FEBS Letters 419 (1997) 276–280 FEBS 19621

Induction of apoptosis and of interleukin-1β secretion by 7β-hydroxycholesterol and 7-ketocholesterol: partial inhibition by Bcl-2 overexpression

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Received 4 November 1997

Abstract The oxysterols, 7β-hydroxycholesterol and 7-ketocholesterol, are involved in the cytotoxicity of oxidized LDL. To elucidate their molecular mechanisms, the human promonocytic leukemia cells U937 and U4 were used. U4 cells overexpressing Bcl-2 were obtained by transfection of U937 cells. 7β-hydroxycholesterol and 7-ketocholesterol induced nuclear condensation and/or fragmentation, internucleosomal DNA fragmentation, and IL-1β secretion, which were partially inhibited by Bcl-2 overexpression. These findings underline that these oxysterols could constitute major risk factors in atherosclerosis by their cytotoxicity and their ability to induce IL-1β release which might favor the recruitment of immunocompetent cells in the atherosclerotic plaque.

Key words: Oxysterol; Apoptosis; Bcl-2; IL-1β

1. Introduction

Oxysterols which result from enzymatic or non-enzymatic oxidation of cholesterol are presumed to mediate cytotoxicity of oxidized low density lipoproteins (LDL) towards different cell types [1,2]. The mode of cell death that they induce on endothelial cells [3,4], smooth muscle cells [5], and monocytic cells [6,7] present typical features of apoptosis which is a highly conserved process in various species and organs [8]. In contrast to necrosis [9], initiation of apoptosis is an active and gene-directed mode of cell death which requires de novo gene expression and new protein synthesis [10]. Some death-regulating genes (*ced* = cell death abnormal) have been identified in the nematode *Caenorhabditis elegans*, and homologous nucleic acid sequences are present in humans [8]. These particular *ced* genes which regulate cell death encode either for proteins which promote or protect from cell death [11]. Some of these gene products are involved in apoptosis induced by oxysterols. Thus, apoptosis triggered by 7-ketocholesterol or 25-hydroxycholesterol on vascular smooth muscle cells from rabbit aorta or on murine macrophage-like P388-D1 cells activates the CPP32 protease [5,7] which is a cysteinyl aspartate-specific proteinase (caspase) [12] homolog of *ced-3* gene product, and which belongs to the interleukin-1β-converting enzyme (ICE) family [13]. CPP32 promotes apoptosis by cleaving and inactivating the poly-(ADP ribose) polymerase (PARP) [14], and its activity can be inhibited with synthetic tetrapeptides such as Ac-DEVD-CHO which reduces apoptosis [5,7]. On the murine macrophage P388-D1 cells treated either with 7-ketocholesterol or with 25-hydroxycholesterol it was also reported that Bcl-2 (homolog of *ced-9* gene product which can protect from cell death in multiple contexts [15]) partially blocked apoptosis induced by oxysterols [7].

As 7β-hydroxycholesterol and 7-ketocholesterol are the main cytotoxic oxysterols present in oxidized LDL [1,16], we demonstrated in the present work that they were potent inducers of apoptosis on the human promonocytic leukemia cells U937, and we attempted to elucidate their molecular mechanisms. To this end, U4 cells which overexpressed Bcl-2 protein were established by transfection of U937 cells [17] to investigate the effect of Bcl-2 overexpression on 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis. Since the processing and the release of interleukin-1β can occur during the apoptotic process [18–20], we also asked wether 7β-hydroxycholesterol and 7-ketocholesterol could stimulate IL-1β secretion, and whether this phenomenon could be modulated by Bcl-2 overexpression.

2. Materials and methods

2.1. Transfection and cell culture

Infection of the human promonocytic leukemia cells U937 with the pSFFV vector, containing *Bcl-2* gene and *neo*-resistant gene (U4 cells), was performed by electroporation as previously described [17]. Transfected cells were selected for 14 days in the presence of 400 μg/ml of geneticin (Boehringer Mannheim, Meylan, France) and cloned by limited dilution. U937 cells were grown in suspension in culture medium consisting of RPMI 1640 medium (Gibco, Eragny, France), 2 mmol/l L-glutamine (Gibco), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) (Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Boehringer Mannheim). U4 cells were grown in the previously cultured medium, and cultured every two weeks for one passage with 200 μg/ml of geneticin (Boehringer Mannheim); the functionality of overexpressed Bcl-2 through passages was regularly assessed by increased cell survival in serum deprived medium. U937 and U4 cells were seeded at 2×10^5 per ml of culture medium, passaged twice a week and incubated at 37°C under a 5% CO_2/95% air atmosphere.

2.2. Cell treatments

7β-hydroxycholesterol and 7-ketocholesterol solutions were prepared as previously described [3,4,21], and they were introduced in the culture medium at the beginning of the culture. In all the experiments, cells were treated with 7β-hydroxycholesterol (Steraloids Inc., Wilton, USA) and 7-ketocholesterol (Steraloids Inc.) for 24 h. The purity of 7β-hydroxycholesterol and of 7-ketocholesterol determined by CPG/SM was 99% and 100%, respectively.

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PII S0014-5793(97)01473-7

2.3. Cell counting

Cell counting was performed with a hematocytometer under an inverted phase contrast microscope Laborlux IX 70 (Olympus, Tokyo, Japan).

2.4. Fluorescence microscopy

Nuclear morphology of control and treated cells was studied by fluorescence microscopy after staining with Hoechst 33342 (Sigma); apoptotic cells were essentially characterized by nuclear condensation of chromatin and/or nuclear fragmentation [22]. Hoechst 33342 was prepared extemporaneously in distilled water at 1 mg/ml, and was added in the culture medium at a final concentration of 10 μg/ml. After 1 h of incubation at 37°C, cells were washed twice in PBS, and resuspended at a concentration of 10^6 cells/ml in PBS containing 1% (w/v) paraformaldehyde. Cell deposits of about 40 000 cells were applied to glass slides by cytocentrifugation for 5 min at 15 000 rpm with a cytospin 2 (Shandon, Cheshire, UK), mounted in Fluoprep (Biomérieux, Marcy l'Etoile, France), coverslipped and stored in dark at 4°C. The morphological aspect of cell nuclei was observed with an inverted microscope Laborlux IX70 (Olympus) by using an UV light excitation, and for each sample 300 cells were examined.

2.5. Transmission electron microscopy

For transmission electron microscopy 20×10^6 cells were fixed for 1 h with 2% glutaraldehyde prepared in a 0.1 mol/l cacodylate buffer (pH 7.4), postfixed in osmium tetroxide, dehydrated with graded ethanol series, and finally embedded in Epon. Sections were stained with uranyl acetate and lead citrate, and were examined with an electron microscope H 600 (Hitachi, Tokyo, Japan).

2.6. Flow cytometry analysis of Bcl-2 expression

Intracellular Bcl-2 expression was determined by flow cytometry on U937 and U4 cells as previously described [23]. Briefly, cells were washed twice in PBS, and fixed with PBS containing 4% paraformaldehyde for 15 min at 4°C. After washing in PBS, they were incubated for 2 h at 4°C with the anti bcl-2 monoclonal antibody (Dako, Copenhagen, Denmark) diluted 1/10 in PBS/0.5% BSA/0.1% saponine. The cells were subsequently washed in PBS/0.5% BSA/0.1% saponine, and incubated for 30 min at 4°C with fluorescein-conjugated $F(ab')_2$ fragments of rabbit immunoglobulins directed against mouse immunoglobulins (Dako) diluted 1/100 in PBS/0.5% BSA/0.1% saponine. Finally, the cells were washed in PBS/0.1% saponin, resuspended in PBS, and stored in dark at 4°C until flow cytometry analysis on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Cells only stained with the fluorescent conjugate were used as control conjugate. For each assay 5000 cells were analysed. Green fluorescence signal of fluorescein was collected through a 530/30 nm band pass filter, and fluorescence intensity was measured on a logarithmic scale of 4 decades of log. Data were analyzed with the software LYSYS II (Becton-Dickinson).

2.7. Analysis of interleukin-1β secretion by ELISA

To determine interleukin-1β (IL-1β) secretion, control and treated U937 and U4 cells were used. Briefly, the cells were seeded at 2×10^6 cells per well of 6 well plates (Nunc, Roskilde, Denmark) containing 3 ml of culture medium, and immediately after the beginning of the culture 7β-hydroxycholesterol and 7-ketocholesterol were introduced in the culture medium at 5, 10, 20, 40 and 80 μg/ml. After 24 h of treatment, the culture medium of control and treated cells was collected by centrifugation, and the cells present in the pellet were counted after resuspension in phosphate-buffered saline (PBS). IL-1β concentration in the culture medium was measured according to the manufacturer's procedure with a high sensitivity ELISA Kit (R and D, Abingdon, UK) which permits to detect as few as 0.125 pg per ml of IL-1β. All measurements were performed at least in triplicate, and the data were expressed in pg of IL-1β per 10^6 cells.

2.8. DNA fragmentation assays

DNA fragmentation assays were performed by electrophoresis on 1.8% agarose gel. To this end, cellular DNA was extracted as previously described [24] by using a DNA extraction kit (Stratagene, La Jolla, CA, USA). Briefly, after overnight cell lysis at 37°C in a lysis buffer containing 10 mmol/l EDTA, 400 mmol/l NaCl, 1 mg/ml proteinase K, 35 mmol/l SDS and 10 mmol/l Tris-HCl (pH 8.2), each tube was centrifugated and the supernatant containing the DNA was precipitated by two volumes of 100% ethanol and left overnight at −20°C. After centrifugation, DNA was resuspended in 100 μl TE buffer (10 mmol/l Tris-HCl, 0.2 mmol/l Na_2EDTA, pH 7.5) before quantitation by spectrofluorimetry. The size of DNA standards used to evaluate DNA fragmentation ranged from 100 to 2072 bp (Gibco). Electrophoresis was carried out for 15 h at 20 V in 1.8% agarose gel prepared in TBE buffer (80 mmol/l Tris-Borate (pH 8.0), 2 mmol/l EDTA) and containing 0.1 μg/ml ethidium bromide. After electrophoresis, gels were examined under ultraviolet light, photographed or computerized with an images analysis system (Biocom, Les Ulis, France).

2.9. Statistical methods

Statistical analyses were performed with SYSTAT software (Evanston, IL, USA), by using a two way analysis of variance followed by a Dunnett *t*-test to compare the effects of 7β-hydroxycholesterol and of 7-ketocholesterol on U937 and U4 cells.

3. Results

3.1. 7β-Hydroxycholesterol and 7-ketocholesterol induced apoptosis

As shown in Fig. 1A, B, after 24 h of treatment in the presence of 7β-hydroxycholesterol or of 7-ketocholesterol used in a range of concentrations from 5 to 80 μg/ml, the number of U937 and of U4 cells was decreased, and simultaneously the number of U937 and of U4 apoptotic cells in-

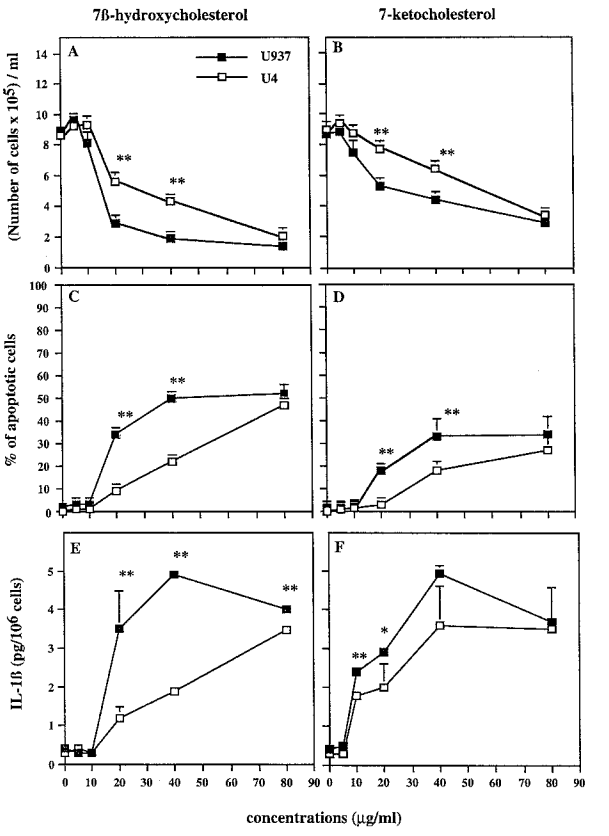

Fig. 1. Cell growth inhibition, induction of apoptotic cells, and of IL-1β secretion under treatment with 7β-hydroxycholesterol or 7-ketocholesterol. U937 cells and U4 cells (overexpressing Bcl-2) cultured in 6 well plates were treated for 24 h with 7β-hydroxycholesterol and 7-ketocholesterol used in a range of concentrations from 5 to 80 μg/ml. At the end of the incubation period, the following parameters were measured: total numbers of cells per well (A–B), proportions of apoptotic cells (C–D), and IL-1β secretion (E–F). Data are means ± S.D. of four independent experiments. Significance of the difference between U937 and U4 cells: $^*P<0.05$; $^{**}P<0.01$.

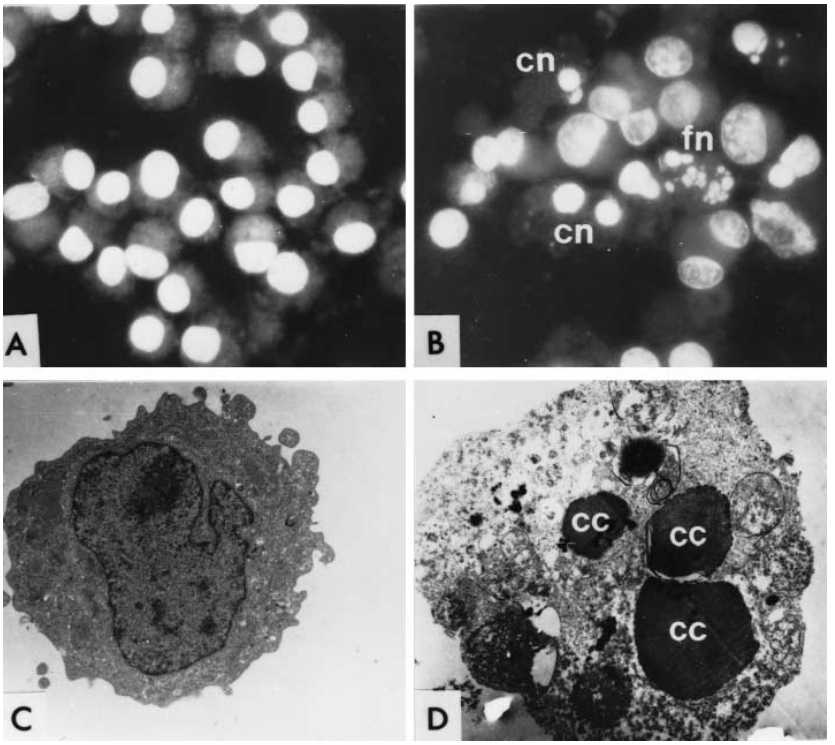

Fig. 2. Morphological characterization of oxysterols treated cells. U937 cells were incubated for 24 h in culture medium with or without oxysterols. Subsequently, U937 cells were observed by fluorescence microscopy after nuclei staining with Hoechst 33342, and by transmission electron microscopy. A and B (×1000): Fluorescence microscopy of untreated U937 cells (A) and of U937 cells treated with 20 μg/ml of 7β-hydroxycholesterol (B), condensed nuclei (cn) and fragmented nuclei (fn) are observed. B and C (×7000): Transmission electron microscopy of untreated U937 cells (C) and of U937 cells treated with 40 μg/ml of 7-ketocholesterol (D), a fragmented nucleus with condensed chromatin (cc) is observed.

creased (Fig. 1C, D). Apoptotic cells were characterized by condensed and/or fragmented nuclei which were observed by fluorescence microscopy after nuclei staining with Hoechst 33342, and by electron microscopy (Fig. 2).

3.2. *Bcl-2 overexpression partially inhibits 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis*

To examine whether Bcl-2 overexpression protect against apoptosis induced by 7β-hydroxycholesterol or 7-ketocholesterol, we compared the cytotoxic effects of these oxysterols on U937 and U4 cells at 5, 10, 20, 40 and 80 μg/ml. Bcl-2 overexpression in U4 cells was demonstrated by flow cytometry (Fig. 3). Under treatment with 7β-hydroxycholesterol or 7-ketocholesterol, we observed a significant ($P < 0.01$) higher number of U4 cells than of U937 cells, and a significant ($P < 0.01$) lower proportion of apoptotic U4 cells than of apoptotic U937 cells only at 20 and 40 μg/ml (Fig. 1A–D).

Furthermore, the ability of Bcl-2 overexpression to protect from apoptosis was assessed by a comparative analysis of the DNA fragmentation pattern on agarose gel (Fig. 4). At 5 and 10 μg/ml no DNA degradation was observed on both cell lines (data not shown). On U937 cells treated with 20 and 40 μg/ml of 7β-hydroxycholesterol or of 7-ketocholesterol an internucleosomal DNA fragmentation typical of apoptosis was detected. On U4 cells, the internucleosomal DNA degradation was suppressed when 7β-hydroxycholesterol or 7-ketocholesterol was used at 20 μg/ml. With 40 μg/ml of 7β-hydroxycholesterol or of 7-ketocholesterol similar internucleosomal DNA fragmentation patterns were revealed in U937 cells and in U4 cells. Similarly, with 80 μg/ml of 7β-hydroxycholesterol or of 7-ketocholesterol, analogous internucleosomal DNA fragmentation patterns were found on U937 and U4 cells (data not shown).

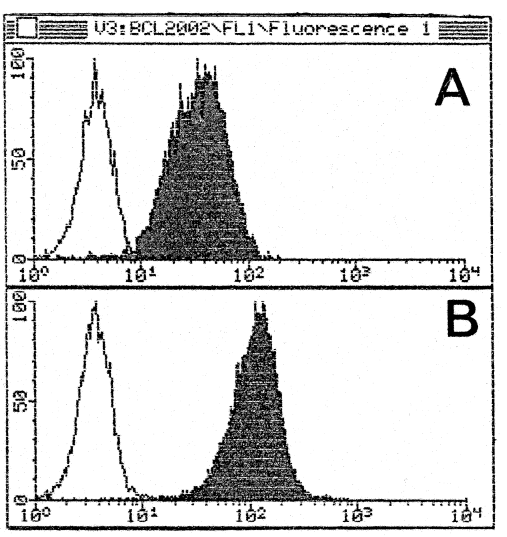

Fig. 3. Flow cytometry analysis of Bcl-2 expression. Bcl-2 expression was quantified by flow cytometry on U937 cells (A), and on U4 cells (B) transfected with the viral vector pSFFV-*neo*-*Bcl-2*. Bcl-2 expression was measured with a FACScan flow cytometer on a logarithmic scale of fluorescence. For each assay, 10000 cells were analyzed. Shaded histograms: Bcl-2 expression; unshaded histograms: conjugated control.

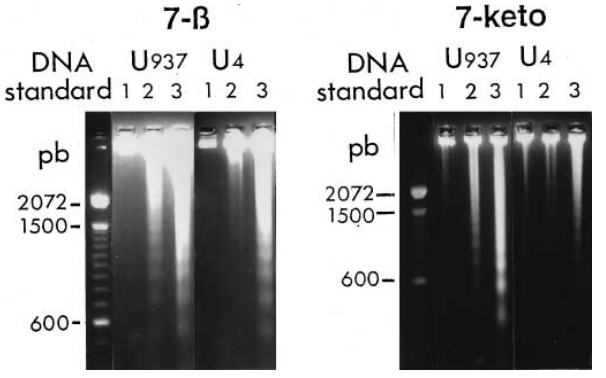

Fig. 4. Effect of Bcl-2 overexpression on the internucleosomal DNA fragmentation induced by 7β-hydroxycholesterol and 7-ketocholesterol. Analysis of DNA fragmentation by electrophoresis on 1.8% agarose gel was performed on U937, and U4 cells (overexpressing Bcl-2) which were untreated (1), or treated with 20 μg/ml (2) or 40 μg/ml (3) of 7β-hydroxycholesterol (7β) or of 7-ketocholesterol (7-keto).

3.3. *7β-Hydroxycholesterol and 7-ketocholesterol induced IL-1β secretion: partial inhibition by Bcl-2 overexpression*

Untreated U937 and U4 cells spontaneously produced low levels of IL-1β, and IL-1β secretion was strongly stimulated both in U937 and U4 cells when 7β-hydroxycholesterol or 7-ketocholesterol was added in the culture medium (Fig. 1E, F). Under treatment with 7β-hydroxycholesterol, IL-1β secretion was increased in U937 and U4 cells at 20, 40 and 80 μg/ml, and at these different concentrations IL-1β secretion was significantly ($P < 0.01$) lower in U4 than in U937 cells (Fig. 1E). With 7-ketocholesterol, IL-1β secretion was increased in U937 and U4 cells at 10, 20, 40 and 80 μg/ml, and IL-1β secretion was significantly lower in U4 than in U937 cells only at 10 μg/ml ($P < 0.01$) and 20 μg/ml ($P < 0.05$) (Fig. 1F).

4. Discussion

Increased plasma levels of LDL are considered as a risk factor for atherosclerosis [25], and oxidized LDL have been shown to induce cytotoxic effects on a variety of cell types [1,2]. Among the principal components of oxidized LDL responsible for cellular injury, oxysterols play a critical role both in vivo and in vitro [26,27], particularly those oxidized in C7 such as 7β-hydroxycholesterol and 7-ketocholesterol [3,21,28]. In the present work, which was performed on U937 and U4 cells (overexpressing Bcl-2 and obtained by transfection of U937 cells with the pSFFV-*neo-Bcl-2* vector [17]), we showed that 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis both on U937 and U4 cells, and that this mode of cell death was associated with IL-1β secretion. The most relevant protective effect of overexpressed Bcl-2 characterized both by a lower proportion of apoptotic cells, by a total inhibition of internucleosomal DNA fragmentation, and by a significant reduction of IL-1β secretion was observed only with 20 μg/ml of 7β-hydroxycholesterol and of 7-ketocholesterol. Therefore, in the range of oxysterol concentrations used (5 to 80 μg/ml), apoptosis as well as IL-1β secretion were partially prevented by Bcl-2 overexpression.

As reported in previous studies, cell death induced by oxysterols share many common features of apoptotic cell death [3,5–7], and the present work demonstrates that 7β-hydroxycholesterol and 7-ketocholesterol are potent inducers of apoptosis, not only on U937 cells but also on U4 cells whereas these later ones overexpressed a functional Bcl-2 protein which protected them from apoptosis when they were used as targets in cytotoxicity assays [17]. Since Bcl-2 overexpression partially inhibits apoptosis induced either by 7-ketocholesterol and 25-hydroxycholesterol in the murine macrophage P388-D1 cells [7] or by 7β-hydroxycholesterol and 7-ketocholesterol on U4 cells (present work), these data strongly suggest that Bcl-2 overexpression is not sufficient to inhibit apoptosis triggered by oxysterols. Thus, in contrast to glucocorticoid induced apoptosis where Bcl-2 possess potent inhibitory effects [29,30] evoking in this case a central role of mitochondria [31,32], the anti-apoptotic effects of Bcl-2 on oxysterol induced cell death are far less remarkable, indicating that oxysterol induced cell death probably involves two pathways: Bcl-2 inhibitable and uninhibitable.

The identification of IL-1β secretion associated with 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis constitutes the major new finding of this study. IL-1β is synthesized as an inactive 33 kDa propeptide [33] that must be processed by proteolytic cleavage in order to be secreted [18]. Interestingly, IL-1β secretion has been reported during apoptosis [19], and the conversion of pro-IL-1β to IL-1β is activated by certain apoptotic signals such as CD95 ligand or TNF [13,14,34]. According to these considerations, our data lead to suppose that 7β-hydroxycholesterol and 7-ketocholesterol could either activate the conversion of pro-IL-1β to IL-1β or favor the neosynthesis of this cytokine, and so stimulate IL-1β release. As interleukin-1β-converting enzyme was detected in apoptotic cells from advanced human atheroma [35], we can suppose, according to our data, that oxysterols present at elevated concentrations in human atherosclerotic lesions [36] could be involved in vivo in the induction of IL-1β synthesis by apoptotic cells. Therefore, in certain conditions and mainly in the present study, IL-1β secretion would be a product of apoptosis [19], and up to now there is no evidence that enhanced IL-1β secretion contributes to apoptosis. Indeed, knockout mice which are caspase1 deficient and therefore do not generate mature IL-1β develop normally and the ex vivo response of cells to various apoptotic stimuli is indistinguishable from cells derived from wild-type animals [12]. In addition, as 7β-hydroxycholesterol and 7-ketocholesterol can stimulate IL-8 production which is chemotactic for T lymphocytes and neutrophils at picomolar and nanomolar concentrations [37], and as IL-1 can induce both in vitro and in vivo endothelial expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 which are involved in the local adhesion and the subendothelial accumulation of T-cells and monocytes [38,39], we speculate that these oxysterols could play a critical role in the recruitment of immunocompetent cells in the atherosclerotic plaque. Interestingly, Bcl-2 overexpression was associated with a lower IL-1β secretion in U4 cells treated with 7β-hydroxycholesterol than in U4 cells treated with 7-ketocholesterol suggesting that the biological effects of oxysterols could be structure dependent as previously supposed by the various potencies of these compounds to induce apoptosis [3,5] and to inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase activity [40].

In conclusion, we confirm that Bcl-2 overexpression partially antagonize oxysterols induced apoptosis [7], and we underline that 7β-hydroxycholesterol and 7-ketocholesterol could constitute major risk factors in atherosclerosis by induc-

ing simultaneously apoptosis and IL-1β secretion. Indeed, in vivo, 7β-hydroxycholesterol and 7-ketocholesterol could similarly induced apoptosis on the cells of the vascular wall and favor IL-1β release to recruit immunocompetent cells which will further contribute to the initiation and to the development of the lesion [39].

Acknowledgements: This work was supported by the Université de Bourgogne, the Conseil Régional de Bourgogne, the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Comité Français de Coordination des Recherches sur l'Athérosclérose et le Cholestérol (ARCOL). We also thank Ms. Maryvonne Moisant and Ms. Colette Bernard (Laboratoire d'Histologie, Faculté de Médecine, Dijon, France) for technical support in electron microscopy, and the assistance of the Centre de Microscopie Appliqué à la Biologie (Université de Bourgogne, Dijon, France) for the observations by transmission electron microscopy. We also express our gratitude to Ms. Laurence Dubrez (Laboratoire d'Oncohématologie; Faculté de Médecine; Dijon; France) for the gift of U937 cells, and to Ms. Jacqueline Bréard (INSERM U341, Chatenay Malabry, France) for the gift of U937 cells transfected with pSFFV-*neo-Bcl-2* (U4 cells).

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