Overexpression of acetylcholinesterase inhibited cell proliferation and promoted apoptosis in NRK cells[1]

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KEY WORDS acetylcholinesterase; gene expression; proliferation; apoptosis

ABSTRACT

AIM: To study the potential function of acetylcholinesterase (AChE) in apoptosis through overexpression of AChE in Normal Rat Kidney (NRK) cells. **METHODS:** AChE activity was detected by the method of Karnovsky and Roots. Activated caspase-3 was analyzed by Western blotting and immunoflurescence with antibody special to activated caspase-3 fragment. The expression plasmids were constructed in pcDNA3.1 containing AChE gene or a fragment of AChE antisense that were got from RT-PCR. Stable expression cell lines were selected by G418 in cells transfected by lipofection. AChE expression was analyzed by RT-PCR and Western blotting. The proliferation rates of transfected cells were examined by the growth curve and cloning efficiency. MTT assay was used to analyze the cell viability. **RESULTS:** The proliferation rate of the cells transfected with AChE was retarded and the cloning efficiency was lower (28.2 %±3.1 % and 48.7 %±2.1 %) than cells transfected with vector (56.1 %±0.3 %) or AChE-antisense (77.7 %±2.2 %). After 2 d the various clone types were deprived of serum, the residue cell viability were 10.4 %±4.6 % and 12.6 %±6.7 % in the cells transfected with AChE, and 27.4 %±3.5 % in cells with vector, and 50.3 %±7.8 % in cells with AChE-antisense. **CONCLUSION:** During apoptosis, increase of AChE protein is to inhibit cell proliferation, and then to promote apoptosis in NRK cells.

INTRODUCTION

Acetylcholinesterase (AChE) is responsible for inactivating acetylcholine at cholinergic synapses in both the central and peripheral nervous systems. AChE is classified into R, H, and T transcripts depending on the structure of the encoded C-terminal domain, formed from alternative splicing of a single gene in vertebrate[1]. However, the enzyme is not restricted to inactivate acetylcholine and accumulated evidence increasingly points to non-catalytic roles[2]. AChE also exists in WRL-10A-cultured fibroblasts[3], human erythrocytes[4], T lymphocytes[5] and murine thrombocytic cells[6]. In these cells, the enzyme has no obvious relations to its classic function.

[1] This work was supported by the National High Technology Program of China ("863 Project", No 2002BA711A07), the National Natural Science Foundation of China (No 30240028), Grant from Chinese Academy of Sciences (No KSCX2-SW-211), and the State Key Basic Research and Development Program of China ("973 Project", G19990-53905).
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Received 2003-12-19 Accepted 2004-05-18

We previously reported that AChE was expressed in a number of cell lines upon induction of apoptosis by various stimuli[7]. γ-Irradiation of erythroleukemic K562 cells caused an increase in AChE activity accompanied by cell differentiation and cessation of cell proliferation[8]. Andrea *et al* also showed that both AChE and apoptosis increased following butyrylcholinesterase antisense transfection in retinospheroids[9]. Moreover, it has been suggested that AChE participates directly in the regulation of bone marrow cell development by reducing proliferation and promoting apoptosis in their progenies[10,11]. We previously showed that AChE expression was increased prior to apoptosis[7]. But the specific function in apoptosis is still unclear. In this article, we investigated the potential function of AChE in apoptosis.

METERIALS AND METHODS

Cell culture and induction of apoptosis Normal Rat Kidney cells (NRK) (from cell bank of Chinese Academic of Sciences, Shanghai, China) were grown as a monolayer in RPMI-1640 (Invitrogen, USA) supplemented with 10 % fetal calf serum, 4 mmol/L glutamine. Cells were grown at 37 ºC in a humidified atmosphere containing 5 % CO_2. To induce NRK cells apoptosis, cells that were grown 80 % confluence were changed with fresh medium and then maintained in culture without changing the medium for 4-12 d to induce apoptosis as previously described[12], or were deprived of serum and continued culturing for 1-2 d to induce apoptosis.

Double staining of AChE activity and activated caspase-3 immunofluorescence AChE activity of NRK cells harvested from long-term cell culture was stained according to the method of Karnovsky *et al*[13]. Briefly, the harvested cells were fixed for 10 min in 1.5 % paraformaldehyde in PBS, rinsed one time with PBS, and incubated in staining solution [0.67 mmol/L acetylthiocholine, 5 mmol/L sodium citrate, 3 mmol/L cupric sulfate, 0.5 mmol/L postassium ferricyanide in 0.1 mol/L sodium phosphate buffer (pH 6.0)] for 6 h at 37 ºC. Then the cells were centrifuged onto slides and permeated with permeabilizing solution (0.1 % Triton X-100, 0.1 % sodium citrate in PBS) for 4 min on ice. The cells were incubated with 50 μL primary antibody (diluted in PBS containing 3 % BSA) to the activated caspase-3 fragment (Cell Signaling Technology, USA), diluted to 1/100 for 24 h at 4 ºC. After washed with PBS three times, the slide was then treated with a secondary antibody (1:100 dilution in PBS containing 3 % BSA, rhodamine conjugated anti-rabbit IgG, Santa Cruz Biotechnology, USA) for 1 h at 37 ºC. The labeled cells were washed with PBS and observed under a fluorescence microscope.

Double staining of AChE immunofluorescence and TUNEL Cells were centrifuged onto slides and fixed with 3 % paraformaldehyde at 4 ºC for 40 min, and then treated with permeabilization solution for 4 min. After blocking with 3 % BSA for 1 h at room temperature, slides were incubated with primary monoclonal antibody to AChE (BD Biosciences, USA), diluted to 1/100 for 2 h and incubated with the second antibody (1:100 dilution, rhodamine conjugated anti-mouse IgG, Santa Cruz Biotechnology, USA) for 1 h at 37 ºC. After washing, apoptosis was detected using 50 μL TUNEL reaction mixture (Roche Applied Science, USA) for 60 min at 37 ºC in the dark. The labeled cells were washed with PBS and observed under a fluorescence microscope.

Visualization of AChE and nuclei by confocal microscopy Cells were grown on chambered coverglass for 12 d. The cells still on the coverglass were washed once with PBS and fixed with 4 % formaldehyde for 1 h at 4 ºC, and then permeated with permeabilizing solution for 4 min. After gently washed three times, the cells were blocked in 3 % BSA in PBS. The cells were then stained with anit-AChE antibody (diluted 1:100 in 3 % BSA in PBS) for 1 h at 37 ºC humidified atmosphere, and followed by washing three times with PBS. The secondary antibody, FITC-conjugated goat anti-mouse Ig G, diluted at 1:100 in 3 % BSA in PBS, was added and the cells were incubated for 1 h at 37 ºC humidified atmosphere, and followed by washing three times with PBS. To double-label nuclei, cells were further stained with 10 μmol/L Hoechst 33258 for 5 min at room temperature. The coverglass were observed using a laser scanning confocal microscopy (Bio-Rad radiance 2100).

Western blot analysis The cultured and transfected cells were harvested and lysed in the 1×SDS PAGE gel loading buffer (Tris 50 mmol/L pH 6.8, DTT 100 mmol/L, 2 % (w/v) SDS, 10 % (v/v) glycerol, aprotinin 1 mg/L, and PMSF 10 mmol/L) and boiled in a water bath for 10 min. The protein concentration was determined by the bicinchoninic acid (BCA) method. Bromophenol Blue (0.01 % concentration) was added to the samples before an equal amount of proteins were loaded in each lane for electrophoresis and electro-trans-

ferred onto nitrocellulose membrane. After blocking with TBST (Tris-HCl 10 mmol/L pH 7.5, NaCl 100 mmol/L, 0.1 % Tween-20) containing 5 % non-fat milk, the membrane was incubated with a primary mouse monoclonal antibody to AChE (BD Biosciences, USA) or rabbit polyclonal activated caspase-3 fragment for 2 h at 37 ºC, and incubated with a corresponding secondary antibody (1:2000 diluted in TBST containing 5 % non-fat milk, HRP conjugated anti-mouse or rabbit IgG, Santa Cruz Biotechnology, USA) at 37 ºC for 1 h. The target protein bands were detected by the ECL method according to the manufacture's instruction (Santa Cruz Biotechnology, USA). A pre-stained molecular weight marker was used to determine the size of the protein (BIO-RAD, USA).

Expression plasmid construction The fragments that expressed full-length coding mRNA and partial antisense mRNA of the AChE gene were obtained by RT-PCR from rat renal mRNA. PCR amplification was preformed in a Model 450 thermal cycler (Perkin-Elmer Cetus, CT) for 35 cycles. The primers designed for full-length coding mRNA were:
1 (+): 5´-GGCGCTAGCCACCATGAGGCCTGGCTG-GTATGG-3´;
1842 (-): 5´-GGGAATTCACAGGTCTGAGCAGCGT-TC-3´. The primers designed for the antisense fragment that included a fraction of exon 3, exon 4, and a fraction of exon 6 were:
1389 (+): 5´-CCGGAATTCCCGTGCCTCCACATTGA-CTTG-3´;
1819 (-): 5´-CCGGCTAGCTGCTATAGTGGTCGAAC-TGGTTCT-3´.

The *Nhe* I site was designed at the 5´ end upstream of the primer and the *EcoR* I site at the 3´ end downstream of the primer for full-length coding mRNA, while the engineered restriction sites were reversed in the primers for the antisense fragment. The PCR conditions for the antisense fragment was denaturing at 96 ºC, 1 min (the first step for 5 min), annealing at 60 ºC, 1 min, and synthesized at 72 ºC, 1 min (the last cycle for 5 min) using Pfu DNA polymerase (Promega, USA). PCR conditions for full-length coding mRNA was the same but the time for synthesis was 4 min and contained 5 % (v/v) Me_2SO in buffer. The fragments digested with *Nhe* I and EcoR I ligated to pCDNA3.1B (Invitrogen, USA) digested with the same restrict-endonuclease. The ligated construct was transferred into competent DH5α *E coli* cells. Plasmid DNA from the bacterial clones with unmodified plasmid and those with sense or antisense inserts were identified by restriction analysis and verified by DNA sequencing. A single bacterial clone for each type of construct was selected for use in future harvesting of plasmid DNA.

Harvesting stable transfectants The sense (AChE-S), antisense (AChE-AS), and pCDNA3.1B expression plasmid was purified by QIAGEN plasmid Midi and Maxi Kits (QIAGEN Companies, German) and transfected into NRK cells by DOTAP liposomal transfection reagent (Roche Applied science, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, 700 mg/L of G418 was used to select clones for a period of 30 d and then maintained with 300 mg/L G418. The clones were further identified by RT-PCR and Western-blot analysis.

RT-PCR for identification of clonal cell populations Total RNA was isolated by TRIzol (Invitrogen, USA) from stable expression clones and reverse-transcribed with Expand Reverse Transcriptase (Boehringen Mannheim, German) according to the manufacturer's instructions, using olig (dT) 18 primer.

To identify clonal cell populations, the following primers were used:
1(+) (described as above) and 563(-): 5´-CCAAAGGT-TCCCACTCGGTAG-3´;
1389(+), and 1819(-) (described as above).
β-actin's primer pairs were designed to act as control of RT-PCR:
β-actin (+): 5´-CAACCGTGAAAAGATGACCCAGAT-3´;
β-actin (-): 5´-CATAGAGGTCTTTACGGATGTCA-ACG-3´.

PCR condition was: denaturation at 96 ºC, 1 min (the first step for 5 min), annealing at 60 ºC, 1 min, and synthesis at 72 ºC, 1 min (the last cycle for 10 min). Me_2SO 5 % (v/v) was in buffer for PCR with primers 1(+) and 563(-).

Growth of tranfected NRK cells The transgenic cells were seeded at 1×10^4 cells/well and grown in 24-well plates for 1- 4 d in duplicate. The cells were then harvested and counted.

Cloning efficiency of transfected NRK cells Cells were sparingly (2×10^3) seeded in 10-cm cell culture dishes with medium containing 300 mg/L G418. Following incubation at 37 ºC for 3 h, non-adherent cells were removed and cultures were further incubated for 14 d, replaced with fresh medium on d 7. At the end of the incubation period, the plates were stained with a Giemsa stain for 30 min after fixing with methanol for 15 min. After the cells were washed and dried

in air, clones in which the number of cells was more than 50 were counted under an inverted microscope. The cloning efficiency (%)=(the number of clones/the number of seed cells)×100. All tests were performed in triplicates.

MTT reduction analysis An MTT reduction analysis was performed to test for the sensitivity of tranfected NRK cells to apoptotic conditions. Cells were seeded in 96-well plates at a density of 1×10^4 cells/200 μL per well. After 1 d cells were deprived of serum. Cell viability was measured by the MTT method. Immediately after serum deprivation, 20 μL MTT 5 g/L was added to each well or added after d 1 or d 2, and subsequently incubated for 4 h at 37 ºC. The culture medium was then removed, Me_2SO was added, and the lysed cells was quantitated at λ=490 nm on an EL311S Automated Microplate Reader (BIO-TEK, USA).

Statistical analysis The data shown were mean values of at least 3 experiments and expressed as mean±SD. Student's *t*-test was used. $P<0.05$ was considered statistically significant.

RESULTS

AChE activity in apoptotic NRK cells induced by long-term culture In living NRK cells, the nuclei were smooth and clear under fluorescence staining, and AChE activity staining was negative. At d 12 without changing the medium, many cells were suspended in the medium. These cells exhibited apoptotic features such as cell shrinkage and nuclei fragment (data not shown). Some round cells were still attached on the bottom of the dish, but their nuclei were condensed and segregated to form apoptotic bodies, and positive AChE activity staining was present in apoptotic nuclei (Fig 1C, 1D). Interestingly, AChE activity was not detected in living NRK cells, but AChE protein was positive stained in plasma by immunofluorescence (Fig 2B). AChE protein presented in both plasma and nuclear (Fig 2E) in apoptotic cells that were marked by TUNEL. The location of AChE protein in apoptotic nucleus was confirmed by observation under confocal microscopy (Fig 3). It suggested that AChE protein existed in plasma of the living NRK cells but no activity, and during apoptosis, part of AChE protein was into nuclei and presented activity.

AChE protein increased before caspase-3 activation Activation of caspase-3 is a marker of apoptosis. In this study, AChE activity was presented in cells that were positive stained (Fig 4A, 4B) by immunofluorescence with the antibody special to activated caspase-3 fragment. It suggested that AChE activity was indeed

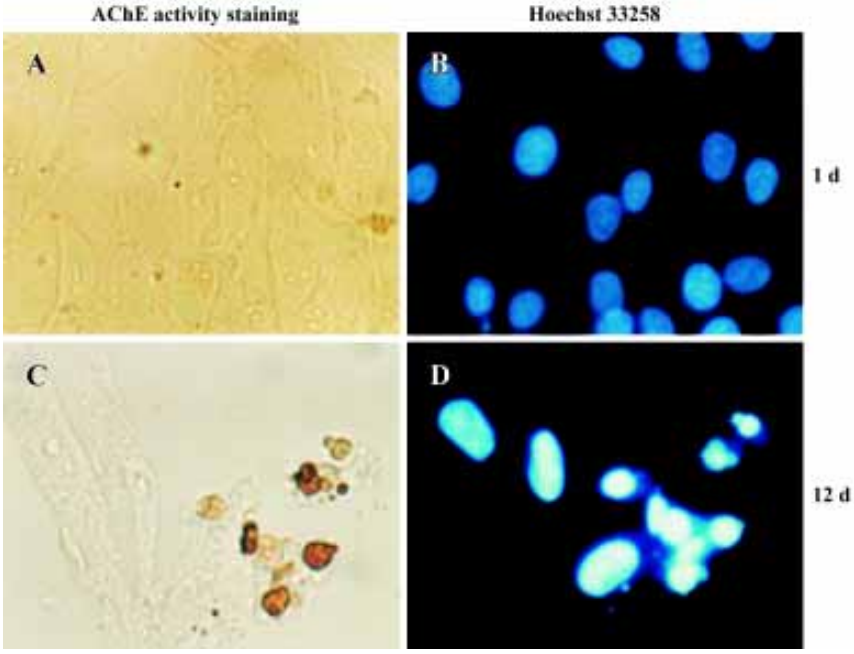

Fig 1. AChE activity in normal and apoptotic NRK cells. (A) Normal NRK cells presented no AChE activity and (B) showed the nuclei of normal cells by Hoechst 33258. (C) AChE activity appeared in apoptotic NRK cells cultured for 12 d without changing the medium and (D) showed condensed nuclei or apoptotic bodies.

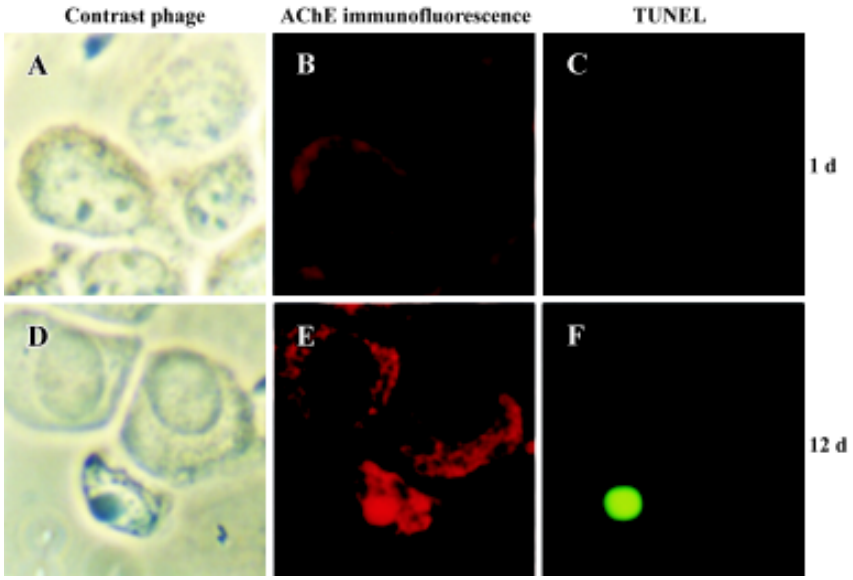

Fig 2. AChE protein in living or apoptotic NRK cells. In living cells, AChE protein existed in plasma (B) and (A) showed contrast phage and (C) showed that TUNEL was negative in living cells. In apoptotic cells, AChE protein existed in both plasma and nuclei (E). Apoptotic cells were marked by TUNEL (F). (D) showed contrast phage.

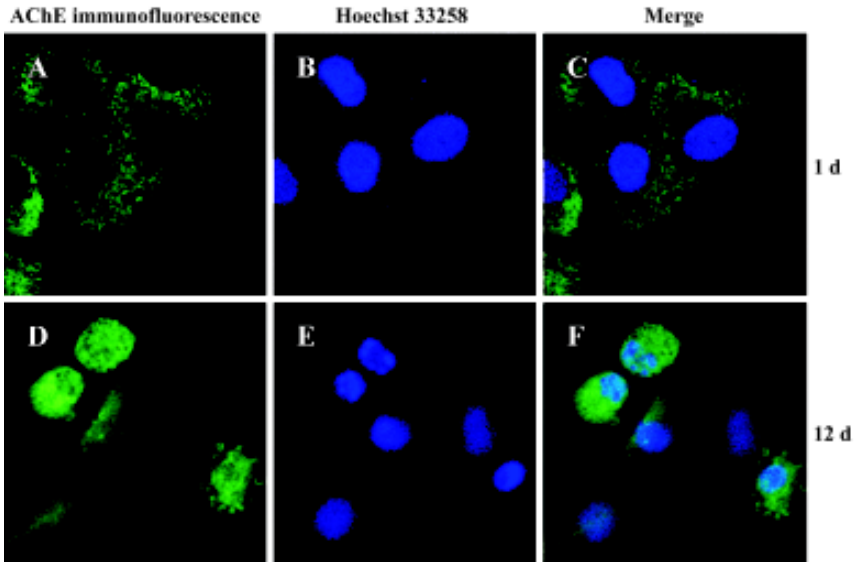

Fig. 3. Location of AChE protein by confocal microscopy. AChE protein was labeled by immunofluorescence (A, D), and the nucleus was labeled by Hoechst 33258 (B, E). In living cells, AChE protein existed in cytoplasm, not in nuclei (C). In apoptotic cells, AChE protein existed in both cytoplasm and nuclei (F).

in apoptotic cells. Previously we have found that AChE expression was increased prior to apoptosis[7]. In this study, AChE protein obviously increased at d 8, but no activated caspase-3 fragment presented in Western blotting results. Up to d 12, the activated caspase-3 fragment was detected (Fig 4C). It indicated that caspase-3 activity increased subsequently after AChE protein increased. In previous studies, caspase-3 activity was present in the late stages of apoptosis, mainly responsible for dismantling essential cell components, resulted in morphological and biochemical changes[14,15]. It indicated that AChE might also play a role in the early stage of apoptosis.

Expression of antisense and sense AChE mRNA in transduced cells To investigate the role of AChE in apoptosis, NRK cells were transfected with either sense

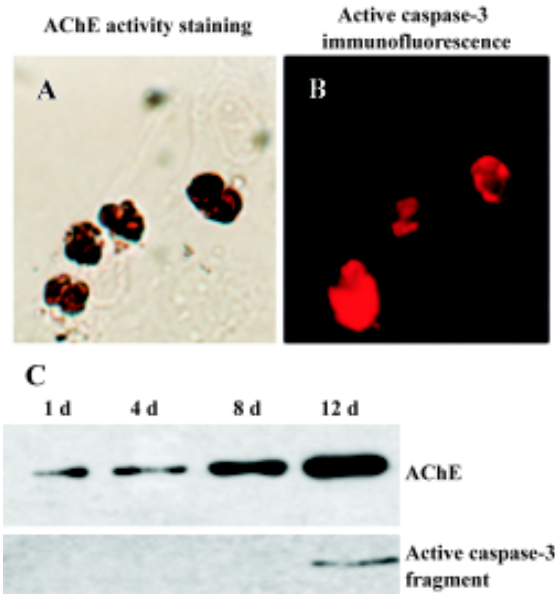

Fig 4. The relation between AChE and activated caspase-3. AChE activity (A) presented in cells that caspase-3 was activated (B). (C) Western blotting analysis of AChE protein and activated caspase-3 fragment in NRK cells were cultured for 1, 4, 8, and 12 d, respectively.

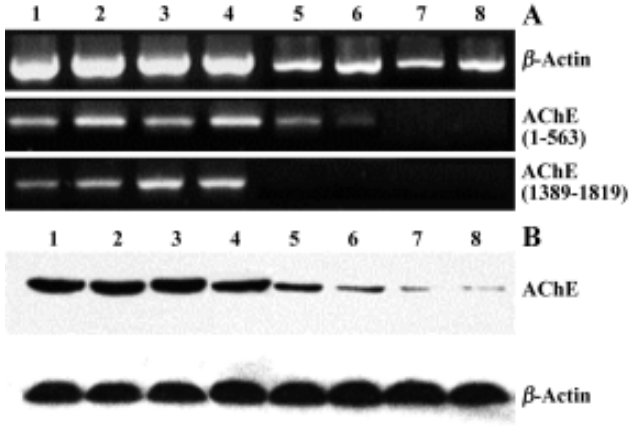

Fig 5. RT-PCR and Western blot analysis for mRNA and protein expression of transfected NRK cells. Agarose gel electrophoresis of RT-PCR products were amplified from mRNA of transgene NRK cells transfected with AChE-S, AChE-AS, and pCDNA3.1B plasmid. For RT-PCR, the primers for β-actin, AChE (1-563), and AChE (1389-1819) fragments were utilized. (A) β-actin RT-PCR products as inner reference; AChE (1-563) RT-PCR products; AChE (1389-1819) RT-PCR products. Lane 1-4: clone AChE-S (1): clone AChE-S (4), lane 5,6: clone AChE-AS (1), clone AChE-AS (2); lane 7,8: clone vector control (1) and vector control (2). (B) Western blot analysis for AChE protein expression. β-Actin as inner reference. Lane 1-4: clone AChE-S (1): clone AChE-S (4), lane 5, 6: clone vector control (1) and vector control (2); lane 7, 8: clone AChE-AS (1), clone AChE-AS (2).

or antisense AChE cDNA. Stable clones were selected by G418. To examine whether the transfection of NRK cells was effective in expressing the corresponding mRNA, total RNA was extracted from four AChE-S clones, two AChE-AS clones and two control clones transfected with pCDNA3.1B vector. The mRNA was analyzed by RT-PCR. A 572 bp fragment with primers 1 (+) and 563 (-), and a 449 bp fragment with primers 1389 (+) and 1819 (-) were obtained for the AChE-S clones. The fragment AChE (1389-1819) was overexpressed, so only a 449 bp fragment was obtained for the AChE-AS clones. Both fragments were absent in the control clones (Fig 5A), suggesting that the AChE sense and AChE antisense fragments that were expressed in the transfected NRK cells, and pCDNA3.1B plasmid had no effect on AChE mRNA expression.

AChE protein expression in transfected NRK cells To further investigate the effect of AChE gene expression in transfected NRK cells, total AChE protein was extracted and detected by Western blotting. AChE increased in the AChE-S clones, and decreased in the AChE-AS clones compared with the vector control clones (Fig 5B). Immunofluorescence showed that the distribution of AChE for the three kinds of clones were all in the cytoplasm as non-transfected NRK cells (data not shown). Although AChE protein was increased in all AChE-S clones, none presented obviously AChE activity by the method of Karnovsky and Roots.

Effect of AChE overexpression on the proliferation and apoptosis in NRK cells Clones maintained in culture with G418 to provide selection pressure, but G418 had an effect on the growth of clones[16]. So cells transfected with pCDNA3.1B vector served as a control. The AChE-S clones showed growth retardation while AChE-AS clones promoted cells proliferation, compared with control clones by directly counting cell numbers (Fig 6A) and cloning efficiency (Fig 6B). Compared with control clones, the cloning efficiency of AChE-S clones was lower. Although the cloning efficiency of AChE-S (4) was similar to the vector control (1), 48.7 %±2.1 % *vs* 56.1 %±0.3 %, the size of the clones were smaller (Fig 6B). The low cloning efficiency of the AChE-S (2) clone (28.2 %±3.1 %) was attributed to many small clones (<50 cells) that were not counted. AChE-AS (1) clones had high cloning efficiency (77.7 %±2.2 %) (mean±SD, n=3) and size. The results showed AChE overexpression did not induce apoptosis in NRK cells, but prevent cell prolifera-

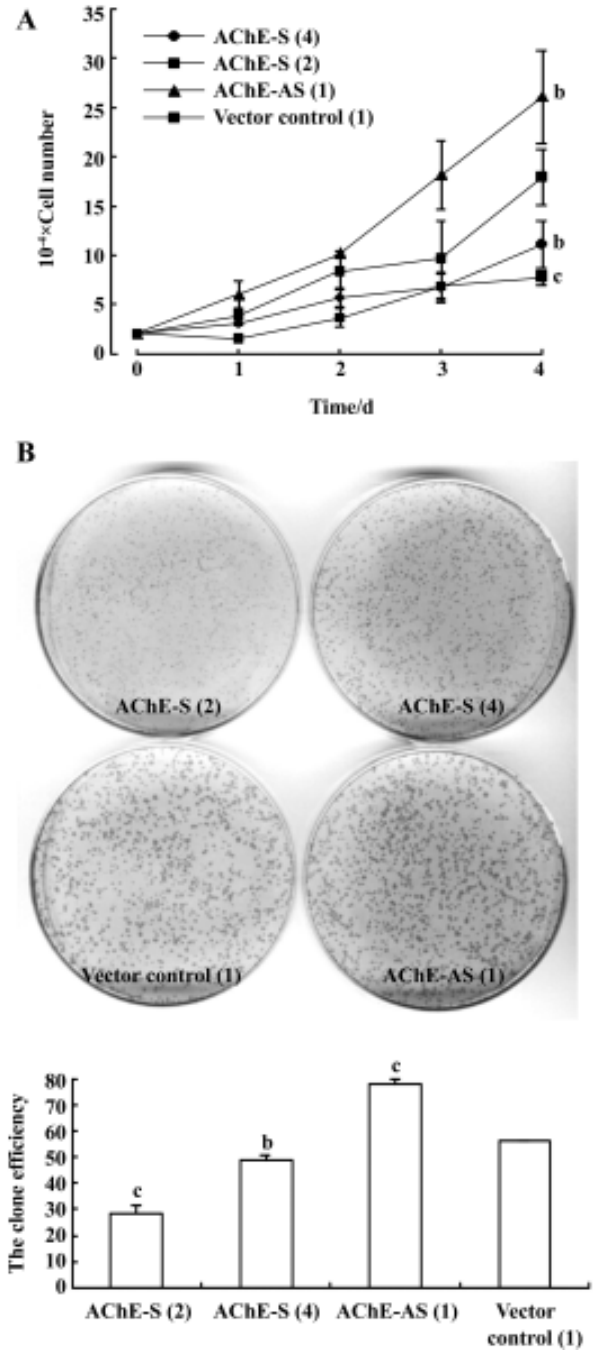

Fig 6. The growth curve and cloning efficiency of transfected cells. (A) 2×10⁴ cells were seeded in 24-well plates and grown for 1-4 d and counted every day. *n*=3. ᵇ*P*<0.05, ᶜ*P*<0.01 *vs* vector control. (B) The dishes of clones of transfected cells, and histogram was the cloning efficiency of them. *n*=3. ᵇ*P*<0.05, ᶜ*P*<0.01 *vs* vector control. [AChE-S (2) and AChE-S (4) were No 2 clone and No 4 clone of cells transfected with AChE sense; AChE-AS (1) was No 1 clone of cells transfected with AChE antisense; Vector control (1) was No 1 clone of cells transfected with vector]

tion, and the inhibition of AChE expression could promote cell proliferation.

To detect whether AChE played a role in apoptosis, transfected NRK cells were deprived of serum for 2 d. The cell viability was measured by MTT assay. The residue of MTT metabolism, ie, the percent of cell survival was counted by the optical absorption value of MTT assay in cells deprived of serum being divided by the value in cells before deprivation of serum. At d 1, mitochondrial MTT metabolism of various clone types had no obviously difference, and at d 2, the residue of mitochondrial MTT metabolism in control clone cells (27.4 %±3.5 %) was higher than that in AChE-S clones cells (10.4 %±4.6 %, 12.6 %±6.7 %), and lower than that in AChE-AS clone cells (50.3 %±7.8 %). It indicated that AChE functioned in promoting apoptosis (Tab 1, Fig 7).

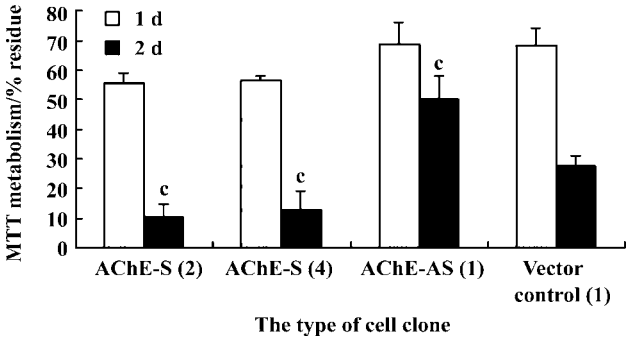

Fig 7. The residue of MTT metabolism in transfected cells deprived of serum over the course of 2 d. *n*=5 separate trials. Mean±SD. ᶜ*P*<0.01 *vs* vector control.

DISCUSSION

Our data showed that AChE activity was not detected in living NRK cells by AChE cytochemical staining, but AChE protein was detected by immunological methods. Several papers have also reported that inactive AChE existed: in chick brain and muscles, a stable pool of inactive AChE, about 70 %-80 % of total enzyme, was found[17]; Tsim and collaborators found that a calcitonin gene-related peptide (CGRP) increased the expression of AChE mRNA and inactive AChE in cultured chick myotubes[18]; inactive AChE protein also appeared in apoptotic HLF cells[19]. However, its existence and function is still unclear. During apoptosis in NRK cells, AChE protein increased and presented activity. AChE activity in apoptotic cells was not contributed to AChE protein increase, because AChE activity was also absent or showed only a slight increase in NRK cells stable overexpressing AChE, although the amount of protein increased. The second explanation

Tab 1. The optical absorption of MTT metabolism in transfected cells deprived of serum over the course of 2 d. Results represent a mean±SD of five separate trials.

Group	0 d (*OD*)	1 d (*OD*)	2 d (*OD*)
AChE-S (2)	0.167±0.012	0.093±0.011	0.017±0.014
AChE-S (4)	0.135±0.014	0.076±0.009	0.017±0.019
AChE-AS (1)	0.294±0.013	0.203±0.080	0.148±0.021
Vector control (1)	0.223±0.027	0.152±0.035	0.061±0.011

was that inactive AChE might have different modifications with active AChE. Although result of Western blot showed only one band in apoptotic cells, it was possible that inactive AChE has little different modification. AChE protein was in cytoplasm of living cells, and in both cytoplasm and nuclei of apoptotic cells, whereas AChE enzyme activity mainly existed in nuclei of apoptotic cells. It resulted in another explanation that the inactive AChE was located in different organelle from active AChE and led to different conformation. A good example is that AChE- Aβ complex changed the biochemical properties of AChE[20].

Overexpression of AChE was not to induce NRK cells apoptosis and only to inhibit the cells growth, and it suggested that during apoptosis increased AChE was not absolutely associated with apoptotic events. During hematopoiesis[21], osteogensis[22], and myogenesis[23], AChE increase was also associated with inhibition of cell proliferation, but not apoptosis. However, AChE promoted the cell death induced by deprivation of serum. It indicated that AChE had no toxic effects under normal conditions, and upon induction by apoptotic stimuli, AChE exerted its role to promote apoptosis. AChE is expressed in endoplasmic reticulum, and transported to Golgi apparatus for modification, and then is secreted or onto cell membrane. It was surprised that AChE protein was into nucleus. A peptide composed of the 67 C-terminal amino acid residues of AChE has nucleus location signal[24], but how did AChE enter into nucleus? One explanation was that the membrane of organelle with AChE was damaged, and AChE was leaked into cytosole and entered into nucleus by nucleus location signal. In nucleus or cytosol, AChE interaction with other molecular or itself promoted apoptosis.

In conclusion, AChE had two functions during apoptosis. During apoptosis, AChE protein increased first to inhibit the cell growth, and this step occurred before execution of apoptosis. With apoptosis proceeding, AChE promoted cell apoptosis, which might be subsequent to the translocation of AChE into nuclei.

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