Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32-mediated pathway

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Abstract Oxysterols are presumed to mediate cytotoxicity of oxidized LDL in atherosclerotic lesions. To elucidate its molecular mechanism, we established murine macrophage-like P388-D1 cells which over-express Bcl-2 protein by retrovirus-mediated gene transfer. Oxysterols (7-ketocholesterol, 25-hydroxycholesterol) induced nuclear condensation and oligonucleosomal DNA fragmentation, which were partially inhibited by Bcl-2 over-expression. Though CPP32 inhibitor suppressed the cell death in control cells, it showed no additive protection in the cells over-expressing Bcl-2. These findings indicate that oxysterols induce apoptosis via Bcl-2-inhibitable and -uninhibitable pathways, and the former depends on CPP32 activation.

Key words: Oxysterol; Apoptosis; Cytotoxicity; Bcl-2

1. Introduction

Oxysterols, or oxygenated derivatives of cholesterol, are produced in vivo by both enzymatic and non-enzymatic reactions, and have emerged as intriguing substances with diverse biological activities. Oxysterols suppress the expression of the genes which are involved in the positive balance of cellular cholesterol (low density lipoprotein (LDL) receptor, HMG-CoA reductase, and HMG-CoA synthase) at both transcriptional and post-transcriptional levels [1]. Furthermore, oxysterols are cytotoxic and inhibit cell growth of various cells [2].

Recently, it has been shown that the cell death induced by oxysterols share many common features of apoptotic cell death [3,4]. On the other hand, atheromatous lesions contain appreciable amounts of apoptotic cell death [5,6]. Together, it is conceivable that oxysterols present in oxidized LDL, at least in part, mediate apoptotic cell death in atherosclerotic lesions.

Apoptosis is a highly conserved process in various species and organs [7]. Genetic analysis of *Caenorhabiditis elegans*, a nematode, has revealed that numerous genes are involved in apoptosis. Ced-9, which negatively regulates apoptosis, has a striking structural similarity to Bcl-2 protein, the product of proto-oncogene *bcl-2* [8,9]. Bcl-2 suppress apoptosis induced by various stimuli [10]. It was postulated that Bcl-2 inhibits apoptosis by preventing lipid oxidation [11], but this theory is still controversial [12]. Recently, Yang et al. and Kluck et al. have reported that Bcl-2 prevent apoptosis by blocking release of cytochrome *c* from mitochondria [13,14]. Ced-3, which positively regulates apoptosis, is homologous to the interleukin-1β converting enzyme (ICE) [15]. Subsequent research has revealed that apoptosis can be induced by other emerging members of ICE-like proteases including CPP32/Yama/Apopain/Caspase-3 (expressed as CPP32 in this report) [16,17].

In the current study, we have characterized the oxysterol-induced cell death of murine macrophage-like cells (P388-D1) by testing the effects of over-expressed Bcl-2 protein and peptide inhibitor of CPP32.

2. Materials and methods

2.1. Reagents

Cholesterol, 7-ketocholesterol, 25-hydroxycholesterol, Hoechst 33342 (Ho33342), rhodamine 123 (Rh123) were purchased from Sigma (St. Louis, MO); propidium iodide (PI) from Nacalai Tesque (Kyoto); synthetic tetrapeptide-aldehyde Ac-DEVD-CHO [16] from Peptide Institute (Osaka). All other chemicals were of analytical grade.

Stock solutions of lipids (5 mM) resolved in ethanol and of Ac-DEVD-CHO (50 mM) in dimethyl sulfoxide (DMSO) were stored at −20°C in the dark. The final concentrations of ethanol and DMSO in the incubation medium were less than 0.5% and 1%, respectively; this concentration range had no effect in the following assays.

2.2. Cells

Murine macrophage-like cell line (P388-D1) was obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan. Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS).

2.3. Infection of cells with recombinant retroviruses

Infections of P388-D1 cells with the retrovirus vectors, containing either *neo*-resistant gene alone or *bcl-2* gene and *neo*-resistant gene controlled by a constitutive promoter, were performed as described previously [18,19]. Stable transfectants were selected based on their resistance to 500 μM of geneticin (G418). Over-expressed Bcl-2 protein was detected by ECL system (Amersham) using antisera specific for human Bcl-2 [18,19].

2.4. Analysis for DNA fragmentation

For assessment of oligonucleosomal laddering of cellular DNA, 2.5×10^5 cells were cultured in 1 ml medium containing various concentrations of lipids. After the indicated periods of incubation, DNA was extracted as described previously. Total cellular DNA was applied in 2% agarose electrophoresis gel and visualized under UV light after staining with ethidium bromide.

2.5. Staining with Hoechst 33342

P388D1 cells (1.0×10^5) on four-well chamber slides (Lab-Tek,

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Abbreviations: LDL, low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutalyl coenzyme A; ICE, interleukin-1β converting enzyme; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-CHO

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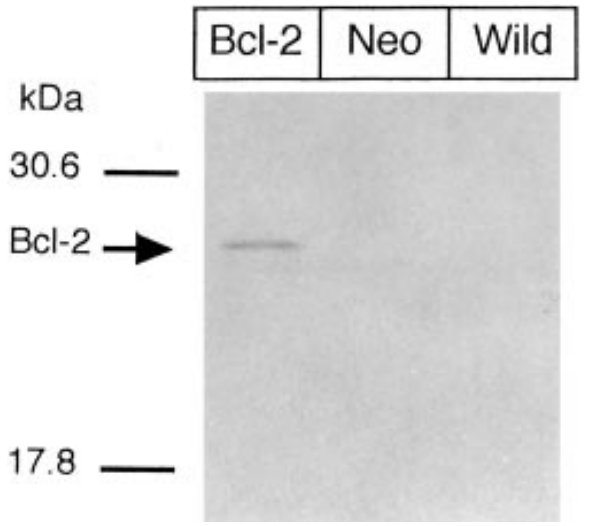

Fig. 1. Immunoblotting for Bcl-2. Over-expression of Bcl-2 were confirmed by immunoblotting (12% SDS-PAGE, 20 μg cell protein/lane). P388-D1 cells infected with retrovirus containing either *bcl-2* gene (Bcl-2) or *neo*-gene alone (neo) and non-infected cells (wild) were shown.

Nunc) were cultured in 0.4 ml RPMI medium with lipids at the conditions indicated in the figure legends. The cells were rinsed with PBS and fixed with 4% formaldehyde in PBS. Nuclei were stained with 16 μg/ml Hoechst 33342 at room temperature for 15 min and visualized under fluorescent microscope. More than 300 cells per each condition were examined and apoptotic cells (with condensed and fragmented nuclei) were detected.

2.6. Flow cytometry

Cell membrane permeability and mitochondrial membrane potential were assessed by flow cytometry using PI and Rh123, respectively [20]. Rh123 (10 μM) was added in the medium 30 min prior to the cell collection, and the cells were washed with PBS, resuspended in PI (10 μg/ml), incubated at room temperature for 5 min. The whole cells kept on ice in the dark were analyzed with a FACSort flow cytometer (Becton Dickinson).

2.7. Statistical analysis

Statistical differences of prevalence rates were determined by the Fisher's exact test. P value < 0.05 was considered significant.

3. Results

3.1. Suppression of oxysterol-induced cell death by over-expression of Bcl-2

To examine whether the over-expressed Bcl-2 protect against oxysterol-induced apoptosis, we compared the cytotoxic effects of oxysterols between P388-D1 cells over-expressing human Bcl-2 protein and non-expressing mock-transfected cells. The expression of Bcl-2 protein was confirmed by immunoblot analysis using antisera specific for the human Bcl-2 (Fig. 1). Oxysterol-induced oligonucleosomal fragmentation of DNA was suppressed in the P388-D1 cells over-expressing Bcl-2 (Fig. 2A). Similarly, the number of cells with fragmented and condensed nuclei revealed by Hoechst 33342 staining was less in the cells expressing Bcl-2 than in the non-expressing cells. Together, it is concluded that the Bcl-2 expressing cells were more resistant against the cytotoxic effects of oxysterols than the non-expressing control cells, albeit that the protective effects of over-expressed Bcl-2 was not complete.

To evaluate the changes in mitochondrial transmembrane potential ($\Delta\Psi$) and the integrity of plasma membrane, flow cytometric analysis was performed using Rh123, a lipophilic cation taken up by mitochondria in proportion to mitochondrial $\Delta\Psi$, and PI (Fig. 3). More than 80% of the cells were with high Rh123 intensity and low PI uptake before incubation with oxysterols, indicating that this cell population is viable. After incubation with oxysterols, there appeared another cluster of cells with low Rh123 intensity and high PI

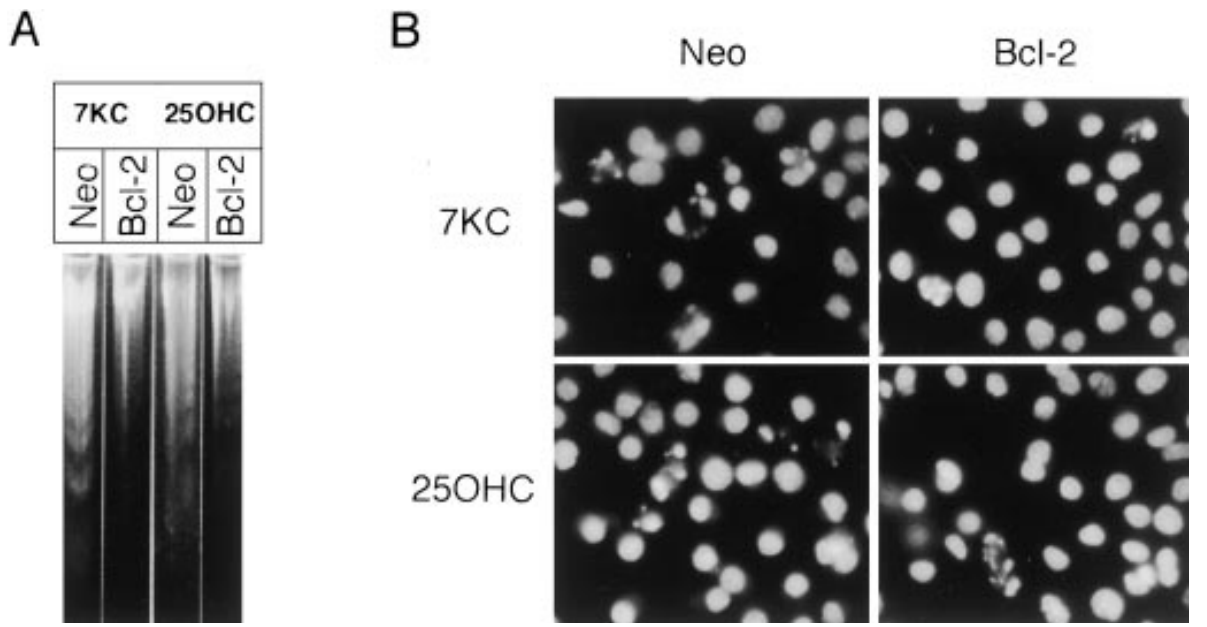

Fig. 2. Suppressive effects of Bcl-2 on DNA fragmentation (A) and nuclear condensation (B) induced by oxysterols. P388-D1 cells were cultured with 25 μM of 7-ketocholesterol (7KC), or 25-hydroxycholesterol (25OHC) for 24 h. DNA fragmentation was evaluated by electrophoresis in 2% agarose gels which were subsequently stained with etidium bromide (A). Nuclear condensation and fragmentation was examined by staining cells with Hoechst 33342 (B).

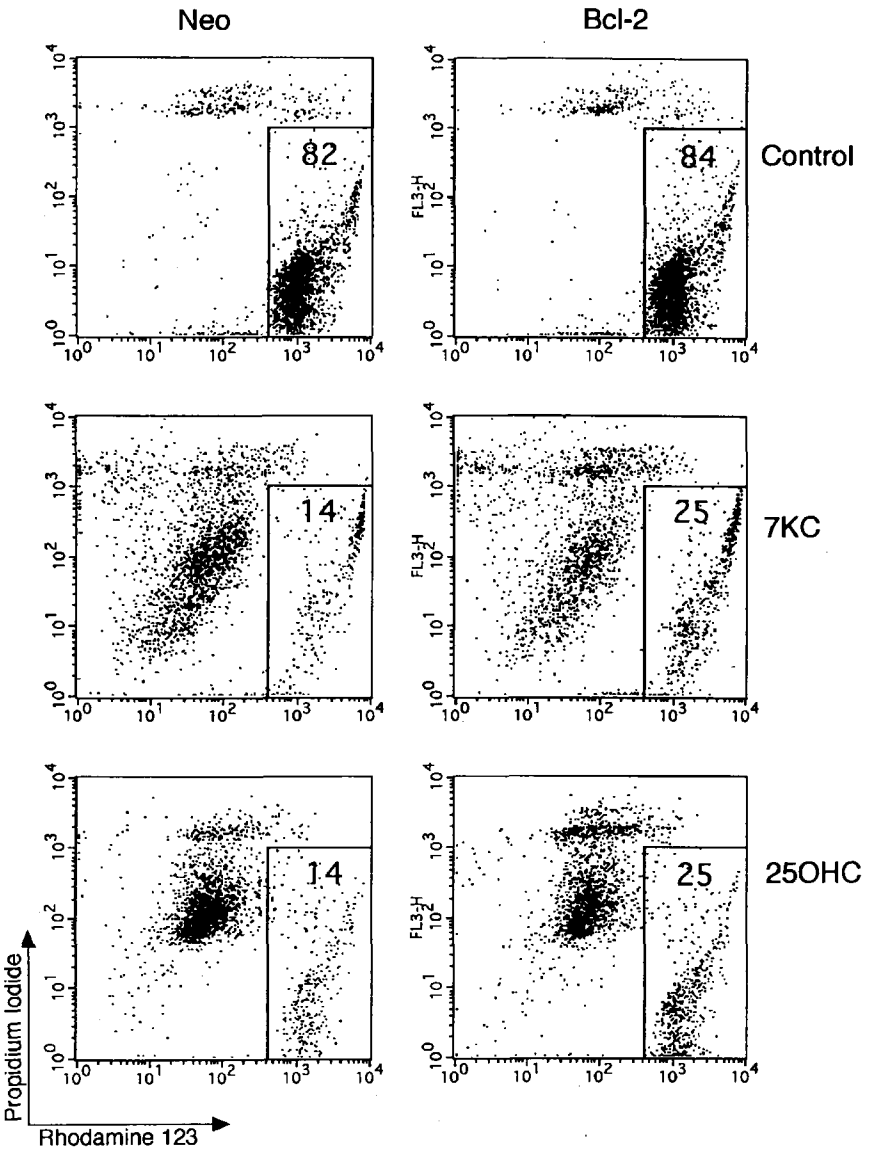

Fig. 3. Flow cytometric analysis of the oxysterol-induced changes in mitochondrial transmembrane potential and plasma membrane permeability. P388D1 cells expressing *neo*-resistant gene alone (Neo) or bcl-2 and *neo*-resistant genes (Bcl-2) were cultured for 24 h in the absence of oxysterols (control, 0.5% ethanol alone), with 7-ketocholesterol (7KC, 25 μM), or with 25-hydroxycholesterol (25OHC, 25 μM). Flow cytometry was performed after staining with Rh123 and PI. High Rh123 and low PI intensity indicates intactness of mitochondrial transmembrane potential and plasma membrane permiability, respectively. The percentage of high Rh123 and low PI intensity cells was shown to indicate the viability.

staining, which represented dead cells with decreased mitochondrial membrane potential and disruption of plasma membrane, respectively. After the treatment with oxysterol, more number of cells with high Rd 123 staining and low PI staining remained in the Bcl-2 over-expressing cells, supporting that the over-expression of Bcl-2 is protective against oxysterol-induced apoptotic cell death.

3.2. *Suppression of oxysterol-induced cell death by specific inhibitor of CPP32*

To determine the roles of CPP32 in the oxysterol-induced apoptotic cell death, the effects of a synthetic protease inhibitor of CPP32 (Ac-DEVD-CHO) on the cytotoxicity of 25-hydroxycholesterol and 7- ketocholesterol were examined (Fig. 4). In the non-over-expressing control cells, Ac-DEVD-CHO inhibited the generation of apoptotic cells to a level attained by the Bcl-2 over-expression. The inhibitor had no anti-apoptotic effect in the cells over-expressing Bcl-2, suggesting that Bcl-2 suppress the cell death by modulating Ac-DEVD-CHO sensitive pathway of cell death.

4. Discussion

LDL oxidation forms numerous lipid-soluble cytotoxic substances, such as oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, epoxycholesterols), 4-hydroxynonenal, malondialdehyde, and lysophosphatidylcholine [21]. In particular, oxysterols possess cytotoxic effects on various cells [3]. Aupeix et al. reported that 7β-hydroxycholesterol and 25-hydroxycholesterol induced apoptosis in monocytic cell lines [4].

The over-expression of Bcl-2 significantly, but partially,

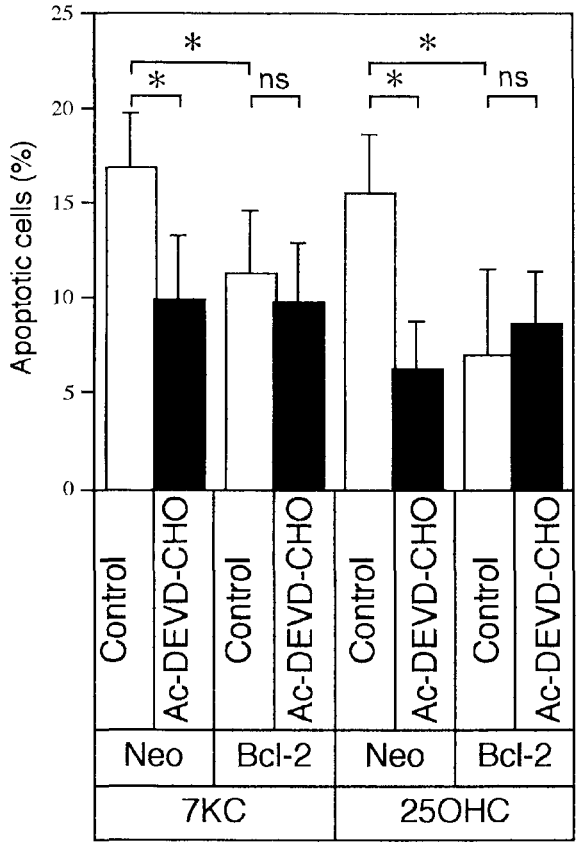

Fig. 4. Effects of the inhibitor of CPP32. P388-D1 cells were cultured in medium containing 25 μM of 25-hydroxycholesterol or 7-ketocholesterol with or without inhibitor of CPP32 (100 μM) for 24 h. Cells were stained with Hoechst 33342 and observed under a fluorescent microscope. More than 300 cells per each condition were examined and cells with condensed and fragmented nuclei were counted as apoptotic. The percentage of apoptotic versus total cells were shown (control: open column; Ac-DEVD-CHO: closed column). Error bars are upper limits of 95% confidence interval. Asterisks indicate a statistically significant difference ($P < 0.05$).

suppressed the apoptotic cell death as shown in Figs. 2 and 3. In contrast to glucocorticoid-induced apoptosis where Bcl-2 possess potent inhibitory effects [18,19], anti-apoptotic effects of Bcl-2 on oxysterol-induced cell death were far less remarkable, indicating that oxysterol-induced cell death involves two pathways; Bcl-2-inhibitable and -uninhibitable. In this concern, other biological effects of oxysterols such as suppression of cholesterol synthesis may account for the presence of multiple pathways towards apoptosis.

Recently, it has been demonstrated that the changes in mitochondrial $\Delta\Psi$ is tightly involved in the progression of apoptosis and is modulated by Bcl-2 [22]. Our results are in agreement with this hypothesis as shown in Fig. 3. Ac-DEVD-CHO, an inhibitor of CPP32, inhibited oxysterol-induced apoptosis in the non-over-expressing cells, indicating that CPP32 is involved in that pathway. In the cells over-expressing Bcl-2, however, Ac-DEVD-CHO did not show further suppression of cell death. We speculate that the over-expression of Bcl-2 directly suppresses the activity of CPP32. In other words, Bcl-2 is a upstream regulator of CPP32. Similar findings have been reported in different experimental systems [23].

Accumulating evidences have suggested that suppression of cholesterol synthesis mediates the oxysterol-induced cell death. Recently, Wang et al. have shown that sterol regulatory element binding proteins (SREBPs), the transcriptional factors which regulate the genes essential for cholesterol metabolism such as LDL receptor and HMG-CoA synthase, is converted to its active form through proteolytic cleavage by CPP32 [24]. It would be tantalizing if CPP32 is a common molecule which links the apoptosis-inducing action of oxysterols to their suppressive effects of cholesterol synthesis. Bcl-2 may antagonize cell death by changing the cellular oxidative reactions particularly in mitochondria [11]. Recently, it has been shown that the dissociation of cytochrome *c* from mitochondria is suppressed by Bcl-2 [13,14]. We propose that oxysterols may change the oxidative status in mitochondrial membrane, thereby predisposing cells to apoptotic cell death.

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