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Cholesterol crystallization and macrophage apoptosis: implication for atherosclerotic plaque instability and rupture

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Abstract

The presence of abundant cholesterol crystals symbolizes the disorder of cholesterol metabolism during the development of atherosclerosis. Examination of cultured human THP-1 macrophages treated with the cholesterol oxide, 7-ketocholesterol, revealed a concentration- and time-dependent increase in formation of cholesterol crystals in the cells. Radioisotope labeling and X-ray diffraction confirmed the presence of 7-ketocholesterol crystalline domains (*d* space 35.8 Å). Under the normal cell culture condition (5% CO_2, 37°), incubation with 7-ketocholesterol induced moderate levels of apoptosis. Elevating temperature from 37 to 40° markedly reduces formation of the crystals in the macrophages. Meanwhile, at high temperatures, significantly increased numbers of apoptotic cells were detected in the cells treated with 7-ketocholesterol but not in those with native free cholesterol. These results suggest that hyperthermia inhibits cholesterol crystallization and promotes apoptotic effects of oxysterols on macrophages.

Keywords: Atherosclerosis; Cholesterol oxidation; Apoptosis; Temperature; Crystallization; Macrophages

1. Introduction

The intimal accumulation of cholesterol in the arterial wall represents one of characteristic pathological alterations in atherosclerosis, a chronic arterial disease with two life-threatening complications, heart attack and stroke. However, cholesterol itself is a normal cellular component but its oxidative products or oxysterols have been suggested to play an active role in the atherosclerotic plaque development [1]. More than decades of investigation have pointed to the fact that the oxysterol:cholesterol ratio in plaque is much higher than in normal tissues or plasma. After conversion into oxysterols, some of the cholesterol molecules become far more bioactive, even though they undertake many of the same reactions as native cholesterol, such as esterification and cross-membrane transport. *In vitro*, oxysterols perturb several aspects of cellular cholesterol homeostasis (including cholesterol biosynthesis, esterification, and efflux), impair vascular reactivity and are cytotoxic and/or induce apoptosis [2,3]. Injection of relatively large doses of oxysterols into animals causes acute angiotoxicity whereas oxysterol-feeding experiments have yielded contrary results as far as their atherogenicity is concerned. There is no direct evidence yet in humans that oxysterols contribute to atherogenesis. However, oxysterol levels are elevated in human LDL subfractions that are considered potentially atherogenic. Indeed, recent data from the studies of oxysterols in human and animal atherosclerosis [1] have indicated that raised plasma levels of certain oxysterols may be associated with an increased risk of atherosclerosis.

Vascular cells including endothelial [4] and smooth muscle [5] cells are sensitive to apoptosis induced by oxysterols. *In vitro* studies have shown that exposure to

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Abbreviations: VSMCs, vascular smooth muscle cells; oxLDL, oxidized low-density lipoprotein; LDL, low-density lipoprotein.

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oxLDL can trigger apoptosis of the vascular cells through a mechanism involved in caspase activation. Harada-Shiba *et al.* [4] studied the apoptosis-inducing activities in lipid fractions of oxLDL. They observed that various oxysterols in the lipid fractions induced human endothelial cell apoptosis. Neither the phospholipid fraction nor its component lysophosphatidylcholine showed the pro-apoptotic effect. Addition of caspase inhibitors into the cell cultures blocked the oxLDL or oxysterol-induced apoptosis, suggesting that activating these cysteine proteases might contribute to the vascular cell death. Similar studies by Nishio and Watanabe [5] on VSMCs indicated that both 7-ketocholesterol and 25-hydroxycholesterol, two predominant oxysterols in plaques, induced VSMC apoptosis, which is followed by a rapid decrease in bcl-2 protein and activation of caspases. The caspase inhibitor prevented apoptosis induced by 7-ketocholesterol and 25-hydroxycholesterol in VSMCs, whereas the exogenous cholesterol, which itself did not have any apoptotic activity, inhibited the cell death induced by 25-hydroxycholesterol but not 7-ketocholesterol, suggesting that the two oxysterols may trigger different death signaling mechanisms in the cells.

In addition to vascular cells, many macrophages accumulate in atherosclerotic lesions. They transform into lipid-laden foam cells after taking up large amounts of lipid-rich lipoproteins via the scavenger receptor pathway. Interestingly, in spite of the lipid cytotoxicity, numerous lipid-loaded macrophages survive the very harsh, atherogenic environment of atherosclerotic lesions. This raises the possibility that the macrophages infiltrating the atherosclerotic lesions may develop an anti-apoptotic mechanism by which they escape the apoptotic attack by atherogenic, cytotoxic factors. Indeed, in our previous studies, we observed that macrophages expressing high levels of scavenger receptors are resistant to apoptosis induced by oxysterols such as 7-ketocholesterol [6].

Recent studies on model macrophages suggest that free cholesterol generated by the hydrolysis of cytoplasmic cholesterol esters is transported through acidic vesicles to the plasma membrane [7]. The membrane accumulation of cholesterol in this pool triggers macrophage death. We have shown that some of the oxysterols form crystals directly in the plasma membrane which are detectable by using the small-angle X-ray diffraction approaches [8]. The interactions of 7-ketocholesterol with other membrane components occur not only in well-defined lipid vesicles but in murine aortic SMC membranes as well. 7-Ketocholesterol-derived extracellular crystals are concomitant with the formation of membrane crystalline domains greater than measured with cholesterol. Interestingly, not all the oxysterols existing in atherosclerotic plaques display the same pro-crystallizing effect as 7-ketocholesterol. For instance, incubation with 25-OH-cholesterol could not generate such signals in SMCs [8].

There are abundant cholesterol crystals in advanced atherosclerotic lesions or fibrous plaques [9]. Morphologically, these crystals display various shapes, including the appearances of needles, plates, and helices, which may reflect differential compositions of cholesterol derivatives. The crystal diversity may reflect the fact that the compositions of the crystals vary from one to another. Cholesterol forms crystals within cultured macrophages loaded with chemically modified lipoproteins [10]. Cholesterol crystallization has been also implicated in association with cell death during the pathogenesis of atherosclerosis. Cholesterol crystals develop upon acceleration of cytoplasmic cholesteryl ester hydrolysis and free cholesterol transport [10,11]. Oxidation of LDL, a reaction leading to formation of oxysterols, may promote cholesterol crystallization and apoptosis in plaques. For example, 7-ketocholesterol, a major oxidative product of free cholesterol, induces apoptosis of lipid-rich macrophages [6,12]. In this study, we examined thermodynamics of crystal formation and apoptosis of macrophages in an inflammatory microenvironment. Our results show a novel mechanism underlying the cholesterol crystallization and apoptosis in lipid-laden macrophages.

2. Materials and methods

2.1. Model human macrophage foam cells and treatment with modified lipoproteins and oxysterols

Human THP-1 monocytic cells obtained from ATCC were cultured in RPMI 1640 medium with 10% fetal bovine serum. The cells were treated with 10 ng/mL of PMA for 24 hr inducing their maturation into macrophages, and then loaded with lipids by incubation with 100 μg/mL acetylated LDL (Panimmune, Inc.) for 48 hr. For treatment with cholesterol and oxysterols, THP-1 macrophages were washed in PBS and then incubated with different concentrations of free cholesterol and 7-ketocholesterol. The lipids were dissolved and stored as the 100× stock solution in ethanol or DMSO. Before addition into the cultures, a small portion of lipid solution was diluted in pre-warmed culture media. In some experiments, the cells were incubated with cholesterol and oxysterols at different temperatures.

2.2. Determination of cell viability

Cell viability was assessed by fluorescent microscopy with the nucleic acid-binding fluorochromes, acridine orange and ethidium bromide, using a previously described method with modification [13,14].

2.3. In situ labeling of DNA fragments

In situ detection of DNA fragments was performed by deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) as previously described

[13,14]. Total DNA was isolated for agarose gel electrophoresis to determine the levels of DNA fragmentation.

2.4. Crystal morphometry

Cholesterol crystals were detected by polarized microscopy. Cells cultured in 6-well plates were treated with cholesterol and oxysterols and their morphology was determined under phase contrast objectives. Images were collected via a digital camera connected to an Olympus fluorescent microscope, and analyzed using the software NIH Image.

2.5. Oxysterol incorporation

THP-1 macrophages (5×10^5 mL^{-1}) in 6-well plates were incubated with 0, 50, 100 and 150 nCi/mL ^{3}H-7-ketocholesterol (Amersham) for 24 hr. The cells were washed in PBS three times and then harvested for assessing the radioactivity of the incorporated radioisotope with a scintillation counter (Microbeta, Perkin-Elmer).

2.6. Small angle X-ray diffraction

The small angle X-ray diffraction assay was performed as previously reported [8]. Briefly, after treatment with cholesterol and oxysterols, cells were harvested for small-angle X-ray diffraction analysis. After centrifugation, cell pellets were analyzed by aligning them at grazing incidence in accord with a collimated monochromatic X-ray beam ($\lambda = 1.54$ Å), produced by a high-brilliance rotating anode microfocus generator (Rigaku Rotaflex RU-200). Data were collected with a one-dimensional, position sensitive electronic detector (Innovative Technologies), and analyzed by using ASA software (Molecular Dynamics). Fourier transformation was generated from Bragg's inflections using Origin (Microcal Software).

3. Results

3.1. Crystal formation in model human THP-1 foamy macrophages treated with 7-ketocholesterol

The presence of large amounts of cholesterol crystals characterizes advanced atherosclerotic lesions [9,15]. Macrophages internalize cholesterol-carrying lipoproteins and store large amounts of cholesterol and its oxidative products or oxysterols. The frequent appearance of cholesterol crystals in the macrophage-rich regions suggests that macrophages may contribute to cholesterol crystal formation. We examined the formation of cholesterol crystals in human THP-1 lipid-laden foam cells, and determined whether oxidized cholesterol treatment may lead to enhanced formation of cholesterol crystals. THP-1 monocytic cells were first induced to mature into macrophages with the phorbol ester PMA, and then converted into lipid-laden foam cells by exposure to acetylated LDL (100 μg/mL). The majority of THP-1 macrophages were transformed into lipid-laden foam cells, after 24–48 hr of incubation with the lipoproteins. After they developed into the lipid-laden foam cells, the cells were then exposed to 7-ketocholesterol (20 μg/mL) or the same amount of free cholesterol. We observed that incubation with 7-ketocholesterol resulted in formation of cholesterol crystals in the macrophage foamy cells (Fig. 1). The crystals became microscopically visible 24 hr after the treatment. With prolonged incubation, the numbers of the crystals increased, and some crystals penetrated through the membrane and extended extracellularly. Some crystals detached from the cells, and floated in the culture media. Interestingly, the crystal formation did not occur in the cells loaded with the same quantities of free cholesterol, suggesting an enhancement of crystallization by cholesterol oxides.

Examination of the viability of human THP-1 macrophages exposed to 7-ketocholesterol showed marked reduction in cell viability, when compared to cholesterol-treated cells. However, many lipid-laden cells survived the 7-ketocholesterol cytotoxic attack, and some of them exhibited intracellular crystals in the cytoplasm as well as in the membrane (Fig. 1). These observations strongly suggest that by crystallization, the cytotoxicity of the cholesterol oxides decline and the resistance of lipid-loaded foam cells to the pro-apoptotic effect of the lipids enhanced.

3.2. Formation of oxysterol crystalline in cellular membranes

Under physiological conditions, the majority of cholesterol molecules are incorporated into the phospholipid layers of cellular membrane. Therefore, it is possible that cholesterol crystals may initially form within the plasma membrane. To determine whether the 7-ketocholesterol crystals were originating in the cellular membranes, we conducted small-angle X-ray diffraction in THP-1 cells treated with free cholesterol and 7-ketocholesterol. As shown in Fig. 2, a diffraction pattern consistent with 7-ketocholesterol domains (d space = 35.8 Å) was clearly detectable in the cells treated with 7-ketocholesterol but not in those treated with free cholesterol. Interestingly, we found that thermal treatment did not diminish the signal for the 7-ketocholesterol-membrane domain. We observed a clear signal for 7-ketocholesterol in the cellular membrane lipid extracts. Thus, the increased membrane accumulation of the oxysterol appeared to be associated with crystallization in human THP-1 macrophages.

To further demonstrate the presence of the oxysterol incorporation into the membrane sterol crystals, we performed radiotracing assays with ^{3}H-7-ketocholesterol. We observed that the radioactive oxysterol entered the cells

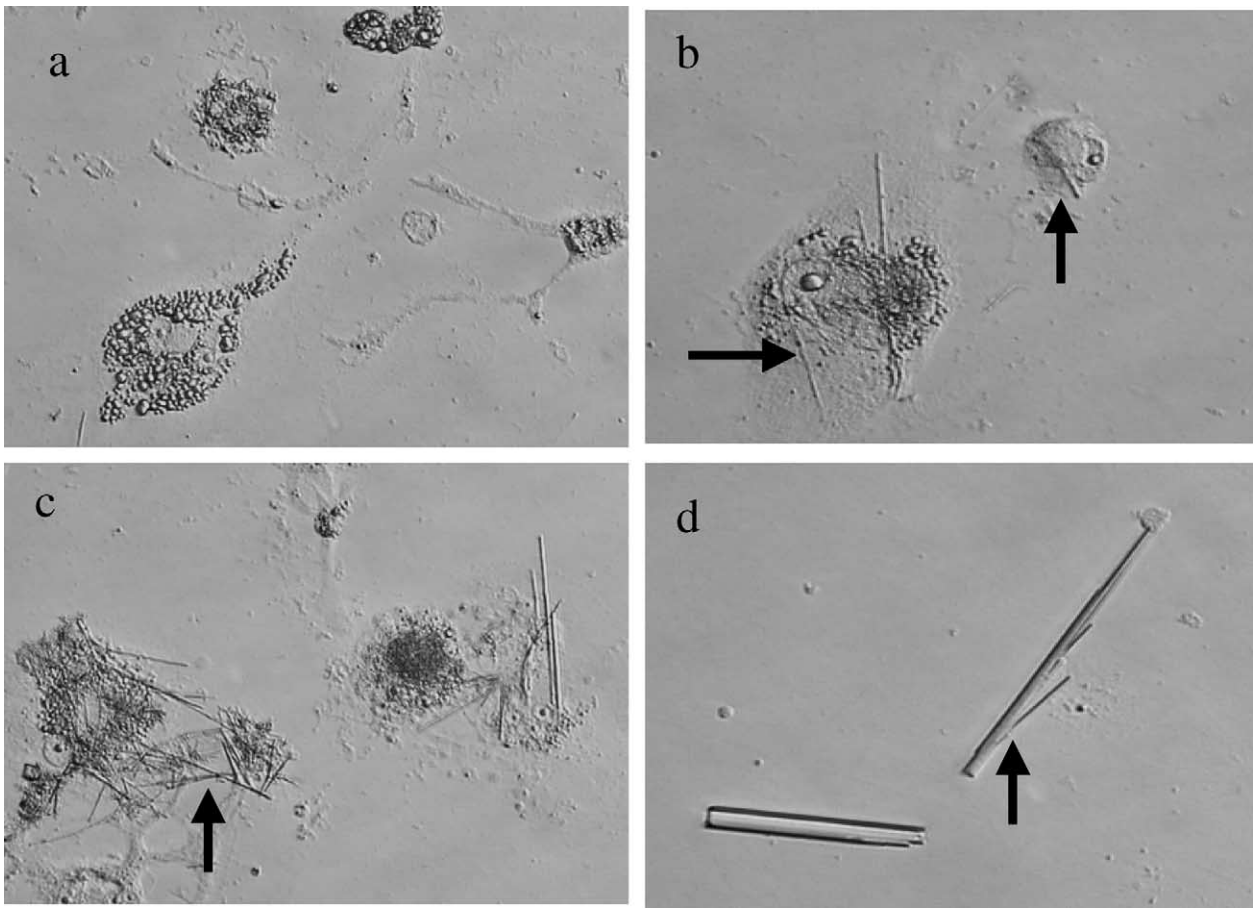

Fig. 1. Cholesterol crystallization in human THP-1 lipid-rich foamy macrophages exposed to 7-ketocholesterol. Human THP-1 macrophages transformed by phorbol ester (PMA) stimulation were loaded with acetylated LDL and then treated with 7-ketocholesterol. Polarized microscopy was performed to image cholesterol crystals (arrows). (a) Foamy macrophages with cholesterol crystals at 37°; (b and c) 7-ketocholesterol-treated foamy macrophages with crystals; and (d) floating crystals.

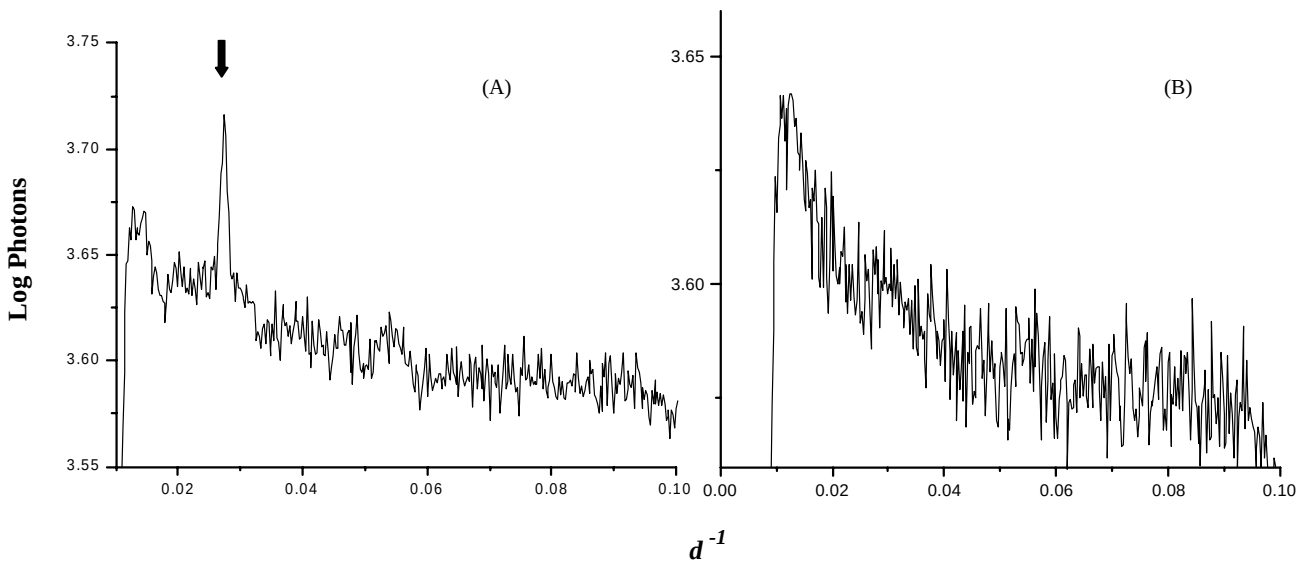

Fig. 2. Small-angle X-ray diffraction analysis of PMA-differentiated, lipid-loaded THP-1 macrophages. Human THP-1 cells treated with or without 7-ketocholesterol at different temperatures were collected for small-angle X-ray diffraction. Samples were aligned at grazing incidence to a collimated monochromatic X-ray beam, Cu Kα, $\lambda = 1.54$ Å, produced by a high-brilliance rotating anode microfocus generator. The peak in these runs is corresponding to a discrete 7-keto domain with a d space of 35.8 Å. Changes in temperature did not appear to alter the presence of the sterol domains in the cells. (A) 7-Ketocholesterol-treated cells; (B) untreated controls.

promptly, and prior to crystallization, the lipid had already existed in the plasma membrane. Lipid extracts from membrane indicated significant amounts (nearly 30–40%) of radioactive 7-ketocholesterol in the membrane fraction. The cholesterol crystals also showed markedly increased radioactivities, suggesting that 7-ketocholesterol or its metabolites could incorporate into the crystals.

3.3. Thermal dynamics of cholesterol crystallization and apoptosis in macrophages

Similar to crystallization of any other molecules, cholesterol crystal formation is a temperature-sensitive biophysical process. We, therefore, examined the thermal effect on the sterol crystallization by treating THP-1 macrophages with 7-ketocholesterol and free cholesterol at different temperatures. We found that gentle heating of the oxysterol-treated cell cultures to 40° significantly reduced 7-ketocholesterol-induced crystallization in the cells (Figs. 3 and 4). Free cholesterol treatment induced only modest crystallization, which was not significantly altered by temperature elevation (Fig. 3). Thus, gentle thermal treatment might attenuate the cholesterol oxide-induced crystallization.

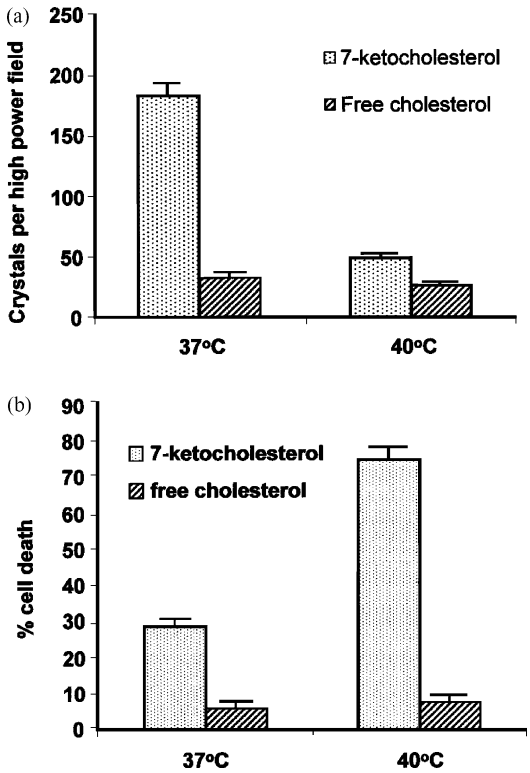

Fig. 3. Thermal effect on cholesterol crystallization and viability in human THP-1 foamy macrophages exposed to 7-ketocholesterol and free cholesterol. THP-1 foamy macrophages exposed to 7-ketocholesterol and free cholesterol at temperatures of 37 and 40° were analyzed under a polarized microscope (Nikon). Cells were stained with a combination of acridine orange and ethidium bromide, and observed under a polarized microscope. Dead cells and crystals were quantified respectively. (a) Crystal formation; (b) cell viability. Data are shown as mean ± SD, N = 6.

In vitro stimulation with oxysterols has been previously demonstrated to induce apoptosis in macrophages. To determine the effect of the oxysterol on apoptosis of macrophage-derived lipid-laden foam cells, we examined morphology and DNA fragmentation in human THP-1 macrophages treated with 7-ketocholesterol. By staining with two DNA-binding fluorochromes, acridine orange and ethidium bromide, we found that the cell viability declined. At the concentrations of 30 μg/mL or higher, significant cell death was detectable (Figs. 3b and 4). The dead cells showed a typical morphology of apoptosis including cellular shrinkage, blebbing, and nuclear fragmentation. Incubation with the concentrations of 7-ketocholesterol (10 μg/mL) or lower for 24 hr did not induce visible cell death in the cultures. However, the intracellular accumulation of cholesterol crystals took place in the cells which excluded ethidium bromide and maintained fine cellular and nuclear structures. Hence, the sterol crystallization appeared to occur in the living cells. We performed DNA electrophoresis with genomic DNA extracted from THP-1 foamy macrophages incubated with or without 7-ketocholesterol, and detected modest fragmentation of genomic DNA isolated from the cells treated with 10 μg/mL of 7-ketocholesterol for 24 hr at different temperatures. Increasing incubation temperature from 37 to 40 or 42° markedly enhanced DNA fragmentation in the cells treated with 7-ketocholesterol but not in the untreated controls (Fig. 4). Cell viability assays further revealed that the gentle heating at 40° significantly increased cell death in the cultures with 7-ketocholesterol but not free cholesterol (Fig. 3). Thus, thermal treatment reduced cholesterol crystallization and enhanced apoptosis of THP-1 lipid-laden foamy cells exposed to 7-ketocholesterol.

4. Discussion

Traditionally, cholesterol crystallization is thought to be due to extracellular accumulation of excess amounts of water-insoluble cholesterol released from the hydrolysis of cholesterol esters [9]. When membrane cholesterol is supersaturated and cholesterol esterification is diminished, nucleation and crystallization of free cholesterol occur in a fashion that coincides with the onset of cell death and plaque formation. Recent studies [10] have, however, suggested that cholesterol crystal formation depends upon cellular lipid metabolism within living cells. Evidence from the studies of model lipid-loaded macrophages in culture and from pathological examination of plaque samples indicates the presence of cholesterol crystals inside, within the membrane or on the surface of the living cells after exposure to high levels of cholesterol inhibiting of cholesterol esterification. Although the crystals can continuously grow extracellularly, our data suggest that intracellular cholesterol crystalline nucleation can be initiated rapidly when the cells are exposed to cholesterol oxides.

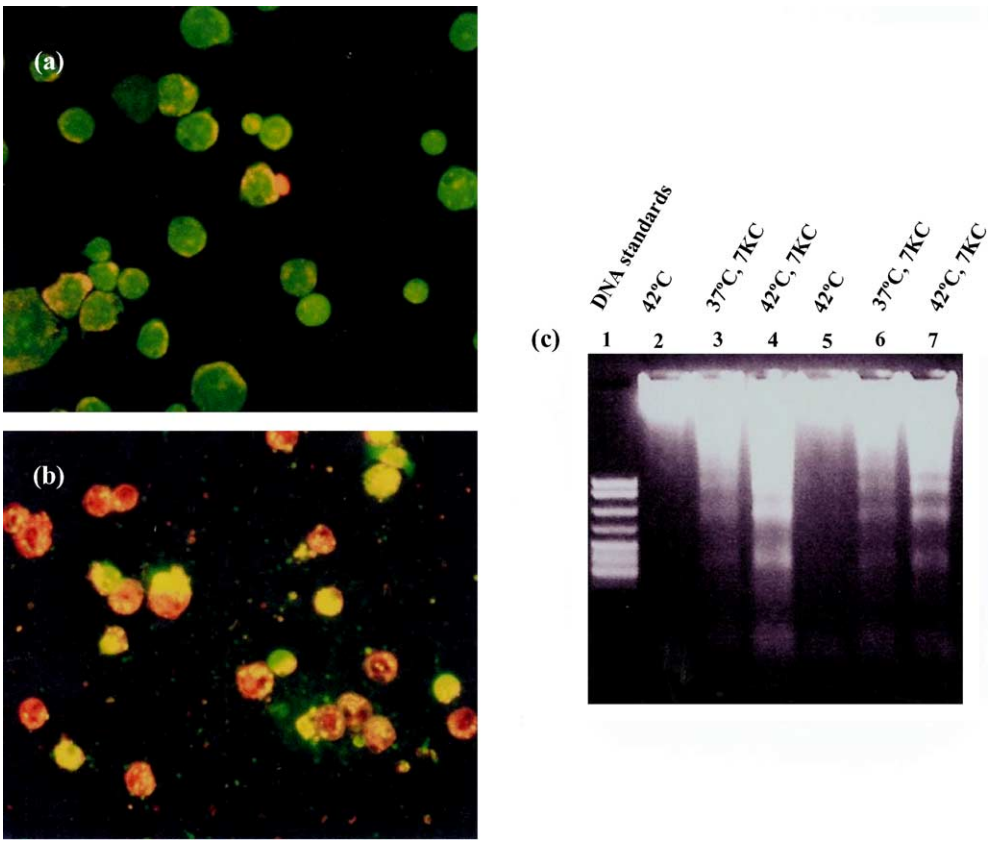

Fig. 4. Apoptosis of human THP-1 foamy macrophages induced by 7-ketocholesterol. Human THP-1 foamy macrophages were treated with or without 7-ketocholesterol at different temperatures. For morphological determination of cell death, double fluorescent nuclear staining was performed with a combination of acridine orange and ethidium bromide. For analysis of DNA fragmentation, DNA was isolated from the cells by chloroform/phenol extraction and fractionated by agarose gel (2%) electrophoresis. (a) Untreated, living THP-1 cells emitting green fluorescence; (b) 7-ketocholesterol-treated THP-1 cells emitting red or yellow-red fluorescence; and (c) DNA agarose gel electrophoresis.

Cholesterol oxidation is markedly enhanced in atherosclerosis, yielding a broad variety of oxysterols in the lesions [1]. There has been, however, limited information regarding the effects of oxysterols, the products of cholesterol oxidation, on cholesterol crystallization. Klinkner *et al.* [16] have reported that long-term incubation of macrophages with oxLDL increases lipid deposition in cultured cells and that, under the conditions studied, cholesterol crystals form in macrophage foam cells. Our current study also demonstrated a crystal-promoting effect of the cholesterol oxidative product, 7-ketocholesterol, one of the most common oxysterols in atherosclerotic plaques [12,17]. By small-angle X-ray diffraction, we detected strong signals for 7-ketocholesterol in the cellular membrane, suggesting that 7-ketocholesterol may incorporate into the phospholipid layer in competition with its parent molecule, cholesterol. The increased oxysterol incorporation in the cellular membrane may result in release of large amounts of free cholesterol that in turn crystallizes in the lack of sufficient high-density lipoproteins. Another possibility is that oxysterols, such as 7-ketocholesterol, themselves form crystals. In this regard, the plaque cholesterol crystals are actually a mixture of crystals from free cholesterol and its oxides. The radioactive 7-ketocholesterol tracing studies provided the evidence supporting this notion.

Oxysterol components play major roles in induction of apoptosis by oxidized lipoproteins [18]. The mechanisms underlying the pro-apoptotic effects of oxysterols remain largely unclear. Recent studies have shown that mitochondrial dysfunction and caspase activation may mediate the apoptotic action of oxysterols. Our recent studies (unpublished observations) indicate that expression of caspase-3 and caspase-8 may be involved in apoptosis of foam cells in advanced atherosclerotic plaques triggered by exposure to oxysterols. However, some of the lipid-rich macrophages in plaque seem resistant to apoptotic stimuli. Highly differentiated, matural macrophages can produce caspase-3 at high levels but they become less sensitive to apoptosis induced by oxLDL and oxysterols [6]. Further experiments showed evidence that expression of high levels of scavenger receptors confers the resistance of macrophages to apoptosis [6,19]. Although no direct linkage between scavenger receptor expression and cholesterol crystal formation has been established through an experimental system, it is likely that lipid-binding or transporting proteins may participate in intracellular sterol crystallization. Functioning as the first line defender in the immune system, macrophages may

obtain an adaptive mechanism in response to the drastic environmental alterations in the atherosclerotic arterial wall. Through such protective mechanisms, macrophages are able to engulf, scavenge, and process cytotoxic substances, such as oxLDL and oxysterols.

Previous studies by Small [9,15] shown the temperature-dependency of cholesterol crystallization. By use of a polarized, hot-stage microscope, they demonstrated different melting temperatures from 40 to 80° for most cholesterol crystals. In this study, we observed that cholesterol crystal formation significantly declined with temperature elevation. The thermal treatment performed in the current study was rather gentle compared with those performed on the heating stage of microscopy by Small [9,15]. The decreased crystallization may not be simply due to the physical effect of temperature, and complicated biochemical interaction between lipids and macromolecules may contribute to crystallization. Among those complex biochemical reactions that may have changed with temperature elevation are the thermal enhancement of cholesterol interaction with other lipids, cholesterol esterification and oxidation.

The Fas ligand/Fas/caspase death-signaling pathway has been implicated in apoptosis of macrophages in atherosclerotic lesions and in culture [20,21]. Stimulation with oxLDL and oxysterols may activate caspase-3. The mechanisms underlying the activation of caspases and the induction of apoptosis in the cells with oxysterol treatment remain largely unclear. Gentle heating may not only help dissolve the crystals and increase free oxysterols but also enhance the enzymatic activities of the caspase pathways. However, differentiated macrophages are resistant to apoptosis induced by the oxysterols. Expression of lipid-transporting proteins (e.g. scavenger receptors) may contribute to the resistance in macrophages.

In summary, cholesterol crystalization and macrophage apoptosis both occur in advanced atherosclerotic lesions. However, in response to cholesterol oxidative products, macrophages develop an adaptive mechanism by which the cells promote the sterol crystallization and subsequently augment their resistance to the cytotoxic effects of the oxysterols. The increased cholesterol crystallization and macrophage survival may contribute to the plaque instability and complication development, including plaque rupture and cholesterol crystal embolisms. Gentle thermal treatment may have beneficial effects as the treatment reduces crystallization and enhances macrophage death. Further clarification of the mechanisms of crystal formation may lead to development of new strategies for prevention and treatment of atherosclerosis and its complications, in particular atheroembolism and acute vascular syndromes.

Acknowledgments

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