Free Radical Research, November 2009; 43(11): 1072–1079

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healthcare

7β-hydroxycholesterol induces natural killer cell death via oxidative lysosomal destabilization

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(Received 18 February 2009; revised 29 May 2009)

Abstract
Peripheral natural killer (NK) cells are reduced in patients with coronary artery disease and highly susceptible to apoptosis induced by oxidized lipids including 7β-hydroxycholesterol (7βOH) *in vitro*. The present study aimed to further explore the mechanisms behind 7βOH-mediated cytotoxicity to human NK cells. Human NK cells were purified and treated with 7βOH in different concentrations and times. Cell death, lysosomal and mitochondrial permeabilization and reactive oxygen species (ROS) production were then analysed. The 7βOH induced time and dose dependent apoptosis and necrosis in human NK cells, which was preceded by loss of lysosomal integrity and enhanced ROS production. At later time points, the mitochondrial membrane permeability in 7βOH-treated cells was significantly increased. The findings indicate that 7βOH induces human NK cell death through early lysosomal permeabilization and consequent oxidative stress. The data further suggest that 7βOH may induce immune disturbances in clinical settings such as atherosclerosis.

Keywords: *Atherosclerosis, cell death, 7*β*-hydroxycholesterol, lysosomal membrane permeabilization, NK cells*

Introduction

Atherosclerosis is a chronic inflammatory and autoimmune disease, characterized by local accumulations of pro-inflammatory cytokines, pro-oxidative lipids and immune cells. A growing body of evidence indicates that both innate and adaptive immune responses participate in atherogenesis [1,2]. Beside other innate immune cells, such as mononuclear phagocytes and neutrophils, natural killer (NK) cells may have participatory roles in several inflammatory disorders, including atherosclerosis. NK constitute up to 15% of all peripheral blood lymphocytes. They have well recognized cytotoxic capacities but also important regulatory roles in the adaptive immune response. Recent studies have found that patients with chronic coronary artery disease (CAD) have a diminished number of NK cells in peripheral blood with a concomitant loss of NK cell function [3,4]. In addition, oxidized low-density lipoproteins (ox-LDL) and oxysterols, which are abundantly found in atherosclerotic lesions [5,6], have been shown to inhibit NK cell activity *in vitro* [7,8]. We recently demonstrated that the number of apoptotic NK cells in peripheral blood was significantly increased in CAD patients compared to controls. Purified NK cells from CAD patients also showed a higher rate of spontaneous apoptosis and were more susceptible to apoptosis induced by oxidized LDL, in particular 7β-hydroxycholesterol (7βOH), *ex vivo* [9].

Oxysterols, especially 7βOH and 7-ketocholesterol (7-keto), are major cytotoxic components of oxidized LDL [10]. Increased plasma levels of oxysterols have been found in a population with a high risk for cardiovascular disease [11] and patients with cardiac vascular disease [12,13]. *In vitro* studies have shown that both 7βOH and 7-keto are cytotoxic to different types of arterial cells, such as smooth muscle cells, macrophages and endothelial cells resulting in either

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760903176919

necrosis, apoptosis or both [13–16]. Moreover, equivalent concentration of oxysterols as found in plasma of vascular disease patients were toxic to cultured endothelial cells as well [13]. We recently reported that 7βOH induced foam cell formation and apoptosis concomitantly with enhanced production of reactive oxygen species (ROS) [17].

Apoptosis is a type of programmed cell death that occurs under both physiological and pathological conditions such as autoimmune disease, cancer and atherosclerosis [18]. It can be mediated either through death receptors or via mitochondrial pathways leading to the cleavage of caspases as the apoptotic executors [19,20]. Lately, lysosomal pathways and their proteolytic enzymes, cathepsins, have been more and more recognized as initiating factors in apoptosis in several cell models [16,17,19–23]. In the present study, we aimed to further characterize the cytotoxic effects of 7βOH in human NK cells by focusing on potential mechanisms involving the lysosomal and mitochondrial membrane integrity.

Materials and methods

Chemical reagents

RPMI 1640 medium, penicillin-G, foetal bovine serum (FBS) and streptomycin were from Gibco (Paisley, UK). Cholesterol, 7β-hydroxycholesterol (7βOH) and propidium iodide (PI) were from Sigma (St Louis, MO). Annexin V-FLOUS (AV) was from Roche (Mannheim, Germany). Dihydroethidine (DHE), tetramethylrhodamine ethyl ester (TMRE) and 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide/chloride (JC-1) were from Molecular Probes (Eugene, OR). Lymphoprep was from Axis-Shield PoC AS (Oslo, Norway) and NK cell isolation kit was from Dynal Biotech ASA (Oslo, Norway).

Isolation of NK cells

Peripheral venous blood was obtained from healthy blood donors and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. Briefly, the blood was mixed with PBS and layered onto Lymphoprep. The PBMC were collected at the interface after centrifugation at 400 × g for 30 min, washed twice in PBS and re-suspended in PBS with 0.1% FBS at 10×10^6 PBMC/100 μL.

NK cell negative isolation kit was used for separation of NK cells from PBMC according to a modification of the protocol reported by the manufacturer. Briefly, the PBMC were incubated for 10 min at 4°C with NK antibody mix (20 μL/10×10^6 PBMC) followed by centrifugation (500×g, 8 min, 4°C). The cells were then re-suspended in PBS with 0.1% FBS, mixed with Dynabeads and further incubated for 10 min at 4°C. The bead-bound cells (all the PBMCs except the NK cells) were removed in two steps with a magnet, leaving the NK cells in the supernatant.

Cell cultures and experimental conditions

NK cells were directly exposed or not to various concentrations of cholesterol or 7βOH for different periods of time and collected by centrifugation. After treatment the cells were used for analysis of cell viability, lysosomal membrane permeabilization (LMP), mitochondrial membrane permeabilization (MMP) and cellular ROS production using different probes.

Apoptosis and necrosis

Cell morphology. The cell morphology was initially examined by morphological assessment directly on living cells by phase contrast light microscopy.

Phosphatidylserine exposure. The numbers of apoptotic cells were assayed by detection of phosphatidylserine exposure using flow cytometry following Annexin V staining [15,16]. Cells scored as early apoptosis when they were predominately positive to Annexin V, while when cells were positive to both Annexin V and PI, they were scored as necrotic cell death, as described previously [15,16]. In brief, control and treated cells were collected, washed once with PBS and stained for 10 min on ice with Annexin V/PI.

Lysosomal membrane permeabilization (LMP)

The integrity of the lysosomal membrane was assessed using the acridine orange uptake technique as established previously [15,16]. In this approach, live cells are incubated with acridine orange (AO) after each time point of treatments. Following different treatments cells were stained with 5 μg/ml AO for 15 min at 37°C and lysosomal AO red fluorescence was analysed by flow cytometry. Percentages of cells with decreased AO red fluorescence were gated and considered as increased lysosomal membrane permeabilization (LMP).

Mitochondria membranes permeability (MMP)

The mitochondrial potential ($\Delta\Psi_m$) was measured using the fluorescent probe JC-1 as described in Blomgran et al. [22]. In brief, following different treatments control and treated cells were incubated with JC-1 (5 μg/ml, 10 min, 37°C) and analysed with flow cytometry. The intensity of green fluorescence from JC-1 monomers was used to represent the cells that lost $\Delta\Psi_m$ and increase in MMP.

Reactive oxygen species (ROS)

Intracellular ROS was assayed by flow cytometry following dihydroethidium (DHE) staining, as described previously [16]. Briefly, cultures were collected, washed once with PBS, incubated for 15 min at 37°C with 10 μmol/L DHE and analysed with flow cytometry.

Statistics

For statistical analysis, one-way ANOVA followed by the post-hoc Bonferroni test was used. Results are given, as means ± SEM. Correlations between LMP, MMP and cell death were analysed using the Spearman correlation coefficient. $p \leq 0.05$ was considered statistically significant.

Results

7βOH caused both apoptosis and necrosis in human NK cells

The potential toxicity of 7βOH to human NK cells was analysed by assessment of morphological changes and flow cytometry following Annexin V and PI staining. As compared to control and cholesterol treated cells, the 20 μM 7βOH caused a significant increase in cell death at 40 and 60 h (Figure 1A). Compared to control cells (Figure 1B, left), nuclear condensation and fragmentation following 20 μM 7βOH were also observed (Figure 1B, right).

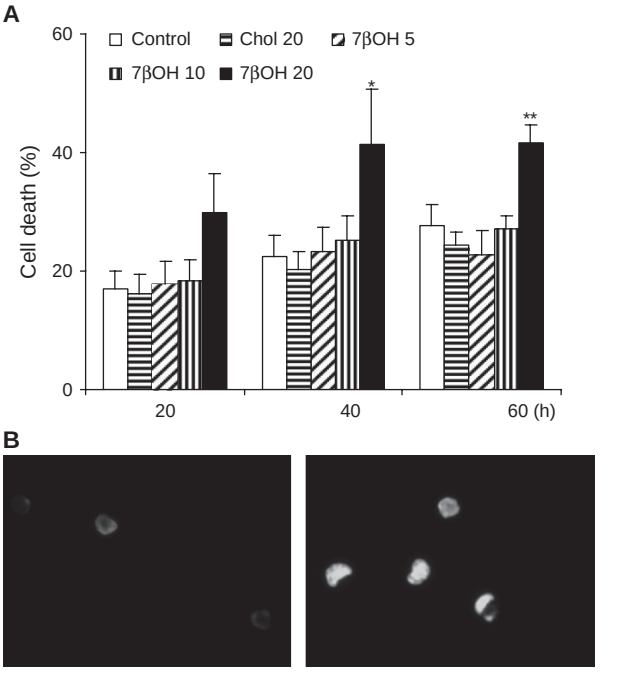

Figure 1. 7βOH causes both apoptosis and necrosis in human NK cells. Cells were stained with AV and PI and analysed by flow cytometry following treatment with 20 μM cholesterol, 5, 10 and 20 μM 7βOH for 20, 40 and 60 h, indicated as control, Chol 20, 7βOH 5, 7βOH 10, 7βOH 20, respectively. Percentages of cell death summarized from flow cytometry results, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ compared to all other groups. Data are means ± SEM of four experiments and each condition with duplicate determinations (A). Nuclear condensation and fragmentation caused by 7βOH. Cells were treated with 20 μM 7βOH for 40 h, stained with Hoechst and viewed with fluorescence microscopy (untreated control cells, left; 7βOH treated cells, right) (B).

The 7βOH induced early lysosomal membrane permeabilization (LMP) in human NK cells

To examine the effects of 7βOH on lysosomal membrane integrity, NK cells were stained with AO and analysed by both flow cytometry and fluorescence microscopy. Microscopy of AO stained control cells showed normal cell morphology (Figure 2A, a) and clear red fluorescence with speckled staining patterns in control cells (Figure 2A, b). 7βOH treatment for 20 h caused cell shrinkage (Figure 2A, c) and reduction of lysosomal red fluorescence with a diffuse pattern in most of the cells (Figure 2A, d). Moreover, the remarkable shrunken cells showed very weak or no red fluorescence (Figure 2A, d). Figure 2B demonstrates the representative histograms from flow cytometry in AO stained control (filled grey histograms) and 20 μM 7βOH treated (black lined histograms) cells at different time points. The percentages of cells with decreased AO red fluorescence were clearly increased after 20 h and even more pronounced after 40 and 60 h. The summarized results from three experiments are shown in Figure 2C, in which a significant 7βOH-induced increase in LMP is observed already after 20 h. A dose- and time-dependent increase in LMP was observed following 7βOH exposure and a marginal statistic increase in LMP was also seen in cells treated with 10 μM 7βOH for a longer time (60 h) (Figure 2C, $p = 0.066$ as compared to control and cholesterol).

The 7βOH caused moderate mitochondrial membrane permeabilization (MMP)

The effects of 7βOH on MMP in human NK cells were analysed with JC-1 staining and flow cytometry. In general, 20 μM 7βOH caused an increase in JC-1 red and slightly in JC-1 green fluorescence after 20 h, indicating that 7βOH enhances polarization of mitochondria. As shown in Figure 3, 20 μM 7βOH caused a significant increase in green fluorescence at 40 h, suggesting that a decrease in mitochondrial membrane potential and an increase in MMP had been initiated in the treated cells. The percentage of cells with increased MMP was significantly higher after exposure to 20 μM 7βOH for 60 h (Figure 3). The results indicated that depolarization of mitochondria predominately occurred after 40 h in cells treated with 20 μM 7βOH.

Since both LMP and MMP are involved in apoptotic cell death, possible correlations between these two parameters and cell death were further

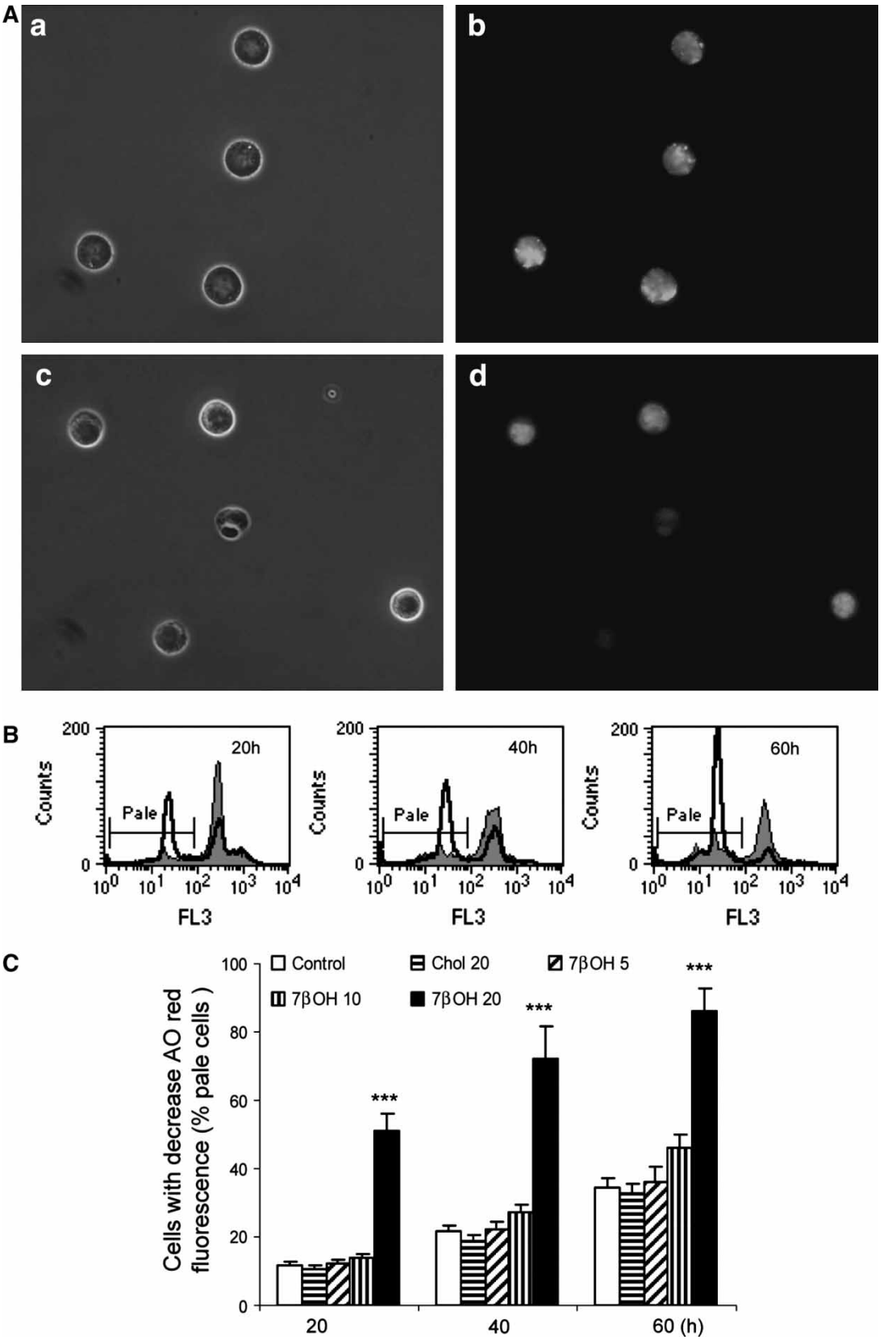

Figure 2. 7βOH reduced red lysosomal fluorescence and increased LMP in human NK cells. Cells were stained with AO and either analysed by phase contrast microscopy (left panel) or fluorescence microscopy (right panel). Upper panel shows untreated cells while cells in lower panel were treated with 20 μM 7βOH for 20 h (A). Representative results of flow cytometry showing time-dependent decreases in red lysosomal fluorescence (gated as pale cells) in 20 μM 7βOH treated cells (black lined histograms) compared to control (filled grey histograms) (B). Histogram showing percentages of cells with decreased lysosomal membrane potential or increased LMP. ***$p \leq 0.0001$ compared to all other groups. Illustrated values are means ± SEM of three experiments and each with duplicate determinations (C).

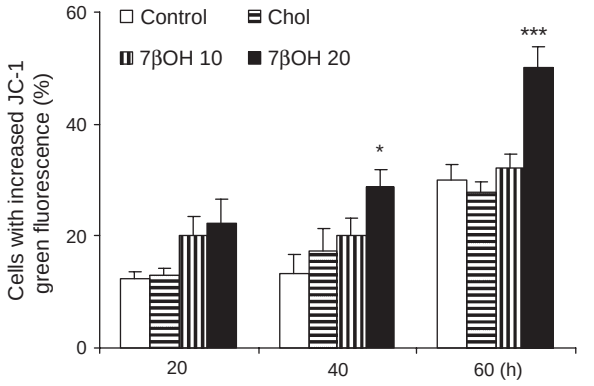

Figure 3. 7βOH caused a relatively late but significant increase in MMP in human NK cells. Cells were stained with JC-1 and assessed by flow cytometry to measure alterations in MMP after treatments with 20 μM cholesterol, 10 or 20 μM 7βOH for 20, 40 and 60 h of incubation (indicated as in Figure 1). Illustrated percentage values are means ± SEM of three experiments and each with duplicate determinations. $^{*}p \leq 0.05$, $^{***}p \leq 0.0001$ compared to all other groups.

explored. Alterations of both LMP (Figure 4A) and MMP (Figure 4B) were significantly associated with percentages of cell death. Furthermore, cell death-related alterations of LMP and MMP were also significantly correlated (Figure 4C).

ROS production was significantly increased as response to 7βOH treatment

DHE stained cells were assessed by flow cytometry to measure ROS production following 7βOH treatment. At 20 h, the levels of ROS were higher in cells treated with 20 μM 7βOH, though not significant. A significant increase in ROS production was seen after 40 and 60 h of exposure to 20 μM 7βOH. After 60 h, more than 55% cells in the 20 μM 7βOH-treated group showed elevated levels of ROS production (Figure 5A). Further correlative tests showed that enhanced ROS production by 7βOH was significantly related to increased LMP, MMP, and percentages of cell death, suggesting that the ROS-associated loss of LMP and MMP may be an important determinant in this cell death model.

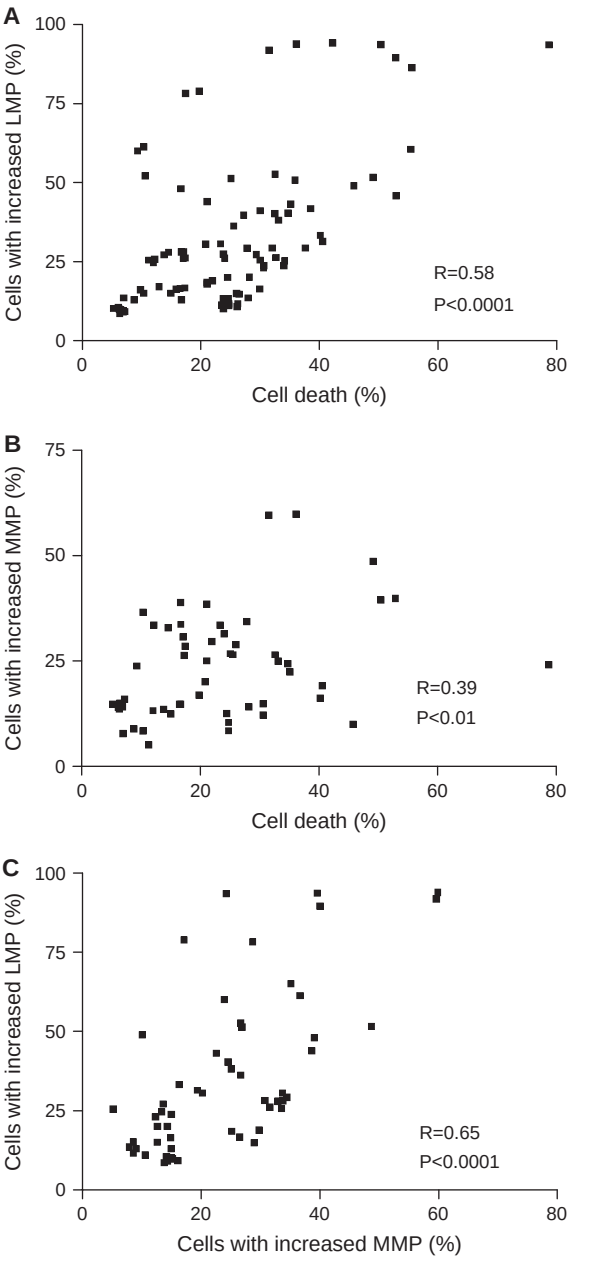

Figure 4. The decreases of LMP and MMP were significantly correlated and significantly related to cell death upon treatment with 7βOH. The cells were treated with or without 7βOH (5, 10 or 20 μM for 20, 40 or 60 h) and analysed for LMP, MMP and cell death, by using AO, JC-1 or AVPI staining, respectively. Correlations between cells with increased LMP (A), and MMP (B) and cell death (C) were examined by Spearman correlation coefficient tests.

Discussion

Oxysterols are abundantly found in atherosclerotic lesions [5,6] and have also been shown to be toxic to vascular cells in experimental models [9,13–17]. Patients with CAD or individuals with higher risk for cardiovascular disease have increased levels of circulating oxysterols, in particular 7βOH, further supporting a role of oxysterols in atherogenesis [11–13]. Moreover, immune perturbations involving dysfunction and apoptosis of peripheral NK cells have been described in atherosclerosis [3,4,9]. In a recent study, we demonstrated that NK cells in CAD patients were more susceptible to apoptosis induced by oxidized lipids than were NK cells in healthy individuals [9]. In the present study we demonstrate that 7βOH at levels as low as 20 μM induced time-dependent apoptosis and necrosis in human NK cells. Cells treated with 20 μM 7βOH showed a significant early loss of lysosomal integrity followed by increased ROS production and a relative late increase in MMP. These findings suggest that the

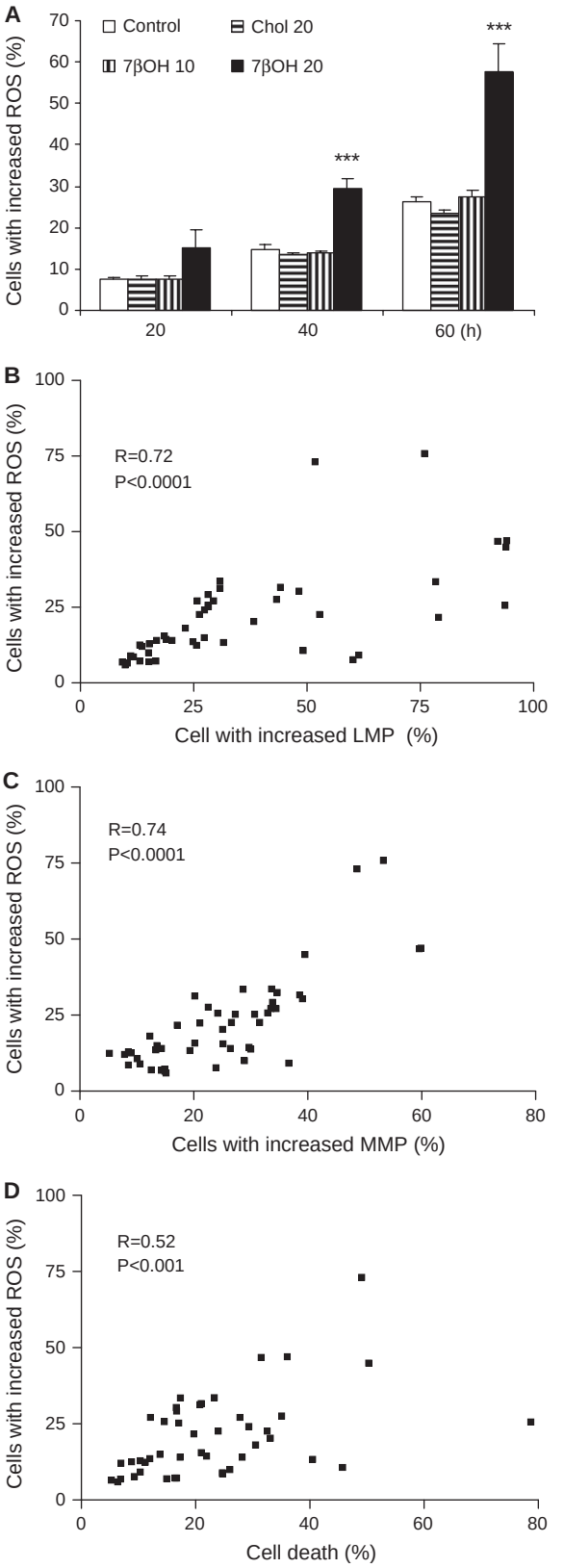

Figure 5 (*Continued*)

activation of oxidative lysosomal pathway by 7βOH and ensuing apoptosis may lead to the diminished number of NK cells seen in patients with CAD [3,9].

The role of lysosomal compartments and related cathepsins as apoptotic initiators has been the subject of interest in a number of study models and is now regarded as an important apoptotic pathway [15–17,19–23]. In the present study we demonstrate that an increased LMP after 20 h exposure to 7βOH preceded a significant apoptotic and necrotic cell death. The findings thus indicate that an increased LMP is an initiating step of apoptotic death in human NK cells. Furthermore, the cytotoxic evaluation experiments showed a decrease in apoptotic cells and a shift to necrosis after exposure to 7βOH for 60 h. This might be a consequence of the further increase in LMP leading to a massive leakage of lysosomal proteases and is also consistent with studies conducted in other cell models [15–17,19–23].

Although lysosomes seemed to be the major pathway in 7βOH induced cell death other reactions, such as the mitochondrial pathway, may occur synergistically. We, therefore, evaluated the role of mitochondrial involvement by measuring mitochondrial membrane potential using JC-1 staining. A late increase of MMP occurred in the study model, suggesting that the increase in MMP was a downstream event of altered LMP in 7βOH-induced NK cell death. The correlation analysis supported that changes in both MMP and LMP contributed to NK cell death.

As previously shown [17], enhanced ROS production is closely related to both apoptosis and necrosis, causing damages to organelle membranes. Accordingly, ROS production may be the consequence rather than the cause of lysosomal breakage. Although further studies are needed to clarify the strong associations between ROS, LMP and MMP, it may still be able to postulate that the formation of ROS and release of lysosomal content interact with each other in a positive feedback circle.

Although oxysterols are enriched in the atherosclerotic lesions, increased plasma concentrations of oxysterols have been associated with atherosclerotic disease in a limited number of studies [12,13,24]. However, oxysterols may still have important local effects in the arterial wall and function as signalling molecules in circulation. Considering that oxidative stress and inflammation including increased levels of

Figure 5. 7βOH caused increases in ROS production in human NK cells. Cells were stained with DHE and analysed by flow cytometry to assess ROS production following treatments with 20 μM cholesterol, 5, 10 and 20 μM 7βOH for 20, 40 and 60 h. ***$p \leq 0.0001$ compared to all other groups. Data are means ± SEM of three separate experiments performed in duplicate (A). The enhanced ROS production by 7βOH was significantly related to the increases in LMP (B), MMP (C) and cell death (D). In (B), (C) and (D), the cells were treated with or without 10 and 20 μM 7β-OH for 20, 40 or 60 h.

malonaldehyde and superoxide anion, and reduced levels of superoxide dismutase, as well as increased levels of C-reactive protein have been demonstrated in plasma of CAD patients [25], we consider that the increased spontaneous NK cell apoptosis *in vivo* [9] may be due to a combined effects of oxidative stress and increased inflammation. Therefore, it might be difficult to mimic cell damage *in vivo* by exposing the cells *in vitro* to doses of oxysterols that are similar to plasma concentrations.

It has been known for some time that oxysterols may exhibit immunosuppressive effects [26,27]. One early study suggested that 7βOH or 25-OH inhibited T-cell responses to different stimuli and induced apoptosis of thymocytes in thymus involution [28]. Our results in the present study further indicate that 7βOH may cause oxidative damage in NK cells, which may perturb immune response in atherogenesis.

To summarize, we show that 7βOH, a cholesterol oxidation product, causes human NK cell death. Apoptosis followed by necrosis was mediated through activation of lysosomal/mitochondrial pathways and enhanced production of ROS. The pathological relevance of NK cell depletion in atherosclerosis is not clarified but may involve a loss of immune regulation and an imbalance between pro- and anti-inflammatory activities. Our findings here together with other previous results raise the intriguing possibility that cholesterol oxidation products may contribute to a state of immune dysfunction [7,25–27].

Acknowledgements

This work was supported by Swedish Heart Lung Foundation and Gamla Tjänarinnor Foundation.

***Declaration of interest*:** The authors have no conflict of interest to declare. The authors alone are responsible for generating the data and preparing the manuscript.

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This paper was first published online on iFirst on 21 August 2009.