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Atherosclerosis and sterol 27-hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages

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ABSTRACT **27-Hydroxycholesterol was found in surprisingly high amounts in atherosclerotic human femoral arteries. When human macrophages were cultured in a medium containing serum, there was a significant transfer of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid from the cells into the medium. Sterol 27-hydroxylase (EC 1.14.13.15) is likely to be responsible for formation of the two products as shown by use of immunoblotting, a specific inhibitor, and the ^{18}O-labeling technique. Sterol 27-hydroxylase has the unusual ability to hydroxylate the same methyl group three times to give a carboxylic acid; thus, 3β-hydroxy-5-cholestenoic acid is likely to be a direct product of the enzyme. The production of these steroids increased after addition of cholesterol to the culture medium. By using deuterium-labeled cholesterol, it was ascertained that most of the oxidized products were formed from exogenous cholesterol taken up by the cells. 27-Hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid are present in the circulation and are efficiently converted into bile acids in human liver. It is suggested that conversion of cholesterol into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid represents a general defence mechanism for macrophages and possibly also other peripheral cells exposed to cholesterol. Absence of this defence mechanism may contribute to the premature atherosclerosis known to occur in patients with sterol 27-hydroxylase deficiency (cerebrotendinous xanthomatosis).**

The sterol 27-hydroxylase (EC 1.14.13.15) has an important role in the degradation of the side-chain in the formation of bile acids from cholesterol in the liver (for a review, see ref. 1). However, this enzyme is present not only in the liver but also in several other organs and tissues (2–5). The abundance of mRNA for the enzyme seems to parallel the cholesterol biosynthesis capacity of the tissues.

The possibility has been discussed that the 27-hydroxylase is of importance for the overall regulation of cholesterol biosynthesis (1, 6). The product, 27-hydroxycholesterol, is a potent inhibitor of the rate-limiting enzyme in cholesterol synthesis, the hydroxymethylglutaryl (HMG)-CoA reductase. Since it is possible to survive a complete or almost complete lack of the enzyme for several decades (7), it is evident that the enzyme cannot be obligatory for the down-regulation of cholesterol synthesis in various tissues. In a recent work from this laboratory, it was shown that a hepatic 27-hydroxylation is not of importance in the down-regulation of HMG-CoA reductase by dietary cholesterol in mice (8).

In the search for a role of the sterol 27-hydroxylase in extrahepatic tissues, we have speculated that the sterol 27-hydroxylase may be involved in elimination of cholesterol from the cells (1). 27-Hydroxycholesterol and, in particular, its oxidation product 3β-hydroxy-5-cholestenoic acid, are considerably more polar than cholesterol. Recently, it was shown that the sterol 27-hydroxylase is responsible not only for formation of the 27-hydroxylated product of a C-27 steroid but also for the further oxidation of this compound into the corresponding acid (5, 9). Thus, the sterol 27-hydroxylase has the unusual capacity to hydroxylate the same methyl group three times. If there is a formation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the cells, these steroids may be transported out from the cell more easily than cholesterol. Intracellular oxysterol-binding protein has a high affinity towards side chain-hydroxylated cholesterol species (10, 11). However, oxysterol-binding protein(s) may be present also in the circulation, and thus there may be an exchange of oxysterols over the cell membranes.

The present work supports the above hypothesis. Cultured human alveolar macrophages are shown to convert exogenous cholesterol into 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, which accumulate in the culture medium. In accordance with previous work (12, 13), we also show that 27-hydroxycholesterol is the dominating oxysterol in the atheromas. It is suggested that the sterol 27-hydroxylase is involved in a general defence mechanism for cells exposed to cholesterol.

MATERIALS AND METHODS

Collection of Material from Atheromas. Two males (ages 53 and 64) and three females (ages 78, 84, and 84) with disabling claudicatio and gangrene of one extremity, underwent surgical vascular reconstruction because of occluded common femoral arteries. At surgery, in which a thrombendarterectomy was performed, a small piece (10 × 10 mm) of the excised portion of the atherosclerotic material was saved for oxysterol analysis. The excised material was frozen in liquid nitrogen and ground with a mortar and pestle. The powder obtained was extracted with chloroform/methanol, 2:1 (vol/vol), and further processed as described below.

Chemicals, Enzymes, and Media. Deuterium-labeled and unlabeled oxysterols and trideuterated cholesterol were those used previously (8, 14). Medium 199, fetal calf serum, and delipidized serum were obtained from GIBCO/BRL. Ampules (100 ml) with 99.5% $^{18}O_2$ were obtained from Larodan Fine Chemicals (Malmö, Sweden). The rabbit antibody towards human sterol 27-hydroxylase was a gift from David Russell (University of Texas Southwestern Medical Center, Dallas). This antibody is directed against amino acids 15–28 of sterol 27-hydroxylase protein. The secondary antibody (goat antirabbit IgG–horseradish peroxidase conjugate) was obtained from Bio-Rad.

Isolation of Alveolar Macrophages and Endothelial Cells. Bronchoalveolar lavage (BAL) was performed on patients as described (15). Aliquots (5 ml) of BAL fluid were removed,

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stained with May Grünewald–Giemsa, and subjected to differential cell counting after cytocentrifugation. Approximately 90% of the recovered cells were alveolar macrophages.

Human endothelial cells were isolated from umbilical veins essentially as described (16, 17). More than 99% of the cells recovered were endothelial cells.

Culture of Macrophages. The lavage fluid was separated from the cells by centrifugation, and the cells obtained were allowed to adhere to plastic bottles containing minimal essential medium (MEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, benzylpenicillin (400 units/ml), and streptomycin (0.2 mg/ml). The cells were cultured (3-ml volumes) in dishes at 37°C in an atmosphere of 5% CO_2 in air. The number of macrophages in each well varied between 1.2 and 3.6 × 10^6 in different experiments. After 4 h, nonadherent cells (mainly lymphocytes) were washed away. The remaining alveolar macrophages were refed with the same medium. In some experiments, different amounts of unlabeled or deuterium-labeled cholesterol were added to the medium dissolved in 20 μl of ethanol. After various periods of time at 37°C in air containing 5% CO_2, the medium was removed and analyzed.

In some experiments macrophages were cultured in closed tubes in an atmosphere containing 20% $^{18}O_2$, 75% N_2, and 5% CO_2. Most of the air in the tube (10 ml) was replaced by bubbling 30 ml of the above gas mixture through the medium. The tube was then closed and incubated as above.

Endothelial Cells. Medium 199 with 20% fetal calf serum and 1% L-glutamine was used as the feeding medium. Each milliliter contained 100 μg of endothelial mitogen, 100 μg of benzylpenicillin, and 100 μg of streptomycin. The medium was adjusted to pH 7.4 with 2.5% $NaHCO_3$ and filter-sterilized.

The cell suspension was transferred to tissue culture dishes coated with gelatine. Feeding medium was added to a final volume of 1.5 ml per well. In general, each well contained 1.5–2 × 10^6 cells. Finally, the dishes were incubated at 37°C in air containing 5% CO_2. After various periods of time, the medium was removed and analyzed.

Analysis of Extracts of Femoral Arteries. After evaporation of the organic solvent from the extracts, 4 ml of ethanol and 0.5 ml of 5 M NaOH were added, and the mixture was stirred at room temperature under argon for 15 h. Neutralization was accomplished by addition of 2.5 ml of 1 M HCl, and the volume was reduced to 4 ml. Lipids were extracted with 20 ml of chloroform/methanol, 2:1 (vol/vol). After evaporation of the solvent, the samples were dissolved in 0.5 ml of chloroform and fractionated on a Bond-Elut NH_2 column as described (14). Only the neutral lipid fraction was collected. The solvent was removed under a stream of argon, and the residue was dissolved in 0.5 ml of methanol/water, 90:10 (vol/vol). The oxysterols were then analyzed by mass spectrometry (14). The assay of 7α-hydroxycholesterol was modified, as compared with the previous work, to use $^2H_5$-substituted 7α-hydroxycholesterol ($^2H_5$-7α-hydroxycholesterol) as internal standard. In addition, the contents of 7β-hydroxycholesterol and 24-hydroxycholesterol were assayed simultaneously with use of $^2H_7$-substituted 7β-hydroxycholesterol and $^2H_7$-substituted 24-hydroxycholesterol, respectively, as internal standards ($^2H_7$-7β- and $^2H_7$-24-hydroxycholesterol). The ions at m/z 456/463 were used in both the latter quantitations.

Analysis of Extracts of Medium from Cell Culture Experiments. To a defined volume (1–3 ml) of the cell medium, 0.5 μg of 3β-hydroxy-5-cholenoic acid and 2 μg of $^2H_5$-27-hydroxycholesterol were added. The medium was acidified with hydrochloric acid, diluted to 10 ml with water, and extracted with 15 ml of diethyl ether. The ether phase was washed with water until neutral, and the solvent was removed under reduced pressure. The residue was dissolved in 0.5 ml of chloroform and purified on a Bond-Elut NH_2 cartridge (18). The neutral lipid fraction, containing 27-hydroxycholesterol, and the fatty acid fraction, containing 3β-hydroxy-5-cholenoic acid and 3β-hydroxy-5-cholestenoic acid, were collected. The fractions were blown to dryness under a stream of argon, the fatty acid fraction was methylated with diazomethane, and both fractions were converted into trimethylsilyl ethers.

Gas Chromatography/Mass Spectrometry. A Hewlett–Packard 5890 GC coupled to a Hewlett–Packard 5970 MSD mass spectrometer was used. The gas chromatograph was equipped with a 25 m × 0.2-mm i.d. × 0.33-μm phase thickness Ultra-1 column (Hewlett–Packard). The temperature program was as follows: 180°C for 1 min, increase at 35°C/min to 270°C, and increase at 20°C/min to 300°C, where the temperature was kept for 20 min. $^2H_5$-27-hydroxycholesterol was used as an internal standard for 27-hydroxycholesterol, and 3β-hydroxy-5-cholenoic acid was the internal standard for 3β-hydroxy-5-cholestenoic acid. The quantitations were performed in the selected ion-monitoring mode with standard curves. Additional full-spectrum analyses were performed to ensure the identity of the compounds recovered. Ions used for quantitations were: 3β-hydroxy-5-cholenoic acid, sum of ions 460, 370, and 331; 3β-hydroxy-5-cholestenoic acid, sum of ions 502, 412, and 373; $^2H_5$-27-hydroxycholesterol, ion 551; and 27-hydroxycholesterol, ion 546. Content of ^{18}O and ^2H in 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid was calculated as described by Biemann (19).

Immunoblotting (Western Blotting). Crude homogenate of the cells was subjected to SDS/polyacrylamide gel electrophoresis as described by Laemmli (20) with 10% gels. The proteins were then transferred to a nitrocellulose membrane by the method of Towbin et al. (21). Blocking was performed with the use of 3% gelatin in phosphate buffered saline for 1.5 h. The nitrocellulose membrane was incubated first with the primary antibody (diluted 1:500) for 2.5 h and then with goat anti-rabbit IgG carrying horseradish peroxidase (after dilution to 1:3000). The bands were visualized with a reagent for horseradish peroxidase conjugates.

RESULTS

Oxysterol Patterns in Human Arteries. Five specimens of human atherosclerotic femoral arteries were analyzed for cholesterol oxidation products by using a highly accurate technique based on isotope-dilution mass spectrometry with individual deuterated standards (14). The results are given in Table 1. The major oxysterol found was 27-hydroxycholesterol, closely followed by 7-oxocholesterol. Also 7α-hydroxycholesterol and 7β-hydroxycholesterol were found in significant amounts as well as 5,6-oxygenated products

Table 1. Distribution of oxysterols in human atherosclerotic femoral arteries

Oxysterol	Weight,* % of total oxysterols
27-Hydroxycholesterol	28 ± 4
25-Hydroxycholesterol	2 ± 0
24-Hydroxycholesterol	1 ± 0
7α-Hydroxycholesterol	12 ± 2
7β-Hydroxycholesterol	14 ± 3
7-Oxocholesterol	24 ± 4
5α,6α-Epoxycholesterol	5 ± 1
5β,6β-Epoxycholesterol	5 ± 1
Cholestane-3β,5α,6β-triol	5 ± 1

*Means ± SEM, n = 5.

(cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide, and cholestane-3β,5α,6β-triol). 24-Hydroxycholesterol and 25-hydroxycholesterol were found in trace amounts only (Table 1).

The ratio between oxysterols and cholesterol in the atheromas was found to be 6.6 ± 3.0 ng per μg of tissue in which the cholesterol content was 5.4 ± 1.4 μg/mg of tissue (means + SEM, n = 5).

Experiments with Macrophages. When human pulmonar alveolar macrophages were cultured as described, there was a significant accumulation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the medium (Fig. 1). The identity of the two products was confirmed by gas chromatography/mass spectrometry. The rate of formation of these products was almost linear with time (Fig. 1). The ratio between the two products varied in different experiments, but in general was about 1. There was no significant cell death up to 48 h. The content of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in macrophages isolated after the experiments was in general less than 20% and 5%, respectively, of the content of these compounds in the medium.

The formation of the two products was linear with the amounts of macrophages (results not shown).

The presence of sterol 27-hydroxylase protein in the macrophages was ascertained by Western blotting. A clear band was obtained with the same mobility as human hepatic sterol 27-hydroxylase at 53 kDa.

We have previously shown that 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D catalyzed by sterol 27-hydroxylase is markedly inhibited by cyclosporin A in concentrations of 10–50 μM (22). Interestingly, 27-hydroxylation of more polar substrates than cholesterol is not inhibited by the drug (22). Addition of cyclosporin A in the above concentration range markedly inhibited production of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid (Fig. 2). The highest inhibition occurred in the formation of 3β-hydroxy-5-cholestenoic acid. There were no signs of cell death during the experiments, indicating that the reduced formation of the 27-oxygenated products was not due to a general toxic effect on the cells.

After addition of 100 μg of cholesterol to the culture medium, the accumulation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid increased significantly. Addition of more cholesterol did not further increase the formation of 27-hydroxycholesterol and caused a decrease in the formation of 3β-hydroxy-5-cholestenoic acid. In the experiment shown in Fig. 3, trideuterated cholesterol was added to the medium in increasing amounts. The relative content of

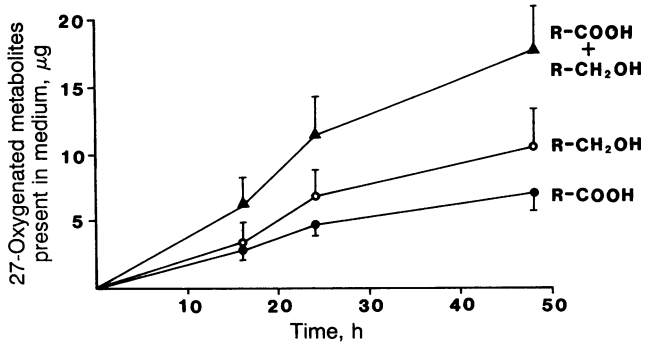

FIG. 1. Accumulation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in medium after incubation of macrophages (1 × 10^6) for different periods of time under the conditions described in *Materials and Methods*. The values given are means ± SEM of three independent experiments with macrophages isolated from different patients.

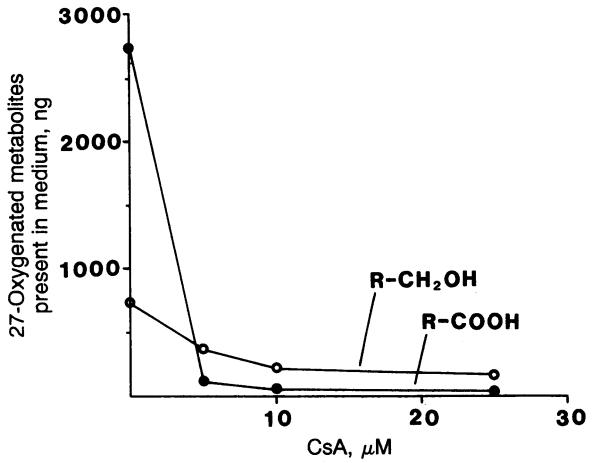

FIG. 2. Effect of cyclosporin A (CsA) on the production of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid by cultured macrophages (3 × 10^6 per incubation).

deuterium in the two products increased with increasing concentration of deuterium-labeled cholesterol in the medium. After addition of 400 μg of deuterated cholesterol, more than 50% of the 27-hydroxycholesterol formed contained the deuterium label.

In all of the experiments above, the culture medium contained fetal calf serum (10%), which contains both cholesterol and proteins that may be of importance for the transport of the oxidized cholesterol metabolites from the cells. Deletion of the calf serum caused a marked reduction in the accumulation of oxidized products in the medium. In the experiment shown in Fig. 4, the macrophages were cultured with deuterated cholesterol in a medium containing increasing amounts of delipidized (cholesterol-free) serum.

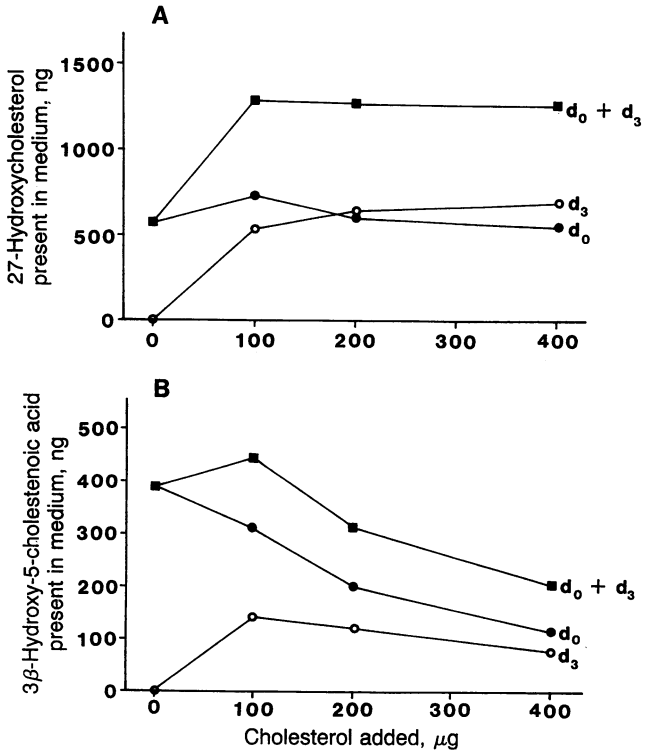

FIG. 3. Accumulation of unlabeled (curve d_0) and deuterium-labeled (curve d_3) 27-hydroxycholesterol (*A*) and 3β-hydroxy-5-cholestenoic acid (*B*) after addition of increasing amounts of trideuterium-labeled cholesterol to cultured macrophages (3 × 10^6 per incubation).

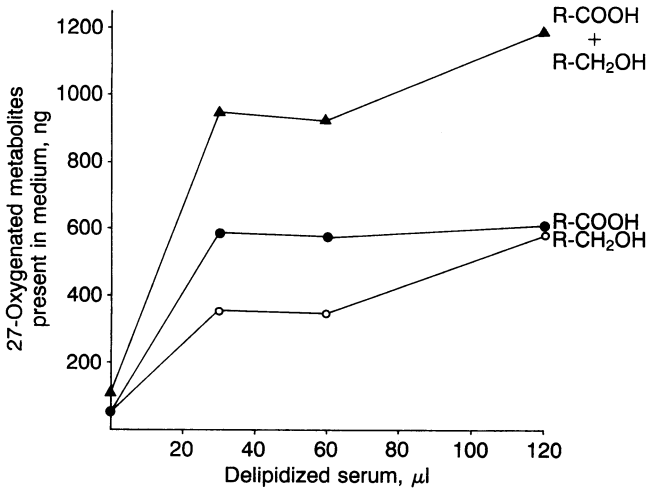

FIG. 4. Effect of delipidized serum on accumulation of deuterium-labeled 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in medium after culture of macrophages (3×10^6) in the presence of 100 μg of deuterated cholesterol. In this experiment, lyophilized dilipidized serum was used that had been reconstituted to a concentration 5-fold higher than normal fetal serum. For other experimental details, see *Materials and Methods*.

Without delipidized serum added to the medium, the accumulation of cholesterol metabolites was very low. Addition of 20–120 μl of delipidized serum increased the accumulation of the two 27-oxygenated sterols almost 10-fold.

If the sterol 27-hydroxylase is involved in the formation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid from cholesterol, the products can be expected to contain ^{18}O-label after exposure of the cells to $^{18}O_2$ gas. Part of the ^{18}O label in the 3β-hydroxy-5-cholestenoic acid can be expected to be lost during the incubation and/or work-up. The 27-hydroxycholesterol isolated from two incubations with macrophages exposed to $^{18}O_2$ contained 27% and 35% ^{18}O, respectively. 3β-Hydroxy-5-cholestenoic acid isolated from these two incubations contained 8% and 12%, respectively, of molecular species with one atom of ^{18}O.

Experiments with Endothelial Cells. Also endothelial cells were shown to produce 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. However, the conversion was less than 2% of that of macrophages. Also in the incubations with endothelial cells, the production of the two oxidized products increased after addition of cholesterol to the medium.

DISCUSSION

The present work shows conclusively that cultured human macrophages are able to produce 27-hydroxycholesterol and its oxidation product, 3β-hydroxy-5-cholestenoic acid. Furthermore, the cells are able to transport these compounds from the cells into the medium. The immunochemical demonstration of sterol 27-hydroxylase in these cells and the effect of the 27-hydroxylase inhibitor cyclosporin A (22) make it likely that the sterol 27-hydroxylase is the enzyme responsible. The incorporation of ^{18}O in 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the experiment with $^{18}O_2$ is also consistent with involvement of an oxygen-dependent hydroxylase in the conversion. The incorporation of less than one atom of ^{18}O per molecule is most probably due to difficulties in equilibrating the cells and the medium completely with the $^{18}O_2$ gas.

When exposed to cholesterol in the medium, part of the exogenous cholesterol was taken up and excreted as 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. The magnitude of the production in the macrophages, more than 0.1 pg per cell per h, is impressive.

We have recently shown that the sterol 27-hydroxylase is able to hydroxylate the terminal methyl group in a C-27 steroid three times to give the corresponding carboxylic acid (9). Thus, it is likely that the sterol 27-hydroxylase is responsible also for the conversion of 27-hydroxycholesterol into 3β-hydroxy-5-cholestenoic acid. The finding that cyclosporin A inhibited production of 3β-hydroxy-5-cholestenoic acid considerably more than production of 27-hydroxycholesterol is consistent with a product–precursor relationship.

The transfer of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid into the medium was highly dependent upon the presence of serum. It is possible that binding protein(s) in serum are of importance for the transfer of the oxysterols over the cell membranes. The presence of a cytosolic oxysterol-binding protein with a high affinity for side chain-hydroxylated cholesterol species is well documented (10, 11). This protein has been extensively characterized, and the corresponding gene has been cloned (23, 24). Whether or not one or more similar proteins are also present in extracellular fluid is not known.

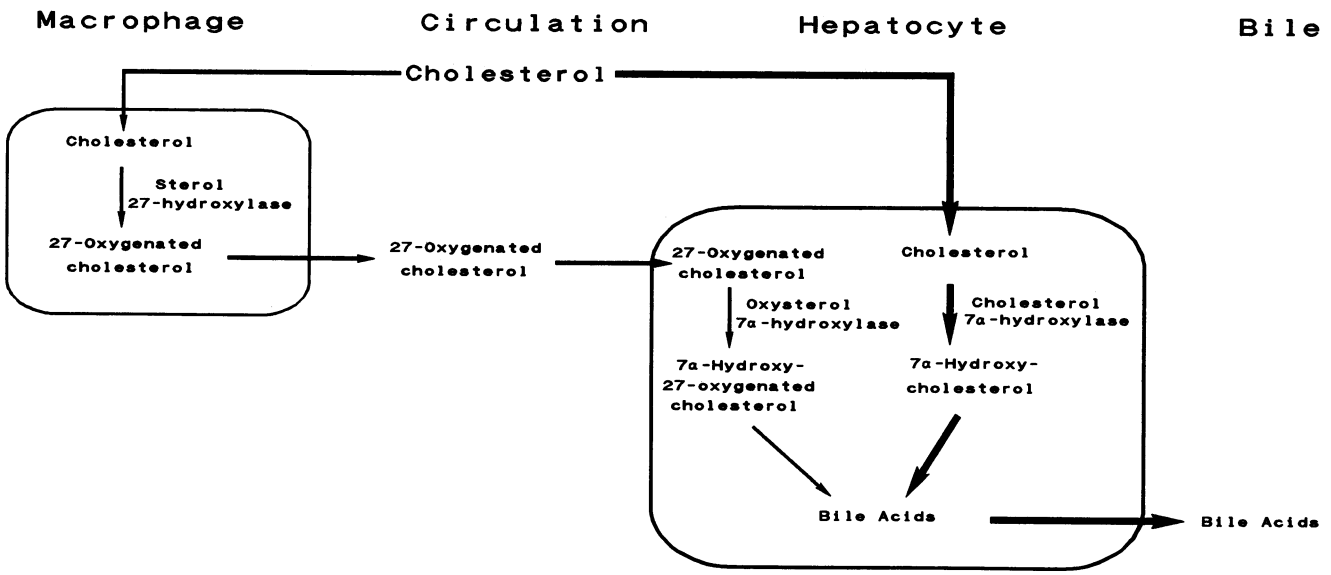

FIG. 5. Suggested mechanism for removal of cholesterol from macrophages and possibly also other peripheral cells.

It has been shown that 27-hydroxycholesterol can be converted into bile acids in man (25). Recently we (26) and others (27) demonstrated the presence of an oxysterol 7α-hydroxylase with a high specificity for side chain-hydroxylated C-27 steroids in human liver. This enzyme has properties different from those of the cholesterol 7α-hydroxylase involved in the classical pathway for formation of bile acids. Since there are significant levels of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in the circulation (28, 29), it is tempting to suggest that there is a continuous flux of these compounds from peripheral tissues to the liver (1, 6). After 7α-hydroxylation in the liver or elsewhere, the side chain-hydroxylated steroids are rapidly converted into bile acids and excreted in bile (1). This may represent a general defence mechanism for removal of excess cholesterol from some tissues (Fig. 5).

Uptake of modified low density lipoprotein by the scavenger receptor in macrophages and the resulting transformation of these cells into foam cells is regarded to be a key process in the development of atherosclerosis. The present mechanism, involving transfer of oxidized cholesterol metabolites from the macrophages, is likely to counteract foam cell formation. When advanced lesions have developed, however, the macrophages are likely to die and the possibility to metabolize cholesterol will disappear. The ratio between 27-hydroxycholesterol and cholesterol was found to be about 50-fold higher in the atheromas than in the circulation (cf. ref. 14), indicating that the two pools are not equilibrated with each other. The finding of high concentrations of 27-hydroxycholesterol in human atheromas is in agreement with previous results by Smith and Pandya (30).

Endothelial cells were also shown to excrete 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, indicating that the mechanism is not restricted to macrophages. However, the capacity for the conversion was much lower in these cells than in the macrophages.

We have shown that patients with the rare inborn disease cerebrotendinous xanthomatosis (CTX) have a deficient sterol 27-hydroxylase (31, 32), and mutations in the sterol 27-hydroxylase gene responsible for the defect have been defined (33–35). The CTX patients develop cholestanol- and cholesterol-containing xanthomas and suffer from premature atherosclerosis in spite of normal levels of circulating cholesterol (7). The lack of sterol 27-hydroxylase in these patients excludes the possibility of removing cholesterol from macrophages by the present mechanism. It is tempting to suggest that the lack of the defence mechanism demonstrated here may be part of the explanation for the development of premature atherosclerosis in CTX.

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