Characterization and Comparison of the Mode of Cell Death, Apoptosis Versus Necrosis, Induced by 7β-Hydroxycholesterol and 7-Ketocholesterol in the Cells of the Vascular Wall

Gérard Lizard, Serge Monier, Catherine Cordelet, Laurence Gesquière, Valérie Deckert, Serge Gueldry, Laurent Lagrost, Philippe Gambert

Abstract—Oxidized low density lipoproteins (LDLs) play a central role in atherosclerosis, and their toxicity is due, at least in part, to the formation of oxysterols that have been shown to induce apoptosis in various cell types. As 7β-hydroxycholesterol and 7-ketocholesterol are the major oxysterols found in oxidized LDLs, we have investigated and compared the mode of cell death, apoptosis versus necrosis, that they induce in the cells of the vascular wall, ie, endothelial cells, smooth muscle cells, and fibroblasts. To this end, human vascular endothelial cells from umbilical cord veins (HUVECs), human artery smooth muscle cells, A7R5 rat smooth muscle cells, MRC5 human fibroblasts, and human fibroblasts isolated from umbilical cord veins were taken at confluence and incubated for 48 hours with 7β-hydroxycholesterol or 7-ketocholesterol (concentration range, 5 to 80 µg/mL). In all cells, both 7β-hydroxycholesterol and 7-ketocholesterol exhibited toxic effects characterized by a loss of cell adhesion and an increased permeability to propidium iodide. In oxysterol-treated endothelial and smooth muscle cells, typical features of apoptosis were revealed: condensed and/or fragmented nuclei were detected by fluorescence microscopy after staining with Hoechst 33342, oligonucleosomal DNA fragments were visualized in situ in the cell nuclei by the TdT-mediated dUTP-biotin nick-end labeling (TUNEL) method, and internucleosomal DNA fragmentation was found on agarose gel. In contrast, in oxysterol-treated fibroblasts, fragmented and/or condensed nuclei were never revealed, and no DNA fragmentation was observed either by the TUNEL method or by DNA analysis on agarose gel, indicating that these oxysterols induced necrosis in these cells but not apoptosis. In addition, acetylated Asp-Glu-Val-L-aspartic acid aldehyde (an inhibitor of Asp-Glu-Val-L-aspartic acid–sensitive caspases) prevented 7β-hydroxycholesterol– and 7-ketocholesterol–induced cell death in HUVECs and smooth muscle cells but not in fibroblasts. Thus, 7β-hydroxycholesterol and 7-ketocholesterol have dual cytotoxic effects on the cells of the vascular wall by their ability to induce apoptosis in endothelial and smooth muscle cells and necrosis in fibroblasts. (Arterioscler Thromb Vasc Biol. 1999;19:1190-1200.)

Key Words: cell death ■ endothelial cells ■ smooth muscle cells ■ fibroblasts ■ oxysterols

Oxidized LDLs play a critical role in the development of atherosclerotic lesions,1,2 and in vitro experiments have revealed that they are toxic to different cell types, including endothelial cells,3 smooth muscle cells,4,5 and fibroblasts.6 The cytotoxicity of oxidized LDLs has been alternatively linked to the formation of various aldehydes,7 to the conversion of phospholipids to lysophospholipids,8 as well as to the oxidation of cholesterol to oxysterols.9 More specifically, a number of in vivo and in vitro studies have suggested a determinant role of oxysterols in inducing cytotoxicity in the vascular wall.10–14 In addition, increased levels of oxysterols were reported in hypercholesterolemic human plasma,15–17 in the arterial wall from hypercholesterolemic rabbits,18 and in atheromatous plaques from hypercholesterolemic patients.19 The mode of cell death, ie, apoptosis versus necrosis, mediated by oxysterols in the cells of the vascular wall was only recently addressed. Apoptotic cells were observed in vivo in atherosclerotic lesions,20–22 and in our previous works, we have shown that sterols oxidized at position 7, ie, 7β-hydroxycholesterol and 7-ketocholesterol, were highly cytotoxic to cultured bovine aortic endothelial cells and induced a mode of cell death by apoptosis characterized by the occurrence of cells with condensed and/or fragmented nuclei and internucleosomal DNA fragmentation;23 in human vascular endothelial cells isolated from umbilical cord veins (HUVECs), we described additional similar features of apoptosis under treatment with 7-ketocholesterol.24 However, it is noteworthy that variations in the cytotoxicity of oxysterols...
were reported from one cell type to another, suggesting that the characteristics of apoptosis and of the mode of cell death might differ among cell types of the vascular wall. In addition, cell death by necrosis and apoptosis can lead to the activation of serine proteases and of the cysteine proteases named caspases, respectively, and in vivo necrosis is associated with an inflammatory response, but apoptosis is not. According to these considerations, characterization of cell death induced by oxysterols has some pathophysiologic and therapeutic ramifications for the understanding of the atherosclerosis process as well as for treatment of the lesions.

Therefore, the aim of the present work was to characterize the cytotoxicity of 7β-hydroxycholesterol and 7-ketocholesterol in a comparative study involving the different cell types of the vascular wall, ie, endothelial cells, smooth muscle cells, and fibroblasts. To this end, cultured HUVECs, human artery smooth muscle cells, A7R5 rat smooth muscle cells, MRC5 human fibroblasts, and human fibroblasts from umbilical cord veins were taken at confluence and incubated in the presence of increasing concentrations of either 7β-hydroxycholesterol or 7-ketocholesterol (concentration range, 5 to 80 μg/mL). Subsequently, cellular viability was assessed by counting adherent and nonadherent cells as well as by measuring the cellular permeability to propidium iodide (PI). The mode of cell death, necrosis versus apoptosis, was described. They were isolated by enzymatic digestion from umbilical vein segments filled with 0.5% collagenase type IV–coated tissue-culture plastic flasks were made by incubating 8 μg of collagen type IV in 100 μL PBS per cm² of surface area of tissue-culture flask for 1 hour at room temperature.

**Methods**

**Cell Culture**

HUVECs were obtained from umbilical cord veins as previously described. They were isolated by enzymatic digestion from 20-cm-long umbilical cord vein segments filled with 0.5% collage- nase (Boehringer Mannheim). After a 15-minute incubation at 37°C, umbilical cord vein segments were perfused with 40 mL PBS for collecting the cells. After centrifugation for 5 minutes at 900g, the cell pellet was resuspended in Ham's F10 medium (Gibco) supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin [Gibco], and 0.25 mg/mL amphotericin B [Gibco]) and 10% heat-inactivated FCS (Boehringer Mannheim). For all experiments, the cultures were used at confluence after 10 days of culture, and they were used at either the first or second passage. Rat smooth muscle cells (A7R5) were isolated from the thoracic aortas of 14- to 17-day-old embryonic BDIX rats. The cells were obtained from the American Type Culture Collection (Manassas, Va.). A7R5 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) containing 2.5 mmol/L L-glutamine, 40 U/mL penicillin, 40 mg/mL streptomycin (Gibco), and 10% heat-inactivated FCS (Boehringer Mannheim). They were seeded at 3×10⁴/cm² in the previously described culture medium. For all experiments, cells were used at confluence after 3 days of culture, and they were used between passages 20 and 30.

**Smooth Muscle Cells**

Human artery smooth muscle cells isolated from umbilical arteries (BioWhittaker/Clonetics, Walkersville, Md) were cultured in smooth muscle growth medium 2 (BioWhittaker) containing 10 μg/mL bovine insulin, 2 μg/mL human recombinant fibroblast growth factor, 0.2 μg/mL human recombinant epidermal growth factor, antibiotics (gentamicin, 100 μg/mL; amphotericin B, 10 μg/mL), and 5% FBS (all from BioWhittaker). The cells were seeded at 3×10⁴/cm² in the previously described culture medium. For all experiments, cells were used at confluence after 10 days of culture, and they were used as fresh as possible.

**Fibroblasts**

Human fibroblasts were isolated from umbilical cord veins as follows. After endothelial cells were removed from umbilical cord veins as previously described, the umbilical segments were successively incubated twice with 0.5% collagenase (Boehringer Mannheim) for 15 minutes at 37°C and washed with PBS. At the second incubation with collagenase, cells were collected in PBS by centrifugation for 5 minutes at 900g. The resulting cell pellet was resuspended in modified Eagle’s medium with Earle’s salts (Gibco) containing 2 mmol/L L-glutamine (Gibco) and 10% heat-inactivated FCS (Boehringer Mannheim), and the cells were seeded in tissue-culture flasks at 4×10⁴/cm².

At confluence, cells isolated under these conditions consistently had a spindle shape and exhibited a monolayer pattern of growth without any hill-and-valley features. In addition, immunostaining reactions were negative with both the mouse monoclonal antibody directed against human muscle α-actin (clone HHF35, Dako) and the mouse monoclonal antibody directed against human desmin (clone D33, Dako), but they were positive with the monoclonal antibody directed against vimentin (clone V9, Dako). These morphological and antigenic characteristics, which are specific for fibroblast-like cells, led us to consider that the cells isolated and amplified under our conditions were indeed fibroblasts. At the first passage and later, fibroblasts isolated from umbilical cord veins as well as MRC5 human fibroblasts (Eurobio, Les Ulis, France) were cultured at 3×10⁴/cm² in modified Eagle’s medium with Earle’s salts (Gibco) containing 2 mmol/L L-glutamine (Gibco) and 10% heat-inactivated FCS (Boehringer Mannheim).

For all experiments, the cultures were passaged once a week by trypsinization with a solution of 0.05% trypsin–0.02% EDTA (Gibco); human fibroblasts from umbilical cord veins and MRC5 human fibroblasts were used at confluence after 3 days of culture and were taken between passages 1 and 5 or between passages 26 and 30, respectively. All the cells used were cultured at 37°C in a humidified air atmosphere containing 5% CO₂.
Cell Treatments
For all experiments, 7β-hydroxycholesterol and 7-ketocholesterol stock solutions were freshly prepared at concentrations of 50, 100, 200, 400, and 800 μg/mL as previously described.23-24 To establish the initial solutions, oxysterols were dissolved in 1 volume of absolute ethanol, and 25 volumes of culture medium were added. One volume of these initial solutions was further introduced into 10 volumes of culture medium to obtain 7β-hydroxycholesterol and 7-ketocholesterol at final concentrations of 5, 10, 20, 40, or 80 μg/mL. Under these experimental conditions, the ethanol concentration in the culture medium was 0.4%. The purity of 7β-hydroxycholesterol and 7-ketocholesterol (both from Steraloids Inc) as determined by gaseous phase chromatography–mass spectrometry was 100%.

A stock solution of Ac-DEVD-CHO (BACHEM Biochimie) was prepared in distilled water at 10 mmol/L. Ac-DEVD-CHO was used at a final concentration of 100 μmol/L and was introduced into the culture medium 1 hour before the addition of oxysterols.

Cell Counting
HUVECs, human artery smooth muscle cells, A7R5 rat smooth muscle cells, human fibroblasts isolated from umbilical cord veins, and MRC5 human fibroblasts were seeded at 3×10⁴ cells per well of 6-well plates (Nunc) containing 3 mL of culture medium. Cell counting of adherent and nonadherent cells was performed by using a hemacytometer. Adherent cells were collected by trypsinization with a solution of 0.05% trypsin–0.02% EDTA (Gibco). Under these conditions, cell detachment, which constitutes an index of cytotoxicity, could be quantified.4 IC₅₀ corresponding to the concentration required to reduce by 50% the number of adherent cells was studied by phase-contrast microscopy and by fluorescence microscopy with an inverted IX70 microscope (Olympus). Hoechst 33342 excited by UV light was used to observe nuclear morphology by fluorescence microscopy: apoptotic cells were characterized by fragmented and/or condensed nuclei and necrotic cells by diffuse and irregular nuclei.46 Hoechst 33342 was prepared in distilled water at 1 mg/mL and added to the culture medium at a final concentration of 10 μg/mL for 1 hour at 37°C. Nonadherent cells in the culture medium were collected by centrifugation and adherent cells by trypsinization with a solution of 0.05% trypsin–0.02% EDTA (Gibco). Cells were washed twice in PBS and resuspended at a concentration of 10⁶ cells/mL in PBS containing 1% (wt/vol) paraformaldehyde. Deposits of ~40 000 cells were applied to glass slides by cytocentrifugation for 5 minutes at 1000 rpm with a cytocentrifuge 2 (Shandon), mounted in buffered glycercin (Bio-Mérieux), and coverslipped. For each sample, 300 cells were examined to determine the percentage of apoptotic and necrotic cells on the basis of the morphological aspect of cell nuclei.

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 labeling (TUNEL) method developed by Gavrieli et al31 with the use of the MEBSTAIN apoptosis kit (Immunotech) and according to the manufacturer’s procedure. In brief, cells were trypsinized with a solution of 0.05% trypsin–0.02% EDTA (Gibco), washed twice in PBS, resuspended at a concentration of 10⁶ cells/mL in PBS, and applied to glass slides (40 000 cells per slide) by centrifugation for 5 minutes at 1000 rpm with a cytosipin 2 (Shandon). After fixation at 4°C for 15 minutes with a 4% paraformaldehyde solution, the cells were permeabilized at room temperature for 15 minutes with a 0.5% Tween 20–0.2% BSA solution, washed 3 times with deionized water, and incubated with the TdT solution for 1 hour at 37°C in a humidified atmosphere. The signal of TUNEL was then detected by incubation for 30 minutes at room temperature with peroxidase-conjugated streptavidin (Dako) diluted 1/300 in PBS–0.2% BSA, and revelation of peroxidase activity was performed with 3,3'-diaminobenzidine with the Dako liquid DAB substrate chromogyn system (Dako). Cells were counterstained with methylene blue (RAL/Rhône-Poulenc), and the slides were mounted in buffered glycerol, coverslipped, and stored at 4°C. Observations were made with an inverted Laborlux IX70 microscope (Olympus).

DNA Fragmentation
DNA fragmentation was assessed by electrophoresis on 1.8% agarose gel. To this end, cellular DNA was extracted as previously described by using a DNA extraction kit (Stratagene).25,24-47 After electrophoresis, gels were examined under UV light and photographed or stored digitally with an image analysis system (Biocom).

Statistical Methods
A one-way ANOVA followed by Dunnett’s t test was used to evaluate the effects of 7β-hydroxycholesterol and 7-ketocholesterol. Statistical analyses were performed with SYSTAT software. The cutoff value of significance was P<0.05.

Results
Effect of Ethanol on Cell Growth
In the present study, ethanol was used to dissolved 7β-hydroxycholesterol and 7-ketocholesterol. To ensure that the solvent vehicle, ie, 0.4% ethanol, did not affect cell growth, HUVECs, human artery smooth muscle cells, A7R5 rat smooth muscle cells, MRC5 human fibroblasts, and human fibroblasts from umbilical cord veins were cultured for 48 hours in 6-well plates containing either the appropriate culture medium alone or culture medium supplemented with 0.4% ethanol. Under these conditions, the number of cells per well, the proportion of cell death, as well as the percentage of cells in the different phases of the cell cycle were similar in the absence or presence of 0.4% ethanol. Thus, in the absence...
or presence of 0.4% ethanol, the number of cells per well did not significantly differ: for HUVECs, the control value was 7.73±0.83×10⁵; ethanol treated 8.36±0.39×10⁵; for A7R5 rat smooth muscle cells, control 4.60±0.03×10⁵; ethanol treated 4.54±0.03×10⁵; for human artery smooth muscle cells, control 5.51±0.10×10⁵; ethanol treated 5.60±0.38×10⁵; for human fibroblasts from umbilical cord veins, control 3.90±0.32×10⁵; ethanol treated 4.11±0.03×10⁵; and for MRC5 human fibroblasts, control 6.47±0.54×10⁵; ethanol treated 6.28±0.22×10⁵. Moreover, after the cells were stained with PI, nonsignificant differences were found in the percentages of cell death: for HUVECs, the control value was 7.58±0.53; ethanol treated 8.06±0.30; for A7R5 rat smooth muscle cells, control 2.15±0.39; ethanol treated 1.88±0.54; for human artery smooth muscle cells, control 8.81±1.00; ethanol treated 9.08±1.11; for human fibroblasts from umbilical cord veins, control 8.37±2.34; ethanol treated 7.84±1.06; and for MRC5 human fibroblasts, control 8.81±1.00; ethanol treated 9.08±1.11. In addition, in the absence or presence of 0.4% ethanol, similar proportions of cells in the different phases of the cell cycle were observed: for HUVECs, the control G0/G1, S, and G2+M values were 82.33±0.81, 8.91±0.71, and 8.75±0.28, respectively; ethanol treated G0/G1, S, and G2+M 82.33±0.81, 9.01±0.51, and 8.65±0.40; for A7R5 rat smooth muscle cells, control G0/G1, S, and G2+M 84.02±0.54, 7.36±0.30, and 8.06±0.42; ethanol treated G0/G1, S, and G2+M 83.46±0.87, 7.60±0.96, and 8.56±0.41; for human artery smooth muscle cells, control G0/G1, S, and G2+M 92.00±6.00, 5.00±2.00, and 3.00±1.00; ethanol treated G0/G1, S, and G2+M 93.00±5.00, 5.00±2.00, and 2.00±1.00; for human fibroblasts from umbilical cord veins, control G0/G1, S, and G2+M 81.66±0.57, 10.00±1.00, and 8.33±0.57; ethanol treated G0/G1, S, and G2+M 81.66±0.57, 10.00±1.00, and 8.33±0.57; and for MRC5 human fibroblasts, control G0/G1, S, and G2+M 85.00±0.00, 6.75±0.50, and 8.25±0.50; ethanol treated G0/G1, S, and G2+M 85.50±0.57, 6.25±0.50, and 8.25±0.50. Thus, over the 48-hour experimental period, cell growth of endothelial cells, smooth muscle cells, and fibroblasts was not affected by the presence of 0.4% ethanol, and analysis of the cell cycle indicated that treatments with oxysterols were performed mainly on nonproliferating cells, as shown by the high percentages of cells in the G0/G1 phase of the cell cycle, varying from 81.66% to 93%.

**Effects of 7β-Hydroxycholesterol and 7-Ketocholesterol on the Number of Adherent and Nonadherent Cells**

When 7β-hydroxycholesterol and 7-ketocholesterol were added to confluent endothelial cells, smooth muscle cells, or fibroblasts, a concentration-dependent decrease in the number of adherent cells was observed in all cases (Figure 1). In the meantime, the number of nonadherent cells progressively increased in the culture medium (Figure 1). As determined from concentration-dependent curves, the mean concentrations required to reduce by 50% the number of adherent endothelial cells (IC₅₀) were 15.2 and 19.6 μg/mL for 7β-hydroxycholesterol and 7-ketocholesterol, respectively. The corresponding IC₅₀ values obtained with smooth muscle cells from umbilical artery as well as with rat smooth muscle cells (A7R5) were 21.2 and 28.2 μg/mL for 7β-hydroxycholesterol and 7-ketocholesterol, respectively. In fibroblasts, the decrease in adherent cells clearly appeared at lower concentrations with 7β-hydroxycholesterol than with 7-ketocholesterol, and with each oxysterol, the number of adherent fibroblasts strongly decreased while the number of nonadherent cells slightly increased, probably because rapid cell lysis occurred when fibroblasts were floating in the culture medium. Therefore, in contrast to endothelial and smooth muscle cells, only a few nonadherent fibroblasts were present in the culture medium after treatment with oxysterols, and their number slightly but significantly increased (P<0.01) only with the highest concentration of 7β-hydroxycholesterol or 7-ketocholesterol (80 μg/mL; Figure 1). The corresponding IC₅₀ values with human fibroblasts from umbilical cord veins were 14.8 and 29.5 μg/mL for 7β-hydroxycholesterol and 7-ketocholesterol, respectively, and those obtained with
hydroxycholesterol and 7-ketocholesterol (Figure 2A and 2B). With A7R5 rat smooth muscle cells as well as with human artery smooth muscle cells (Figure 2C and 2D), the proportion of PI-stained adherent cells rose significantly (P<0.01) at a lower concentration of 7β-hydroxycholesterol (5 µg/mL) than of 7-ketocholesterol (40 µg/mL). The maximal proportions of PI-permeable adherent A7R5 cells, ~35%, were reached with 10 µg/mL 7β-hydroxycholesterol, and this plateau value was maintained with the highest concentrations studied (Figure 2C); with human artery smooth muscle cells, the percentage of PI-permeable adherent cells rose progressively, and the highest proportion of PI-permeable adherent cells reached ~50% with 80 µg/mL 7β-hydroxycholesterol (Figure 2C). With 7-ketocholesterol, the proportion of PI-stained adherent human and rat smooth muscle cells rose also progressively, and a maximum of ~35% was reached with the 80 µg/mL concentration (Figure 2D). With human fibroblasts isolated from umbilical cord veins as well as with MRC5 human fibroblasts, PI-stained adherent cells were also detected earlier with 7β-hydroxycholesterol (10 µg/mL) than with 7-ketocholesterol (40 µg/mL). In contrast to endothelial and smooth muscle cells, virtually all adherent fibroblasts were stained with PI in the presence of the highest oxysterol concentrations, and >95% of cells died after treatment with 40 or 80 µg/mL 7β-hydroxycholesterol (Figure 2E) or 7-ketocholesterol, respectively (Figure 2F).

### Characterization of 7β-Hydroxycholesterol– and 7-Ketocholesterol–Induced Cell Death by Phase-Contrast Microscopy, Fluorescence Microscopy After Nuclear Staining With Hoechst 33342, and the TUNEL Method

Endothelial cells, smooth muscle cells, and fibroblasts were incubated for 48 hours in the presence of either 7β-hydroxycholesterol or 7-ketocholesterol, and they were subsequently observed and characterized by phase-contrast microscopy, fluorescence microscopy after nuclear staining with Hoechst 33342, and the TUNEL method to identify in situ DNA fragmentation.

As shown by phase-contrast microscopy, incubation of endothelial cells with 7β-hydroxycholesterol caused some morphological changes (Figure 3A and 3B). Compared with control cultures (Figure 3A), loss of the typical cobblestone aspect of confluent endothelial cells was observed in the presence of 7β-hydroxycholesterol, and the number of round cells floating in the culture medium increased (Figure 3B). The cellular morphology of oxysterol-treated human artery smooth muscle cells and rat smooth muscle cells (A7R5) was similarly and markedly altered, and a 48-hour exposure to 40 µg/mL 7-ketocholesterol induced cell shrinkage, cell detachment, and a loss of cell connections (Figure 3E and 3F). In human fibroblasts isolated from umbilical cord veins or in MRC5 human fibroblasts, 7β-hydroxycholesterol and 7-ketocholesterol also induced morphological changes. Thus, compared with untreated fibroblasts (Figure 3I), some morphological modifications were detected among counterparts treated with 40 µg/mL 7-ketocholesterol, with a loss of cell connections and the appearance of nonbirefringent fibroblasts attached to the plastic culture dish (Figure 3J); in addition, some cells and cellular debris floating in the culture medium were observed.
Characterization of cell death, apoptosis versus necrosis, was also performed by examining the morphological aspect of cell nuclei as assessed by fluorescence microscopy after staining with Hoechst 33342 and by the TUNEL method, which allows detection of in situ DNA fragmentation. Under these conditions, as shown in Figure 3D, cells with fragmented and/or condensed nuclei typical of apoptotic cells appeared among nonadherent endothelial cells treated with 40 μg/mL 7β-hydroxycholesterol, whereas cells with regular nuclei were observed in control cultures (Figure 3C). In 7β-hydroxycholesterol- and 7-ketocholesterol-treated rat smooth muscle cells (A7R5), as well as in human artery smooth muscle cells, typical features of apoptosis were also found by the TUNEL method. This latter method revealed the presence of fragmented DNA in some condensed and/or fragmented nuclei among nonadherent, oxysterol-treated smooth muscle cells (Figure 3H), whereas no DNA fragmentation was found in untreated cells (Figure 3G). In contrast, in untreated as well as oxysterol-treated fibroblasts, no DNA fragmentation was detected by the TUNEL method (Figure 3K and 3L); in addition, oxysterol-treated fibroblasts had no fragmented or condensed nuclei, but they did exhibit irregular cell contours and diffuse nuclei, as visualized by counterstaining with methylene blue (Figure 3L), resembling necrotic cells previously characterized by staining with Hoechst 33342.

Quantitation of Apoptotic or Necrotic Cells After Treatment With 7β-Hydroxycholesterol and 7-Ketocholesterol

Apoptotic and necrotic cells were counted either after nuclei staining with Hoechst 33342 or by the TUNEL method. Under these conditions, in untreated cells the proportions of apoptotic or necrotic cells was <5% by simultaneously taking into account both adherent and nonadherent cells (Figures 4 and 5). Apoptotic endothelial cells were mainly present among nonadherent cells after treatment with 7β-hydroxycholesterol or 7-ketocholesterol (Figure 4A and 4B), and the increase in nonadherent apoptotic cells became significant with 10 μg/mL 7β-hydroxycholesterol (P<0.01) or with 20 μg/mL 7-ketocholesterol (P<0.05). The maximal proportion of apoptosis among nonadherent endothelial cells was obtained with an oxysterol concentration of 40 μg/mL, and this value was ≈50% with 7β-hydroxycholesterol and 40% with 7-ketocholesterol. Among adherent endothelial
cells, the proportion of apoptotic cells was significantly increased with 7β-hydroxycholesterol (P<0.01) and with 7-ketocholesterol (P<0.05) at 40 and 80 μg/mL, respectively. With smooth muscle cells, both 7β-hydroxycholesterol and 7-ketocholesterol induced apoptosis mainly among nonadherent cells (Figure 4C and 4D). The increase in nonadherent, apoptotic smooth muscle cells became significant with 10 μg/mL 7β-hydroxycholesterol (P<0.01) and with 40 μg/mL 7-ketocholesterol (P<0.01). The maximal proportion of apoptotic, nonadherent smooth muscle cells tended to be higher after treatment with 7β-hydroxycholesterol (~40%) than with 7-ketocholesterol (~20%); Figure 4).

In contrast to endothelial and smooth muscle cells, necrosis was identified among adherent and nonadherent fibroblasts after treatment with 7β-hydroxycholesterol or 7-ketocholesterol (Figure 5). The increase in necrotic cells became significant with 10 μg/mL 7β-hydroxycholesterol or 40 μg/mL 7-ketocholesterol among nonadherent fibroblasts and with 20 μg/mL or 40 μg/mL 7β-hydroxycholesterol among adherent fibroblasts. Virtually all of the fibroblasts became necrotic with the highest concentrations of oxysterols (40 and 80 μg/mL; Figure 5).

**Analysis of DNA Fragmentation by Electrophoresis on Agarose Gel**

Because the highest proportions of apoptotic endothelial and smooth muscle cells were present among nonadherent cells, DNA was extracted from nonadherent 7β-hydroxycholesterol- or 7-ketocholesterol–treated cells. To this end, endothelial cells, smooth muscle cells, and fibroblasts were treated or not with 20 μg/mL 7β-hydroxycholesterol or 40 μg/mL 7-ketocholesterol for 48 hours, because these concentrations gave simultaneously high proportions of apoptotic and necrotic cells, and the DNA was analyzed by electrophoresis on 1.8% agarose gel. As shown in Figure 6, a typical internucleosomal DNA fragmentation into 180 to 200 bp and multiples thereof was observed in nonadherent, treated endothelial and smooth muscle cells. In contrast, no DNA fragmentation was found among nonadherent, treated human fibroblasts isolated from umbilical cord veins or in MRC5 human fibroblasts exposed to either 7β-hydroxycholesterol or 7-ketocholesterol (Figure 6).

**Effect of Ac-DEVD-CHO, an Inhibitor of DEVD-Sensitive Caspases, on 7β-Hydroxycholesterol– and 7-Ketocholesterol–Induced Cell Death**

The effect of the synthetic inhibitor Ac-DEVD-CHO used at 200 μM on DEVD-sensitive caspases such as caspase-3 was investigated in 7β-hydroxycholesterol- or 7-ketocholesterol–treated HUVECs, smooth muscle cells, and fibroblasts by quantifying the proportion of apoptotic or necrotic cells by the TUNEL method. 7β-Hydroxycholesterol and 7-ketocholesterol were used at 20 and 40 μg/mL, respectively, because these concentrations yielded similar high proportions of apoptosis in HUVECs and smooth muscle cells and high proportions of necrotic cells in fibroblasts. Interestingly, Ac-DEVD-CHO provided an effective protection against 7β-hydroxycholesterol– and 7-ketocholesterol–induced apoptosis, as shown by the significantly decreased proportions of apoptotic HUVECs and smooth muscle cells; however, no reduction in the proportions of necrotic cells was observed (the Table).

**Discussion**

The cores of advanced atherosclerotic lesions have been shown to contain lipid deposits, apoptotic and/or necrotic cells, and cell debris. In addition, fibrosis also occurs as atherosclerosis progresses, often yielding a lesion containing a dense, extracellular matrix with a relatively low cell density. Although in the latter process cell death provides a means for conversion of a hypercellular lesion to more fibrotic atheroma, little is known about the agents inducing cell death in the vascular wall and about its mechanisms, but...
In this complex process, LDLs probably play a critical role. Indeed, LDLs accumulate at the subendothelial level, and their exposure to not only endothelial cells but also macrophages localized in the vessel wall (which produces various toxic lipid oxidation products such as oxysterols). The present in vitro study on the different cells of the vascular wall, ie, endothelial cells, smooth muscle cells, and fibroblasts, brings new insight to the role of oxysterols in promoting cytotoxicity, and the current results indicate that the mode of cell death induced by oxysterols oxidized on carbon 7, ie, 7β-hydroxycholesterol and 7-ketocholesterol, can vary according to the cell type in the vascular wall being considered.

In agreement with our previous studies, treatment of HUVECs with 7β-hydroxycholesterol and 7-ketocholesterol enhanced cell detachment, reduced cellular viability, and was accompanied by the appearance of condensed and/or fragmented nuclei characteristic of apoptotic cells (mainly among nonadherent cells), as well as by intermuclesomal DNA fragmentation. Overall, these latter observations indicated that 7β-hydroxycholesterol and 7-ketocholesterol constitute potent inducers of apoptosis in HUVECs. In contrast to previous studies in which the toxicity of oxysterols was investigated in endothelial cells that were cultured directly on plastic culture plates, we chose to culture HUVECs on collagen IV. Indeed, collagen IV was reported to protect cultured sheep pulmonary artery endothelial cells from apoptosis induced by lipopolysaccharides, indicating that experimental conditions, in particular, the culture substrate used, might influence the cytotoxicity of exogenous compounds. In fact, we did not observe any protective effect of collagen IV on the apoptotic potency of 7β-hydroxycholesterol and 7-ketocholesterol, and very similar observations were made when HUVECs were cultured either on plastic or on a collagen IV substrate. However, the possibility of protective effects of some other components of the extracellular matrix cannot be excluded. For instance, laminin but not fibronectin was reported to protect HR9 mouse endodermal carcinoma cells from apoptosis when cultured in the absence of serum. Because apoptosis of endothelial cells can be associated with thrombin activation, well as P21 WAF1/CIP1 and P27 KIP1. Because apoptosis of endothelial cells can be associated with thrombin activation, well as P21 WAF1/CIP1 and P27 KIP1. Nevertheless, some components of the extracellular matrix may also be able to regulate the behavior of specific cell types, possibly by acting on apoptosis-related genes encoding interleukin-1β-converting enzyme and Bcl-2, as well as P21WAF1/CIP1 and P27KIP1. Because apoptosis of endothelial cells can be associated with thrombin activation, IL-1β secretion, and paracrine induction of the adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, we can envision a link between apoptosis at the endothelial cell level, procoagulant activity, and mononuclear infiltration of the vessel wall. Taken together, these data reinforce the hypothesis that apoptosis of endothelial cells certainly plays a critical role at different stages of development of atherosclerotic plaques.

As with endothelial cells, smooth muscle cells can also die by apoptosis when exposed to either 7β-hydroxycholesterol or 7-ketocholesterol. Thus, 7β-hydroxycholesterol and 7-ketocholesterol induced the appearance of fragmented and

### Table: Effect of the Tetrapeptide Ac-DEVD-CHO on the Percentages of Apoptosis or Necrosis Induced by 7β-Hydroxycholesterol (7β) and 7-Ketocholesterol (7-Keto) on Cells of the Vascular Wall

<table>
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<th>Cells</th>
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<th>7β + DEVD</th>
<th>7-Keto</th>
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<td>10±5†</td>
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The percentages of apoptotic and necrotic cells were determined on 300 cells by the TUNEL method. Data are mean±SEM of 4 independent experiments. 7β: 20 μg/mL; 7-Keto: 40 μg/mL; DEVD: 100 μmol/L.

Significance of the difference between oxysterol and (oxysterol + Ac-DEVD-CHO)–treated cells: *P<0.05, †P<0.01.
condensed nuclei typical of apoptosis, both among treated human artery smooth muscle cells taken at the first passage and among A7R5 rat smooth muscle cells, whereas these latter cells could display different characteristics than smooth muscle cells taken at earlier passages. Like oxysterol-treated endothelial cells, oxysterol-treated smooth muscle cells exhibited loss of cell adhesion and increased permeability to PI, revealing the occurrence of some membrane damage similar to that previously reported by studies involving increased permeability to albumin of porcine endothelial cells\(^{12}\) or modification of gap junctions in human smooth muscle cells after oxysterol treatments.\(^{14}\) It is, however, noteworthy that only a fraction of adherent smooth muscle cells, \(\sim 40\%\) to \(50\%\) of the total, became permeable to PI while the remaining cells were not stained, even with the highest oxysterol concentrations used. These observations would suggest either that adherent smooth muscle cells constitute a heterogeneous population of cells with different sensitivities to oxysterols or, more probably, that an atherogenic stimulus recruits only a subset of smooth muscle cells.\(^{62}\) In addition, in the present study, internucleosomal DNA fragmentation into 180 to 200 bp and multiples thereof was detected among nonadherent human and rat smooth muscle cells, as has also been observed in smooth muscle cells from thoracic aortas of Japanese white rabbits treated with 7-ketocholesterol or 25-hydroxycholesterol.\(^{32}\) By contrast, rat smooth muscle cells exposed to calphostin C,\(^{63}\) interferon-\(\gamma\), heparin, and cyclic nucleotide analogues\(^{64}\) did not show any signs of internucleosomal fragmentation, despite the detection of morphologically apoptotic cells. Therefore, the DNA fragmentation pattern could vary not only from one cell type to another\(^{65}\) but also, with smooth muscle cells, on the inducer of cell death used. As it has been previously reported that in atherosclerotic plaques of cholesterol-fed rabbits the foam cells of macrophage origin express cell proliferation markers while adjacent smooth muscle cells show apoptosis,\(^{66}\) foam cells could be relatively more resistant to oxysterols when compared with smooth muscle cells. In addition, as the smooth muscle cells in atherosclerotic plaques show increased expression of the proapoptotic protein Bax\(^{67}\) and as apoptosis triggered by 7-ketocholesterol is associated in vitro with a decrease of Bcl-2,\(^{32}\) we hypothesize that dysregulation of the Bax to Bcl-2 ratio could be involved in oxysterol-induced cell death.

The present study also demonstrated that 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol can exert a potent cytotoxic effect on the third cell type investigated, i.e., fibroblasts (fibroblasts from human umbilical cord veins and MRC5 human fibroblasts). However, in contrast to endothelial and smooth muscle cells, no evidence for apoptosis was observed among oxysterol-treated fibroblasts. In fact, both 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol treatment induced the emergence of necrotic fibroblasts, which exhibited irregular and diffuse nuclei after being stained with Hoechst 33342. In further support of the absence of apoptotic cells among oxysterol-treated fibroblasts, no DNA fragmentation was observed in situ with the TUNEL method and by agarose gel electrophoresis. It is noteworthy that the results obtained in oxysterol-treated fibroblasts are rather puzzling, but in a previous work it had been shown that exposure of human fetal lung fibroblasts to oxidized LDLs, known to contain high levels of 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol, induced only 10\% apoptotic cells;\(^{68}\) therefore, we cannot exclude the possibility that the remaining 90\% dead cells might correspond to necrotic ones, as suggested by aspects of the cells presented by phase-contrast and bright-field microscopy. Interestingly, the membrane damage revealed in our study by PI staining constitutes a common feature of both the apoptotic and necrotic process induced by 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol. However, major differences in the shape of the PI permeability curves were observed with increasing concentrations of oxysterols between endothelial cells, smooth muscle cells, and fibroblasts. In particular, whereas all adherent fibroblasts became permeable to PI after a threshold cytotoxic concentration of oxysterol was reached, the proportion of permeable endothelial and smooth muscle cells increased progressively along the oxysterol concentration range, and significant proportions of both adherent endothelial and adherent smooth muscle cells remained unstained with PI, even with the highest oxysterol concentrations studied. These observations strongly suggest that the metabolic pathways leading to increased permeability to PI might differ between apoptotic and necrotic cells. Therefore, we attempted to define the molecular mechanisms implicated in oxysterol-induced cell death.

As caspase-3 is highly expressed within apoptotic cells of human atherosclerotic plaques\(^{69}\) and 7-ketocholesterol– and 25-hydroxycholesterol–induced apoptosis is inhibited by Ac-DEVD-CHO,\(^{32}\) a tetrapeptide inhibiting DEVD-sensitive caspases such as caspase-3,\(^{34}\) we investigated the effect of this molecule on 7\(\beta\)-hydroxycholesterol– and 7-ketocholesterol–induced cell death. Interestingly, 7\(\beta\)-hydroxycholesterol– and 7-ketocholesterol–induced apoptosis in HUVECs and smooth muscle cells was significantly inhibited by Ac-DEVD-CHO, whereas no effect was observed on necrosis in oxysterol-treated fibroblasts. Thus, these data suggest the involvement of DEVD-sensitive caspases\(^{34,70}\) in the apoptotic process triggered by these oxysterols in HUVECs and smooth muscle cells but not in the necrosis observed in fibroblasts. It is noteworthy that these observations are in agreement with previous investigations reporting that necrosis, unlike apoptosis, was not accompanied by caspase-mediated proteolysis.\(^{26}\)

In conclusion, the cytotoxic concentrations of 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol were in the range of those measured in plasma from hypercholesterolemic patients\(^{71}\) as well as of those found in atherosclerotic plaques.\(^{19}\) Therefore, our data not only reinforce the hypothesis that 7\(\beta\)-hydroxycholesterol and 7-ketocholesterol probably play critical roles at different stages of the atherosclerotic plaques\(^{71,72}\) but also demonstrate that these oxysterols can induce both apoptosis and necrosis depending on the cell type of the arterial wall being considered.

Acknowledgments

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References


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Characterization and Comparison of the Mode of Cell Death, Apoptosis Versus Necrosis, Induced by 7 β-Hydroxycholesterol and 7-Ketocholesterol in the Cells of the Vascular Wall

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