Interactions of acetylcholinesterase with caveolin-1 and subsequently with cytochrome c are required for apoptosome formation

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Acetylcholinesterase (AChE) is emerging as an important component in leading to apoptosis. Our previous study demonstrated that silencing of the AChE gene blocked the interaction between cytochrome c and apoptotic protease-activating factor-1 (Apaf-1) in etoposide-induced apoptosis of HT-29 cells. We undertook this study to further dissect the molecular role of AChE in apoptosome formation. The present study elicited that small interfering RNA (siRNA) to cytochrome c gene blocked the interaction of AChE with Apaf-1, whereas siRNA to Apaf-1 gene did not block the interaction of AChE with cytochrome c, indicating that the interaction of AChE with cytochrome c is required for the interaction between cytochrome c and protease-activating factor-1. We further observed that AChE is localized to caveolae via interacting with caveolin-1 during apoptosis and that the disruption of caveolae prevented apoptosome formation. These data indicate that the interactions of AChE with caveolin-1 and subsequently with cytochrome c appear to be indispensable for apoptosome formation.

Introduction

Cholinesterase is a member of the serine hydrolase family using a serine residue at their active site (1). Other than cleaving acetylcholine, it has been revealed that this enzyme may also be active in peptide hydrolysis (2,3), although some reservations regarding this activity have been raised (4). In addition, these enzymes have also been shown to exhibit aryl acylamidase activity (5). There are two major types of cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase. Since butyrylcholinesterase has no known specific natural substrate and its mutation does not result in significant physiological consequences, the function of butyrylcholinesterase remains a puzzle. In contrast, AChE is known to hydrolyze acetylcholine at cholinergic synapses as well as at neuromuscular junctions (6).

Although the key role of AChE is terminating neurotransmission at cholinergic synapses, it is also expressed in tissues devoid of cholinergic responses (1) and several types of hematopoietic cells including erythrocytes and megakaryocytes (7,8). Several studies show abnormality of the AChE gene in several tumors and agricultural use of AChE inhibitors is known to induce several tumors (9). These findings indicated its potential functions beyond neurotransmission.

Recently, the increasing evidence has shown that AChE may be involved in apoptosis. A study demonstrated the presence of AChE activity in various types of apoptotic cells (10). The study also showed that not only pharmacological inhibitors of AChE prevented apoptosis but also blocking the expression of AChE with antisense inhibited apoptosis. Another recent study documented that etoposide or exisatin A-induced expression of AChE messenger RNA and protein in SW620 colon cancer cell line was blocked by a specific inhibitor of stress-activated protein kinase/c-Jun N-terminal kinase and small interfering RNA (siRNA) directed against c-jun N-terminal kinase 1/2. The study also showed that transfection with adenovirus-mediated dominant-negative c-jun blocked the upregulation of AChE expression. These results suggest that AChE expression may be mediated by the activation of c-jun N-terminal kinase pathway during apoptosis through a c-Jun-dependent mechanism (11).

We reported previously that silencing of the AChE gene prevented etoposide-induced apoptosis of HT-29 human colon adenocarcinoma cells by blocking the interaction between apoptotic protease-activating factor-1 (Apaf-1) and cytochrome c, which suggested the pivotal role of AChE in apoptosome formation (12). However, detailed molecular mechanism explaining the function of AChE in apoptosome formation is remained largely unknown.

We undertook this study to further dissect the molecular role of AChE in apoptosome formation. The present study demonstrates that AChE interacts with caveolin-1 and subsequently cytochrome c in cells undergoing apoptosis, which interactions appear to be indispensable for apoptosome formation.

Materials and methods

Apoptosis systems

Most experiments to dissect the molecular role of AChE in apoptosome formation were mainly conducted by employing 40 μg/ml etoposide-induced apoptosis in HT-29 cells. In addition, several other apoptosis systems were also used. Apoptosis types tested are as follows: HT-29 cells treated with 1 mM salinade for 48 h, with 2.5 μM thapsigargin for 24 h or with 100 μM LY294002; a Phosphoinositide-3-kinase (PI3K) inhibitor for 48 h; Rapa256.7 cells treated with a nitric oxide donor SIN-1 at 1 mM for 24 h; SK-MEL-5 cells treated with 10 μg/ml etoposide for 48 h; Malme-3M cells treated with 50 μg/ml etoposide for 24 h; TE671 cells treated with 50 μg/ml etoposide for 24 h; U373MG human brain tumor cells treated with 40 μg/ml etoposide for 24 h; interleukin-3 deprived (murine) bone marrow-derived mast cells and primary cultured rat articular chondrocytes treated with 1.5 mM sodium nitroprusside, a nitric oxide donor, or infected with 100 multiplicity of infection adenoviral TRAIL for 72 h.

siRNA transfection

Twenty-one nucleotide RNA with 3′-dTdT overhangs was synthesized by Dharmacon Research (Lafayette, CO) in the ‘ready-to-use’ option. Sense sequences for AA-N19 messenger RNA targets are as follows: AChE sequence 1, 5′-GCTACGATCTGCAGTATTTGGA-3′; AChE sequence 2, 5′-CCAAATAGTGGAACACCTCTATAT-3′; AChE sense sequence 3, 5′-AATTGTGGACACCTATACTGCA-3′; Apaf-1 sense sequence 1, 5′-CATGATGGATGAGTATTC-3′; Apaf-1 sense sequence 2, 5′-CAGAAGCTTCTAAATGGTA-3′; Apaf-1 antisense sequence 3, 5′-TGGGAAGGGTGCACTTTGAGGA-3′; cytochrome c sense sequence 1, 5′-GAAGGCCAGGGAGATTGTTT-3′; cytochrome c sense sequence 2, 5′-CAGGATTGTTGGAACCACTGGA-3′ and cytochrome c sense sequence 3, 5′-TGGGAGAGGATACACTGTA-3′. As a negative control, the same nucleotides were scrambled to form a non-genomic combination (controlled by local alignment search tool). Transfection of siRNA was performed by using siPORT Amine (Ambion, Austin, TX) and Opti-MEM media according to the manufacturer’s recommendations. Cells grown to a confluency of 40–50% in six-well plates were transfected with 1 nM final siRNA concentration per well. Twenty-four hours after transfection, apoptotic stimuli were applied. Among three sense sequences of each target, every sequence 1 showed highly efficient silencing effect. Thus, this single sequence was used for following assays.
Human full-length AChE complementary DNA (accession number: NM_000665) was purchased from 21C Frontier Human Gene Bank (21C Frontier Human Gene Bank, Taejeon, Korea) and cloned into pDs XB-EGFP destination vectors (a gift from Dr. Tobias Meyer, Stanford University) by Gateway Cloning System (Invitrogen, Carlsbad, CA). The N-terminal primer 5’-GCGGATCCGACACCTTTGTACAAAGCAGGCTC-3’ and the C-terminal primer 5’-GGGGCCGCAGTTGTCTG-3’ were used to introduce an amphi sequence (underlined) followed by a Kozak sequence (bold) upstream of the coding sequences of AChE. Similarly, the C-terminal primer 5’-GGGGCCGCAGTTGTCTGACAAAGCAGGCTC-3’ was used to introduce amphi sequences immediately after the last amino acid of these two genes. The full-length AChE gene was amplified on a thermal cycler using DNA polymerase, HiPi Plus PCR premix (Elpis-Biotech, Taejeon, Korea). Polymerase chain reaction products were cloned into pDONR207 vectors using the Gateway Cloning System (Invitrogen). Sequence identity was confirmed by dyeoxy sequencing analysis (Macrogen, Seoul, Korea). These entry clones were then added to pDs XB-EGFP destination vectors in the presence of LR clonase (Invitrogen), and the resulting C-terminal enhanced green fluorescent protein (EGFP)-tagged expression vector (AChE-EGFP) was selected.

To visualize the localization of green fluorescent protein (GFP) fusion AChE protein, HT-29 cells were plated on poly-l-lysine-coated coverslips at a density of 5 x 10^4 per well (which were cultured in six-well plates) and transfected with Lipofectamine 2000 reagent (Invitrogen) with expression vectors, AChE-EGFP. Twenty-four hours after transfection, cells were treated with 40 μg/ml etoposide for 0–24 h. Intracellular localization of AChE was analyzed according to the EGFP fluorescent detection method under Zeiss LSM 510 laser scanning confocal microscope (Göttingen, Germany).

Cell viability assay
Cell viability was determined by the Vi-Cell (Beckman Coulter, Fullerton, CA) cell counter that performs an automated trypan blue exclusion assay.

Confocal immunofluorescence microscopic analysis
Cells were seeded on coverslips at a density of 5 x 10^4 per well, fixed in 3% paraformaldehyde, incubated in primary antibody and fluorescent-tagged secondary antibody. In addition, the nuclei were visualized by Hoechst 33342. Fluorescent images were also observed and analyzed under Zeiss LSM 510 laser scanning confocal microscope.

Quantification of colocalization
Quantification of colocalization was performed using LSM 510 colocalization software program. Briefly, background was subtracted from the images, and the percentage of overlapping pixels between AChE and caveolin-1 or other marker pixels was determined. Eight cells from three experiments were analyzed for n = 24. All data were analyzed by r-test.

Flow cytometric analysis
Cells were trypsinized, washed with phosphate-buffered saline and fixed in 75% ethanol at 4°C for 30 min. Prior to analyses, cells were again washed with phosphate-buffered saline, suspended in cold propidium iodide (Sigma, St. Louis, MO) solution and incubated at room temperature in the dark for 30 min. Flow cytometry analyses were performed on a flow cytometry system (Becton Dickinson, San Jose, CA).

Antibodies and antibody array screening
Antibodies against apoptosis molecules used for making the antibody arrays and for the immunoprecipitations were obtained from Santa Cruz Biotechnolog (Santa Cruz, CA). Antibodies (0.5 mg each) were immobilized on polyvinylidene difluoride membranes (5 x 2 cm) at predetermined positions. The antibody array membranes were then incubated with whole-cell lysates from HT-29 cells treated or untreated with 40 μg/ml etoposide for 40 h. After incubation for 2 h, the membranes were blotted with horseradish peroxidase-conjugated anti-AChE polyclonal antibody (against AChE C-terminal; Santa Cruz Biotechnology) for an additional 2 h and followed by enhanced chemiluminescence detection.

Western blot analysis and coimmunoprecipitation
These procedures were performed as described by Park et al. (12). Coimmunoprecipitation assay was reciprocally conducted.

Molecular weight cutoff study
Cell lysate was loaded on a Vivaspin 300 000 MWCO column (Vivascience, Hannover, Germany) and centrifuged at 20 000g. Lower fraction containing monomeric-free Apaf-1 (~130 kDa) and upper fraction containing oligomerized Apaf-1 (300–400 kDa) were collected and aliquots of 50 μl from each fraction were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western blotting with Apaf-1 antibody.

Statistical analysis
Data are expressed as means ± SDs. Statistical significance of differences was determined by the paired Kruskal–Wallis non-parametric test.

Results
AChE plays a critical role in apoptosis formation
To generalize the involvement of AChE in apoptosis formation, we first examined whether upregulation of AChE protein is observed in a variety of apoptosis systems. We performed western blot analysis and demonstrated the upregulation of AChE in all types of apoptotic cell lines tested (supplementary Figure S1A is available at Carcinogenesis Online). We next examined whether the prevention of the interaction between Apaf-1 and cytochrome c by siRNA directed against AChE is a commonly occurring event in apoptosis. Our data show that siRNA-directed against AChE prevented apoptosis formation in all types of apoptosis models we tested (Figure 1A).

We next tried to reveal the interaction of AChE with various apoptosis constituents by antibody array and coimmunoprecipitation assays. Our data show that AChE interacts with apoptosis constituents, such as Apaf-1, cytochrome c, c-IAP-1, c-IAP-2, caspase-3 and caspase-9 in etoposide-induced apoptosis of HT-29 cells (Figure 1B, supplementary Figure S1B is available at carcinogenesis Online and Figure 1C). Similar results were observed in other several types of apoptosis (supplementary Figure S1C is available at Carcinogenesis Online).

We next examined whether AChE plays a role in the oligomerization of Apaf-1. We undertook molecular weight cutoff study. Lower and upper fractions on 300 000 MWCO column should contain monomeric free AChE (~130 kDa) and oligomerized Apaf-1, respectively. Our molecular weight cutoff study shows that siRNA to AChE gene prevented etoposide-induced oligomerization of Apaf-1 (Figure 1D).

Interaction between AChE and cytochrome c is required for the assembly of AChE/cytochrome c/Apaf-1 three-component complex in etoposide-treated HT-29 cells
We demonstrated before that the formation of the AChE/cytochrome c/Apaf-1 complex is required for apoptosis formation. We present the recent study tried to unveil which interaction among these three components is critical for apoptosis formation. Our data indicate that the silencing of Apaf-1 gene efficiently downregulated Apaf-1, resulting in the prevention of apoptosis (Figure 2A). Noticeably, the interaction between AChE and cytochrome c was maintained throughout the tested period. The presence of the interaction of AChE with cytochrome c, under this condition, was also observed not only in Apaf-1-negative SK-MEL-5 cells undergoing apoptosis after etoposide treatment but also in Apaf-1−/− knockout cells (kindly provided by Dr. David Vaux, The Walter and Eliza Hall Institute, Parkville, Victoria, Australia) undergoing apoptosis after interleukin-3 deprivation (13, Figure 2B).

On the other hand, the silencing of cytochrome c gene efficiently downregulated cytochrome c, resulting in the prevention of apoptosis (Figure 2C and supplementary Figure S2A is available at carcinogenesis Online). Importantly, the silencing of cytochrome c gene prevented the interactions of AChE not only with cytochrome c but also with Apaf-1 (Figure 2D). Our molecular weight cutoff study also showed that siRNA to cytochrome c gene prevented the oligomerization of Apaf-1 (Figure 2E). These data indicate that the interaction between AChE and cytochrome c is required for the interaction between cytochrome c and Apaf-1. We also observed that the silencing of cytochrome c gene prevented the interaction between AChE and Apaf-1 in several other apoptosis systems (supplementary Figure S2B is available at carcinogenesis Online).

AChE shuttles between the nucleus and the cytoplasm during apoptosis
Laser scanning confocal microscopy at different time points using the fusion protein AChE-GFP in HT-29 cells treated with etoposide
showed dynamic subcellular localization of fusion protein AChE–GFP. The fusion protein was observed out of the nucleus in control cells (0 h). However, etoposide treatment induced the shuttling of AChE. During the early phases of apoptosis (1–4 h), AChE was first translocated into nucleus. AChE was then observed out of the nucleus to the cytosol (8–16 h), forming a ring structure around the nucleus. At the later phases, AChE was localized on the periphery of cells (24–40 h). At the end time point of apoptosis (72 h), in which most cells showed the phenotype of apoptotic body with disrupted nucleus, AChE appeared to be present on the nucleus as well as in the cytosol (Figure 3A).

We then examined whether blocking nuclear export of AChE prevented apoptosis or not. AChE was observed on the periphery of cells treated with etoposide for 40 h. However, in cells pretreated with leptomycin B (LMB), an inhibitor of nuclear export receptor chromosome region maintenance 1, AChE was observed on the nucleus, indicating that LMB efficiently induced accumulation of AChE on the nucleus. Noticeably, DNA hypoploidy determination by flow cytometry elucidated that the induction of apoptosis was prevented by LMB pretreatment (Figure 3B). These data suggest that blocking nuclear export of AChE prevents apoptosis.

**AChE colocalizes with caveolin-1 during apoptosis**

We further determined subcellular localization of AChE by double immunofluorescence utilizing several cell organelle markers. Our data obtained by employing an antibody directed against the endoplasmic reticulum-associated protein disulfide isomerase and a lysosomal fluorescent probe LysoTracker DND-99 showed that AChE was neither localized on endoplasmic reticulum nor on lysosome. Rather, immunofluorescent staining to AChE appeared uneven and punctuate over the plasma membrane (Figure 4A).

Thus, we examined whether AChE was localized to a specific plasma membrane compartment, caveolae, via its interaction with caveolin-1. As shown in the upper panel of Figure 4B, confocal microscopic study demonstrated the colocalization of AChE with caveolin-1. The merged image obtained by anti-AChE antibody together with an anti-caveolin-1 antibody clearly revealed yellow-orange areas, resulting from the overlap of green and red fluoroses, which corresponded to nearly complete colocalization, suggesting their interaction. The validity of this interaction was confirmed by coimmunoprecipitation assay (lower panel of Figure 4B and supplementary Figure S3A is available at *cancer* Online). This colocalization was also evidently observed in other types of apoptosis (Figure 4C).

**Interaction of AChE with caveolin-1 is indispensable for apoptosome formation**

We next asked whether the interaction of AChE with caveolin-1 is indispensable for apoptosome formation. Thus, we used methyl-β-cyclodextrin (MβCD) which not only disrupts caveolae structure...
Fig. 2. Interaction between AChE and cyttochrome c is required for the interaction between cyttochrome c and Apaf-1 in etoposide-treated HT-29 cells. (A) Silencing of the Apaf-1 gene efficiently downregulated the expression of Apaf-1 and significantly prevented the reduction of cell viability induced by 40 μg/ml etoposide. Viability was determined by a cell counter performing an automated trypan blue exclusion assay. Statistically significance: "*" P < 0.01. (B) AChE maintains the interaction with cyttochrome c irrespective of the silencing of Apaf-1 gene by si Apaf-1. Although si Apaf-1 blocks the interaction between AChE and Apaf-1, it does not prevent the interaction of AChE with cyttochrome c. The presence of the interaction of AChE with cyttochrome c is also observed not only in Apaf-1-negative SK-MEL-5 cells treated with 10 μg/ml etoposide but also in interleukin-3-deprived Apaf-1−/− knockout cells. Western blot of immunoprecipitated AChE from the cell lysates obtained 24 h after application of apoptotic stimuli is presented as a positive control. (C) Silencing of cyttochrome c gene efficiently downregulated the expression of cyttochrome c and significantly prevented the reduction of cell viability induced by 40 μg/ml etoposide. Viability was determined by a cell counter performing an automated trypan blue exclusion assay. Statistically significance: "*" P < 0.01. Data are presented as mean ± SD (n = 4). (D) siRNA to cyttochrome c gene (si Cyt c) prevents the interactions of AChE not only with cyttochrome c but also with Apaf-1. IgG is shown as a loading control. Western blot of immunoprecipitated Apaf-1 or AChE from the cell lysates obtained 24 h after etoposide treatment is presented as positive controls. (E) Western blotting of upper (U) and lower (L) fractions collected with a molecular weight cutoff method shows that silencing cyttochrome c gene by si Cyt c followed by 40 μg/ml etoposide treatment for 40 h prevented the oligomerization of Apaf-1. PC, positive control.

but also reduces caveolin-1 expression. We observed that MβCD pretreatment prevented the reduction of viability of cells (Figure 5A). Confocal microscopy showed that AChE was accumulated not on the periphery but in the cytosol of cells pretreated with MβCD in which caveolin-1 was hardly detected (Figure 5B). Moreover, MβCD pretreatment prevented not only the interaction between AChE and caveolin-1 but also the interaction of cyttochrome c with AChE or with Apaf-1 (Figure 5C and supplementary Figure S3B is available at carcinogenesis Online). Noticeably, our molecular weight cutoff study revealed that MβCD pretreatment prevented the oligomerization of Apaf-1 (Figure 5D). In addition, we observed that silencing of cyttochrome c gene did not prevent the interaction between AChE and caveolin-1 (Figure 5E and supplementary Figure S3C is available at carcinogenesis Online). To this end, we examined the relevance of apoptosome localization at the caveolea. Our coimmunoprecipitation data show that neither cyttochrome c nor Apaf-1 interacts with caveolin-1 in etoposide-treated HT-29 cells, which indicates that apoptosome does not localize on the caveolea (supplementary Figure S3D is available at carcinogenesis Online). Taken together, our data suggest that the interaction between AChE and caveolin-1 precedes apoptosome formation, indicating that the interaction of AChE with caveolin-1 is indispensable for obtaining its capability pertaining to apoptosome formation.

We then employed caveolin-negative cell lines, LNCaP and PC-3 cells, to prove the role of the interaction between AChE and caveolin-1 in apoptosome formation. Although AChE was also upregulated in etoposide-treated LNCaP and PC-3 cells (Figure 6A), no interaction of AChE and cyttochrome c, AChE and Apaf-1 or Apaf-1 and cyttochrome c is observed in these cells (Figure 6B and supplementary Figure S3E is available at carcinogenesis Online). Accordingly, the interaction between AChE and caveolin-1 appears to be indispensable for apoptosome formation.

Discussion

In our previous study, we reported that AChE exerted its pivotal role in apoptosis by participating in the formation of apoptosome (12). To dissect further the molecular role of AChE in apoptosome formation, we first tried to corroborate that the upregulation of AChE is commonly observable in apoptosis. Our western blotting data, in addition to reverse transcription–polymerase chain reaction data from a previous study which demonstrated the expression of AChE messenger RNA in various types of apoptosis (10), suggest that the upregulation of AChE is commonly observable in apoptosis. Furthermore, we demonstrated that siRNA directed against AChE prevented apoptosome formation in all types of apoptosis systems we tested. Therefore, we
Acetylcholinesterase in apoptosome formation

...apoptosome constituents, which include caspase-3, caspase-7, XIAP, and Apaf-1. Reconstitution experiments using purified recombinant proteins indicate that the apoptosome is a 700 kDa complex. However, the specific mechanisms that govern these processes remain unclear. Our data show that AChE not only interacts with various apoptosome constituents but also plays a pivotal role in the oligomerization of Apaf-1. Noticeably, our data indicate that the interaction between AChE and cytochrome c is required for the interaction between cytochrome c and Apaf-1. Thus, we assume that the interaction between AChE and cytochrome c is indispensable for the interaction between cytochrome c and Apaf-1.

Nucleocytoplasmic transport of signal transducers and execution factors may be a crucial and even essential aspect of apoptosis. Several proteins implicated in apoptotic cell death have been shown to migrate in and out of the nucleus following apoptosis induction. Active transport signals have been identified in an increasing number of cellular proteins executing apoptosis and most of proapoptotic nuclear factors are known to be activated in the cytoplasm and to gain access to the nucleoplasm during apoptotic process (20). Accordingly, the nuclear uptake and release of apoptotic factors appear to play important roles in the execution as well as in the initiation of the apoptotic program. The nucleus is also the main target for genotoxic insult. Signals generated in the nucleus by DNA damage have to propagate to all cellular compartments, ensuring the coordinated execution of apoptosis. Thus, the nucleocytoplasmic shuttling of signaling and execution factors is thus an integral part of the apoptotic program.

Previous studies demonstrated that AChE was found in the cytoplasm in the nucleus or apoptotic bodies upon commitment to cell death (10,21). We here demonstrated the localization of AChE out of the nucleus in healthy cells, which suggests that the nuclear export of AChE predominates over the import process. Etoposide treatment to HT-29 cells is probably to alter this equilibrium. After etoposide treatment, AChE shuttled between the nucleus and the cytoplasm during apoptosis. Noticeably, LMB induced accumulation of AChE on the nucleus, which results in the prevention of apoptosis. These findings indicate that nuclear export of AChE via exportin-1 is an indispensable step in apoptosis. AChE is known to have a nucleus localization signal (22). Thus, it could translocate onto nucleus on which it seems to play a certain role contributing to the apoptotic process. Although a previous study suggested that AChE on the nucleus may participate in the modulation of nuclear components leading to chromatin condensation and fragmentation (10), it remains an open question why AChE first enter to nucleus and then export into cytoplasm during apoptosis.

Caveolae are specialized invaginated microdomains of the plasma membrane (23), which are composed mainly of cholesterol and sphingolipids. Their associated proteins, the caveolins, are a family of 21–25 kDa integral membrane proteins and the primary structural constituent of caveolae. Currently, the functional roles attributed to caveolae and caveolin-1 are quite diverse, ranging from vesicular transport (transcytosis, endocytosis and potocytosis) and cholesterol homeostasis to the suppression of cell transformation and the regulation of signal transduction (24,25). It has been shown that many signaling molecules directly interact with caveolin-1 through a defined modular protein domain, known as the caveolin-scaffolding domain (residues 82–101) (26). Thus, the ability of caveolin-1 and caveoleae to modulate signaling has important implications for the process of cell transformation and tumor formation.

To date, the role of caveolin-1 in apoptosis remains enigmatic. The role of caveolin-1 in apoptosis appears to be antigenic in different systems. A study showed that caveolin-1 overexpression sensitizes fibroblasts to ceramide-induced death through a P38-dependent mechanism (27). Another study elicited that caveolin-1 expression sensitizes both NIH-3T3 fibroblasts and T24 bladder carcinoma cells to apoptosis initiated by staurosporine (28). In contrast, disruption of caveolae showed to block interleukin-6- and insulin-like growth factor-1-induced activation of the PI3K/Akt survival signaling pathway (29). It was also depicted that caveolin-1 mediates cell survival
by sustaining Akt activation through the binding and inhibition of the serine/threonine protein phosphatases, namely PP1 and PP2A (30). This incongruity of the proapoptotic or antiapoptotic functions of caveolin-1 may be explained by cell-type-specific effects. Alternatively, the disparate effects of caveolin-1 may be due to the use of different apoptotic inducers. Further future studies will be necessary to distinguish between these two possibilities.

Our data indicate that AChE interacts with caveolin-1 in cells undergoing apoptosis. We observed not only disruption of caveolae and downregulation of caveolin-1 prevented apoptosis formation but also apoptosis was not formed in caveolin-negative cells. In addition, we also demonstrated that the interaction between AChE and caveolin-1 is required for the interaction between AChE and cytochrome c. These data indicate that the interaction between AChE and caveolin-1 appears to be indispensable for apoptosis formation. However, we observed that neither cytochrome c nor Apaf-1 interacts with caveolin-1. These data indicate that the interaction between AChE and caveolin-1 precedes apoptosis formation and that AChE

Fig. 4. AChE colocalizes with caveolin-1. (A) Confocal microscopy using cell organelle markers shows the subcellular location of AChE in HT-29 cells treated with 40 μg/ml etoposide for 40 h. Confocal microscopy using antiprotein endoplasmic reticulum (ER)-associated protein disulfide isomerase antibody to detect endoplasmic reticulum or fluorescent probes LysoTracker DND-99 to detect lysosome demonstrates that AChE is neither localized on endoplasmic reticulum nor on lysosome. The lowest confocal microscopy using FM 1-43FX to detect plasma membrane shows that AChE appeared uneven and punctuate over the plasma membrane. Hoechst 33342 to detect the nucleus. LT, LysoTracker; PM, plasma membrane. Quantification of confocal images demonstrates that colocalization of AChE with plasma membrane is significantly enhanced in treated cells (40 h) compared with the control (0 h). **P < 0.01. (B) Confocal microscopy shows the colocalization of AChE with caveolin-1 in HT-29 cells treated with 40 μg/ml etoposide. Quantification of confocal images demonstrates that colocalization of AChE with caveolin-1 is significantly enhanced in treated cells (40 h) compared with the control (0 h). **P < 0.01. Coimmunoprecipitation shows a time-sequenced interaction of AChE with caveolin-1 in HT-29 cells treated with 40 μg/ml etoposide. IgG is shown as a loading control. Hoechst 33342 to detect the nucleus. (C) Confocal microscopy also shows the colocalization of AChE with caveolin-1 in HT-29 cells treated with 40 μg/ml etoposide for 4 h, with 1 mM sulindac for 48 h or with 2.5 μM thapsigargin for 24 h. Scale bar, 10 μm. Quantification of confocal images demonstrates that colocalization of AChE with caveolin-1 is significantly enhanced in treated cells (40 h) compared with the control (0 h) of each treatment. **P < 0.01.
Fig. 5. Interaction of AChE with caveolin-1 is indispensable for apoptosome formation. (A) MβCD (100 mg) was dissolved in 2 ml of distilled water prior to the experiments. Pretreatment of HT-29 cells with 0.5 mg/ml MβCD for 1/12, 1, 2 or 24 h followed by 40 μg/ml etoposide treatment for 40 h prevents cell death. Viability was determined by a cell counter performing an automated trypan blue exclusion assay. Statistically significance: *P < 0.05; **P < 0.01. Data are presented as mean ± SD (n = 4). (B) Confocal microscopy shows that pretreatment of HT-29 cells with 0.5 mg/ml MβCD for 24 h followed by 40 μg/ml etoposide treatment for 40 h induces accumulation of AChE in the cytosol. Hoechst 33342 to detect the nucleus. Scale bar, 10 μm. Quantification of confocal images demonstrates that MβCD significantly reduced colocalization of AChE with caveolin-1 in treated cells compared with the experimental control. **P < 0.01. (C) Coimmunoprecipitation shows that pretreatment of HT-29 cells with 0.5 mg/ml MβCD for 1/12, 1, 2 or 24 h followed by 40 μg/ml etoposide treatment for 40 h prevents the interactions of AChE with caveolin-1 or cytochrome c and of cytochrome c with Apaf-1. IgG is shown as a loading control. (D) Western blotting of upper (U) and lower (L) fractions collected with a molecular weight cutoff method shows that MβCD pretreatment for 24 h followed by etoposide treatment for 40 h prevents the oligomerization of Apaf-1. (E) Coimmunoprecipitation shows that silencing of cytochrome c gene does not prevent the interaction between AChE and caveolin-1. IgG is shown as a loading control.
obtains its capability pertaining to apoptosis formation via the interaction with caveolin-1. Thus, we assume that the interactions of AChE with caveolin-1 and subsequently cytochrome c in cells undergoing apoptosis appear to precede apoptosis formation.

We propose that regulated subcellular localization of AChE and its interaction with other apoptosis constituents are a crucial and even essential aspect of apoptosis, mainly related with apoptosis formation. However, the relationships between AChE protein and classical apoptotic molecules during apoptosis remain to be elucidated. Improved understanding of this unresolved regulation will yield new insights of general interest in apoptosis biology and identify pathways that might be targeted for the design of anticancer therapeutics.

Supplementary material

Supplementary Figures S1–3 can be found at http://carcin.oxfordjournals.org/

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