Ac-hE-18A-NH₂, a novel dual-domain apolipoprotein mimetic peptide, inhibits apoptosis in macrophages by promoting cholesterol efflux

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Abstract. A novel synthetic dual-domain apolipoprotein (apo)-mimetic peptide, Ac-hE-18A-NH₂, has been proposed to possess several apo A-I- and apo E-mimetic properties. This study investigated the protective effect of this peptide on oxidized low-density lipoprotein (ox-LDL)-induced apoptosis in RAW264.7 cells. For this purpose, RAW264.7 cells were exposed to 50 µg/ml ox-LDL for 48 h, and then incubated with the peptide Ac-hE-18A-NH₂ at various concentrations. Apoptosis was detected using annexin V-fluorescein isothiocyanate staining and flow cytometric analysis. The study revealed that the peptide Ac-hE-18A-NH₂ (1, 10 and 50 µg/ml) inhibited ox-LDL-mediated apoptosis, and this was accompanied by an increased rate of intracellular cholesterol efflux, and decreased total cholesterol levels in the cells in a concentration-dependent manner. The peptide also decreased caspase-3 activity and increased B-cell lymphoma 2 protein (Bcl-2) expression in macrophages in a dose-dependent manner. Moreover, blockage of cholesterol efflux by brefeldin A decreased the protective effect of Ac-hE-18A-NH₂ against ox-LDL induced apoptosis, while increasing the cholesterol efflux by β-cyclodextrin administration led to a marked decrease in the rate of apoptosis of the cells. These findings demonstrate that the apo-mimetic peptide Ac-hE-18A-NH₂ exerts a protective effect against apoptosis by reducing the accumulation of cholesterol.

Introduction

Apoptosis is a key event in the progression of advanced atherosclerosis. Macrophage apoptosis occurs throughout all stages of atherosclerosis. Research directed at understanding the consequences of macrophage death in atherosclerosis has revealed opposing roles of apoptosis in atherosclerotic plaque progression (1). Apoptosis of macrophages is particularly important in plaque development and breakdown and may be induced by uptake of oxidized low-density lipoprotein (ox-LDL) or by loading with free cholesterol (FC) (2). Increasing evidence suggests that elevation of cellular cholesterol has a critical role in the regulation of ox-LDL-mediated apoptosis, and factors regulating the accumulation or elimination of lipids from macrophages are critical in the formation of early atherosclerotic lesions and progression to the chronic stage of atherosclerosis (3-6). Thus, the acceleration of cholesterol efflux or prevention of the accumulation of excess cholesterol in cells may inhibit ox-LDL-induced apoptosis, which may provide new strategies for the prevention and reversal of atherosclerosis.

A dual-domain peptide has been designed that possesses the 141- to 150-residue arginine-rich domain (LRKLRKRLLRLR), of apolipoprotein (apo) E, covalently linked to the well-characterized class A amphipathic helical peptide 18A, a high-affinity lipid-associating peptide (DWLKAFYDKVA-EKLKEAF) (7). The resulting peptide (Ac-hE-18A-NH₂) has been shown to promote the rapid uptake and clearance of atherogenic apo B-containing lipoproteins in vitro and in dyslipidemic mouse models (8-10). Furthermore, in the Watanabe heritable hyperlipidemic rabbit, a single administration of the peptide Ac-hE-18A-NH₂ not only reduced plasma cholesterol levels, but also restored endothelial function (11-12).

The association of peptide Ac-hE-18A-NH₂ to apo B-containing lipoproteins is a prerequisite for the clearance of these lipoproteins (10). Certain class A peptides have been shown to remove ‘seeding molecules’ that are oxidized products of arachidonic and linoleic acids from the LDL surface (13-14). As the peptide Ac-hE-18A-NH₂ possesses a class A amphipathic helical domain that has been shown to possess several apo A-I-mimetic properties, it was
hypothesized in the present study that this peptide, in addition to significantly reducing plasma cholesterol levels, was also likely to exert a protective effect against ox-LDL-induced apoptosis of macrophages, and that this effect was likely to be associated with cholesterol efflux.

Materials and methods

Peptide synthesis. The peptide Ac-hE-18A-NH₂, with the sequence Ac-LRKLRRKLLR-DWLKAFYDKVAEKLEAF-NH₂ was synthesized using 9-fluorenlymethoxy carbonyl chemistry in an automated peptide synthesizer (PE Biosystems, Foster City, CA, USA) according to the previously described procedure (9). The purity of the synthetic peptides (typically >98%) was established by analytical high-performance liquid chromatography and confirmed by mass spectral analysis.

Cell culture. RAW264.7 (Xiangya Medical College, Changsha, China), a murine macrophage cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA) and 1% antibiotics (penicillin and streptomycin). Cells were grown at 37°C and 5% CO₂ in humidified air. To further investigate the role of cholesterol efflux on apoptosis induced by ox-LDL, in certain experiments, the cells were co-treated with Ac-hE-18A-NH₂ and β-cyclodextrin (a cholesterol efflux stimulator) or BFA (a cholesterol efflux blocker) for 24 h.

Cell viability assay. Following pre-incubation of the RAW264.7 cells (1x10⁸ cells/ml) in DMEM for 24 h, the peptide Ac-hE-18A-NH₂ (0-100 µg/ml) was added to the cells prior to incubation for 24 and 48 h, respectively. The cytotoxic effect of the peptide was then evaluated using the conventional MTT assay. A total of 25 µl MTT (Sigma, St. Louis, MO, USA) solution [5 mg/ml in phosphate-buffered saline (PBS), pH 7.4] was added and the cells were incubated for 4 h. The incubation was terminated by addition of 150 µl dimethylsulfoxide (Sigma). The absorbance at 492 nm was assessed using a Varioskan Flash (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Flow cytometric assessment of apoptosis. Cultured RAW264.7 cells were collected and washed twice with PBS. The measurement of phosphatidylserine redistribution in a plasma membrane was conducted according to the manufacturer's instructions for the Annexin V-FITC Apoptosis Detection kit (FITC, fluorescein isothiocyanate; Bender MedSystems, Vienna, Austria). Cells were resuspended in binding buffer (1X), and the cell density was adjusted to 1-10x10⁶/ml, prior to addition of 5 µl annexin V-FITC and 10 µl propidium iodide (PI; 20 µg/ml) to 100 µl cell suspension. The suspension was agitated and incubated for 15 min at room temperature, under exclusion of light. Following the addition of 400 µl binding buffer (1X), fluorescence-activated cell sorting (FACS) analysis was performed using the flow cytometer (Becton Dickinson, Franklin, NJ, USA). Annexin V⁺/PI cells were considered to be apoptotic cells.

Caspase-3 activity assay. The activity of caspase-3 was determined using the Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the activity of caspase-3, cell lysates were prepared following their respective incubation with various designated agents. Assays were performed in 96-well microtiter plates by incubating 10 µl cell lysate per sample in 80 µl reaction buffer containing 10 µl caspase-3 substrate (Ac-DEVD-pNA, 2 mM). Lysates were incubated at 37°C for 4 h. The concentration of the p-nitroanilide (pNA) released from the caspase-3 substrate was measured at 405 nm by a Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The detailed analysis procedure is described in the manufacturer's protocol.

Western blot analysis for assessment of B-cell lymphoma 2 (Bel-2) protein. Total cell lysates were separated using SDS-PAGE and the proteins were transferred onto polyvinylidene fluoride membranes (Invitrogen). The gels (Invitrogen) were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated overnight at 4°C with primary antibodies (rabbit anti-mouse monoclonal anti-Bel-2 antibody, Abcam, Cambridge, UK; anti-β-actin, Cell Signaling Technology, Inc., Danvers, MA, USA) at the appropriate dilution recommended in the product datasheet. Following three washes for 10-15 min each with TBS [0.1% (v/v)] in Tween 20 (TBST; Xiangya Medical College), the gels were incubated with horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signaling Technology, Inc.) in blocking buffer for 1 h at room temperature. Following three washes with TBST, the gels were developed with a chemiluminescence reagent (Cell Signaling Technology, Inc.) and exposed to X-rays. The expression of Bcl-2 was semi-quantitatively evaluated by Gel-Pro Analyzer software, version 4.5 (Media Cybernetics, Rockville, MD, USA) and compared with the expression of β-actin.

Cholesterol efflux assays. Experiments were performed as described by Marcil et al (15) with minor modifications. Cells were plated at a density of 5x10⁵ cells/ml in 24-well culture dishes. Following starvation for 24 h in DMEM (high glucose) with 0.2% bovine serum albumin (BSA; Invitrogen), cells were incubated in medium containing 0.5 µCi/well [³H]-labeled cholesterol and 50 µg/ml ox-LDL for 48 h. The cells were then incubated in the absence or presence of the peptide Ac-hE-18A-NH₂ at various concentrations (1, 10 and 50 µg/ml). The radioactivity in the supernatants and total cell extracts was measured by scintillation counting. The efflux was quantified as the percentage of total radioactive counts removed from the cells during the efflux period.

Cellular cholesterol/cholesteryl ester quantitation analysis. Cellular cholesterol/cholesteryl ester content was measured using a commercially available quantitation kit (Cholesterol/Cholesteryl Ester Quantiﬁcation Colorimetric Kit II; BioVision, Inc., Mountain View, CA, USA), following the manufacturer's instructions.

Statistical analysis. All results are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Differences between the groups were assessed by one-way
Results

Ac-hE-18A-NH$_2$ does not affect the viability of RAW264.7 cells. The potential cytotoxic effect of the peptide Ac-hE-18A-NH$_2$ on the viability of RAW264.7 cells was evaluated. There was no cytotoxic activity induced by the peptide at the concentrations examined (1, 10, 50 and 100 µg/ml) within 24 h (data not shown).

Ox-LDL-induced apoptosis of RAW264.7 cells and effect of Ac-hE-18A-NH$_2$ on ox-LDL-induced cell apoptosis. Ox-LDL induced apoptosis in RAW264.7 cells in a time- and concentration-dependent manner in the present study (Table I). In the absence of ox-LDL, a rate of apoptosis of merely 3.39±0.35% was detected following 48 h of incubation. Compared with the control group, a 6.5-fold increase in the rate of apoptosis in the 50 µg/ml ox-LDL group was observed, whereas a 12.2-fold increase was observed in the 100-µg/ml ox-LDL group following 48 h of incubation (Table I). Significant levels of apoptosis were obtained with ox-LDL treatment (50 µg/ml) for 24 h (24.4%) and 48 h (40.4%) (Table I).

Incubation of macrophages with Ac-hE-18A-NH$_2$ for 24 h significantly reduced the toxic effect of ox-LDL, and the protective effect was dose-dependent at ≤50 µg/ml (Fig. 1).

Table I. Ox-LDL-induced apoptosis of RAW264.7 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>% apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-24 h</td>
<td>2.57±0.25</td>
</tr>
<tr>
<td>Control-48 h</td>
<td>3.39±0.35</td>
</tr>
<tr>
<td>Ox-LDL 50 µg/ml-12 h</td>
<td>13.69±0.66</td>
</tr>
<tr>
<td>Ox-LDL 50 µg/ml-24 h</td>
<td>24.40±2.57</td>
</tr>
<tr>
<td>Ox-LDL 50 µg/ml-48 h</td>
<td>40.36±3.37</td>
</tr>
<tr>
<td>Ox-LDL 100 µg/ml-48 h</td>
<td>70.74±4.35</td>
</tr>
</tbody>
</table>

Table II. Effect of Ac-hE-18A-NH$_2$ on total cholesterol level in RAW264.7 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cholesterol (mg/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>201.2±22.2</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>590.6±23.3*</td>
</tr>
<tr>
<td>Ox-LDL+Ac-hE-18A-NH$_2$ 1 µg/ml</td>
<td>490.5±12.8*</td>
</tr>
<tr>
<td>Ox-LDL+Ac-hE-18A-NH$_2$ 10 µg/ml</td>
<td>415.0±17.8*</td>
</tr>
<tr>
<td>Ox-LDL+Ac-hE-18A-NH$_2$ 50 µg/ml</td>
<td>299.2±16.4*</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD (n=3). *P<0.001, versus control; **P<0.01 and ***P<0.001, versus ox-LDL. Ox-LDL, oxidized low-density lipoprotein.

Effect of Ac-hE-18A-NH$_2$ on caspase-3 activity and Bcl-2 protein expression. The activation of caspase-3 was analyzed by measuring the levels of pNA cleaved from the substrate N-Ac-DEVD-pNA. The activity assay showed that, following exposure to 50 µg/ml ox-LDL for 48 h, Ac-DEVD-pNA cleavage was significantly reduced in the macrophages treated with the peptide Ac-hE-18A-NH$_2$ compared with that in the cells that were not treated with the peptide. Furthermore, Ac-hE-18A-NH$_2$ reduced ox-LDL-induced caspase-3 activity in a dose-dependent manner (Fig. 2A).

Bcl-2 protein expression was confirmed by western blot analysis. The results indicated that exposure of RAW264.7 cells...
to ox-LDL reduced Bcl-2 protein expression, while treatment with Ac-hE-18A-NH$_2$ increased Bcl-2 protein expression levels in a concentration-dependent manner (Fig. 2B).

**Effect of Ac-hE-18A-NH$_2$ on cholesterol efflux and cholesterol levels in RAW264.7 cells.** Exposure of RAW264.7 cells to ox-LDL for 48 h increased total cellular cholesterol levels from 201.2±22.2 mg/g protein to 590.6±23.3 mg/g protein without significantly affecting the rate of cholesterol efflux (Table II). Co-treatment with Ac-hE-18A-NH$_2$ and ox-LDL increased the rate of cholesterol efflux (Fig. 3A), and decreased the cellular total cholesterol level (Table II). The two alterations were concentration-dependent. In addition, apo A-I was selected as a positive control in the efflux experiment in order to evaluate the efficiency of the peptide. Of note, Ac-hE-18A-NH$_2$ (50 µg/ml) was more efficient than apo A-I (10 µg/ml) in inducing cholesterol efflux (Fig. 3B).

**Effect of brefeldin A (BFA) and β-cyclodextrin (β-CD) on apoptosis and cholesterol efflux in RAW264.7 cells.** To further investigate the role of cholesterol efflux on apoptosis induced by

![Figure 2. Effect of Ac-hE-18A-NH$_2$ on caspase-3 activity and Bcl-2 protein expression levels](image)

![Figure 3. Effect of Ac-hE-18A-NH$_2$ on cholesterol efflux](image)

![Figure 4. Effect of BFA and β-CD on the apoptosis and cholesterol efflux in RAW264.7 cells](image)
ox-LDL, β-CD and BFA were utilized. β-CD has been shown to be capable of stimulating efficient cholesterol efflux from cultured human fibroblasts (16). However, BFA, an antibiotic, has been shown to inhibit high-density lipoprotein (HDL)-mediated cholesterol efflux from cholesterol-enriched cells (17).

Flow cytometric analysis demonstrated that BFA alone did not exert any effect on cellular apoptosis (data not shown). However, both the inhibition of ox-LDL-induced apoptosis and the facilitation of cholesterol efflux by Ac-hE-18A-NH₂ (50 µg/ml) were significantly attenuated by BFA. Addition of β-CD decreased the ox-LDL-induced rate of apoptosis from 17.53 to 10.89% (Fig. 4B) and increased the rate of cholesterol efflux from 25.56 to 43.03% (Fig. 4A).

Discussion

Ox-LDL has been shown to be taken up by macrophages in a rapid and uncontrolled manner, leading to the formation of cholesterol-loaded foam cells, the major cellular component of fatty streaks (18). However, ox-LDL may also modulate atherogenesis by inducing apoptosis in a variety of tissues and cell types, including human coronary artery endothelial cells (19), vascular smooth muscle cells (20) and monocyte-macrophages (21). Among these, the apoptosis of macrophages has been proposed to be crucial in the evolution of atherosclerotic plaques, and apoptotic macrophages are often concentrated in areas of plaque rupture (2). Furthermore, increasing evidence suggests that ox-LDL and oxysterols are able to induce the activation of the executioner caspase-3 (22) via the mitochondrial apoptotic pathway. In addition, ox-LDL is able to promote the overexpression of the Bcl-2-associated X-protein (Bax) (19) and reduce the expression of Bcl-2 (23), thereby promoting susceptibility to apoptosis. The present study demonstrated that exposure of macrophages to ox-LDL increases the apoptosis rate of cells in a time- and dose-dependent manner. It was also observed that ox-LDL downregulated Bcl-2 protein expression and promoted caspase-3 activity.

Although the exact mechanism of ox-LDL-induced apoptosis in macrophages remains to be elucidated, it has been suggested that treatment with ox-LDL induces accumulation of large amounts of cholesterol in macrophages and leads to failure of lipid homeostasis (24). Furthermore, cellular accumulation of excess cholesterol may serve as a trigger of apoptosis and promote ongoing inflammation, calcification, thrombosis and plaque rupture, which are the major sequelae of advanced atherosclerosis. Plasma HDL levels are inversely correlated with the risk of atherosclerotic cardiovascular disease (25). One of the most important atheroprotective roles of HDL is reverse cholesterol transport, in which excess cholesterol in macrophage foam cells undergoes efflux followed by being transported to the liver for excretion in the bile. Terasaka et al (26) demonstrated that HDL protects macrophages from FC- or ox-LDL-induced apoptosis by promoting efflux of cholesterol via the ATP binding cassette sub-family G member 1 (ABCG1) transporter (26). In addition, Jiang et al (27) reported that HDL₄ antagonizes ox-LDL-induced apoptosis in RAW264.7 cells through reducing the accumulation of toxic cholesterol (28).

Recently, HDL/apo-based mimetic peptides have been increasingly considered to be potential effective treatments for atherosclerosis. Their atheroprotective activity is also attributed to their unique ability to facilitate the reverse cholesterol transport process. A previous study demonstrated that the dual-domain peptide Ac-hE-18A-NH₂ not only reduces plasma cholesterol in dyslipidemic animal models but also exerts anti-inflammatory/antioxidant effects through its ability to scavenge ‘seeding molecules’ (28). The present study revealed that Ac-hE-18A-NH₂ inhibits apoptosis induced by ox-LDL in a dose-dependent manner. Concomitantly, Ac-hE-18A-NH₂ decreased caspase-3 activity and increased Bcl-2 expression in a similarly concentration-dependent manner. An important question that arose from this was whether the protection of macrophages from ox-LDL-induced apoptosis by Ac-hE-18A-NH₂ proceeds via promoting the intracellular cholesterol efflux in a similar manner to that of HDL. To elucidate this, cholesterol efflux and intracellular cholesterol were assessed in macrophages treated with ox-LDL and Ac-hE-18A-NH₂. The results indicated that Ac-hE-18A-NH₂ dose-dependently increased the rate of cholesterol efflux, and significantly decreased intracellular cholesterol levels in ox-LDL-treated RAW264.7 cells. However, it was not elucidated whether the protective effect of this peptide on ox-LDL-induced apoptosis was mediated via the promotion of cholesterol efflux. To investigate this possibility, β-CD and BFA were administered to stimulate or inhibit the cholesterol efflux from macrophages. β-CD is a cyclic oligomer of glucose that has the ability to sequester cholesterol in its hydrophobic core. A series of studies have demonstrated that β-CD is particularly efficient in stimulating the removal of cholesterol from a variety of cells in culture (28,29). The exposure of cells to high concentrations of β-CD (10-100 mM) results in rates of cholesterol efflux far in excess of those achieved with physiological cholesterol efflux in a similar manner.

Acknowledgements

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References


