacrine, eserine, and BW284c51 were used at 10 μ M

gland tumors.⁵³ Based on our finding of the role of AChE in apoptosis, it is probable that the alteration of AChE function may render tumor cells resistant to apoptosis and thus allow uncontrolled cell expansion. Although this hypothesis has not been tested, further investigations in this direction could lead to a better understanding of the harmful effects of environmental contamination by organophosphorus compounds.

In our studies we have seen the expression of AChE in various cell types upon treatment with different apoptosis inducers. The expression of AChE was also seen in cells treated with cycloheximide and TNF in the presence of 10 μ g/ml cycloheximide in several cell lines examined (Figure 1B). We also examined the effect of cycloheximide on global protein synthesis. We found that complete inhibition of protein synthesis needs a much higher level of cycloheximide than 10 μ g/ml. In fact, at 10 μ g/ml, cycloheximide inhibited the synthesis of some proteins (data not shown). These data, in addition to the results with acetylcholinesterase inhibitors, strongly suggest that AChE is an important component in the common pathway leading to apoptosis induced by various stimuli.

AChE expression has also been found in the nervous system during terminal differentiation of fetal neurones⁵⁴ when apoptosis takes place to shape the nervous tissue. Importantly, acetylcholinesterase is also believed to play a critical role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease.⁵⁵ AChE inhibi-

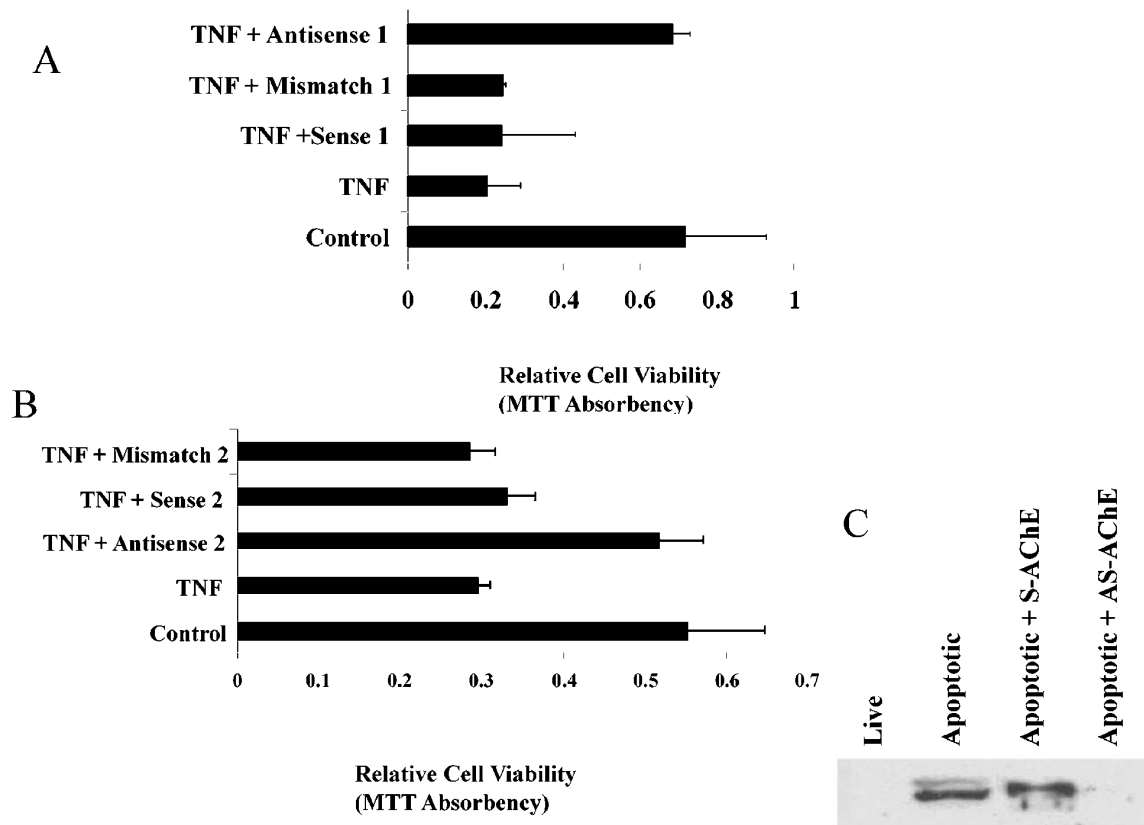


Figure 9 Effects of antisense oligodeoxynucleotides against AChE mRNA on the induction of apoptosis. The effects of AChE antisense on apoptosis were examined in several cell lines including SK-N-SH, HLF and NIH/3T3. (A) Antisense to exon 1 (AS-AChE1: 5'-cgatgttccccggcg-3'; S-AChE1: 5'-cgccggggaacatcg-3'; MS-AChE1, 5'-tcggcagtcgctccg-3'). Three phosphorothioate-modified nucleotides are at both ends which blocks apoptosis in SK-N-SH cells induced by TNF- α and cycloheximide. (B) Antisense to exon 2 (AS-AChE2: 5'-ctgcgggggcctcat-3'; S-AChE2, 5'-atgaggccccgcag-3'; MS-AChE1, 5'-gctgcggcgctcgat-3'). All nucleotides are phosphorothioate-modified which blocks apoptosis in HLF cells induced by TNF- α and cycloheximide. (C). Western blot analysis of the effect of antisense to exon 2 on the expression of AChE protein in SK-N-SH cells. The AS-AChE was effective at the concentrations between 5 to 20 μ M. In the presented experiments, all oligos were used at 10 μ M

tors,⁵⁶ such as tacrine,⁵⁵ have been shown to be able to slow down the development of Alzheimer's disease. This therapeutic effect has been attributed to the role of AChE in fibril formation, our demonstration of the induction of AChE during apoptosis may lead to understanding the origin of this enzyme in the amyloid plaque of the Alzheimer's brain. We believe that further determination of whether AChE is derived from apoptotic neurons could lead to a better prevention and treatment of Alzheimer's disease and other disorders manifested by excessive cell death.

It is unclear how AChE contributes to the apoptotic process. It is interesting to note that the expression of AChE is detected at early stages of apoptosis induction. We have found that AChE first appears in the cytoplasm and then moves to the nucleus before significant nuclear morphological changes take place. At late stages prior cytoplasmic membrane rupture, AChE is only present in the fragmented nuclei. Clearly, an interesting possibility is that AChE participates in the modulation of nuclear components leading to chromatin condensation and fragmentation. In this respect, it is relevant that AChE also possesses activities besides its pivotal role in the hydrolysis of acetylcholine.⁵⁷ Since activation of proteases, especially

the caspase cascade, has been shown to be a vital step in the execution of apoptosis, we believe it will be interesting to further investigate how caspases and acetylcholinesterase are related in regulating apoptosis. We believe that further identification of the targets of acetylcholinesterase in apoptotic cells may provide important information for understanding the mechanisms of apoptosis.

Materials and Methods

Induction of apoptosis

All cells were cultured in DMEM plus 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO₂/95% air. In the GM-CSF-dependent cell line M07e, apoptosis was induced by deprivation of GM-CSF and FCS for 18 h.⁵⁸ Apoptosis in HeLa, endothelial cell lines, and primary rat aorta SMC was induced by 1 ng/ml recombinant human TNF- α (Shanghai Central of Bioengineering, Shanghai, China) in the presence of cycloheximide (10 μ g/ml, Sigma)⁵⁹ or TGF- β (5 ng/ml) (R&D Systems, UK).⁶⁰ Daunorubicin (1 μ M, Farmitalia Carlo Erba, Italy) was used to induce apoptosis in HL-60 cells.⁶¹ Torriglia's long-term culture method was employed to induce apoptosis in several cell

lines.³³ Briefly, cells were incubated at a cell density of 1×10^5 /ml. The cultures were maintained for up to 10 days without medium change. Portions of cells were harvested at different times and analyzed for apoptosis by AChE staining and TUNEL assay.

Antibodies

Antibody to AChE (C-16) (catalog number, sc-6430, Santa Cruz Biotechnology) is an affinity-purified goat anti-the carboxy terminus of human acetylcholinesterase. Antibody against cleaved caspase-3 (D-175) was obtained from Cell Signaling TECHNOLOGY[®] (catalog number 9661).

MTT staining

In some experiments cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO, USA] conversion. Briefly, cells were incubated in 100 μ l media in 96-well plates with various treatments. After treatments as indicated, 20 μ l of MTT solution (5 mg MTT/ml in H₂O) was introduced and cells were incubated at 37°C for 3 h. Each well then received 100 μ l of lysis buffer (20% sodium dodecyl sulfate in 50% N,N-dimethylformamide, 0.4% acetic acid, and 0.04N HCl). Reduced MTT crystals were dissolved by pipetting. The relative cell viability was obtained by scanning with an ELISA reader with a 590 nm filter. Since MTT is only a measure of the viable mitochondria, we always examine cell viability by morphology (cell bulging, cytoplasm and nuclear condensation) and biochemical analysis (TUNEL and caspase assay) prior MTT assay. We have found that the reduction in MTT conversion correlates with the apoptotic changes morphologically and biochemically. Nevertheless, the MTT assay provides us with an opportunity to quantify cell viability and compare different treatments in a large number of samples.

Apoptosis detection by TUNEL

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) was performed using the fluorescein-dUTP-*in situ* death detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cells were fixed in 4% paraformaldehyde for 5 min and then washed twice in PBS. Cells were loaded on glass slides and permeabilized in a solution containing 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C. Cells were then incubated with 100 μ l TUNEL reaction mixture for 60 min at 37°C. Microphotographs were taken under fluorescence microscopy. For cytochemical staining for AChE activity, cells were further treated with thiocholine complexed with copper and ferrous ions created according to the established method.³⁰ Megakaryocytes from mouse bone marrow cells were used as a positive control. For immunocytochemistry staining, cell samples fixed as above and incubated for 1 h with a rabbit anti-rat AChE polyclonal antibody (a gift from Professor Jean Massoulie, Ecole Normale Supérieure, France). The samples were washed three times in PBS, incubated with secondary FITC-conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) for 30 min in a dark and humidified chamber. Slides were analyzed under a fluorescence microscope.

Western blot analysis

Equal numbers of cells were lysed in a lysis buffer composed of 1% NP-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 μ M orthovan-

date, 50 mM ZnCl₂, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1% SDS, and 0.1% deoxycholate. Samples were incubated at 4°C for 10 min and then centrifuged at 10,000 $\times g$ for 15 min at 4°C. The supernatants were transferred, mixed, and boiled in SDS sample buffer. The lysates were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Biorad, Hercules, CA, USA). The membrane was then incubated at room temperature in a blocking solution composed of 5% skim milk powder dissolved in 1 \times TBST (10 mM Tris, pH 8.0, 140 mM NaCl, and 0.1% Tween-20) for 1 h followed by incubation with the blocking solution containing anti-human AChE (AChE c-16) at 1 μ g/ml for 4 h at room temperature. The membrane was washed three times in TBS (5 min each), and then incubated with a horse radish peroxidase conjugated protein A in the blocking solution. The blot was then exposed to ECL (Amersham, Arlington Heights, IL, USA) after another three washes in TBS.

Double staining of AChE cytochemical and DNA break

To ensure that expression of AChE indeed occurs in apoptotic cells, we performed AChE cytochemical staining and TUNEL assay in the same cells. The method of AChE cytochemical staining was originally described by Karnovsky and Roots²⁸ for the demonstration of acetylcholinesterase (AChE) and modified by Hanker *et al.*³¹ and Kobayashi *et al.*³⁰ Briefly, cells were transferred onto glass slides by cytospin. Upon being dried in the air, the slides were incubated in 15 ml 0.1 M sodium phosphate pH 6.0 containing 10 mg acetylthiocholine iodide, 1 ml of 0.1 M sodium citrate solution, 2 ml of 30 mM copper sulphate solution, 2 ml of 5 mM potassium ferricyanide solution at room temperature for 4–8 h. When only AChE cytochemical staining was required, the slides were incubated in Harris' hematoxylin solution for another 30 s. The samples were then dehydrated with ethanol and sealed in neural balsam. When the samples were for electronic microscopy, upon AChE cytochemical staining, cells were postfixed by 1% osmium tetroxide. Double staining of both AChE and TUNEL were performed using the fluorescein-dUTP-*in situ* death detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

Double staining of AChE and TUNEL

To ensure that the AChE protein indeed exists in apoptotic cells, we performed double staining with immunocytochemistry for AChE and TUNEL reaction for DNA breaks. Briefly, after 20 min fixation at 4°C with paraformaldehyde solution (3% in PBS, pH 7.4), harvested cells were washed three times with TBST [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween]. The cells were incubated with 1 ml of Blocking buffer (5.5% Normal Goat Serum in TBST) for 45 min and then incubated with 100 μ l primary antibody (1 : 100 dilution in TBST containing 2% BSA) for 24 h at 4°C. Following incubation, the cells were washed and incubated with 100 μ l of secondary antibody (1 : 100 dilution, Rhodamine conjugated anti-mouse IgG-R, Santa Cruz) for 60 min at 37°C in the dark. The cells were then washed and resuspended in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing, the pellet was resuspended in 50 μ l TUNEL reaction mixture (Roche) and incubated 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.

Purification of AChE from apoptotic cells

Isolation of AChE was performed according to the established procedure.³⁷ Briefly, tacrine was coupled to epoxy-activated Sephar-

ose 6B beads. Cell lysate (in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 1 g/ml protinin, and 1% Triton X-100) was applied onto the tacrine affinity column pre-equilibrated with 250 ml buffer containing 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0. The column was washed with a buffer containing 1 M NaCl, 50 mM Tris-HCl, pH 8.0, and the AChE was eluted with 100 ml of 50 mM Tris-HCl, 10 mM tacrine, pH 8.0. The AChE-containing fractions were identified using the Ellman's method.³⁵

Determination of AChE activity

AChE activity was determined spectrophotometrically in a 96-well microtiter plate using a modified Ellman's assay as described.³⁵ The assays were 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. OD value at 405 nm were determined.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from 7×10^6 cells with the Tripure TM isolation reagent (Boehringer Mannheim, Mannheim, Germany) and was reverse transcribed into cDNA by random priming. PCR analysis was performed using specific primers for AChE mRNA. To detect the mRNA of the synaptic AChE including exons E2-E3-E4-E6, we employed the 1522 (+)/2003(-) primer pair (5'-CGG GTC TAC GCC TAC GTC TTT GAA CAC CGT GCT TC; 5'-CAC AGG TCT GAG CAG CGA TCC TGC TTG CTG) designed to generate a fragment of 482 bp. For the AChE mRNA encoding the glycopospholipid-anchored AChE (exons E2-E3-E4-E5-E6), we used the primers 1522(+) and 1917 (-) pair (5'-ATG GGT GAA GCC TGG GCA GGT G). Two different fragments, 399 and 478 bp, could be generated. Primer 1 (5'-CCA CCC ATG GCA AAT TCC ATG GCA) and 2 (5'-TCT AGA CGG CAG GTC AGG TCC ACC) were used to generate a 588 bp fragment of GAPDH as internal control. RT-PCR products were electrophoresed on a 1.5% agarose gel.

Antisense oligodeoxynucleotides

Two phosphorothioate-modified oligodeoxynucleotides were designed to target the human AChE mRNA corresponding to exons 1 and 2 and designated AS-AChE1 and AS-AChE2. For each antisense, the sense (S-) and mismatched (MS-) sequences were used as controls. AS-AChE1: 5'-CGA TGT TCC CCG GCG-3', S-AChE1: 5'-CGC CGG GGA ACA TCG-3', and MS-AChE1, 5'-CTG GCA GTC GCT CCG-3' contain three phosphorothioate-modified nucleotides at each end. In AS-AChE2: 5'-CTGCGGGGGCCTCAT-3', S-AChE2, 5'-ATG AGG CCC CCG CAG-3', and MS-AChE1, 5'-GCT GCG GCG CTC GAT-3', all nucleotides were phosphorothioate-modified. All oligos were dissolved in H₂O at a stock concentration of 1 mM. Different concentrations of the oligos were added directly to the tissue culture.

Acknowledgments

The authors are grateful to Drs. Daniel Lawrence, Sidney Pestka, Achsah Keegan, Allan Mufson and Miss Jennifer Solomon for their critiques of the manuscript. This work was supported by the Basic Research Fund and the Targeted Biotechnology Support of the Chinese Academy of Sciences, G1999053905, by the Programme Franco-Chinois de Recherches Avancees, and by National Institutes of Health Grants CA76492 and AI43384.

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