Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol

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Abstract **Oxidized LDL (OxLDL) is composed of many potentially proatherogenic molecules, including oxysterols. Of the oxysterols, 7-ketocholesterol (7-KC) is found in relatively large abundance in OxLDL, as well as in atherosclerotic plaque and foam cells in vivo. Although there is evidence that 7-KC activates endothelial cells, its effect on monocytes is unknown. We tested the hypothesis that 7-KC may induce monocyte differentiation and promote foam cell formation. THP-1 cells were used as a monocyte model system and were treated with 7-KC over a range of concentrations from 0.5 to 10 μg/ml. Changes in cell adhesion properties, cell morphology, and expression of antigens characteristic of differentiated macrophages were monitored over a 7-day period. 7-KC promoted cells to firmly adhere and display morphologic features of differentiated macrophages; this effect was time and dose dependent and was markedly more potent than cholesterol treatment (45% of cells became adherent after 7 days of treatment with 7-KC at 10 μg/ml vs. less then 5% for control cells, $P < 0.01$). Similar effects were obtained when LDL enriched with 7-KC or OxLDL were added to THP-1 cells. 7-KC-differentiated cells expressed CD11b, CD36, and CD68, phagocytized latex beads, and formed lipid-laden foam cells after exposure to acetylated LDL or OxLDL. In contrast to 7-KC, oxysterols with known cell regulatory effects such as 25-hydroxycholesterol, 7β-hydroxycholesterol, and (22*R*)-hydroxycholesterol did not effectively promote THP-1 differentiation. In conclusion, these results demonstrate for the first time that 7-KC, a prominent oxysterol formed in OxLDL by peroxidation of cholesterol, may play an important role in promoting monocyte differentiation and foam cell formation. These studies also suggest that 7-KC induces monocyte differentiation through a sterol-mediated regulatory pathway that remains to be characterized.**—Hayden, J. M., L. Brachova, K. Higgins, L. Obermiller, A. Sevanian, S. Khandrika, and P. D. Reaven. **Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol.** ***J. Lipid Res.*** **2002.** 43: **26–35.**

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It is now widely recognized that monocytes play an integral role in the development and progression of atherosclerosis (1, 2). Monocytes are among the first cells present in lesion-prone areas, and they continue to accumulate during plaque formation. On exposure to a variety of regulatory signals, monocytes rapidly differentiate into tissue macrophages in the vascular intima. When activated, monocyte-macrophages may secrete multiple cytokines and free radicals that promote inflammation and atherosclerosis (1, 3, 4). Macrophages also express multiple scavenger receptors that facilitate internalization of modified lipoproteins, leading to the development of cholesterol-laden foam cells and plaque formation in arteries (5–7). The primary role of monocyte-macrophages in atherogenesis has been confirmed in gene knockout mice lacking monocyte chemoattractant protein or its receptor, CCR2. In these respective mouse models it was demonstrated that aortic atherosclerosis was significantly reduced in comparison with wild-type controls under experimental conditions that predispose to atherosclerosis (8, 9).

Although the consequences of macrophage accumulation in lesion-susceptible regions of the artery wall are now evident, the regulatory signals that induce monocyte differentiation in this environment are less completely understood. Although it has been demonstrated that oxidized low density lipoprotein (OxLDL) can induce the differentiation of monocytes (10), the components of OxLDL that actually promote this process are not fully elucidated. Initial studies have suggested that oxidized phospholipids are remarkably proinflammatory, and at least one report has implicated specific oxidized phospholipids in the regulation of monocyte differentiation (11). Other components of OxLDL, such as oxysterols, have also been shown

Abbreviations: AcLDL, acetylated low density lipoprotein; D-PBS, Dulbecco's phosphate-buffered saline; 7-KC, 7-ketocholesterol; LDH, lactate dehydrogenase; MM-LDL, minimally modified low density lipoprotein; nLDL, native low density lipoprotein; OxLDL, oxidized low density lipoprotein; SRA-1, macrophage scavenger receptor class A-1.

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to promote a variety of potentially proatherogenic events. Of the oxysterols, 7-ketocholesterol (7-KC) has been found in relatively high concentrations in OxLDL produced in vitro (12–14) and is enriched in arterial foam cells and atherosclerotic plaque in vivo (15, 16). Furthermore, 7-KC induces apoptosis in vascular cells (17, 18), stimulates adhesion molecule expression in endothelial cells (19), and inhibits cholesterol efflux in macrophages (20). At present, the effects of oxysterols on monocyte-macrophage differentiation have not been examined. Herein we demonstrate for the first time that 7-KC promotes differentiation of THP-1 cells and primary human monocytes, and enhances foam cell formation in vitro.

EXPERIMENTAL PROCEDURES

Cells and general experimental protocols

THP-1 cells (American Type Tissue Culture Collection, Rockville, MD) were grown in RPMI 1640 containing 10% heat-inactivated FBS (Irvine Scientific, CA), 10 mM HEPES, 1 mM sodium pyruvate, sodium bicarbonate (1.5 g/l), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. In each experiment, cells were plated at a density of approximately 2.0×10^5 cells/ml. Initial experimentation demonstrated that THP-1 cells were more predisposed to 7-KC-mediated differentiation after pretreatment with serum-free medium (containing 0.1% BSA); therefore, in all experiments described in this study THP-1 cells were treated with serum-free RPMI 1640 medium containing 0.1% BSA for 24 h before exposure to oxysterols. After pretreatment with serum-free medium, heat-inactivated FBS was added to a final concentration of 10% in the medium and oxysterols were added (0.5 to 10 μg/ml) to the cells for various periods (24 h to 7 days). All stock solutions of oxysterols {5-cholesten-3β-ol-7-one (7-KC); 5-cholestene-3β,7β-diol (7β-hydroxycholesterol); 5-cholestene-3β,22[*R*]-diol [22(*R*)-hydroxycholesterol], and 5-cholestene-3β,25-diol (25-hydroxycholesterol); Sigma, St. Louis, MO} were prepared in absolute ethanol. The majority of experiments were performed with ethanol at a concentration ≤0.1% (v/v). At the highest 7-KC concentration used in the dose-response experiments ethanol concentrations were ≤0.5% in culture. At this dose, ethanol did not affect any parameter of differentiation that was examined in the study. Ethanol alone or cholesterol (Sigma) dissolved in ethanol served as negative controls in all experiments. In addition, phorbol 12-myristate 13-acetate (PMA, 0.01 μg/ml) was added to THP-1 cells to serve as a positive control for differentiation. In addition, we confirmed that oxysterol stimulation of THP-1 cells did not result from potential endotoxin contamination (*Limulus amebocyte* lysate assay; Charles River Laboratories, Charleston, SC) in 7-KC stock solutions or cell culture reagents.

Primary human monocytes were isolated from sodium EDTA-treated blood that was obtained from healthy volunteers. The blood was diluted 1:1 with Dulbecco's PBS (D-PBS) and layered on Histopaque-1077 (Sigma) and centrifuged at 400 *g* for 30 min at room temperature. The resulting monocyte-enriched layer was collected, washed with D-PBS, and plated in medium (THP-1 growth medium minus 2-mercaptoethanol) that was supplemented with 10% autologous serum. After 2 h the nonadherent cells were removed by gentle washing with D-PBS and the remaining adherent monocyte-enriched cells were provided complete growth medium for 2 days. Monocytes were then exposed to ethanol alone or to 7-KC (5 and 20 μg/ml) for 7 days before subsequent analyses.

Assessment of morphologic changes and quantification of adherent THP-1 cells

Under normal culture conditions THP-1 cells remain in suspension; however, after treatment with 7-KC a subpopulation of cells becomes adherent within 3 to 7 days. During this period, morphologic changes of the adherent cells were assessed by phase-contrast microscopy (×20 or ×40 objective). In addition, adherent cells were released from the plates by treatment with trypsin and directly counted with a hemacytometer (Hausser Scientific, Horsham, PA) or a particle counter (model Z1 D/T; Beckman Coulter, Miami, FL). In additional experiments relative cell adherence was also assessed by the quantification of total cellular protein concentration (MicroBCA; Pierce, Rockford, IL).

Immunohistochemical staining of cells

Control cells and THP-1 cells that remained in suspension (undifferentiated cells) after 7-KC exposure were applied to slides by centrifugation (Cytospin-2; Shandon, Pittsburgh, PA) at 500 rpm for 5 min at room temperature. Cells that were applied to the slides, and cells that were induced to adhere to plastic by 7-KC, were fixed by immersion in ice-cold methanol for 10 min at −20°C. After fixation, the cells were washed several times with PBS and endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxide diluted in 50% methanol-PBS 1:1 (v/v) for 5 min. The cells were then pretreated with either normal mouse serum (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) or 3% BSA (Sigma) for 1 h before incubation with primary monoclonal antibodies [CD11b and CD68, 1:50 and 1:100 dilution, respectively (Dako, Carpinteria, CA), and CD36, 1:50 dilution (Novocastra Laboratories, Ltd., Burlingame, CA)] for 2 h at room temperature. After additional washing, bound primary antibodies were detected by incubation with a diluted (1:50) biotinylated anti-mouse immunoglobulin antibody (Vector Laboratories), followed by diaminobenzidine (DAB) treatment as per standard ABC/DAB immunohistochemistry methodology (Vectastain ABC kit). To exclude the possibility of nonspecific binding of primary antibodies, additional cells were treated with a nonspecific monoclonal antibody (von Willebrand factor, diluted 1:50; Sigma) or with PBS (no primary antibody addition) before detection.

Determination of phagocytosis

To assess whether oxysterol-treated adherent THP-1 cells demonstrate the typical phagocytic activity of macrophages, latex beads (averaging 0.8 μm in diameter; Sigma) were added to cells for 1 h at 37°C and 5% CO_2. The cells were then rinsed several times with D-PBS and internalized latex beads were examined by phase-contrast microscopy (×40 objective).

Assessment of cellular cytotoxicity

To evaluate the potential cytotoxic effect of 7-KC on THP-1 cells, lactate dehydrogenase (LDH) released in the supernatant was determined (CytoTox 96; Promega, Madison, WI) from nonadherent cells and adherent cells treated up to 7 days with 7-KC. The percentage of cellular LDH release was calculated as the portion of LDH detected in the conditioned medium divided by total LDH from conditioned medium plus LDH from the cell lysate.

In additional experiments, trypan blue (0.4% solution) staining was used to document the viability of suspension and adherent THP-1 cells after exposure to 7-KC. All cells (stained and unstained) were counted as described above.

Isolation and modifications of LDL

LDL (1.021–1.063 g/ml) was isolated from plasma provided by healthy volunteers by sequential gradient ultracentrifugation as described previously (21). 7-KC-loaded or vehicle (ethanol)-

treated LDL were also prepared from plasma that was first incubated at 37°C for 4 h with a small volume of 7-KC (2.4 mM final concentration of 7-KC) or ethanol (same volume as used for 7-KC) (20). The amount of 7-KC in LDL was determined by gas chromatography as described previously (13). After isolation, all LDL were dialyzed against PBS containing 75 μM EDTA, pH 7.4, and stored in the dark at 4°C until use. Before use, LDL was further dialyzed against D-PBS alone. Acetylated LDL (AcLDL) was prepared by the repetitive addition of acetic anhydride to LDL (22) and OxLDL was prepared by treatment with $CuSO_4$ at 37°C for 24 h as described previously (21). The extent of LDL oxidation was assessed by thiobarbituric acid-reactive substance (TBARS) formation (23). In addition, oxidation and acetylation of LDL samples were also confirmed by comparison with native LDL by electrophoretic mobility (1% agarose gels; Helena Laboratories, Beaumont, TX). In these assays LDL bands were visualized by staining with Fat Red 7B (Sigma).

Acetylated LDL uptake and foam cell generation

Differentiated THP-1 cells (after 7 days of 7-KC treatment at 5 μg/ml) were treated with trypsin and replated in 24-well plates at a density of 2.5×10^5 cells/well for 24 h. The readherent cells were then incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled AcLDL (5 μg of protein per ml; Biomedical Technologies, Stoughton, MA) with or without excess unlabeled competitors [AcLDL or native LDL (nLDL), 10–200 μg of protein per ml, for 4 h at 37°C and 5% CO_2]. The cells were then washed with D-PBS and fluorescence was measured at 520 nm (excitation) and 575 nm (emission) with a Victor2 multilabel counter (EG&G Wallac, Turku, Finland) at 37°C. The specific fluorescence intensity resulting from each treatment was corrected by subtracting autofluorescence intensity obtained from nontreated THP-1 cells.

To assess the effect of 7-KC exposure on foam cell formation, THP-1 cells were treated with 7-KC (5 μg/ml) for 7 days. At this time, the nonadherent cells were removed, and the adherent cells remaining were washed and growth medium was replenished with AcLDL or OxLDL (50 μg of protein per ml) for 24 h. The cells were then washed with D-PBS, fixed with 4% paraformaldehyde, and stained with oil red O (Sigma). Stained neutral intracellular lipids were then examined by light microscopy (×40 objective).

Cellular protein isolation and Western blot analyses

Whole cell lysates were collected 7 days after treatment with 7-KC, PMA, or vehicle as described above. The cells were lysed in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, phenylmethylsulfonyl fluoride (100 μg/ml), aprotinin (30 μl/ml; Sigma, A6279) and 1 mM sodium orthovanadate. The protein content of each sample was determined by the bicinchoninic acid method (BCA; Pierce) before storage at −70°C.

Protein samples (50 to 100 μg/lane) were separated by electrophoresis (4 to 20% acrylamide gradient gels; Invitrogen/Novex, Carlsbad, CA), and transferred to nitrocellulose (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, NJ) by standard electroblotting procedures with use of a buffer containing 0.25 M Tris, 1.92 M glycine, 0.04% sodium dodecyl sulfate, and 20% methanol. The blots were pretreated with a solution containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5% nonfat dried milk for at least 1 h before the addition of the primary antibodies (dilution range varying from 1:500 to 1:1000) for CD68 (Dako), or CD36 (Novocastra Laboratories, Ltd.). After incubation overnight, the primary antibodies were removed and appropriate horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN) were added in a dilution range of 1:2500 to 1:5000 for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and by exposure to X-ray film (Hyper ECL; Amersham Pharmacia Biotech) for various times. Quantification of the protein bands was then accomplished by densitometry (ChemiImager 4400; Alpha Innotech, San Leandro, CA).

Total RNA isolation and mRNA analyses

THP-1 and primary monocytes were treated with 7-KC and PMA for 7 days as described above. Total RNA was extracted from the cells by the TRIzol method (GIBCO-BRL, Grand Island, NY). After acid-phenol-chloroform extraction and isopropyl alcohol precipitation, the resulting RNA pellet was washed with ice-cold 80% ethanol and redissolved in nuclease-free water. The quantity of RNA was then determined by absorbance at 260 nm before storage at −70°C.

The effect of 7-KC on CD68, CD36, and macrophage scavenger receptor class A-1 (SRA-1) mRNA abundance was determined by reverse transcription-polymerase chain reaction procedures (Access RT-PCR system; Promega Corp., Madison WI). Briefly, this system supports synthesis and amplification of cDNA products in a single tube by a one-step reaction that provides a buffer system allowing for optimal activity of combined avian myeloblastosis virus reverse transcriptase and *Thermus flavus* (*Tfl*) DNA polymerase. Total RNA (0.1 to 1.0 μg) was added in the system along with forward and reverse primers for each of the following genes: CD68, 5′-CGTCACAGTTCATCCAACAAGC-3′ and 5′-TTGGGGTTCAGTACAGAGATGC-3′ (332-bp product); CD36, 5′-GCTGAGGACAACACAGTCTC-3′ and 5′-GCTGATGTCTAGC ACACCAT-3′ (674-bp product); and SRA-1, 5′-TCTCATTGGA ATAGTGGCAGC-3′ and 5′-TATTGGACCTGGAAATCCTCG-3′ (699-bp product). For each set of analyses, genomic β-actin transcripts (331-bp product) were also amplified and examined as an internal control (24). Reverse transcription was performed at 48°C for 45 min, and the cDNA amplification program consisted of an initial denaturation step (94°C for 2 min) followed by 35 amplification cycles of denaturation (94°C) for 30 s, annealing (59°C for CD36 and 63°C for CD68 and SRA-1) for 1 min, and extension (68°C) for 7 min. Each amplification reaction was analyzed by electrophoresis (10 μl/lane) in gels containing 1.5% agarose and stained with ethidium bromide.

Statistical analyses

Analysis of variance was conducted to examine whether significant ($P < 0.05$) main treatment and time effects occurred. Additional post hoc comparisons of treatment means were conducted by using the Dunnett's *t*-test (treatments vs. controls) and Bonferroni *t*-test (selected comparisons) as indicated. Data given represent means ± standard deviation.

RESULTS

Evidence of THP-1 cell differentiation

Initial studies examining the effect of 7-KC on THP-1 cell differentiation demonstrated that these cells, originally in suspension, began to aggregate in clusters of 7 to 15 cells after 24 h of 7-KC treatment. With longer duration of exposure to this oxysterol (~3 days), a population of the cells began to adhere. Within 7 days of culture, the adherent THP-1 cells displayed hypertrophy, developed vacuoles in the cytoplasm, and formed extended processes; all characteristics of mature macrophages (**Fig. 1B**). In

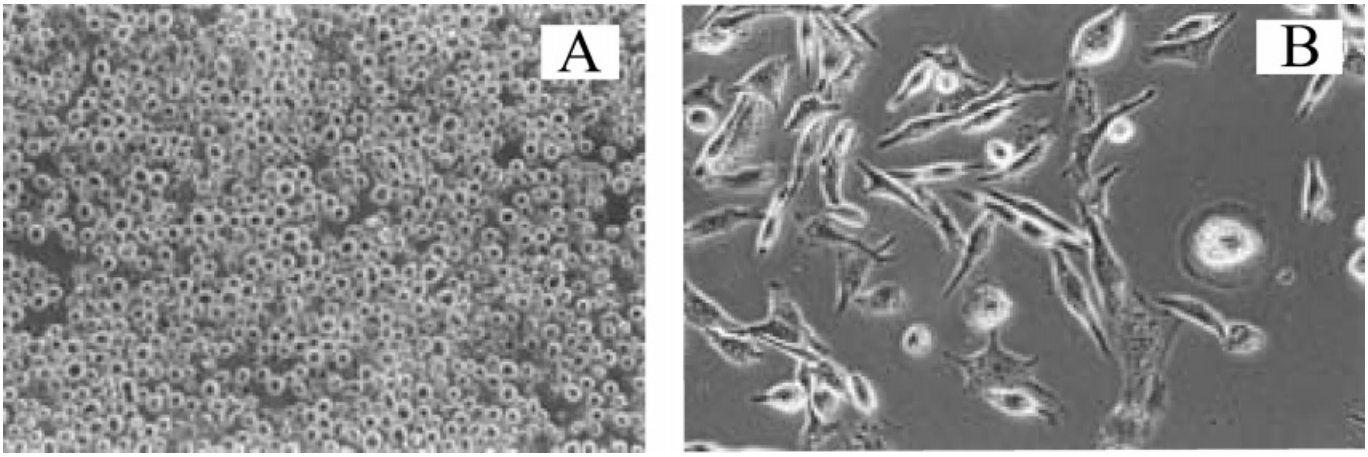

Fig. 1. THP-1 cells develop characteristic macrophage morphology after exposure to 7-KC. THP-1 suspension cells were treated with either ethanol vehicle (A) or 7-KC at 5 μg/ml (B) for a period of 7 days. THP-1 cells began to adhere after 3 days of 7-KC treatment and maximal effects on adherence were demonstrated after 7 days. In contrast, cholesterol treatment did not induce cell adherence over the same time frame (data not shown). Shown are representative phase-contrast microscopy (×20 objective) images.

contrast, THP-1 cells treated with vehicle alone (Fig. 1A) and cells treated with cholesterol (results not shown) did not develop these aforementioned morphological changes.

We also directly counted the adherent THP-1 cells resulting from exposure to 7-KC (range, 0.5 to 10 μg/ml) for 7 days. The number and percentage of adherent cells increased in proportion to increasing concentration and time of exposure to 7-KC (ANOVA, $P < 0.0001$; linear effect, $P < 0.001$). 7-KC at 10 μg/ml induced nearly 45% of the initial cell population to become adherent (**Fig. 2**).

To more accurately assess the effects of low doses of 7-KC on THP-1 cell adherence, total protein in cell lysates taken from each well was determined. The results from this procedure showed that total protein content of adherent cells also increased after 7-KC treatment in a dose-dependent manner ($P < 0.001$; data not shown). In addition, a strong correlation between cell number and protein concentration of adherent cells ($r = 0.98$; $P < 0.001$) was present in these experiments, indicating that the increase in adherent cell protein was not due to an increase in cellular hypertrophy, but actually resulted from an increase in cell number. On the basis of these results, the amount of adherent cell protein was used in the majority of subsequent experiments to assess effects of 7-KC treatment on cell adherence.

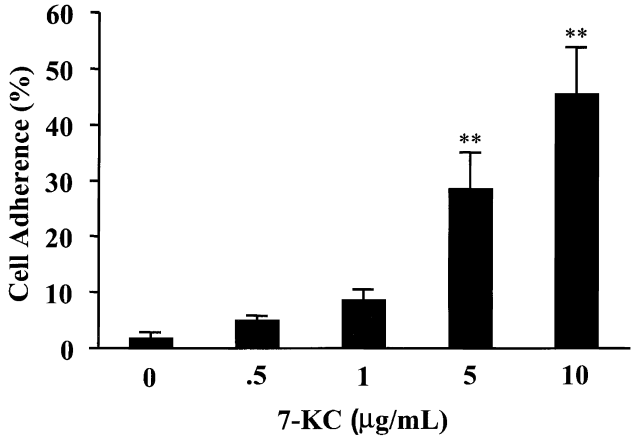

Fig. 2. The effect of 7-KC on THP-1 cell adherence. THP-1 cells were treated with vehicle control (ethanol) or 7-KC (0.5 to 10 μg/ml) for 7 days. Nonadherent cells were washed off and the remaining adherent cells were released by trypsin and counted. Data shown represent the percentages of initially plated cells that have adhered and represent means ± SD of three individual experiments. ANOVA, main treatment effect, $P < 0.001$. ** $P < 0.01$ versus control by Dunnett's test.

Similar experiments were performed with LDL that was isolated from plasma exposed to vehicle only or 7-KC before isolation. Vehicle-treated LDL (TBARS = 6 nmol/mg protein, 7-KC concentration = 15.5 nmol/mg protein) and LDL loaded with 7-KC (TBARS = 4 nmol/mg protein, 7-KC concentration = 283 nmol/mg protein) were added to THP-1 cells in a concentration that ranged between 12.5 and 50 μg of LDL protein per ml for 7 days. Whereas vehicle-treated LDL had little effect on THP-1 cell adherence, LDL that was enriched with 7-KC induced marked changes in cell adherence and morphology that are characteristic of differentiation. These effects occurred in a dose-related fashion (data not shown) and maximal cell adherence for the 7-KC-loaded LDL treatment occurred at 50 μg of protein per ml (**Fig. 3**). In addition, the effect on cell adherence produced by 7-KC-enriched LDL matched those induced by 7-KC that was solubilized in ethanol. In separate experiments, OxLDL (TBARS = 80 to 100 nmol/mg protein) also induced cell adherence; however, this effect appeared slightly less than that of 7-KC treatment alone. Interestingly, the effect of 7-KC-loaded LDL on THP-1 cell adherence occurred between days 3 and 7, a time frame that is comparable to induction of monocyte differentiation by OxLDL or 7-KC in solution.

Because previous reports have shown that oxysterols may produce cytotoxicity in other cell types, we examined

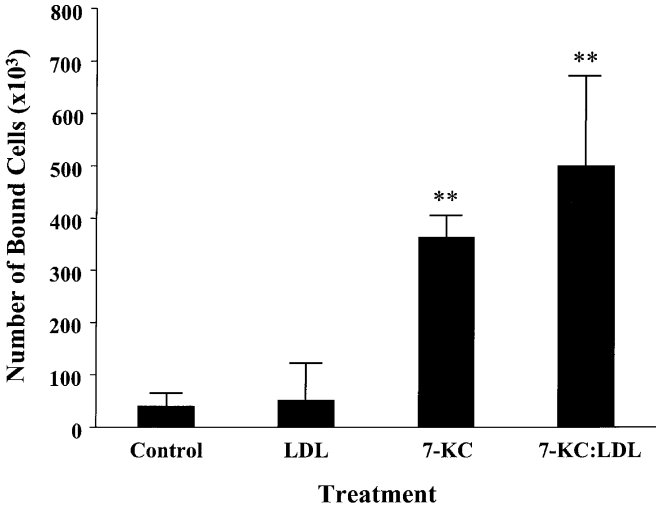

Fig. 3. The effect of 7-KC-loaded LDL on THP-1 cell adherence. Plasma from healthy human volunteers was treated with 7-KC or vehicle (ethanol) alone before isolation of LDL (see Experimental Procedures). Subsequently, vehicle alone (Control), vehicle treated LDL (LDL; 50 μg/ml), 7-KC solubilized in vehicle (7-KC; 5 μg/ml), and 7-KC loaded LDL (7-KC:LDL; 50 μg/ml) were added to THP-1 cells for 7 days. Nonadherent cells were washed off and the remaining bound cells were released by trypsin and counted. Data shown represent means ± SD of adherent cells counted from three individual experiments. ANOVA, main treatment effect, $P < 0.01$. ** $P < 0.01$ versus control and LDL treatment by Bonferroni test.

whether the dose range of 7-KC used in the present study may induce THP-1 cell death. Experiments examining the extent of trypan blue staining in THP-1 cells remaining in suspension after 7-KC treatment demonstrated that concentrations of 7-KC ranging from 0.5 to 5.0 μg/ml did not induce significant cell death when compared with control cells. In contrast, exposure of THP-1 cells to 7-KC at 10 μg/ml for 7 days significantly increased the number of trypan blue-stained nonadherent cells (~34% nonviable cells vs. 2.4% for controls, $P < 0.05$). In contrast to the THP-1 cells remaining in suspension, adherent cells did not stain positive for trypan blue even when this population of cells was exposed to levels of 7-KC up to 25 μg/ml.

The release of LDH from THP-1 cells exposed to 7-KC followed a similar trend as trypan blue staining. Only the 10 μg/ml dose of 7-KC significantly increased membrane leakage and release of LDH in the total population of THP-1 cells after 7 days of treatment (3.9-fold increase in LDH vs. nontreated controls, $P < 0.01$). In contrast, in a separate set of experiments, LDH release was not induced by 7-KC treatment (up to 25 μg/ml) in the adherent population of THP-1 cells. On the basis of these data, the concentration of 7-KC used in subsequent cell culture experiments did not exceed 5 μg/ml.

Expression of proteins of macrophage differentiation

To further establish whether 7-KC induced THP-1 cell differentiation, THP-1 cells that became adherent subsequent to this treatment were examined for the expression of selected protein markers (CD68, CD36, and CD11b) that characterize mature macrophages. The entire population of adherent cells demonstrated high levels of CD68 and CD11b expression (**Fig. 4B** and **D**, respectively), whereas only ~75% of the cells expressed CD36 (Fig. 4C). In contrast, control cells exposed to a nonspecific antibody (Fig. 4A), and cells that were treated with cholesterol (data not shown) did not demonstrate expression of these proteins. We also performed preliminary studies to determine whether 7-KC would also facilitate differentiation of human peripheral monocytes. As with THP-1 cells, human monocytes treated with 7-KC appeared to undergo morphologic changes characteristic of differentiation to macrophages at an accelerated rate as compared with untreated cells (data not shown). Moreover, in comparison with untreated monocytes (Fig. 4G), cells treated for 1 week with 7-KC at 5 μg/ml demonstrated striking expression of CD36 (Fig. 4H).

To quantify and confirm expression of the macrophage markers, Western blot analyses were performed on THP-1 cells treated with 7-KC. In these experiments we confined our analysis to the scavenger receptors CD68 and CD36 because of their purported role in oxidized lipoprotein uptake and subsequent foam cell development. As shown in **Fig. 5**, 7-KC greatly increased CD68 abundance in differentiated THP-1 cells ($P < 0.01$ for 1 μg/ml and $P < 0.05$ for 5 μg/ml vs. controls). Moreover, CD36 protein expression was also significantly increased by 7-KC treatment (5 μg/ml vs. control, $P < 0.01$; Fig. 5).

Expression of scavenger receptor mRNA

RT-PCR analysis was employed to determine whether 7-KC treatment increased the expression of CD68, CD36, and SRA-1 mRNAs (**Fig. 6**). Similar to the level of protein, transcripts for CD68 and CD36 were increased by 7-KC treatment. In addition, there was a moderate increase in the level of SRA-1 mRNA, indicating that this class of scavenger receptor may also be upregulated by 7-KC treatment. Unlike the scavenger receptors, the expression of genomic β-actin transcripts was not enhanced by 7-KC treatment. These results further confirm that 7-KC treatment stimulates the expression of a variety of scavenger receptors, and suggest that regulation by this oxysterol may occur, in part, at the level of transcription.

Biological activity of THP-1-derived macrophages

To establish that 7-KC-induced differentiated cells demonstrate typical biological activity of macrophages, we examined whether these cells can phagocytose inert particles, take up modified LDL, and generate lipid-laden foam cells. As demonstrated in Fig. 4F, 7-KC-differentiated cells demonstrated a great capacity to take up multiple latex beads. These experiments also provide further evidence that prolonged exposure to 7-KC does not reduce the viability of differentiated THP-1 cells.

AcLDL is a well-recognized ligand for multiple scavenger receptors including SRA-1, CD36, and CD68. To demonstrate the ability of 7-KC-differentiated cells to bind and internalize modified lipoproteins we incubated adherent cells with either DiI-labeled AcLDL or DiI-labeled nLDL (5 μg of protein per ml) for 4 h at 37°C. In additional ex-

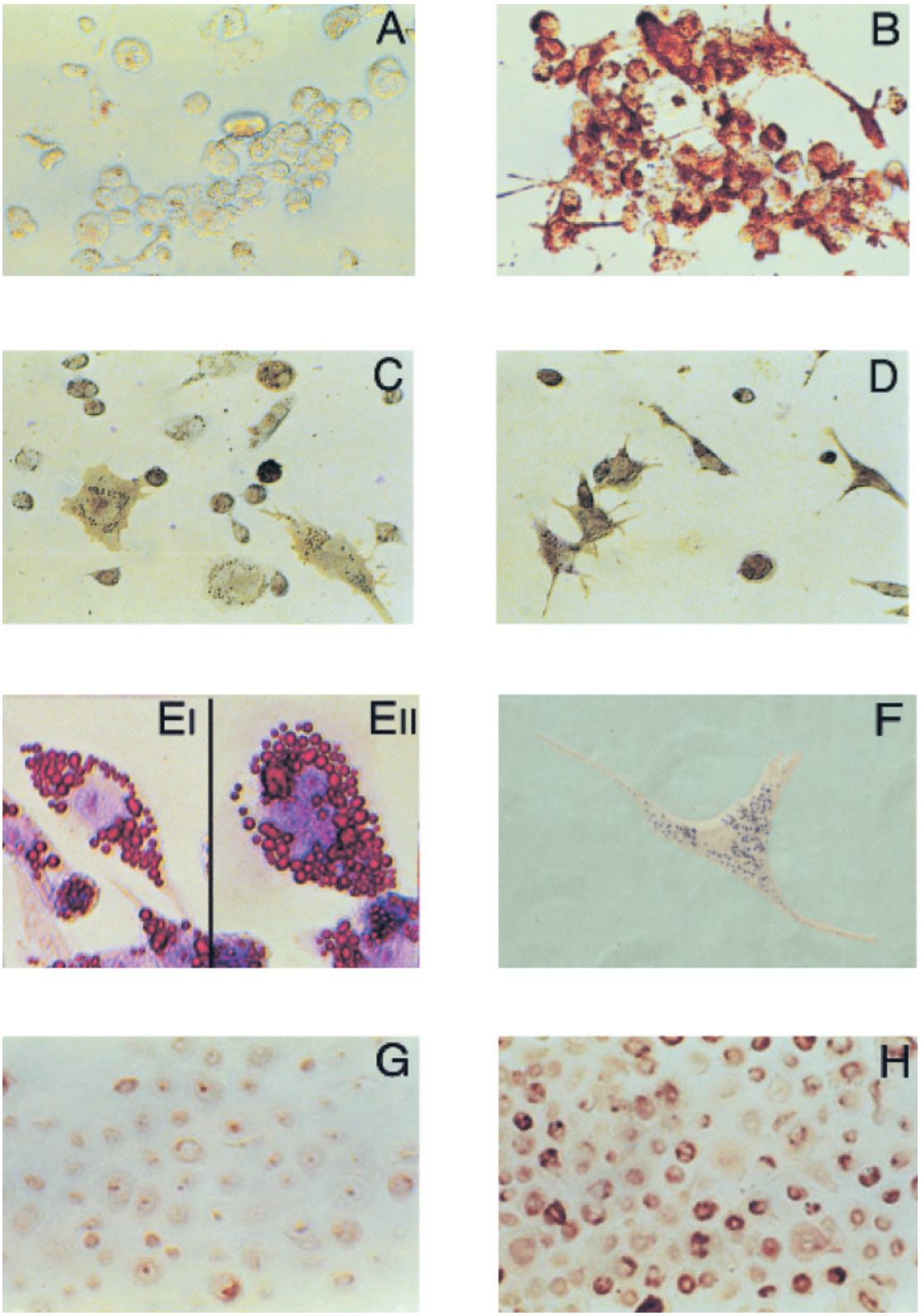

Fig. 4. Monocytes treated with 7-KC express markers of macrophage differentiation, phagocytose particles, and form foam cells. A to F: Demonstrated are characteristics of THP-1 cells induced to adhere after treatment with 7-KC (5 μg/ml) for 7 days. Immunohistochemical analyses (×40 images) were performed with adherent cells to examine the expression of the scavenger receptors CD68 (B) and CD36 (C), and the integrin CD11b (D). Control cells (A) were treated with a nonspecific antibody of the same IgG class as used for the other proteins described above. Adherent THP-1 cells accumulate numerous lipid droplets (stained with oil red O) after exposure to a 50 μg/ml concentration of oxidized LDL (E, panel I) or acetylated LDL(E, panel II) for 24 h; cresyl violet was used for nuclear staining. F: Adherent THP-1 cells actively phagocytose inert particles within 1 h of exposure to latex beads. Human primary monocyte-macrophages were treated with vehicle alone (G) or with 7-KC at 5 μg/ml (H) for 7 days and immunohistochemical analysis was performed to examine the expression of CD36.

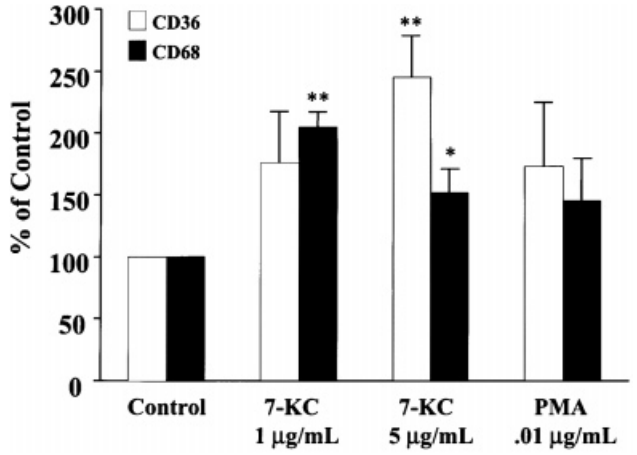

Fig. 5. CD68 and CD36 expression is stimulated in macrophages generated by 7-KC treatment of THP-1 cells. THP-1 cells were treated with vehicle (control) or 7-KC at 1 or 5 μg/ml or with PMA at 0.01 μg/ml for 7 days. Total cell protein was collected from adherent cells and protein expression was determined by Western blot analyses. Laser density units were determined from autoradiographs. Data presented represent means ± SD of three individual experiments that have been normalized for β-actin expression. * $P < 0.05$ versus control, ** $P < 0.01$ versus control.

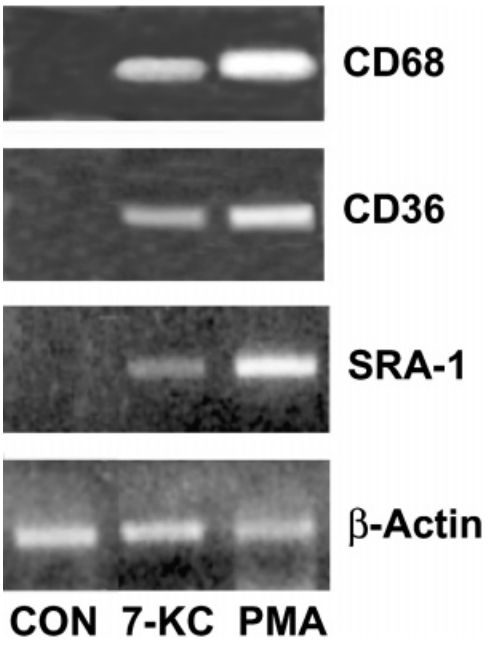

Fig. 6. 7-KC increases the level of CD68, CD36, and SRA-1 mRNA in macrophages generated by 7-KC treatment of THP-1 cells. THP-1 cells were treated with vehicle alone (control; CON), 7-KC at 5 μg/ml, or PMA at 0.01 μg/ml for 7 days. Total RNA was extracted from cells and used for reverse transcriptase-polymerase chain reaction analyses for determination of CD68, CD36, SRA-1, and genomic β-actin transcript production. Ten microliters of each reaction was separated on a 1.5% agarose gel that was stained with ethidium bromide.

periments unlabeled AcLDL (20–200 μg of protein per ml) or nLDL (200 μg of protein per ml) were added as competitors for labeled AcLDL binding and uptake. In the absence of competitors, cell-associated (bound or internalized) AcLDL was significantly greater than cell-associated nLDL (solid columns, 7.6-fold increase, $P < 0.001$; **Fig. 7**). In addition, unlabeled AcLDL, but not nLDL, effectively reduced the cellular association of labeled AcLDL in a dose-dependent manner (open columns; Fig. 7), thus demonstrating specificity of the THP-1 macrophages in recognizing and binding AcLDL.

Additional experiments were conducted to examine whether prolonged incubation of 7-KC-induced differentiated cells with AcLDL would promote development of lipid-laden foam cells. The addition of AcLDL at 50 μg of protein per ml to these cells for 24 h led to marked lipid internalization and foam cell development (Fig. 4E, panel II). This effect was also mimicked by the addition of OxLDL at the same concentration for 24 h (Fig. 4E, panel I).

Specificity of oxysterol induction of differentiation

Previous work has demonstrated that other oxysterols, for example, 25-hydroxycholestrol, may regulate important cellular processes (5, 25). Thus, we examined whether other forms of oxysterols may also induce THP-1 differentiation. A panel of oxysterols, including 7β-hydroxycholesterol, 7α-hydroxycholesterol, *22*(*R*)-hydroxycholesterol, and 25-hydroxycholesterol, was added to THP-1 cells over the range of concentrations (0.5 to 5 μg/ml) that was previously established for 7-KC. As shown in **Fig. 8**, 7-KC had the greatest capacity to induce THP-1 cell differentiation, and this effect occurred in a dose-dependent manner. Treatment with 7β-hydroxycholesterol and 22(*R*)-hydroxycholesterol induced minimal THP-1 cell differentiation after 7 days of treatment. Furthermore, increasing concentrations of 7β-hydroxycholesterol and 22(*R*)-hydroxycholesterol (>5 μg/ml) markedly increased cell toxicity and did not further enhance monocyte differentiation. In contrast to the other oxysterols, 25-hydroxycholestrol did not effect THP-1 differentiation when provided at any level (Fig. 8). Lower concentrations of all the above-described oxysterol compounds (<0.5 μg/ml) were also tested, and these levels did not induce THP-1 cell differentiation (data not shown).

DISCUSSION

The factors that contribute to the transformation of monocytes to macrophages in the artery wall are not well understood. Previous studies have indicated that OxLDL may play a role in promoting differentiation of monocytes to macrophages (10, 11). It has been demonstrated that oxidized phospholipids from minimally oxidized/modified LDL (MM-LDL) particles are proinflammatory and may be responsible for numerous atherogenic consequences. Many of the proposed effects of MM-LDL occur through activation of neighboring vascular wall cells (2). One such example is the stimulation by MM-LDL of macrophage colony-stimulating factor (M-CSF) from endothelial cells (11). This cytokine is a lineage-specific growth factor responsible for the proliferation and differentiation of bone

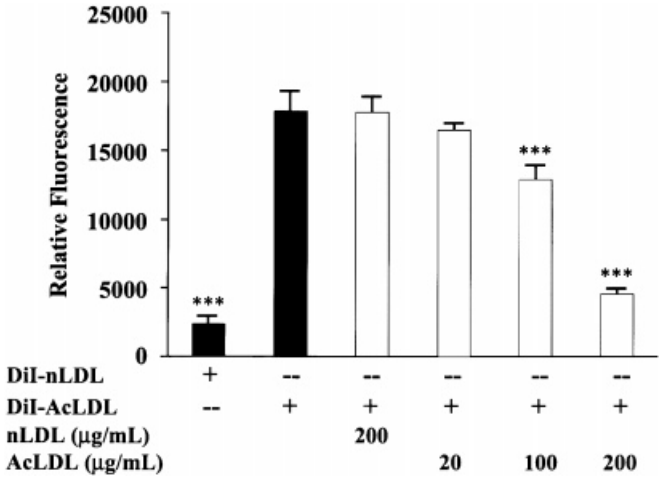

Fig. 7. Binding/internalization of acetylated LDL (AcLDL) in macrophages generated by 7-KC treatment of THP-1 cells. THP-1 cells were treated with 7-KC (5 μg/ml) for 7 days. Adherent cells were then treated with DiI-labeled nLDL (DiI-nLDL, 5 μg of protein per ml) or DiI-labeled AcLDL (DiI-AcLDL, 5 μg of protein per ml) (solid columns) and unlabeled competitors (open columns) for 4 h at 37°C before the determination of relative fluorescence (see Experimental Procedures). Data represent means ± SD of three separate experiments. ANOVA main effect, $P < 0.001$. Selected post hoc comparisons were performed by the Bonferroni test: *** $P < 0.001$ for DiI-labeled nLDL versus DiI-labeled AcLDL, and for unlabeled LDL competitors versus DiI-labeled AcLDL.

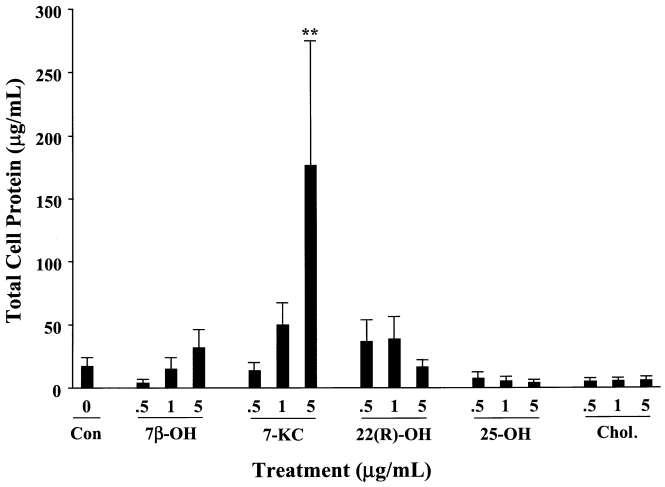

Fig. 8. The effects of selected oxysterols on THP-1 cell differentiation. THP-1 cells were treated with vehicle (control; Con) or with 0.5-, 1-, or 5-μg/ml concentrations of 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-KC), 22(*R*)-hydroxycholesterol [22(*R*)-OH], 25-hydroxycholesterol (25-OH), or cholesterol (Chol.) for 7 days. At this time, total cell protein from adherent cells was assessed. Data represent mean total protein levels ± SD obtained from multiple experiments (n = 3–6). ** Significant at $P < 0.01$ versus control.

marrow-derived progenitor cells into mononuclear phagocytes and may promote differentiation of mature monocytes (26). The consequences of MM-LDL formation are likely to be particularly relevant in early lesion formation. As atherosclerotic lesions become more complex, oxidative stress presumably increases, leading to more extensive oxidation of retained lipoproteins and cell membrane lipids and greater generation of oxysterols, such as 7-KC (13, 14, 27). Indeed, these products of cholesterol oxidation are found in increased concentrations in foam cells and in advanced lesions (15, 16).

In this study, we demonstrate that 7-KC can directly promote differentiation of THP-1 cells, a well-characterized monocytic cell line (28, 29). THP-1 cells treated with 7-KC demonstrated enhanced adherence to tissue culture plates, and developed morphologic changes and expression of cellular markers that are characteristic of mature macrophages. Similar to the time course of human monocyte differentiation in vitro, this process occurred gradually, requiring 3 to 7 days for THP-1 cell-derived macrophages to develop. Importantly, cells treated with 7-KC-loaded LDL were also induced to adhere and develop the same morphologic changes of differentiation. As these LDL particles were not oxidized and were enriched only in 7-KC, these experiments clearly demonstrate that 7-KC, whether free in solution or carried within lipoproteins, is a potent stimulus of monocyte differentiation. THP-1 cells exposed to OxLDL also differentiated over the same time frame as cells exposed to 7-KC. Although many products are formed during LDL oxidation, it has been repeatedly demonstrated that a predominant oxysterol resulting from this process is 7-KC (12–14). Therefore, these studies suggest that 7-KC may be one of the components within this form of LDL that actively induces monocyte differentiation.

THP-1 cells induced to adhere to tissue culture plates after treatment with 7-KC displayed the typical biological functions of macrophage cells. They demonstrated active phagocytosis and were able to take up modified lipoproteins through scavenger receptors in an unregulated fashion, leading to foam cell formation in vitro. Human peripheral monocytes treated with 7-KC also appeared to undergo morphologic changes characteristic of differentiation at an accelerated rate as compared with untreated cells. Moreover, we also established that 7-KC can promote increased scavenger receptor expression in these human monocytes. These latter experiments suggest that the effect of 7-KC is not limited to the THP-1 cell line.

The mechanism by which 7-KC induces macrophage differentiation is unclear. One may speculate that 7-KC may induce release of cytokines that directly influence monocyte differentiation. The relatively slow induction of cell adherence and morphologic changes seen in 7-KC-treated cells is consistent with this hypothesis and is in sharp contrast to the rapid effects produced by PMA, which utilizes the protein kinase C pathway. It has also been previously demonstrated that OxLDL and oxysterols, including 7-KC, are capable of inducing proinflammatory cytokines in other vascular cells (2, 4, 19). In preliminary studies we have shown that 7-KC stimulates the production of M-CSF and granulocyte-macrophage (GM)-CSF in THP-1 cells (data not shown). As noted above, these cytokines are potent stimulators of differentiation and proliferation of bone marrow mononuclear cell precursors and may contribute to the differentiation of mature monocytes. Alter-

natively, 7-KC may also have a direct regulatory effect on THP-1 cell differentiation. Oxysterols have been shown to have a multitude of effects on gene regulation (5, 30, 31). LXR and SF-1 are two sterol receptors identified to date that preferentially bind 22(*R*)-hydroxycholesterol and 25-hydroxycholesterol, respectively, and facilitate oxysterol-mediated regulation of cell lipid metabolism (30, 31). Of importance, 22(*R*)-hydroxycholesterol and 25-hydroxycholesterol, along with several other forms of oxysterols and cholesterol, were relatively ineffective in inducing THP-1 differentiation, suggesting that 7-KC may act through a unique pathway. Current studies in our laboratory are focusing on elucidation of potential mechanisms of 7-KC action.

Evidence that oxysterols may contribute to atherosclerosis has accumulated over several decades. Plasma levels of oxysterols appear higher in individuals with atherosclerotic disease than in healthy controls (32). Moreover, plasma levels of 7-KC are elevated in animals displaying hypercholesterolemia (15), and these levels are comparable to those used in culture in the present study. A consistent finding reported in the literature has also been the presence of increased amounts of oxysterols, including 7-KC, in foam cells and atherosclerotic plaque (4, 15, 16). Concentrations of oxysterols in these microenvironments are substantially greater than in the plasma, suggesting that oxysterol enrichment may occur in the atherosclerotic plaque. Perhaps the strongest evidence supporting a role for oxysterols in atherosclerotic disease has been that animals fed or injected with oxysterols have frequently developed greater atherosclerosis than their control counterparts (16, 33–35).

As the concept that oxidation of lipoproteins may contribute to atherosclerosis has developed, there has been increasing interest in the role of different components of OxLDL in these events. With more extensive oxidation of LDL, as would occur in more advanced lesions or within foam cells, greater accumulation of 7-KC is present (14, 27). This oxysterol has already been shown to be bioactive and presumably is atherogenic. The current study suggests that 7-KC may also accelerate monocyte differentiation and foam cell formation. Moreover, OxLDL (and perhaps one or more oxysterol components) appears to have the ability to increase the proliferation and survival of macrophages (36). This combination of increased generation, proliferation, and prolonged survival of macrophages exposed to environments that are enriched with oxysterols may certainly contribute to progression of atherosclerosis. Greater understanding of the signaling mechanisms that underlie these events may provide additional therapeutic targets for prevention of atherosclerosis.

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