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Different patterns of IL-1β secretion, adhesion molecule expression and apoptosis induction in human endothelial cells treated with 7α-, 7β-hydroxycholesterol, or 7-ketocholesterol

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Abstract Among oxysterols oxidized at C7 (7α-, 7β-hydroxycholesterol, and 7-ketocholesterol), 7β-hydroxycholesterol and 7-ketocholesterol involved in the cytotoxicity of oxidized low density lipoproteins (LDL) are potent inducers of apoptosis. Here, we asked whether all oxysterols oxidized at C7 were able to trigger apoptosis, to stimulate interleukin (IL)-1β and/or tumor necrosis factor (TNF)-α secretion, and to enhance adhesion molecule expression (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin) on human umbilical venous endothelial cells (HUVECs). Only 7β-hydroxycholesterol and 7-ketocholesterol were potent inducers of apoptosis and of IL-1β secretion. TNF-α secretion was never detected. Depending on the oxysterol considered, various levels of ICAM-1, VCAM-1 and E-selectin expression were observed. So, oxysterols oxidized at C7 differently injure and activate HUVECs, and the α- or β-hydroxyl radical position plays a key role in apoptosis and IL-1β secretion.

Key words: Adhesion molecule; Apoptosis; Human endothelial cell; Interleukin-1β; Tumor necrosis factor-α; Oxysterol

1. Introduction

It is now well established that early events occurring in the pathogenesis of atherosclerosis include oxidation of low density lipoproteins (LDL), accumulation of oxidized LDL (LDLox) at the sub-endothelial level [1], extravasation of leukocytes such as monocytes or lymphocytes [2,3] and accumulation of LDLox in tissue monocytes/macrophages to form foam cells that pile up in the atherosclerotic lesion [4]. It has also been demonstrated that LDLox are strongly cytotoxic on cultured endothelial cells [5], and that they induce on these cells massive apoptosis [6] as well as adhesion molecule expression [7,8] such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin which are overexpressed on endothelial cells from hypercholesterolemic fed rabbits and from hypercholesterolemic patients [9,10], and which also play a major role in extravasation of monocytes and lymphocytes recruited during the atherosclerotic process [11]. Since LDLox are a complex mixture of lipid hydroperoxides, aldehydes, and oxysterols [4] it remains to be determined what components can mimic the various effects of LDLox at the endothelial level.

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To date, it is well known that oxysterols which are a family of molecules resulting either from peroxidation of cholesterol [12], from enzyme-catalyzed transformation [13] or from autooxidation of cholesterol in air [14], have a wide range of biological activities similar to those of LDLox [15–17].

Particularly, aorta of rabbits fed with concentrated oxysterols exhibit diffuse fibrous lesions [18]; toxic effects of oxysterols have also been identified in cultured endothelial cells [19], and we have demonstrated in our laboratory that 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis in bovine aortic endothelial cells [20]. Similar features of cell death were also observed on human umbilical venous endothelial cells (HUVECs) treated with 7-ketocholesterol [21] or with 7β-hydroxycholesterol [22]. It has also been reported that the human promonocytic leukemia cells U937 incubated with 7β-hydroxycholesterol and 7-ketocholesterol released interleukin (IL)-1β in the culture medium [23]. However, up to now, the cytotoxic effects of 7α-hydroxycholesterol have not been studied.

Since processing and release of IL-1β and/or of tumor necrosis factor (TNF)-α (known to modulate adhesion molecule expression involved in the extravasation of immunocompetent cells [8]) can occur during the apoptotic process [23,24], we investigated in the present work whether all oxysterols oxidized at C7 (7α-, 7β-hydroxycholesterol, and 7-ketocholesterol) were able to induce apoptosis, to stimulate IL-1β and/or TNF-α secretion, and to enhance the expression of adhesion molecules (i.e. ICAM-1, VCAM-1 and E-selectin) involved in the recruitment of immunocompetent cells especially at the early stages of atherosclerosis [4].

2. Materials and methods

2.1. Cultures of human umbilical venous endothelial cells (HUVECs)

HUVECs were obtained from umbilical cord veins as previously described [25]. HUVECs were seeded at 2.5×10^6 cells in 75-cm^2 tissue culture flasks (Falcon/Becton-Dickinson, Plymouth, UK) containing 15 ml of culture medium consisting of medium 199 with Earle's salts, 2.2 g/l $NaHCO_3$, amino acids and Glutamax I (Gibco, Eragny, France), supplemented with antibiotics (10 U/ml penicillin, 10 μg/ml streptomycin) (Gibco), and 20% heat-inactivated fetal calf serum (Boehringer-Mannheim, Meylan, France). At confluence, HUVECs were treated with a solution of 0.05% trypsin/0.02% EDTA (Gibco). At the first passage and further, they were cultured in the above growth medium plus 100 μg/ml endothelial cell growth supplement (Sigma) and 90 μg/ml heparin (Sigma) [26]. HUVECs were grown at 37°C in a humidified atmosphere containing 5% CO_2, and all subsequent experiments were done at the second passage. To check that HUVECs were used in a non-proliferating state, analysis of cell proliferation was performed by flow cytometry [27], and for all experiments, at least 80% of the cells were in the G0/G1 phase of the cell cycle.

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2.2. Cell treatments

7α- and 7β-hydroxycholesterol were purchased from Steraloids (Wilton, USA), and 7-ketocholesterol was provided by Sigma-Aldrich (L'Isle d'Abeau-Chesnes, France). The purity of oxysterols determined by CPG/GSM was 100%. 7α-, 7β-hydroxycholesterol, and 7-ketocholesterol solutions were prepared as previously described [20,21,23], and used at 10, 20, and 40 μg/ml final concentrations. Treatments with oxysterols were performed either for 20 or 48 h on confluent non-proliferating HUVECs.

2.3. Quantitative determination of internucleosomal DNA fragmentation by ELISA

DNA fragmentation analysis was carried out by a photometric enzyme immunoassay for the quantitative and qualitative in vitro determination of cytoplasmic histone-associated DNA fragments with the cell death detection ELISA^{plus} kit (Boehringer-Mannheim, Meylan, France) according to the manufacturer's protocol. Data were expressed by the ratio: enzyme activity in treated cells/enzyme activity in untreated cells (% control) [28]. All assays were performed at least in triplicate, and the absorbance was read on a Biomek 2000 (Beckman, Palo Alto, CA, USA) at 405 nm.

2.4. Nuclear staining with Hoechst 33342

Nuclear morphology of control and treated cells was studied after staining with Hoechst 33342 (Sigma, St. Louis, MO, USA) as previously described [20,21]. Apoptotic cells were characterized by condensed and/or fragmented nuclei [29]. The morphological aspect of nuclei was observed with an inverted Laborlux IX70 microscope (Olympus, Tokyo, Japan) by using UV light excitation, and 300 cells were examined per sample.

2.5. In situ detection of DNA fragmentation

In situ visualization of DNA fragmentation at the single cell level was performed by the TdT-mediated dUTP-biotin nick end-labelling method (TUNEL method) developed by Gavrieli et al. [30] by using the MEBSTAIN apoptosis kit (Immunotech, Marseille, France) according to the manufacturer's procedure. The signal of TdT-mediated dUTP-biotin nick end-labelling was detected by incubation for 30 min at room temperature with peroxidase-conjugated streptavidin (Dako, Copenhagen, Denmark) diluted 1:300 in PBS/0.2% BSA, and determination of peroxidase activity was performed with 3,3′-diaminobenzidine using the Dako Liquid DAB Substrate Chromogen System (Dako). HUVECs were counterstained with methylene blue (RAL/Rhône-Poulenc, Villers Saint-Paul, France). The slides were mounted in Fluoprep (Biomérieux, Marcy l'Etoile, France), coverslipped, and stored in the dark at 4°C. Observations were made with an inverted Laborlux IX70 microscope (Olympus).

2.6. Analysis of IL-1β and TNF-α by ELISA

To determine the secretion level of IL-1β and TNF-α, control and treated HUVECs were used. Briefly, the cells were seeded at 25 000 cells per well of 96-well plates (Falcon/Becton-Dickinson) containing 120 μl of culture medium, and 7α-, 7β-hydroxycholesterols and 7-ketocholesterol were then introduced in the culture medium at 10, 20 or 40 μg/ml. After 20 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 PBS. IL-1β and TNF-α concentrations in the culture medium were measured according to the manufacturer's procedure with high sensitivity ELISA kits Quantikines H.S. (R&D, Abingdon, UK) which allows a detection level as low as 0.125 pg/ml of IL-1β, and 0.5 pg/ml of TNF-α. All assays were performed at least in triplicate, the absorbance was read at 490 nm on a Biomek 2000 (Beckman, Palo Alto, CA, USA), and the data were expressed in pg of IL-1β or TNF-α per 10^6 cells.

2.7. Quantification of cell adhesion molecules (VCAM-1, ICAM-1, and E-selectin) by ELISA

To quantify adhesion molecule expression, control and treated HUVECs were used. Briefly, the cells were seeded at 25 000 cells per well of 96-well plates (Falcon/Becton-Dickinson) containing 120 μl of culture medium, and 7α-, 7β-hydroxycholesterols and 7-ketocholesterol were then introduced in the culture medium at 10, 20 or 40 μg/ml. After 20 h of treatment, the culture medium of control and treated cells was collected by centrifugation, the cells were washed twice in PBS, and the primary antibodies (mouse anti-human VCAM-1, mouse anti-human ICAM-1, or mouse anti-human E-selectin from R&D, Abingdon, UK) were added to each well, and incubated for 1 h at 4°C (50 μl per microwell of a 10 μg/ml primary antibody solution diluted in PBS/1% BSA/0.01% NaN_3). At the end of the incubation period, the cells were washed 4 times with PBS/1% BSA, and then incubated for 1 h at 4°C with a peroxidase conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA, USA) diluted 1:500 in PBS/1% BSA. The microwells were further washed 4 times with PBS, and the enzymatic activity was detected by the addition of 100 μl of a orthophenylenediamine solution (0.4 mg/ml) containing 0.002% hydrogen peroxide. After 15 min of incubation at room temperature, the reaction was stopped by addition of 50 μl of H_2SO_4 (2N). All assays were performed at least in triplicate, and the absorbance was read on a Biomek 2000 (Beckman, Palo Alto, CA, USA) at 490 nm. Cells only stained with the peroxidase-conjugated goat anti-mouse IgG were used as a conjugated control. To estimate the involvement of IL-1β secretion in adhesion molecules expression, control HUVECs were treated with various concentrations of recombinant IL-1β (R&D, Abingdon, UK): 200, 2000 and 20 000 pg of recombinant IL-1β for 10^6 cells.

2.8. Statistical methods

Statistical analyses were performed with SYSTAT software (Evanston, IL, USA) by using a one-way analysis of variance (ANOVA) followed by a Dunett *t*-test.

3. Results

3.1. Induction of apoptosis by oxysterols oxidized at C7 position

The mode of cell death, apoptosis vs. necrosis, induced on HUVECs treated with 7α-, 7β-hydroxycholesterol or 7-ketocholesterol for 20 and 48 h was defined with biochemical and morphological criteria. Whatever the time of treatment, exposure of HUVECs to 7α-hydroxycholesterol (10, 20 and 40 μg/ml) did not induce internucleosomal DNA fragmentation quantified by ELISA (Fig. 1), and the morphological aspect of 7α-hydroxycholesterol-treated cell nuclei observed after staining with Hoechst 33342 was the same as those of untreated cells (Fig. 2A). Similarly, when HUVECs were incubated with 7-ketocholesterol (10, 20 μg/ml) or 7β-hydroxy-

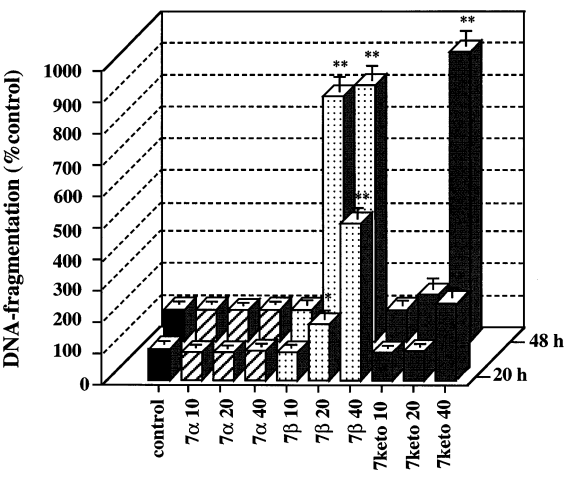

Fig. 1. Effects of oxysterols oxidized at C7 on DNA fragmentation. HUVECs were treated for 20 and 48 h with 10, 20 or 40 μg/ml of 7α-hydroxycholesterol (7α), 7β-hydroxycholesterol (7β) and 7-ketocholesterol (7keto), and DNA fragmentation was determined by a quantitative determination of internucleosomal DNA fragmentation by ELISA. Values are means ± S.E.M. and reflect 3 separate experiments, each performed in triplicate. ** and * indicate the values different ($P < 0.01$ and $P < 0.05$, respectively) from control.

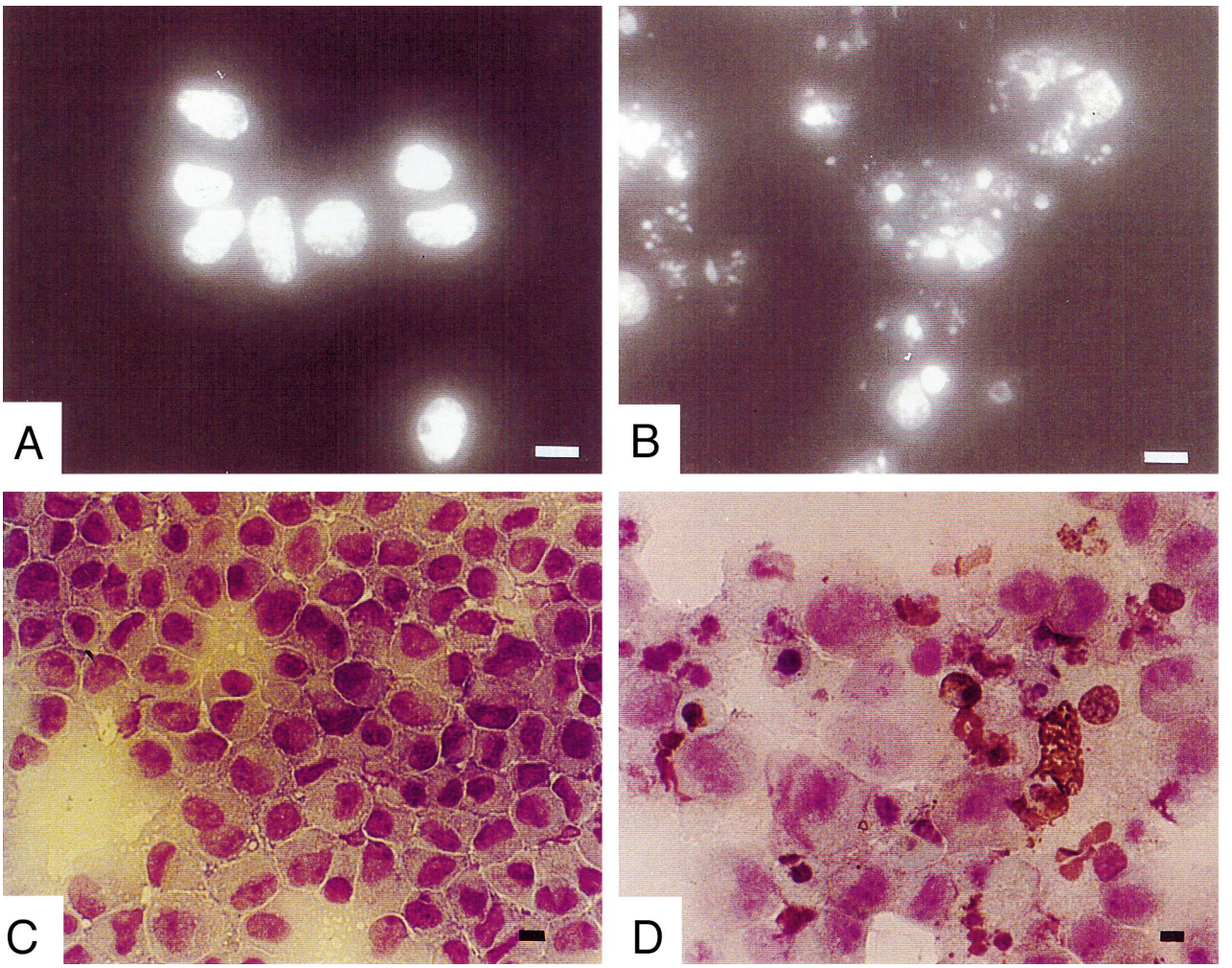

Fig. 2. Morphological characterization of cell death by nuclear staining with Hoechst 33342 and by the TUNEL method. HUVECs treated for 20 or 48 h with 7α-, 7β-hydroxycholesterol and 7-ketocholesterol used at 10, 20 or 40 μg/ml were characterized by fluorescence microscopy after nuclear staining with Hoechst 33342, and by brightfield microscopy after in situ identification of internucleosomal DNA fragmentation with the TUNEL method. Fluorescence microscopy of untreated HUVECs (A), and of HUVECs treated for 48 h with 7-ketocholesterol (40 μg/ml) (B) (apoptotic cells with condensed and/or fragmented nuclei are observed). Brightfield microscopy of untreated HUVECs (C) and of HUVECs treated for 20 h with 40 μg/ml of 7β-hydroxycholesterol (D) (the fragmented DNA revealed by the TUNEL method in the nuclei of apoptotic cells is colored in brown; it is only detected in treated cells). Scale bar = 5 μm.

cholesterol (10 μg/ml) no internucleosomal DNA fragmentation (Fig. 1) and no morphological changes of the nuclei were observed. In contrast, as early as after 20 h of treatment, 7β-hydroxycholesterol (20 and 40 μg/ml) and 7-ketocholesterol (40 μg/ml) were potent inducers of DNA fragmentation known to occur during apoptosis (Fig. 1), but the highest percentages of DNA fragmentation were observed at 48 h. Cell death by apoptosis was confirmed by nuclei staining with Hoechst 33342, and by the in situ detection of DNA fragmentation with the TUNEL method. Thus, as compared with untreated HUVECs exhibiting regular nuclei (Fig. 2A), 7β-hydroxycholesterol (20 and 40 μg/ml) and 7-ketocholesterol (40 μg/ml) induced apoptotic cells characterized by condensed and/or fragmented nuclei after Hoechst staining (Fig. 2B), and the TUNEL method revealed the presence of fragmented DNA in some condensed and/or fragmented nuclei (Fig. 2D) whereas no DNA fragmentation was found in untreated cells (Fig. 2C). According to these data, further experiments related to IL-β and TNF-α secretion, as well as to adhesion molecule expression, were performed at 20 h of treatment to define the effects of oxysterols at an early time of the apoptotic process.

3.2. *Effects of 7α-, 7β-hydroxycholesterol and 7-ketocholesterol on the secretion of IL-1β and TNF-α*

To investigate the possibility of cytokine release in oxysterol-treated endothelial cells, we measured the secretion of TNF-α and IL-1β in the supernatants of HUVECs incubated either with 7α-, 7β-hydroxycholesterol or 7-ketocholesterol (10, 20 or 40 μg/ml) for 20 h. No secretion of TNF-α was detected for any of the oxysterols considered, and with 7α-hydroxycholesterol a slight but not significant increase of IL-1β was found (Fig. 3). However, when compared to untreated HUVECs, IL-1β production was significantly higher in endothelial cells treated with 7-ketocholesterol (20 and 40 μg/ml), and the most important effects were obtained with 7β-hydroxycholesterol which induced a 30-fold increase of IL-1β secretion at 40 μg/ml and a 10-fold increase at 20 μg/ml (Fig. 3).

3.3. Effects of 7α-, 7β-hydroxycholesterol and 7-ketocholesterol on ICAM-1, VCAM-1 and E-selectin expression

To define whether oxysterols oxidized at C7 position induced the expression of adhesion molecules on the surface of endothelial cells, HUVECs were treated with 7α-, 7β-hydroxycholesterol and 7-ketocholesterol at concentrations of 10, 20 or 40 μg/ml for 20 h, and the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin was measured using an ELISA assay. The surface expression of ICAM-1 was increased by oxysterol treatments but varied according to the oxysterol considered (Fig. 4A). Therefore, 7β-hydroxycholesterol had a stronger effect on ICAM-1 expression than either 7α-hydroxycholesterol or 7-ketocholesterol. Indeed, a 40-μg/ml concentration of 7β-hydroxycholesterol induced a 5-fold increase of ICAM-1 expression vs. 3-fold and 1.5-fold increases with 7α-hydroxycholesterol and 7-ketocholesterol, respectively. With 10 and 20 μg/ml, only 7β-hydroxycholesterol treated cells exhibited a significant increase of ICAM-1 expression with 2- and 3.5-fold increases, respectively. VCAM-1 expression was significantly induced by all oxysterols at the concentration of 40 μg/ml (Fig. 4B). However, VCAM-1 expression was also significantly increased at a concentration of 20 μg/ml on 7β-hydroxycholesterol-treated cells. As for E-selectin, all oxysterols induced its expression at 20 and 40 μg/ml but at levels varying according to the type of oxysterol considered (Fig. 4C). Therefore, 7β-hydroxycholesterol appeared to be the most potent inducer of E-selectin expression and 7-ketocholesterol the least.

3.4. Effects of recombinant IL-1β on ICAM-1, VCAM-1 and E-selectin expression

To define whether enhanced IL-1β secretion was sufficient to increase ICAM-1, VCAM-1 and E-selectin expression, HUVECs were treated for 20 h with 200, 2000 and 20 000 pg of recombinant IL-1β per 10^6 cells. In those conditions, incubation of HUVECs with recombinant IL-1β did not induce sufficient expression of ICAM-1, VCAM-1 and E-selectin when compared to the secretion of IL-1β (around 1–4 pg per 10^6 cells) found in oxysterol-treated cells. Indeed, at least 2000 pg of recombinant IL-1β were required to induce similar levels of VCAM-1 expression compared to those observed in 7β-hydroxycholesterol treated HUVECs. In addition, even 20 000 pg of recombinant IL-1β remained insufficient to reach the ICAM-1 and E-selectin expression levels found especially in 7β-hydroxycholesterol-treated cells. Therefore, IL-1β is certainly not the only factor controlling adhesion molecule expression in oxysterol-treated HUVECs.

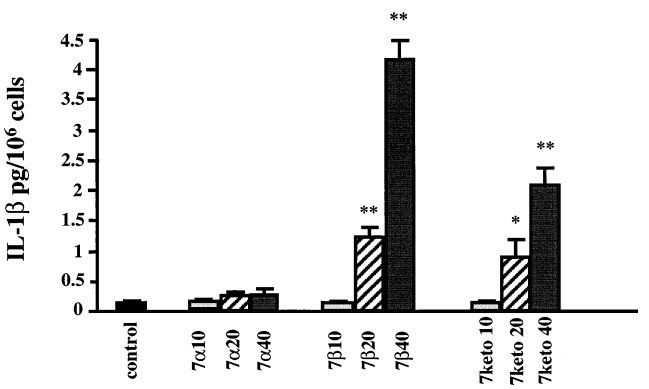

Fig. 3. Quantification of IL-1β secretion in oxysterol-treated HUVECs. Cells were incubated for 20 h with 10, 20 or 40 μg/ml of 7α-hydroxycholesterol (7α), 7β-hydroxycholesterol (7β) and 7-ketocholesterol (7keto), and the supernatants were used to determine IL-1β secretion. Values are means ± S.E.M. and reflect 3 separate experiments, each performed in triplicate. ** and * indicate the values different ($P < 0.01$ and $P < 0.05$, respectively) from control.

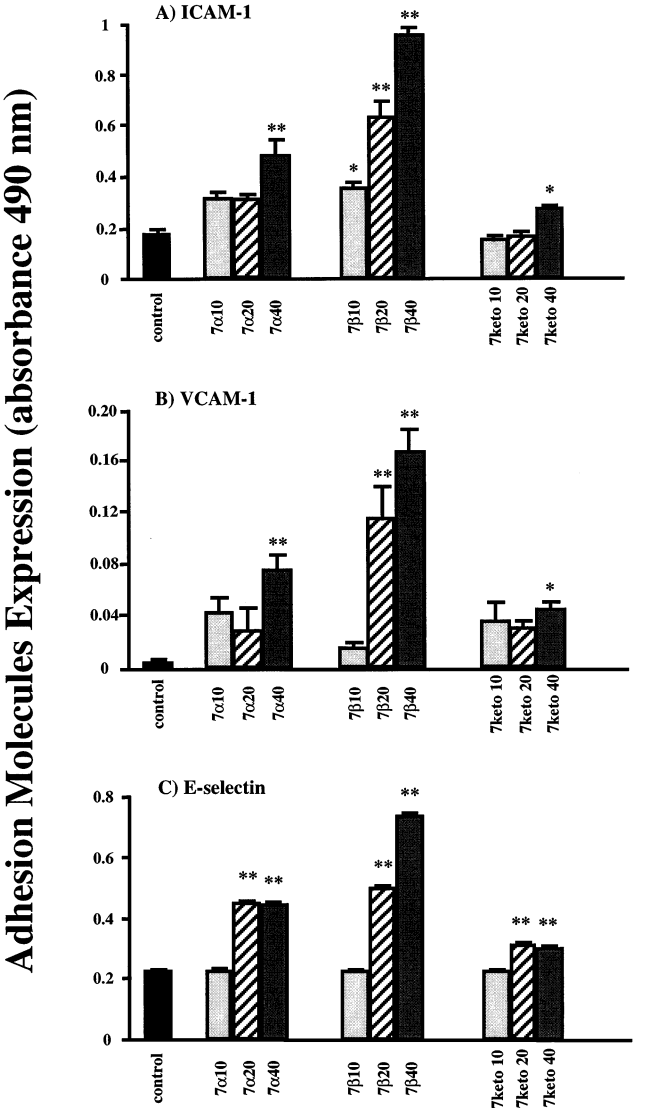

Fig. 4. Effect of 7α, 7β-hydroxycholesterol and 7-ketocholesterol on ICAM-1, VCAM-1 and E-selectin expression. HUVECs were plated in 96-well plates, and incubated for 20 h with 10, 20 or 40 μg/ml of 7α-hydroxycholesterol (7α), 7β-hydroxycholesterol (7β) and 7-ketocholesterol (7keto). The expression of adhesion molecules ICAM-1 (A), VCAM-1 (B) and E-selectin (C) was determined by ELISA. Values are means ± S.E.M. and reflect 3 separate experiments, each performed in triplicate. ** and * indicate the values different ($P < 0.01$ and $P < 0.05$, respectively) from control.

4. Discussion

The early steps of atherosclerosis need the interaction of monocytes and T lymphocytes with vascular endothelial cells. The recruitment of these circulating blood cells is mediated by specific adhesion molecules such as ICAM-1, VCAM-1 or E-selectin expressed by activated endothelial cells in a process analogous to acute or chronic inflammation [31] where

VCAM-1 interacts with very late antigen-4 (VLA-4), which is a β1 integrin that is differentially expressed on lymphocytes and monocytes but not on polymorphonuclear leukocytes [32]. This process requires the release of IL-1β and/or TNF-α involved in monocyte and lymphocyte recruitment by stimulating the endothelial expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin [33]. We hypothesized that this release of cytokines could be mediated by the endothelial cells under the action of oxysterols since we have previously reported on U937 cells that 7β-hydroxycholesterol and 7-ketocholesterol-induced apoptosis was associated with an enhanced secretion of IL-1β [23]. Taken together, these different observations have led us to consider whether all oxysterols oxidized at C7 (7α-, 7β-hydroxycholesterol, and 7-ketocholesterol) were able to induce apoptosis, to stimulate IL-1β and/or TNF-α secretion as well as the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin on human umbilical venous endothelial cells (HUVECs). In the present work, we demonstrated on HUVECs that only 7β-hydroxycholesterol and 7-ketocholesterol, but not 7α-hydroxycholesterol, were potent inducers of apoptosis and of IL-1β secretion, and that the levels of adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin) depend on the oxysterol considered. Noteworthy, an induction of TNF-α secretion was never observed.

Interestingly, in agreement with previous reports, 7β-hydroxycholesterol and at a lower extent 7-ketocholesterol induced apoptosis on endothelial cells [20,21]. However, 7α-hydroxycholesterol was not an inducer of apoptosis, and had no cytotoxic effects (data not shown) when cell death was characterized at 20 and 48 h with criteria previously used to demonstrate the toxicity of oxysterols, i.e. loss of cell adhesion, and increased permeability to propidium iodide [20,21]. So, these observations provide new arguments indicating that 7β-hydroxycholesterol and 7-ketocholesterol are able to mimic the cytotoxic effects of LDLox by inducing apoptosis [20–22] but they also suggest that the biological effects of oxysterols oxidized at C7 vary according to the nature of C7 bond, and that the position (α or β) of the hydroxyl radical plays a critical role in the induction of the apoptotic process.

As for cytokine secretion, only 7β-hydroxycholesterol and 7-ketocholesterol induced significant IL-1β secretion, but no TNF-α secretion was observed either with 7α-, 7β-hydroxycholesterol and 7-ketocholesterol. Noteworthy, early in the apoptotic process (20 h of treatment), we observed with 7β-hydroxycholesterol and 7-ketocholesterol (especially at 40 μg/ml) a positive correlation between the percentage of DNA fragmentation (corresponding to the ability of the oxysterol to induce apoptosis) and the level of IL-1β secretion per cell. Thus, 7β-hydroxycholesterol and 7-ketocholesterol, which are potent inducers of apoptosis, induced also the highest levels of IL-1β secretion whereas only a slight but not significant secretion of IL-1β was observed under treatment with 7α-hydroxycholesterol which had no cytotoxic effects. As IL-1β is synthesized as a promolecule which is proteolytically processed by the interleukin-1β-converting enzyme (ICE) belonging to the cysteinyl aspartate-specific proteinase family (caspases) which are activated during apoptosis [34], our data lead to suppose that 7β-hydroxycholesterol and 7-ketocholesterol could activate the conversion of pro-IL-1β to IL-1β during cell death. This provides additional arguments indicating that 7β-hydroxycholesterol and 7-ketocholesterol are able to mimic, at least in part, the effects of LDLox [35,36]. As LDLox containing high levels of 7β-hydrocholesterol and of 7-ketocholesterol [37] have also been described to induce TNF-α secretion on human monocytes/macrophages [38], we asked whether a similar phenomenon could occur on HUVECs treated with oxysterols oxidized at C7. The inability of 7α-, 7β-hydroxycholesterol and 7-ketocholesterol to induce the secretion of TNF-α suggests that the expression and/or the secretion of this cytokine is either under the control of other compounds present in the LDLox, or depends on the cell type considered.

The quantification of ICAM-1, VCAM-1, and E-selectin on HUVECS treated with oxysterols oxidized at C7 constitutes the major new finding of this study. Indeed, we report for the first time that oxysterols can induce adhesion molecule expression on endothelial cells, and that this phenomenon occurs early (after 20 h of treatment) during apoptosis. Therefore, the overexpression of adhesion molecules observed on endothelial cells from hypercholesterolemic subjects [9,10] could be due to enhanced concentrations of oxysterols present in the plasma [39], and/or to oxysterol deposits found in the atherosclerotic lesions of these patients [40]. It is obvious that 7β-hydroxycholesterol, which is the most potent inducer of apoptosis and of IL-1β secretion, also stimulates the highest level of adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin). However, 7-ketocholesterol which also triggered apoptosis and stimulated IL-1β secretion, induced a lower expression of adhesion molecule than 7α-hydroxycholesterol which has no cytotoxic effects. Therefore, on HUVECs treated with 7α-, 7β-hydroxycholesterol or 7-ketocholesterol it seems that IL-1β secretion could not be directly related to adhesion molecules expression in contrast to what has been previously suggested for macrophages [24] or HUVECs deprived of growth factors [41]. This hypothesis is reinforced by our observations performed with recombinant IL-1β, which has biological activities similar to the naturally occurring forms of the molecule [42]. Thus, similar levels of VCAM-1 were obtained only when HUVECs were treated with high concentrations of recombinant IL-1β, but even at very high concentrations of recombinant IL-1β the levels of ICAM-1 and E-selectin were lower than those obtained on 7β-hydroxycholesterol-treated cells. Therefore, in oxysterol-treated cells, IL-1β is probably not the only component involved in the expression of ICAM-1, VCAM-1, and E-selectin. As apoptosis of HUVECs induced by growth factor deprivation or mitomycin C is associated with a paracrine induction of ICAM-1 and VCAM-1 under the action of IL-1β but also of other unidentified inflammatory mediators [41], it is possible that oxysterol-induced adhesion molecules on HUVECs could be mediated by a similar manner by some cytokines secreted during cell death. Indeed, as the percentage of DNA fragmentation was higher at 48 h of treatment than at 20 h, this leads us to suggest that some viable cells are still present at 20 h. Therefore, our data raise the possibility that mediators released by apoptotic cells may act in a paracrine fashion to induce adhesion molecule expression by adjacent viable cells [41].

In conclusion, the biological activities of oxysterols oxidized at C7 are tightly regulated since the different effects observed on HUVECs depend on the radical (hydroxyl or keto) present at C7, but also on the α or β position of the hydroxyl radical. According to our observations, 7α-, 7β-hydroxycholesterol

and 7-ketocholesterol could play a critical role in atherosclerosis by inducing functional and antigenic modifications at the endothelium level. Thus, by triggering apoptosis of endothelial cells, IL-1β secretion and/or adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin), oxysterols oxidized at C7 could not only increase the permeability of the vascular endothelium to LDL, but also favor the adhesion and extravasation of monocytes and lymphocytes, which are the main leukocytes found in atheromatous lesions.

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References

[1] Ross, R. (1995) Annu. Rev. Physiol. 57, 791–804.
[2] Khan, B.V., Parthasarathy, S.S., Alexander, R.W. and Medford, R.M. (1995) J. Clin. Invest. 95, 1262–1270.
[3] Holvoet, P. and Collen, D. (1994) FASEB J. 8, 1279–1284.
[4] Berliner, J.A. and Heinecke, J. (1996) Free Radic. Biol. Med. 20, 707–727.
[5] Sevanian, A., Hodis, H.N., Hwang, J., McLeod, L.L. and Peterson, H. (1995) J. Lipid Res. 36, 1971–1986.
[6] Exargueil-Blanc, I., Meilhac, O., Pieraggi, M.T., Arnal, J.F., Salvayre, R. and Nègre-Salvayre, A. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 331–339.
[7] Amberger, A., Maczek, C., Jurgens, G., Midraetis, D., Schelt, G., Taieb, K., Eberl, T., Tindal, S., Xo, Q. and Wick, G. (1997) Cell Stress Chaperones 2, 94–103.
[8] Pober, J.S. and Cotran, R.S. (1990) Physiol. Rev. 70, 427–451.
[9] Cybulsky, M.I. and Gimbrone, M.A. (1991) Science 251, 788–791.
[10] Davies, M.J., Gordon, J.L., Gearing, A.J.H., Piggott, R., Woolf, N., Katz, D. and Kyriakopoulos, A. (1993) J. Pathol. 171, 223–229.
[11] Springer, T.A. (1994) Cell 76, 301–314.
[12] Kamido, H., Kuksis, A., Marai, L. and Myher, J.J. (1992) FEBS Lett. 304, 269–272.
[13] Martin, C.M. and Nicholas, H.J. (1973) J. Lipid Res. 14, 618–624.
[14] Smith, L.L. (1987) Chem. Phys. Lipids 44, 87–125.
[15] Hughes, H., Mathews, B., Lenz, M.L. and Guyton, J.R. (1994) Arterioscler. Thromb. Vasc. Biol. 14, 1177–1185.
[16] Sevanian, A., Hodis, H.N., Hwang, J., McLeod, L.L. and Peterson, H. (1995) J. Lipid Res. 36, 1971–1986.
[17] Guardiola, F., Codony, R., Addis, P.B., Rofecas, M. and Boatella, J. (1996) Food Chem. Toxicol. 7, 285–332.
[18] Imai, H., Werlthessen, N.T., Taylor, B. and Lee, K.T. (1976) Arch. Pathol. Lab. Med. 100, 565–572.
[19] Henning, B. and Boissonneault, G.A. (1987) Atherosclerosis 68, 255–261.
[20] Lizard, G., Deckert, V., Dubrez, L., Moisant, M., Gambert, P. and Lagrost, L. (1996) Am. J. Pathol. 148, 1625–1638.
[21] Lizard, G., Moisant, M., Cordelet, C., Monier, S., Gambert, P. and Lagrost, L. (1997) J. Pathol. 183, 330–338.
[22] Harada Shiba, M., Kinoshita, M., Kamido, H. and Shinokado, K. (1998) J. Biol. Chem. 273, 9681–9687.
[23] Lizard, G., Lemaire, S., Monier, S., Gueldry, S., Néel, D. and Gambert, P. (1997) FEBS Lett. 419, 276–280.
[24] Hogquist, K.A., Nett, M.A., Unanue, E.R. and Chaplin, P.D. (1991) Proc. Natl. Acad. Sci. USA 88, 8485–8489.
[25] Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. (1973) J. Clin. Invest. 52, 2745–2756.
[26] Maciag, T., Cerundolo, J., Isley, S., Kelley, P.R. and Forand, R. (1979) Proc. Natl. Acad. Sci. USA 76, 5674–5678.
[27] Vindelow, L.L., Christensen, I.J. and Nissen, N.I. (1983) Cytometry 3, 323–327.
[28] Dimmeler, S., Haendeler, J., Nehls, M. and Zecher, A.M. (1997) J. Exp. Med. 185, 601–607.
[29] Lizard, G., Fournel, S., Genestier, L., Dhedin, N., Chaput, C., Flacher, M., Mutin, M., Panaye, G. and Revillard, J.P. (1995) Cytometry 21, 275–283.
[30] Gavrieli, Y., Sherman, Y. and Ben Sasson, S.A. (1992) J. Cell Biol. 119, 493–501.
[31] Springer, T.A. (1990) Nature 396, 425–434.
[32] Bochner, B.S., Luscinskas, F.W., Gimbrone Jr., M.A., Newman, W., Sterbusky, S.A., Derse-Anthony, C.P., Klunle, D. and Schleimer, R.P. (1991) J. Exp. Med. 173, 1553–1556.
[33] Carlos, T.M. and Harlan, J.M. (1994) Blood 84, 2068–2101.
[34] Nicholson, D.W. and Thornberry, N.A. (1997) Trends Biochem. Sci. 22, 299–306.
[35] Thomas, C.E., Jackson, R.L., Ohlweiler, D.F. and Ku, G.J. (1994) J. Lipid Res. 35, 417–427.
[36] Grasing, K., Rifici, V., Patel, M., Patel, A., Dennis, R. and Kostis, J.B. (1997) J. Invest. Med. 45, 474–482.
[37] Colles, S.M., Irwin, K.C. and Chisolm, G.M. (1996) J. Lipid Res. 37, 2018–2028.
[38] Jovinge, S., Ares, M.P., Kallin, B. and Nilsson, J. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 1573–1579.
[39] Koopman, B.J., Van Der Molen, J.C., Wolthers, B.G. and Vanderpas, J.B. (1987) J. Chromatogr. 416, 1–13.
[40] Carpenter, K.L.H., Taylor, S.E., van der Veen, C., Williamson, B.K., Ballantine, J.A. and Mitchinson, M.J. (1995) Biochim. Biophys. Acta 1256, 141–150.
[41] Hebert, M.J., Gullans, S.R., Mackemie, H.S. and Brady, H.R. (1998) Am. J. Pathol. 152, 523–532.
[42] Casagli, M.C., Borri, M.G., Bigio, M., Rossi, R., Nucci, D., Bossu, P., Boraschi, D. and Antoni, G. (1989) Biochem. Biophys. Res. Commun. 162, 357–363.