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Identification of apoptosis-inducing factor in human coronary artery endothelial cells

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Abstract

Apoptosis has been observed in vascular tissues in response to pro-inflammatory and pro-atherosclerotic stimuli, such as oxidized low density lipoproteins (ox-LDL), angiotensin II (Ang II), and tumor necrosis factor-α (TNF-α). Apoptosis is believed to be mediated via caspase-dependent pathway. Recently, a 57 kDa molecule, apoptosis-inducing factor (AIF), has been described as a basis for cell injury via a caspase-independent pathway. This study was designed to identify the presence of AIF and the regulation of its gene expression in human coronary artery endothelial cells (HCAECs). Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were used to determine AIF mRNA and protein expression. Cultured HCAECs were treated with ox-LDL (10–40 μg/ml), angiotensin II (10^{-9}–10^{-6} M), or TNF-α (0.1–10 ng/ml). AIF was barely detectable in unstimulated HCAECs; however, treatment with ox-LDL, but not with Ang II or TNF-α, significantly increased the expression of AIF in a concentration- and time-dependent manner. DNA sequencing analysis substantiated the existence of AIF in the HCAECs. Treatment of cells with the caspase inhibitor with Z–VAD–fmk did not change ox-LDL-mediated AIF protein expression. Ox-LDL-mediated upregulation of AIF expression was inhibited by actinomycin D, suggesting transcriptional regulation. Further, upon treatment of cells with ox-LDL AIF translocated from mitochondria to the nucleus, as determined by immunocytochemistry. These data suggest that AIF is expressed in HCAECs and is upregulated by ox-LDL.

Keywords: Apoptosis; Apoptosis-inducing factor; Endothelium; Ox-LDL

Programmed cell death (apoptosis) is a critical feature of coronary endothelial and myocardial injury following ischemia [1]. Apoptosis of endothelial cells is also evident in the early stages of atherosclerosis [2]. Strategies that limit apoptosis reduce vascular and myocardial damage as a result of ischemia. Therefore, it is important to identify the expression and regulation of steps leading to apoptosis.

Generally apoptosis is believed to be a result of activation of caspases. Apoptosis-inducing factor (AIF) is a newly described pathway, which leads to cell injury independent of caspase activation [3]. AIF is a flavoprotein of 57 kDa molecular mass that shares homology with the bacterial oxidoreductases and can induce apoptosis of isolated nuclei [4]. AIF is normally confined to the mitochondrial inter-membrane space, but translocates to the nucleus when apoptosis is induced. AIF plays a complementary or cooperative role along with caspases that leads to nuclear apoptosis. Microinjection of AIF into the cytoplasm of intact cells induces condensation of chromatin, dissipation of the mitochondrial transmembrane potential, and exposure of phosphatidylserine in the plasma membrane. None of these effects is prevented by wide-ranging caspase inhibitors [5]. Several investigators have demonstrated a pathological role of AIF in lung carcinomas [6], its spread to adjacent somites [7], meningitis [8,9], drug resistant variants [10], neural tumor [11], leukemia [12], HIV [13], and renal ischemia [14]. The role of AIF or its presence in human endothelial injury has not yet been examined.

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The goal of this study was to delineate the presence of AIF in HCAECs. We studied its regulation by three unrelated pro-inflammatory and pro-atherosclerotic stimuli angiotensin II (Ang II), the cytokine tumor necrosis factor-α (TNF-α), and oxidized-low density lipoprotein (ox-LDL).

Methods

Materials. The caspase inhibitor zenzyloxycarbonyl–Val–Ala–Asp–fluoromethylketone (Z–VAD–fmk) was obtained from Enzyme System Products (Livermore, CA). The goat anti-human AIF polyclonal antiserum was obtained from Santa Cruz (San Diego, CA). The horseradish peroxidase (HRP)-labeled rabbit polyclonal antibody against goat IgG and HRP-labeled monoclonal antibodies against mouse IgG were from Calbiochem Biosciences (La Jolla, CA). TOPO TA cloning kit was from Invitrogen (San Diego, CA). FITC conjugatd anti-goat IgG antibody, TNF-α, and all other reagents, unless indicated, were from Sigma Chemical (St. Louis, MO).

Cell culture. Original batch of HCAECs was purchased from Clonetics (East Rutherford, NJ). The cells were cultured in endothelial basal medium 2 (EBM-2) (Clonetics) that consisted of 500 ml endothelial cell basal medium, 5 ng human recombinant epidermal growth factor, 0.5 mg hydrocortisone, 25 mg gentamicin, 50 μg amphotericin B, 6 mg bovine brain extract, and 25 ml FBS, as described earlier [15–17]. Fourth-generation HCAECs were used in this study. HCAECs were incubated with ox-LDL (10–40 μg protein/ml) for 3–24 h, or with Ang II (10^{-9}–10^{-6} M) or TNF-α (0.1–10 ng/ml) for 24 h. The expression of AIF was then determined.

Parallel groups of HCAECs were pretreated with Z–VAD–fmk (50 μM; 30 min) for 1 h, and the HCAECs were then exposed to ox-LDL (40 μg protein/ml) to examine the expression of AIF.

Preparation of ox-LDL. Native-LDL was prepared as described earlier, oxidized at 37 °C in the presence of 5 μmol/L $CuSO_4$ for up to 24 h, and then dialyzed against three changes of PBS for 24 h [15,16]. Ox-LDL samples were filter sterilized and incorporated in the culture medium. The integrity of the lipoproteins was confirmed by agarose gel electrophoresis [18].

RT-PCR analysis. Total RNA was isolated with RNeasy Minikit (Qiagen, Valencia, CA). One μg of total RNA was reverse-transcripted with oligo(dT) and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) at 37 °C for 1 h; 1.5 μL of the reverse-transcripted material was amplified with *Taq* DNA polymerase with use of a primer pair (forward primer, 5′-GGA TCC TGG GGC CAG GGT ACT GAT-3′, reverse primer 5′-CTC GGG GAA GAG TTG AAT CAC TTC-3′). For PCR, 35 cycles were used at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min. Human β-actin was amplified as an internal reference [3,18].

DNA sequencing. cDNA products of whole length AIF mRNA were amplified by PCR with use of a primer pair unique to human AIF [3,8]. The PCR product was incorporated into pCR2.1-TOPO vector by using TOPO TA Cloning Kit (Invitrogen). The nucleotide sequence of AIF cDNA was examined by ABI 377 DNA Sequencer by serial primers. The nucleotide sequence was compared to human AIF from GenBank.

Western blots. HCAECs were homogenized in ice-cold buffer (10 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$, and 0.5 mM PMSF). The homogenate was centrifuged at 2000*g* for 10 min. Twenty micrograms of supernatant was loaded on a 10% SDS–PAGE and blotted on nitrocellulose filters. Immunoblots were performed with goat antibody against human AIF (Santa Cruz, CA) [18].

Immunocytochemistry. The cells were fixed on slides in cold methanol for 10 min at −20 °C and the specimen was washed twice with PBS. The specimen with 3% BSA in PBS for 15 min at room temperature. The specimens were washed with PBS and covered with anti-AIF polyclonal antibody (dilution 1:20) in PBS for 2 h at room temperature. After washing, FITC-conjugated secondary antibody (dilution 1:40) was applied for 1 h at room temperature. The slides were washed and visualized under fluorescent microscopy [18].

Statistical analysis. All data are representative of at least three independent experiments. Data values are presented as means ± SD. The data were subjected to analysis of variance. Difference of $P < 0.05$ was considered to be statistically significant.

Results

Characterization of AIF in HCAECs and its upregulation with ox-LDL

As shown in Fig. 1, unstimulated HCAECs expressed very little AIF. Treatment with ox-LDL induced marked upregulation of AIF protein ($P < 0.05$). Upregulation of AIF protein by ox-LDL was concentration-dependent. The upregulation of AIF proteins was also time-dependent.

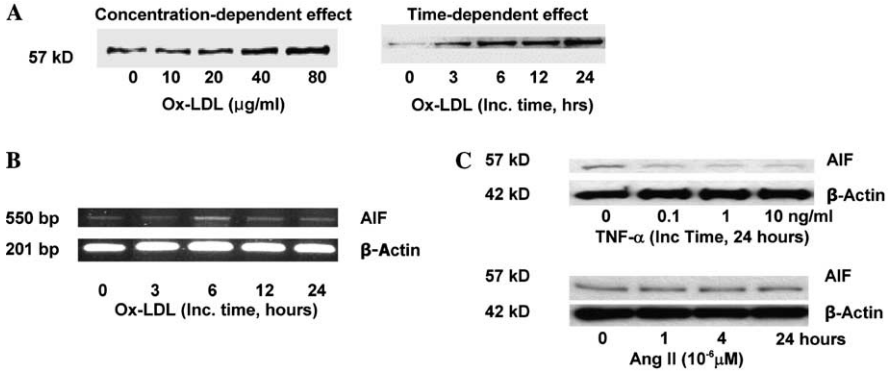

Fig. 1. (A) Concentration (left) and time-dependent (right) effects of ox-LDL on the expression of AIF protein. HCAECs were incubated with ox-LDL at different concentrations for varying time intervals. Expression of AIF proteins was determined by Western blots. (B) AIF mRNA expression also increased upon treatment with ox-LDL. Note that the upregulation of AIF is evident at 3 h, is maximal at 6 h, and then begins to decline. These examples are representative of five independent experiments. (C) Absence of effect of TNF-α or angiotensin (Ang) II on AIF expression. HCAECs were incubated with TNF-α or Ang II for 24 h. These examples are representative of 3–5 independent experiments.

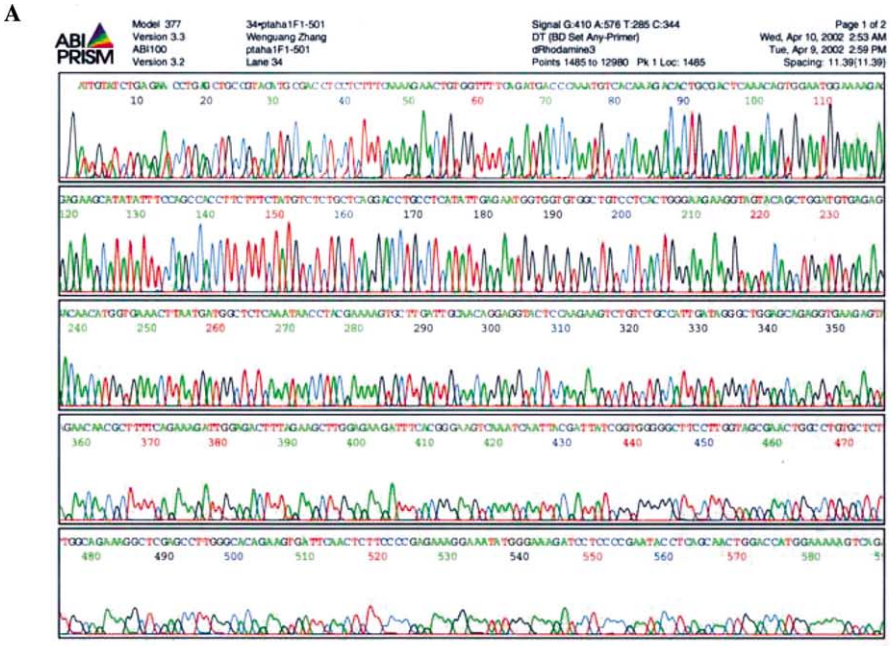

Fig. 2. Nucleotide sequences of AIF cDNA from HCAECs. Compared to original human AIF sequence, number 1001 nucleotide G is changed to A and number 1–10 nucleotides are lost.

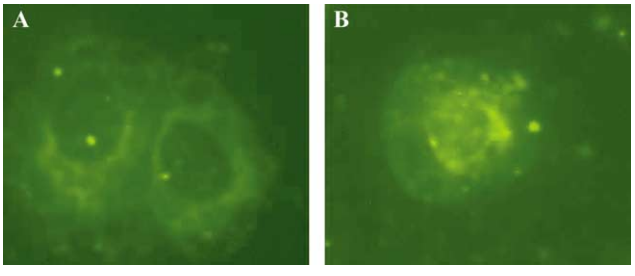

Fig. 4. Immunolocalization of AIF in HCAECs in basal state and after treatment with ox-LDL. The cells were treated with ox-LDL (40 μg/ml) for 24 h. Note that the expression of AIF protein was mainly restricted to outside the nucleus in the basal state and it was redistributed into nucleus following treatment of cells with ox-LDL.

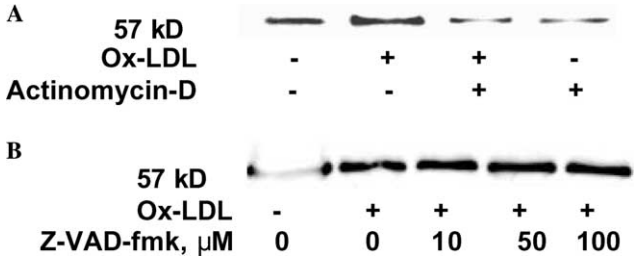

Fig. 3. (A) The inhibition of AIF expression with actinomycin-D. HCAECs were incubated with 40 μg/ml ox-LDL in the absence or presence of actinomycin-D (5 μg/ml) at 37 °C for 24 h. (B) Absence of effects of the caspase inhibitor Z–VAD–fmk on AIF expression. HCAECs were treated for 1 h with Z–VAD–fmk (10–100 μM) and then treated with ox-LDL. These Western blots are representative of three independent experiments.

As shown in Fig. 1B, ox-LDL treatment significantly induced expression of AIF mRNA. Induction of expression was first evident at 3 h, became maximal at 6 h, and then appeared to fall.

AIF expression did not appear to increase when HCAECs were treated with Ang II or TNFα (Fig. 1C).

Analysis of the nucleotide sequences of AIF isolated from HCAECs revealed that the number 1001 nucleotide G was changed to A and number 1–10 nucleotides were missing compared to the human AIF sequence obtained from GenBank (Fig. 2). Thus there was 99% similarity between AF recovered fro the HCAECs and other human tissues.

AIF expression and actinomycin-D

To determine whether or not ox-LDL activates AIF transcription, HCAECs were incubated with ox-LDL in the absence or presence of actinomycin-D (5 μg/ml). Western blotting showed that pretreatment of cells with actinomycin-D markedly reduced AIF expression in HCAECs (Fig. 3A), suggesting that ox-LDL-induced AIF expression occurs at transcriptional level.

AIF expression and caspase-inhibitor

HCAECs were pretreated with the caspase inhibitor Z–VAD–fmk (10–100 μM) for 1 h and then stimulated with ox-LDL (40 μg/ml) for 24 h. Western blotting showed that Z–VAD–fmk failed to alter AIF expression in response to ox-LDL (Fig. 3B).

Translocation of AIF to HCAEC nuclei

The cells were treated with ox-LDL (40 μg/ml) for 24 h or left untreated. The immunolocation of AIF protein was restricted mainly to outside the nuclei in the basal state. The AIF translocated partially into nuclei following treatment of HCAECs with ox-LDL (Fig. 4).

Discussion

The present study provides initial evidence that AIF is expressed in small amounts in HCAECs in the baseline state; however, it is rapidly upregulated when the cells are exposed to ox-LDL. DNA sequencing showed a marked similarity of AIF in the HCAECs to that in other human tissues. We also show that treatment with ox-LDL causes translocation from the cytoplasm to the nuclei.

Our RT-PCR data clearly show that ox-LDL upregulates the level of AIF mRNA, suggesting that the modulation of gene transcription and/or mRNA processing or stability of mRNA are involved in this effect of ox-LDL. Upregulation of AIF expression in HCAECs treated with ox-LDL may be due to the augmentation of gene transcription changes in processing or the stability of mRNA and/or AIF recruitment from intracellular stores.

We observed that the upregulation of AIF mRNA occurred as early as 3 h of exposure to ox-LDL, was maximal at 6 h of exposure, and then began to decline. The basis for decrease in mRNA at 24 h is not known, but it is possible that as AIF started to translocate to the nuclei, it bound to the DNA and caused injury to DNA. This resulted in slowing of the process of transcription. The upregulation of AIF protein would still be expected to continue as preformed AIF mRNA templates continued to be translated.

We also observed that actinomycin-D inhibited ox-LDL-mediated increase in AIF mRNA, suggesting that the modulation of gene transcription and/or mRNA processing or stability of mRNA are involved in this effect of ox-LDL. We also found that the ox-LDL-induced upregulation of AIF could not blocked by the caspase inhibitor, which is consistent with the phenomenon that this caspase-independent pathway of cell injury is present in human endothelial cells.

We examined the effect of two other mediators of tissue inflammation and atherosclerosis, Ang II and TNF-α on AIF expression. Both these mediators have previously been shown to activate caspase-dependent pathway of tissue injury by upregulating a specialized ox-LDL receptor LOX-1 [18]. However, we failed to show upregulation of AIF expression in HCAECs. It is possible that while ox-LDL induces injury by both caspase-dependent and independent pathways, Ang II and TNF-α predominantly affect the caspase-dependent pathway.

Ox-LDL, which is present in atherosclerotic lesions [17], plays an important role in the development of atherosclerosis. Ox-LDL is cytotoxic to many cell types, including endothelial cells [19–21]. We and others have shown that ox-LDL is involved in endothelial injury, including apoptosis [15,16,22]. Our preliminary studies suggest that ox-LDL is capable of inducing injury to

HCAECs that are not entirely blocked by inhibition of caspase pathway. Identification of AIF shown in this study may well be another important pathway operative in endothelial injury when cells are exposed to ox-LDL.

AIF in some respects is similar to cytochrome *C*. Both proteins contain prosthetic groups and are found in the intermembrane space of mitochondria, and the apoptotic functions of both proteins appear to be independent of their redox capacities. Whereas cytochrome *C* clearly leads to the activation of procaspase-9, the effects of AIF appear to be largely caspase-independent.

In summary, we have demonstrated that ox-LDL upregulates AIF expression in HCAECs in a concentration- and time-dependent manner, and the upregulated expression of AIF by ox-LDL is associated with translocation of AIF from cytoplasm to the nuclei.

Acknowledgments

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