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IL-4 induces apoptosis of endothelial cells through the caspase-3-dependent pathway

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Abstract The present study was designed to investigate the hypothesis that interleukin-4 (IL-4) can induce apoptosis of human endothelial cells and to study regulatory pathways of this process. Indeed, DNA ladder assay and flow cytometry study showed that IL-4 can induce apoptosis of endothelial cells in a time- and dose-dependent manner. In addition, IL-4 markedly increased activity of caspase-3, and inhibition of this enzyme suppressed IL-4-induced apoptosis in a dose-dependent manner. These results provide the first evidence that IL-4 can induce apoptosis of human endothelial cells. In addition, the data indicate that the caspase-3-dependent pathway is critically involved in this process.

Key words: Interleukin-4; Apoptosis; Human endothelial cell; Caspase-3; Atherosclerosis

1. Introduction

Apoptosis of vascular cells can be involved in the progression of atherosclerosis [1–3]. For example, apoptotic cells can be detected in neointima of atherosclerotic vessels, suggesting that apoptosis might participate in remodeling of the vessel wall during atherogenesis [2]. Endothelial cells may undergo apoptosis in response to the proinflammatory cytokines produced by activated macrophages and T-lymphocytes as a consequence of the ongoing local immune and inflammatory response characteristic of atherogenesis [4]. Selected cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) or interferon-γ, can promote apoptosis of endothelial cells [5,6].

Interleukin-4 (IL-4) is a pleiotropic immunomodulatory cytokine secreted by T-helper 2 (TH2) lymphocytes, eosinophils, and mast cells [7,8]. Normally, IL-4 promotes the differentiation of premature lymphocytes to the TH2 subset and induces immunoglobulin class switching in B-lymphocytes. In addition, IL-4 is present at high levels in tissues of patients with chronic inflammatory diseases [9], including atherosclerosis [10]. Evidence indicates that IL-4 may play a role in atherogenesis through induction of inflammatory responses, such as upregulation of vascular cell adhesion molecule-1 (VCAM-1) [11]. IL-4 also can synergize IL-1β, TNF-α or lipopolysaccharide (LPS)-induced VCAM-1 gene expression in endothelial cells [12–14]. Although recent evidence suggests that IL-4 can induce apoptosis of cancer cells [15], the possible involvement of this cytokine in apoptotic death of endothelial cells is unknown.

Intracellular proteases are enzymes critically involved in apoptotic cell death. Caspase-3, also known as CPP32, is a member of the IL-1β converting enzyme family of cysteine proteases [16]. Caspase-3 is composed of 17 and 12 kDa subunits derived from a common enzyme, pro-caspase-3 [17,18], and it cleaves substrates at aspartate residues. Induction of caspase-3 proteolytic activity appears to be one of the most important events in apoptosis [19,20]. In addition, caspase-3-mediated apoptosis may be modulated by cellular oxidative stress. For example, an increase in caspase-3-like protease activity in human endothelial cells was associated with the induction of apoptosis by oxidized LDL [21]. Because exposure of endothelial cells to IL-4 also can increase cellular oxidation [22], it is possible that caspase-3 may be activated by this cytokine. Therefore, the aim of the present study was to study the hypothesis that IL-4 can induce apoptosis of human endothelial cells through the caspase-3-dependent pathway.

2. Materials and methods

2.1. Cell culture and reagents

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously [23]. Cells were determined to be endothelial by their cobblestone morphology and uptake of fluorescent-labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc., Eugene, OR, USA). HUVEC from passage 2 were used in the present study. Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN, USA). Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Calbiochem (La Jolla, CA, USA).

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Abbreviations: CHX, cycloheximide; HUVEC, human umbilical vein endothelial cells; IL-1β, interleukin-1β; IL-4, interleukin-4; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; zVAD-fmk, z-Val-Ala-Asp-fluoromethylketone

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2.2. DNA ladder assay

Treated HUVEC were collected by trypsinization and suspended in 100 μl of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% NP-40. After centrifugation (3000 rpm, 5 min), supernatants were collected and treated with 1% sodium dodecyl sulfate and RNase A (5 mg/ml) for 2 h at 56°C, followed by proteinase K (2.5 mg/ml) for 2 h at 37°C. The samples were mixed with 0.5 volume of 10 M ammonium acetate and 2.5 volumes of ice-cold ethanol, and incubated for 1 h at −80°C. The DNA was precipitated by centrifugation (12 000 rpm, 20 min), washed with 80% ice-cold ethanol, air-dried, and resuspended in 50 μl of TE buffer. Aliquots of 4 μg of cellular DNA were fractionated by electrophoresis on a 2% agarose gel. After staining with SYBR® Green I (Molecular Probes, Eugene, OR, USA), the internucleosomal DNA cleavage (DNA laddering) was visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN, USA).

2.3. Flow cytometry (annexin V staining)

Annexin V binding was measured using a commercially available kit (Annexin-V-FLUOS; Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's recommendations. The positive binding was determined using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.4. Caspase-3 activity assay

Caspase-3 activity in cell extracts was measured using a Caspase-3 Cellular Activity Assay kit PLUS (Biomol, Plymouth Meeting, PA, USA). Briefly, cells were collected and lysed in an ice-cold lysis buffer containing 50 mM HEPES at pH 7.4, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1% CHAPS, and 0.1% NP-40. The cell lysate was centrifuged at 10 000 × *g* for 5 min at 4°C and the supernatant fraction was used to quantitate caspase-3 activity. Aliquots of 20 μg of cell lysate protein were incubated for 1 h at 37°C in assay buffer containing 50 mM HEPES at pH 7.4, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% glycerol and 0.2 mM Ac-Asp-Glu-Val-Asp-*p*-nitroaniline substrate. The amount of *p*-nitroaniline released by caspase-3 activity was quantitated by measuring the optical density at 405 nm. Caspase-3 activity was expressed as pmol *p*-nitroaniline released per min per μg cellular protein.

2.5. Statistical analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $P < 0.05$ was considered significant.

3. Results

3.1. IL-4 can induce endothelial cell apoptosis

To determine whether IL-4 can induce endothelial cell apoptosis, DNA fragmentation was studied after exposure of HUVEC to IL-4. As shown in Fig. 1, IL-4 in the presence of cycloheximide (CHX), which inhibits protein synthesis, dramatically induced internucleosomal DNA fragmentation. Significant DNA laddering was detected as early as 2 h after treatment with 10 ng/ml of IL-4 and reached maximum after a 4 h exposure to this cytokine (Fig. 1A).

Effects of different doses of IL-4 on DNA fragmentation are shown in Fig. 1B. DNA laddering was observed in cells treated with IL-4 at the dose of 1 ng/ml. Exposure of HUVEC to 10 ng/ml of IL-4 caused a slightly more markedly DNA fragmentation as compared to 1 ng IL-4/ml. However, an increase in IL-4 dose to 50 ng/ml did not further potentiate internucleosomal DNA fragmentation.

Flow cytometry analysis was performed to confirm that IL-4 can induce endothelial cell apoptosis. The appearance of phosphatidylserine in the outer leaflet of the plasma membrane, as measured by binding of FITC-labeled annexin V,

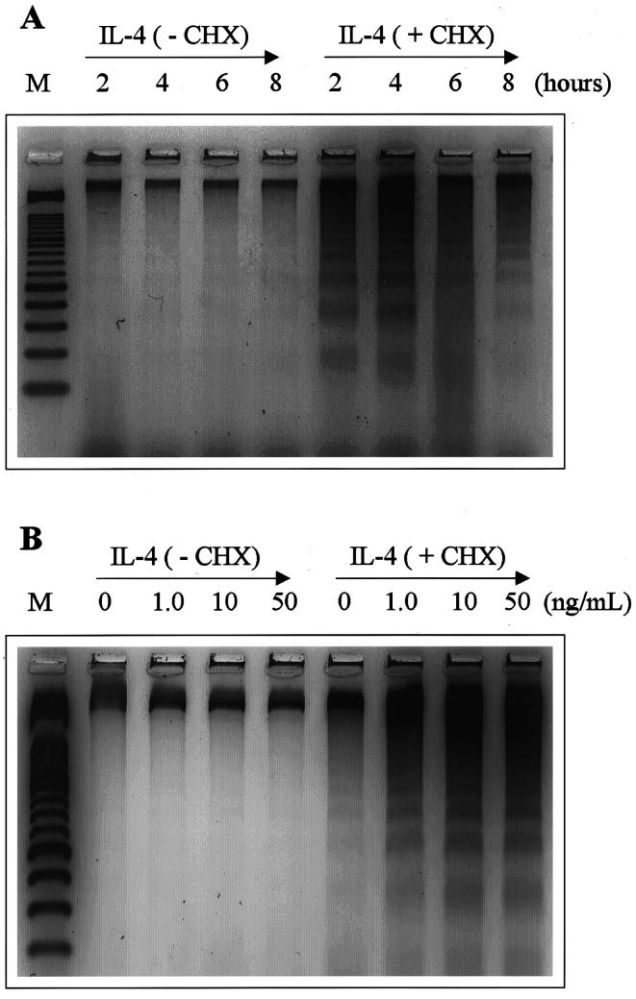

Fig. 1. IL-4 induces apoptosis of human endothelial cells. HUVEC were incubated with or without CHX (10 μg/ml) for 0.5 h and exposed to 10 ng/ml of IL-4 for the indicated time (A). In parallel experiments, HUVEC were treated with different doses of IL-4 for 4 h (B). Following treatment exposures, DNA was extracted, fractionated by 2% agarose gel electrophoresis and visualized using phosphoimaging technology. M, molecular weight markers (100 bp DNA ladder).

was increased in cells incubated with IL-4 in a dose-dependent manner as compared to controls (Fig. 2A). Treatment of endothelial cells with IL-4 changed the percentage of apoptotic cells from 22.2 ± 7.4% (control cultures) to 31.5 ± 1.62% (0.1 ng IL-4/ml), 44.8 ± 1.66% (1.0 ng IL-4/ml), and 42.9 ± 2.30% (10 ng IL-4/ml). Fig. 2B shows typical examples of histograms obtained in HUVEC treated with different doses of IL-4 as well as in control cells. In IL-4-treated cultures, populations of apoptotic cells were markedly distinguished from the overall cell population.

3.2. Caspase-3 pathway is involved in IL-4-induced endothelial cell apoptosis

To identify the regulatory pathways of endothelial cell apoptosis induced by IL-4, activity of caspase-3 was measured in HUVEC treated with this cytokine for 4 h. As indicated in Fig. 3, exposure to IL-4 resulted in a dose-dependent increase in caspase-3 activity. To further prove involvement of caspase-3 in IL-4-induced apoptosis of endothelial cells, HUVEC

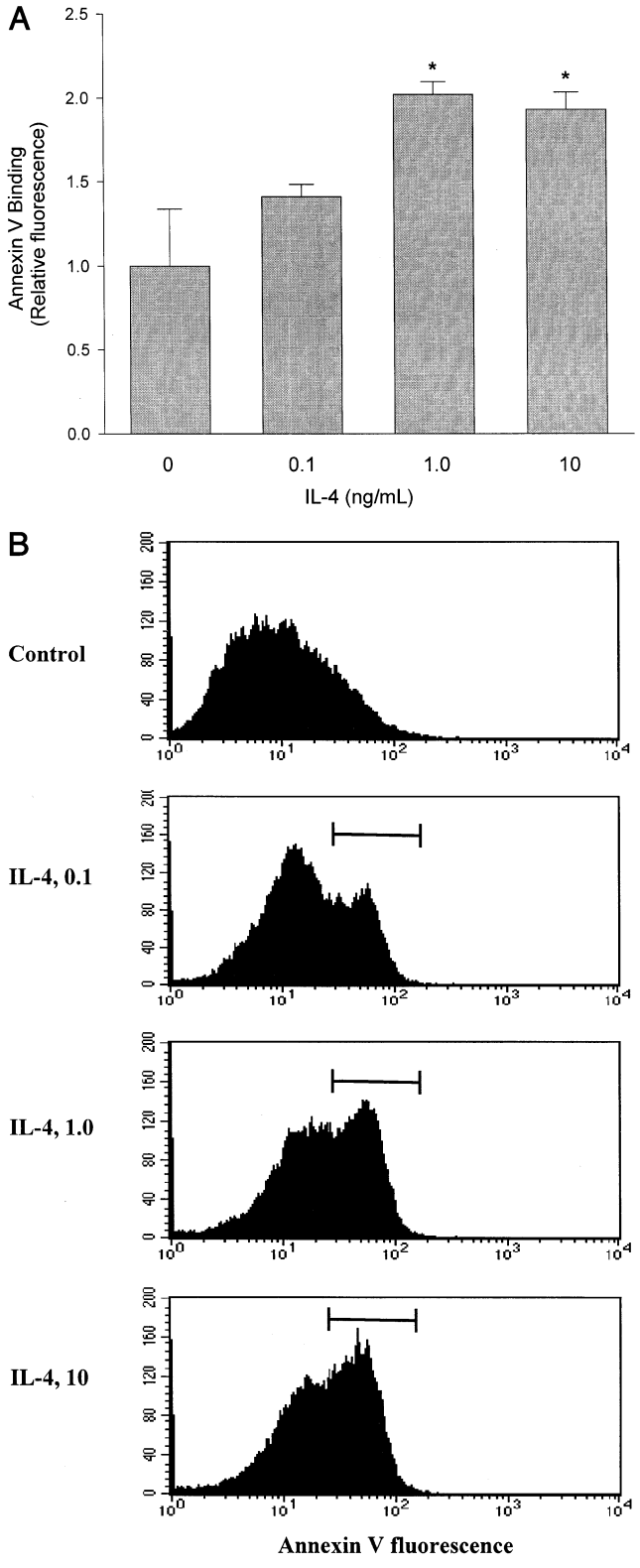

Fig. 2. A: Annexin V binding to human endothelial cells in response to IL-4 as detected by flow cytometry. HUVEC were pretreated with CHX (10 μg/ml) for 0.5 h and exposed to the indicated amounts of IL-4 for 4 h. Data are expressed as mean ± S.D. *Statistically significant as compared to the control group ($P < 0.05$). B: Representative histograms of annexin V binding to human endothelial cells in response to IL-4. HUVEC were treated as indicated to the legend to A. Populations of apoptotic cells are indicated by vertical bars.

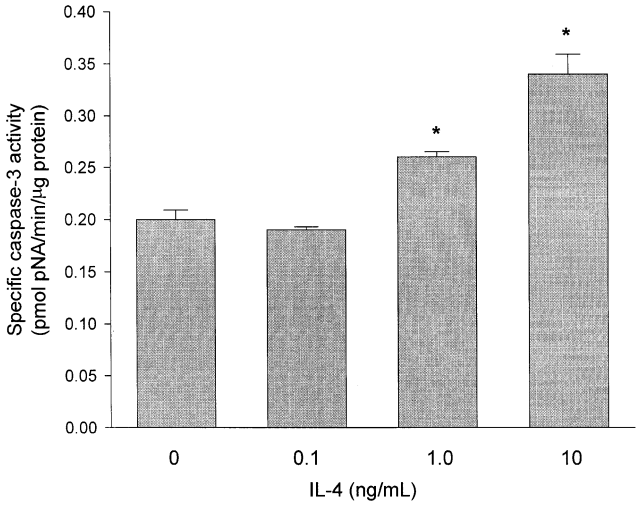

Fig. 3. Dose-dependent increase in caspase-3 activity in human endothelial cells in response to IL-4. HUVEC were pretreated with CHX (10 μg/ml) for 0.5 h and exposed to the indicated amounts of IL-4 for 4 h. Data are expressed as mean ± S.D. *Statistically significant as compared to the control group ($P < 0.05$).

were pretreated with zVAD-fmk, a specific inhibitor of this enzyme, before exposure to 10 ng/ml of IL-4. Fig. 4 indicates that zVAD-fmk markedly reversed the ability of IL-4 to induce endothelial cell apoptosis in a dose-dependent manner. Indeed, the IL-4-induced apoptosis of HUVEC was completely prevented by pretreatment with 100 μM of this inhibitor.

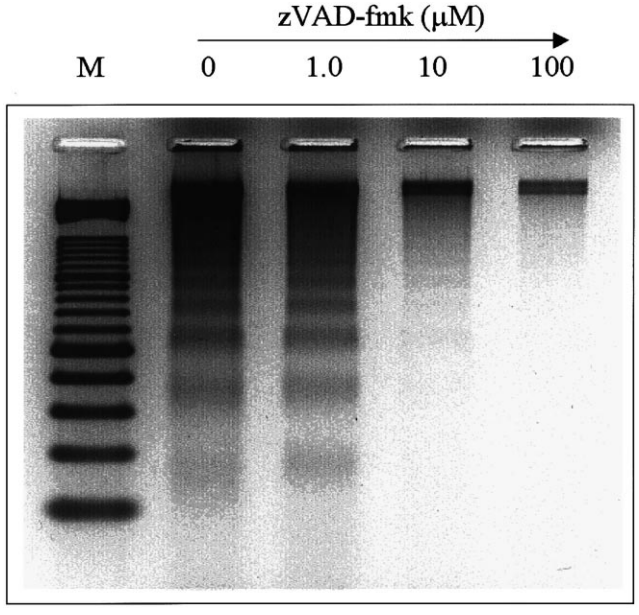

Fig. 4. Inhibition of caspase-3 prevents IL-4-induced apoptosis of human endothelial cells. HUVEC were pretreated with the indicated concentrations of zVAD-fmk for 2 h, incubated with CHX (10 μg/ml) for 0.5 h, and exposed to IL-4 (10 ng/ml) for 4 h. Following treatment exposures, DNA was extracted, fractionated by 2% agarose gel electrophoresis and visualized using phosphoimaging technology. M, molecular weight markers (100 bp DNA ladder).

4. Discussion

Results of the present study provide compelling evidence that IL-4 induces apoptosis of cultured endothelial cells via caspase-3-dependent pathway(s). Apoptosis has been characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or their multiples. These fragments are readily detected as a DNA ladder by agarose gel electrophoresis and, in fact, such a ladder pattern has been regarded as the most characteristic hallmark of apoptosis [24–26]. Therefore, to study endothelial cell apoptosis, we evaluated internucleosomal DNA fragmentation by assessing the size of DNA isolated from IL-4-treated and untreated HUVEC. Typical internucleosomal DNA fragments appeared in HUVEC treated with IL-4 in the presence of CHX. These results are consistent with previous studies, which have demonstrated that inhibition of protein synthesis may be required to promote complete DNA fragmentation of vascular endothelial cells. For example, a variety of stimuli including IL-1β, LPS, and TNF-α have been reported to induce endothelial cell apoptosis in the presence of a protein synthesis inhibitor [5,6,27]. Results of these studies, including current research report, suggest that inducible or constitutive cytoprotective proteins control endothelial cell survival.

Apart from DNA fragmentation, rearrangements of cellular plasma membranes are another marker of apoptotic cell death. Indeed, in the early stages of apoptosis, phosphatidylserine is translocated from the plasma membrane inner leaflet to the outer leaflet where it triggers recognition and phagocytosis of the apoptotic cells [28,29]. Annexin V is a Ca^{2+}-dependent phospholipid binding protein with high affinity for phosphatidylserine. Therefore, flow cytometry analysis of annexin V binding was used as a sensitive marker of apoptotic cell death [28,30–32]. Results of these studies confirm that IL-4 can markedly induce apoptosis of human endothelial cells in the presence of CHX. Because the endothelium controls several vital functions of the vascular endothelium, such as vascular permeability, tone, homeostasis, and inflammatory responses [33], apoptosis of endothelial cells can significantly affect normal metabolism of the vessel wall. In addition, apoptosis can contribute to endothelial cell loss, a prominent feature of atherosclerosis, and apoptotic endothelial cells express marked pro-coagulatory properties [3].

Caspases have been recognized to play a major role in the execution of apoptosis induced by a variety of stimuli [34]. They are normally present as inactive zymogens in the cellular cytoplasm, and their activation during cell apoptosis is achieved hierarchically, so that activation of the upstream members of this family initiates the caspase cascade by cleavage and subsequent activation of downstream caspases [35]. Among individual members of the caspase family, it appears that caspase-3 is critically involved in apoptosis. In fact, recent evidence provides a direct link between caspase-3 activation and DNA fragmentation. It was demonstrated that the cytosolic endonuclease CAD (caspase-activated deoxyribonuclease) or DFF40 (DNA fragmentation factor, 40 kDa subunit) is activated as a result of caspase-3-catalyzed cleavage of the associated inhibitor ICAD or DFF45 [19,36,37]. Our findings, especially with the specific caspase-3 inhibitor (Fig. 4), confirm that caspase-3 activation by IL-4 is involved in the regulatory pathway of endothelial cell apoptosis induced by this cytokine.

In summary, the present study provides the first evidence that IL-4 can induce apoptosis of human vascular endothelial cells. In addition, the data indicated that the caspase-3-dependent pathway is critically involved in this process.

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