Apop-1, a Novel Protein Inducing Cyclophilin D-dependent but Bax/Bak-related Channel-independent Apoptosis*

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In the intrinsic pathway of apoptosis, mitochondria play a crucial role by releasing cytochrome c from the intermembrane space into the cytoplasm. Cytochrome c release through Bax/Bak-dependent channels in mitochondria has been well documented. In contrast, cyclophilin D (CypD), an important component of permeability transition pore-dependent protein release, remains largely undefined, and no apoptogenic proteins that act specifically in a CypD-dependent manner have been reported to date. Here, we describe a novel and evolutionarily conserved protein, apoptogenic protein (Apop). Mouse Apop-1 expression induces apoptotic death by releasing cytochrome c from mitochondria into the cytosolic space followed by activation of caspase-9 and -3. Apop-1-induced apoptosis is not blocked by Bcl-2 or Bcl-xL, inhibitors of Bax/Bak-dependent channels, whereas it is completely blocked by cyclosporin A, an inhibitor of permeability transition pore. Cells lacking CypD were resistant to Apop-induced apoptosis. Moreover, inhibition of Apop expression prevented the cell death induced by apoptosis-inducing substances. Our findings, thus, indicate that the expression of Apop-1 induces apoptosis through CypD-dependent pathway and that Apop-1 plays roles in cell death under physiological conditions.

Apoposis, which is programmed cell death, is essential in normal development and is involved in many diseases and pathophysiological conditions including cancer, neurodegenerative disorders, and atherosclerosis (1–3). There are two major pathways for the induction of caspase-dependent apoptosis; one is an extrinsic or receptor-mediated pathway which involves Fas and tumor necrosis factor receptor, whereas the other is an intrinsic pathway in which mitochondria play a crucial role by releasing cytochrome c from the intermembrane space into the cytoplasm after mitochondrial outer membrane permeabilization (4). Mitochondrial outer membrane permeabilization can be initiated by opening of the permeability transition (PT)2 pore, an incompletely characterized protein complex presumably consisting of a voltage-dependent anion channel, adenine nucleotide translocator (ANT), and cyclophilin D (CypD) (5), although recent findings have shown that PT pores can form in the absence of ANT (6, 7). Normally, PT pores allow materials of up to 1.5 kDa to be released from mitochondria (8). Sustained opening of PT pores allows equilibration of ions between the matrix and cytoplasm and dissipation of the inner membrane potential (9). However, determination of whether PT pores are independent mediators of apoptosis has been hampered by the lack of an apoptogen that acts solely through them.

Mitochondrial outer membrane permeabilization is also mediated by Bcl-2 family proteins. In this type of apoptosis, Bcl-2 family proteins act directly on the outer mitochondrial membrane, and PT and the inner membrane do not play major roles, although there appear to be physical and/or functional interactions between PT and Bcl-2 family proteins.

Regulation of cytochrome c release by both pro- and anti-apoptotic members (10) of the Bcl-2 family of proteins is well documented (11). The anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, and Bcl-w, share four Bcl-2 homology (BH) domains (BH1 through BH4), although some members of this group appear not to possess a BH4 domain. The pro-apoptotic Bcl-2 family members can be classified into two subgroups based on their structure, i.e. BH3-only members and multidomain members. Bad, Bid, and Bim, which share only the BH3 domain, belong to the BH3 only protein subgroup, whereas Bax and Bak, which share three BH regions (BH1, BH2, and BH3), belong to the multidomain subgroup. The members of these two subgroups are functionally distinct. BH3-only proteins appear to function as death signal sensors (12, 13), whereas multidomain members function as gateways for many types of apoptotic signals, since cells lacking both Bax and Bak are resistant to many apoptotic stimuli (14). Beside the BH regions, many Bcl-2 family members possess a C-terminal hydrophobic domain, which is predicted to facilitate membrane localization of these proteins (15, 16).

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2 The abbreviations used are: PT, permeability transition; ANT, adenine nucleotide translocator; CypD, cyclophilin D; BH domain, Bcl-2 homology domain; SMC, smooth muscle cell(s); P, plaque; NP, non-plaque; GFP, green fluorescent protein; tBid, truncated BID; SNP, sodium nitroprusside; PBS, phosphate-buffered saline; z-, benzoyloxycarbonyl; fmk, fluoromethyl ketone; CyA, cyclosporin A.
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After being released from the intermembrane space of mitochondria into the cytosol, cytochrome c associates with Apaf-1 and pro-caspase-9 and forms a complex termed an apoptosome, which leads to the activation of pro-caspase-9 (17). Subsequently, the active form of caspase-9 cleaves and activates downstream executioner caspases, such as caspase-3 (18). Besides cytochrome c, Smac/DIABLO is also released from the intermembrane space of mitochondria and inhibits the activity of IAP, itself is an inhibitor of caspase-3, -7, and -9 (19–21).

Dysregulation of apoptosis results in deficient or excessive cell death and is implicated in many human diseases. Insufficient apoptosis may be involved in carcinogenesis, whereas excessive apoptosis is implicated in the pathogenesis of stroke, myocardial infarction, and heart failure. For example, atherosclerotic plaque tends to rupture at sites of increased macrophages and reduced numbers of vascular smooth muscle cells (SMC), suggesting that apoptosis of SMC, possibly induced by activated macrophages, promotes the rupture of atherosclerotic plaque (22). In the present study, we cloned a novel apoptogenic gene from atherosclerotic SMC in culture. We named this protein apoptogenic protein (Apop) and demonstrate that it induces apoptosis through the CypD-dependent but Bax/Bak-related channel-independent mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—SMC cultures were established from plaque (P) and non-plaque (NP) areas of the descending thoracic aorta of ApoE-deficient mice by the explant method as described previously (23). SMC were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. HeLa cells (a human cervical carcinoma cell line) were transfected with a pUC-CAGGS vector bearing human Bcl-2 to obtain a stable transfectant (24), which was designated HeLa-Bcl-2. Empty vector was used to obtain control HeLa cells (designated HeLa-V). HeLa cells and transfected HeLa cells were maintained in RPMI1640 containing 10% fetal calf serum. Hepatocytes were isolated by in situ collagenase perfusion from wild-type and CypD-deficient mice and cultured on collagen-coated dishes (Iwaki). CypD-deficient mice were generated in collaboration with Lexicon Genetics Inc, and the hepatocytes were kindly provided by Dr. Shimizu of Osaka University. Human umbilical vein endothelial cells were cultured in endothelial basal medium 2 (Sanko Junyaku) supplemented with 2% fetal calf serum.

**Differential Display**—Total RNA isolated from P and NP cultures was analyzed by differential display essentially as described by Liang and Pardee (25) with some modifications. First-strand cDNA synthesis and PCR reaction were performed as previously described (23). The DNA fragment differentially expressed by P and NP cultures was excised from the gel, reamplified by PCR, and cloned into a pGEM-T vector (Promega) for sequencing.

**Quantitative Real-time Reverse Transcription-PCR**—RNA was isolated from mouse tissues using ISOGEN (Nippon Gene) according to the manufacturer’s instructions and converted to cDNA by reverse transcription. A TaqMan MGB® gene expression kit was used according to the manufacturer’s instructions (Applied Biosystems). Quantitative PCR was performed using real-time detection technology and analyzed on a model 7900 sequence detector (Applied Biosystems). The PCR primers and fluorescent probe for Apop-1 were as follows: forward, 5′-GCCAGGATGTTGGATTGTGTGTTG-3′; reverse, 5′-AAGGGCGAAGCTTGTGAA-3′; probe, 5′-CAAGTGCTTCTTTTGCCAGCTGCA-3′. For Apop-2 the primers and probe were as follows: forward, 5′-AGTCCAGGCAAAAGAGCAACG-3′; reverse, 5′-CTTCCCCATGGAGAAGAAGTGAT-3′; probe, 5′-CATGTGCTTCTGGAAAT-3′. Levels of mRNA were compared after normalization to concurrent 18 S rRNA amplification.

**Plasmid Construction**—Apop-1 cDNA was cloned from cultured SMC by reverse transcription-PCR using specific primers and high fidelity DNA polymerase, Pyrobest (Takara). The amplified Apop-1 cDNA was ligated with pcDNA3.1/CT-GFP-TOPO (Invitrogen) to make Apop-1 and green fluorescent protein (GFP) fusion protein expression vector, pcDNA3Apop-1/GFP. Apop-1 cDNA including a stop codon was inserted into pcDNA3.1/mychis (Invitrogen) for construction of the Apop-1 expression vector, pcDNA3Apop-1/GFP. Apop-1 cDNA encoding human truncated Bid (tBid) and Bcl-xL were generated from human genomic DNA using 18 S rRNA as an internal control. A, aorta; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen. Values are the mean ± S.D. of three mice.

**Identification of Apop protein.**—A differential display comparing expression profiles for plaque (P) and non-plaque (NP) cultures. Differential display reaction was performed in the presence of 35S-labeled UTP, and labeled DNA was analyzed on 6% denaturing polyacrylamide gel. Results for three pairs of P and NP cultures from individual animals (1–3) are shown. B, genomic structure of the mouse Apop gene. Boxes E1 through E6 indicate exons. White boxes indicate untranslated regions, and black boxes indicate coding regions. Alternative splicing produces two transcripts; Apop-1 is composed of E1–E4, E5’, and E6, and Apop-2 is composed of E1–E4 and E5. C, expression of the Apop gene. Real-time PCR was performed using RNA isolated from mouse organs. Levels of Apop-1 and Apop-2 mRNA were corrected using 18 S rRNA as an internal control. A, aorta; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen. Values are the mean ± S.D. of three mice.
reagents (Invitrogen) according to the manufacturer’s instructions. Antisense plasmid DNA was introduced into SMC using a Nucleofector Basic kit for primary mammalian smooth muscle cells (Amaxa Biosystems) according to the manufacturer’s instructions. For treatment of cells with cyclosporin A (CyA), HeLa cells were cultured and treated with this drug after transfection of pcDNA3Apop-1 or from 2 h before initiation of treatment with etoposide. For endogenous Apop inhibition experiments, SMC cultures were transfected with control (pcDNA3.1) or antisense plasmid (pcDNA3AS) and incubated at 37 °C for 24 h. Cells were then supplied with serum-free medium containing 200 μM lysophosphatidylcholine or 50 μM sodium nitroprusside (SNP) and then cultured for an additional 24 h.

Immunocytochemical Study—Cells were rinsed with PBS and fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) for 5 min at room temperature. After washing with PBS and blocking with 1% BSA in PBS for 30 min, the cells were incubated with anti-HSP60 (Stressgen) or anti-cytochrome c antibody (Lab Vision) overnight at 4 °C and then washed with PBS. Tetramethylrhodamine-labeled anti-mouse IgG antibody (Rockland) was added to the cells and incubated for 1 h. For staining of nuclei, cells were rinsed with PBS and exposed to 10 μM Hoechst 33342 (Dojindo) in PBS for 5 min. After washing with PBS, cells were examined under a laser scanning confocal microscope.

Western Blot Analysis—The mitochondrial fraction was isolated using digitonin, as described previously (27). Briefly, after washing twice with phosphate-buffered saline, the cultured cells were collected and treated with 10 μM digitonin for 5 min at 37 °C. The cytosolic and organelle fractions were separated by centrifugation and lysed with radioimmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS). Aliquots of each fraction were subjected to Western blot analysis.

Enzymatic Assays—Protein concentration was determined by DC protein assay (Bio-Rad) using bovine serum albumin as a standard. Caspase-3 and -9 activities in cells cultured for 24 h after transfection were measured using a colorimetric assay kit (BioVision) according to the manufacturer’s instructions. For measurement of caspase activity, cells were trypsinized (0.25%, Nacalai Tesque) and combined with medium, centrifuged, washed, and fixed in cold 90% ethanol for 20 min. Cells were then resuspended in staining buffer consisting of 1 mg/ml RNase A, 20 μg/ml RNA
ml propidium iodide (Medical and Biological Laboratories), and 0.01% Nonidet P-40. DNA content was determined by fluorescence-activated cell sorting analysis on the FL-2 channel, and gating was set to exclude debris and cellular aggregates. A total of $1 \times 10^4$ cells were counted for each sample. In the endogenous Apop expression inhibition experiment, cell viability was assayed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay kit (Sigma).

**Statistical Analysis**—Statistical analysis was performed by the unpaired $t$ test. Results are expressed as mean ± S.D.

**RESULTS**

**Identification of Apop-1**—In a previous study of atherosclerotic SMC, we compared SMC cultured from atherosclerotic plaque (P) with those from non-plaque (NP) areas of aorta of apolipoprotein E-deficient mice (23). Differential display using P and NP cell pairs from three animals identified several transcripts that differed in level of expression. A 140-bp cDNA fragment was one of these transcripts and was more intense in P than in NP lines in two pairs (2P/2NP, 3P/3NP) but similar in P and NP in the third (1P/1NP) (Fig. 1A).

The nucleotide sequence of the 140-bp fragment was nearly identical to AA546169 in the mouse EST database. The 5′ portion of AA546169 is identical to that of AK002873, a cDNA clone of unknown function in the database. Analysis of the mouse genome revealed that these sequences represent two alternatively spliced transcripts from an **Apop** locus, RIKEN cDNA 2810002N01, on mouse chromosome 12. Our clone (which we designated Apop-1) and AA546169 consisted of exons 1–4, 5′, and 6, whereas AK002873 (Apop-2) consisted of exons 1–4 and 5 (Fig. 1B). Reverse transcription-PCR showed that both Apop-1 and Apop-2 were expressed in all mouse organs tested (not shown), indicating ubiquitous expression of the **Apop**

**FIGURE 3.** Apop-1/GFP is located in mitochondria and releases cytochrome c. *A*, fluorescence microscopic picture of a SMC transfected with pcDNA3-Apop-1/GFP. GFP expression (left panel) and immunostaining with Hsp60-specific antibody (middle panel) are shown. Merged picture (right panel) shows colocalization of Apop-1 and Hsp60. Scale bar, 30 μm. *B*, localization of Apop-1/GFP and release of cytochrome c into the cytosolic space. SMC were transfected with pcDNA3-Apop-1/GFP and cultured for 24 h. Cytosolic (S) and mitochondria-containing (M) fractions were isolated by differential centrifugation and analyzed by immunoblotting using anti-cytochrome c (Cyt c), GFP, tubulin (cytosol marker), or Hsp60 (mitochondrial marker) antibodies. CTR, control cells; ETP, etoposide-treated cells. C, release of cytochrome c from mitochondria into the cytosolic space. SMC transfected with control vector or pcDNA3-Apop-1 were immunostained with a cytochrome c-specific antibody (anti-cytochrome c) or stained with Hoechst 33342. The cell transfected with control vector exhibits cytochrome c in mitochondria and a normal nucleus, whereas the Apop-1-transfected cell exhibits cytochrome c released into the cytosol and has an apoptotic nucleus. Scale bar, 30 μm.

**FIGURE 4.** Expression of Apop-1 induces apoptosis. *A*, apoptosis induced by expression of Apop-1 or Apop-2. Cultured SMC were transfected with control (CTR), pcDNA3-Apop-1, or pcDNA3-Apop-2. Microscopic photographs were taken 24 h after transfection. *B*, apoptosis of SMC transfected with pcDNA3-Apop-1/GFP. At 24 h after transfection, cells were photographed. Fluorescence microscopic pictures of cells stained with Hoechst 33342 exhibit condensed nuclei of Apop-1-GFP-transfected cells. C, fragmentation of DNA in cells transfected with Apop-1-expression vector. HeLa cells were transfected with control vector or pcDNA3-Apop-1 and cultured for 24 h. Nuclear fragmentation was assayed by propidium iodide staining of nuclei and fluorescence-activated cell sorter analysis. D, apoptosis of human umbilical vein endothelial cells induced by expression of Apop-1. Cultured human umbilical vein endothelial cells were transfected with control or pcDNA3-Apop-1. Microscopic photographs were taken 24 h after transfection.
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sequence analysis suggested that there are two tyrosine residues potentially available for phosphorylation and two asparagines potentially available for glycosylation in the mouse Apop-1 sequence. Of these amino acid residues, one tyrosine and one asparagine are highly conserved across species (Fig. 2).

In addition, loose similarities to bipartite nuclear localization signal sequence and coiled-coil sequence are present in an Apop homologue in C. elegans. The C-terminal sequence of Apop-2, which is enriched in arginine and lysine residues, also suggests potential nuclear localization in addition to mitochondrial localization.

**Mitochondrial Localization**—To investigate the mitochondrial localization of Apop-1 predicted from the analysis of amino acid sequence, SMC were transfected with pcDNA3Apop-1/GFP, an expression vector of Apop-1 fused to GFP at its C terminus. On fluorescence microscopic examination, GFP was predominantly in the cytosol and exhibited a punctate distribution similar to that of mitochondria (Fig. 3A). GFP was colocalized with HSP60, a mitochondrial marker, in immunocytochemical staining with an antibody to HSP60. To further examine the localization, cytosolic and mitochondrial proteins were extracted from cells after transfection of pcDNA3Apop-1/GFP (see below for cell morphology). Consistent with the results of immunocytochemical observation, GFP immunoreactivity was localized in the mitochondrial fraction, suggesting that Apop-1 is located in mitochondria (Fig. 3B).

**Apop-1-induced Apoptotic Cell Death**—To clarify the function of this novel protein, we prepared Apop-1 and Apop-2 expression vectors, pcDNA3Apop-1 and -2, and introduced them into cultured wild-type mouse SMC. Remarkably, cells expressing Apop-1 or Apop-2 died within 24 h after transfection (Fig. 4A). More than half of cells transfected with pcDNA3Apop-1/GFP also died within 24 h (Fig. 4B). Cells exhibited morphological features of apoptosis (30), i.e. cell shrinkage and condensed/fragmented nuclei on staining with Hoechst 33342 (Fig. 4B). Fragmentation of DNA in Apop-expressing HeLa cells was apparent on flow cytometric analysis of propidium iodide-stained cells (Fig. 4C). Human umbilical vein endothelial cells also died within 24 h after the transfection of Apop-1 expression vector (Fig. 4D).

**Apop-1-induced Caspase Activation**—Conditioned medium of pcDNA3Apop-1-transfected SMC failed to induce SMC death in culture, indicating that Apop-1 does not induce the expression of secreted apoptogenic substances such as Fas ligand (Not shown). Apoptosis was confirmed by the inhibition of cell death by a broad-spectrum caspase inhibitor, z-VAD-fmk, which was partial at 50 μM and complete at 100 μM (Fig. 5A). This indicates that Apop-1-induced death is mediated by the activation of caspases. Consistent with this finding, the activity of DEVDase (caspase-3), a representative effector molecule in the apoptosis cascade, was elevated ~10-fold in pcDNA3Apop-1-transfected cells compared with that in vector-transfected control (Fig. 5B). DEVDAse activation was completely inhibited by Ac-DQTD-CHO, a casapase-3/7 inhibitor (Fig. 5B). To examine whether activation of caspase-9, the best characterized upstream caspase, is involved in Apop-1-induced apoptosis, we assessed the activity of LEHDase.
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(caspase-9) and found that it also was elevated, ~7-fold, in pcDNA3Apop-1-transfected cells (Fig. 5C). Consistent with this, a caspase-9 inhibitor, Ac-LEHD-CHO, inhibited DEVDase activation as well as apoptosis (Fig. 5D). These findings indicate that Apop-1-induced apoptosis is mediated by caspase-9, an essential component of the apoptosome, which mediates mitochondria-induced apoptosis.

Apop-1 Releases Cytochrome c—To examine whether cytochrome c, a key molecule in the mitochondria-mediated apoptotic cascade, is released from mitochondria, SMC were transfected with control vector or pcDNA3Apop-1, and cytochrome c was stained with a specific antibody. Immunostaining of cytochrome c yielded a spotty pattern in control cells, confirming mitochondrial localization (Fig. 3C). In contrast, SMC transfected with pcDNA3Apop-1 exhibited a diffuse pattern of staining, suggesting release of cytochrome c from the mitochondria into the cytosol (Fig. 3C). Western blot analysis confirmed the distribution of cytochrome c in both mitochondrial and cytosolic fractions of the transfected cells (Fig. 3B). Translocation to the cytosol of cytochrome c was apparent at 24 h after transfection with pcDNA3Apop-1/GFP. Etoposide, a chemotherapeutic drug that can initiate the mitochondrial pathway of apoptosis, was used as a positive control and induced similar translocation of cytochrome c from mitochondria to the cytosol.

Anti-apoptotic Bcl-2 Family Members Are Unable to Inhibit Apop-1-induced Apoptosis—Apop-1 is structurally distinct from the members of the Bcl-2 family in that it possesses no BH domains, has a mitochondrial localization signal at the N terminus, and does not have the C-terminal transmembrane domain, which many Bcl-2 family proteins possess. To investigate whether Apop-1-induced apoptosis involves Bcl-2 family proteins, we examined whether Bcl-2, an anti-apoptotic Bcl-2 family member, can inhibit apoptosis induced by Apop-1. Both pcDNA3Apop-1 and a vector with tBid, a pro-apoptotic Bcl-2 family member, induced apoptosis in control HeLa cells (HeLa-V). However, although the introduction of tBid was unable to cause apoptosis of cells that constitutively express Bcl-2 (HeLa-Bcl-2), the apoptosis induced by Apop-1 was not inhibited by expression of Bcl-2 (Fig. 6A). Apop-1 induced comparable degrees of apoptosis in HeLa-V and HeLa-Bcl-2 cells in a DNA-dose-dependent manner, whereas tBid-induced apoptosis was completely eliminated in HeLa-Bcl-2 cells (Fig. 6B). Bcl-xL is another anti-apoptotic member of the Bcl-2 family. Consistent results were obtained when Bcl-xL expression vector was co-transfected together with tBid or Apop-1 expression vector. Apoptosis was not observed when tBid expression vector was introduced into HeLa cells together with Bcl-xL expression vector, whereas apoptosis was apparent when Apop-1 expression vector was co-transfected with Bcl-xL (Fig. 6C). Quantitative analysis of apoptosis showed that co-transfection of HeLa cells with the Bcl-xL expression vector and the tBid expression vector rescued them from apoptosis in a Bcl-xL DNA-concentration-dependent manner. In contrast, Bcl-xL expression had no inhibitory effect on Apop-1-induced apoptosis (Fig. 6D). These findings indicate that Apop-1-induced apoptosis is independent of Bcl-2 and Bcl-xL, which inhibit cytochrome c release from mitochondria.

Inhibition of Cyclophilin D Prevents Apop-1-induced Apoptosis—CypD is a mitochondrial member of the cyclophilin family (31) that catalyzes cis-trans isomerization of peptidylprolyl bonds (32, 33). The immunosuppressive drug CyA binds specifically to cyclophilins and strongly inhibits their activity (34).
CypD is thought to be an integral constituent of the mitochondrial PT pore (5), and its interaction with ANT presumably promotes change in its conformation to open inner membrane space proteins, including cytochrome c (35, 36). To examine whether Apop-1 triggers efflux of mitochondrial proteins in CypD-dependent fashion, we examined the inhibitory effect of CyA on the release of cytochrome c by Western blot analysis. As shown in Fig. 7A, CyA reduced the release of cytochrome c by Apop-1, indicating that Apop-1 induces CypD-dependent release of cytochrome c. FACScan analysis demonstrated that the apoptosis induced by Apop-1 was significantly inhibited by CyA, which was able to reduce apoptosis to nearly basal level (Fig. 7B). In contrast, CyA did not affect etoposide-induced apoptosis, which is mediated by proteolytically activated Bid, a pro-apoptotic Bcl-2 family member (37). Apop-1, thus, acts through a CypD-dependent mechanism distinct from etoposide-induced apoptosis involving Bcl-2 family members.

Cyclophilin D Deficiency Prevents Apop-1-induced Apoptosis—To confirm CypD-related apoptosis of Apop-1, we introduced Apop-1 into primary culture of hepatocytes of CypD-deficient mice (38). At 24 h after transfection, numerous cells with shrunken and apoptotic morphology was detected in wild-type cells but not in CypD-deficient cells (Fig. 7C). In contrast, tBid induced death of both wild-type and CypD-deficient cells. The percentage of CypD-deficient cells that were apoptotic after transfection with pcDNA3Apop-1 did not differ from that for untransfected cells, whereas tBid increased apoptotic death of CypD-deficient cells to a level comparable with that for wild-type cells (Fig. 7D). These findings indicate that Apop-1 induces apoptosis in a CypD-dependent fashion that is quite different from the Bax/Bak-dependent apoptosis mediated by tBid.

Inhibition of Apop Expression Prevents Cell Death Induced by Apoptogenic Substances—To assess the physiological function of Apop, we examined the effects of inhibition of Apop gene expression using an antisense plasmid, pcDNA3AS. The inhibitory effect of the antisense plasmid was confirmed by reduction of green fluorescence expressed by the co-transfected pcDNA3Apop-1/GFP. GFP signal was not affected by the control plasmid (Fig. 8A). We confirmed this result by Western blot analysis. The expression of Apop-1/GFP was prevented by co-transfection of pcDNA3AS (Fig. 8B). Cells were transfected with pcDNA3AS or control vector and cultured in the presence or absence of apoptosis-inducing reagents. Inhibition of endogenous Apop gene expression using antisense plasmid prevented the cell death induced by lysoosphatidylcholine or SNP (Fig. 8C). This finding is consistent with the results in Fig. 4 showing that Apop-1 induces cell death. Suppression of Apop gene expression using antisense plasmid did not completely inhibit the death induced by SNP (Fig. 8D). This suggests that SNP-induced death is partially mediated by Apop signaling, or Apop protein sensitizes cells to death. These findings together indicate that Apop plays roles in cell death under physiological conditions.

DISCUSSION

We have identified a novel protein, Apop, that induces CypD-dependent apoptosis when expressed using an expression vector. Because Apop is highly conserved evolutionarily and lacks other family members, it probably plays a crucial function in the physiology of cells that has remained unrecognized. Indeed, overexpression of Apop induces apoptotic cell death of cultured SMC, and inhibition of Apop expression prevents cell death by apoptosis-inducing stimuli. These findings indicate that Apop is physiologically concerned with cell death and that tight control of Apop activity must be important for cell viability.

Recent studies suggest that mitochondria can release intermembrane space proteins, including cytochrome c and Smac/DIABLO, through two different mechanisms (4, 9, 39). One involves surgically precise permeabilization of the outer membrane mediated by Bcl-2 family members, whereas the other involves induction of the mitochondrial PT, which occurs mainly in response to the release of Ca2+ from endoplasmic reticulum stores. It is reported that Bcl-2 family member-in-
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We found that Apop-1-induced apoptosis was not prevented by anti-apoptotic Bcl-2 family members, whereas it was inhibited by CyA or CypD deficiency. We also showed that Apop-1 is a CypD-dependent apoptogenic protein that does not act on Bax/Bak-dependent channels. Unlike Bcl-2 family members, Apop-1 possesses neither BH domains nor a C-terminal transmembrane domain. Instead, it possesses a mitochondrial localization signal (mitochondrial targeting sequence) at the N terminus. Apop-1 is, thus, a novel pro-apoptotic protein that is not considered a member of the Bcl-2 family. It is at present the only protein known to act specifically in a CypD-dependent fashion and may be useful for study of the CypD-mediated apoptotic pathway.

Notably, although transcripts for both Apop-1 and Apop-2 are present in various tissues of mice, the significance of alternative splicing of Apop is yet to be determined. The C-terminal 34-amino acid sequence of Apop-1 differs from the C terminus of Apop-2 and lacks the positively charged amino acids that are highly conserved among the Apop homologues present in other species. A data base search failed to identify any EST sequences that share homology with the 34-amino acid sequence. Thus, although it is apoptosis-inducing, Apop-1 may not have all of the normal functions of Apop. Nevertheless, although we have not demonstrated CypD-dependent apoptosis by Apop-2, it is very likely that Apop-2 induces apoptosis by the same mechanism as Apop-1 and that CypD-dependent apoptosis is mediated by the first 160 amino acids of Apop. The importance of the N-terminal 160-amino acid sequence for the function of Apop may be consistent with our finding that the fusion of GFP at the C terminus of Apop-1 does not substantially affect the apoptogenic activity of Apop-1.

We also note that Apop-1 was originally isolated because expression of it was higher in atherosclerotic plaque-derived SMC than in non-plaque-derived SMC by differential display. Apoptotic death of SMC is an important process during the development of atherosclerotic plaque. Decrease in SMC due to apoptosis could also contribute to the formation of vulnerable plaque, which is prone to rupture and causes cardiovascular events (41). Indeed, there is increased SMC apoptosis in symptomatic plaque (35, 42, 43) in patients presenting with unstable angina compared with patients with stable angina, and apoptotic macrophages are co-localized with sites of plaque rupture (44). Direct evidence of a causal relationship between plaque rupture and apoptosis in an animal model was obtained with the finding that induction of SMC apoptosis in fibrous cap of mouse lesions induces plaque rupture and thrombosis of plaque (45). Several studies have examined the effects on experimental atherosclerosis of CyA and FK506, which are immunosuppressive drugs used for transplant rejection. The results obtained are somewhat contradictory; CyA accelerated atherosclerosis in hyperlipidemic mice (46), whereas a recent study found that low dose FK506 blocked atherosclerosis and stabilized plaque in ApoE-deficient mice (47). Because they are both immunosuppressive drugs, the mechanisms by which they exert effects on atherosclerosis would be more complex than merely by affecting apoptosis. Nevertheless, we showed in the present study that Apop-1 expression induces apoptosis of SMC and that CyA prevents apoptosis induced by Apop-1. It will, therefore, be important to assess the level of expression of...
Apop in atherosclerotic plaque in vivo and to determine the significance of the Apop proteins in atherosclerosis and other pathophysiological conditions.

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