The Role of Cholesterol Oxidation Products in the Pathogenesis of Atherosclerosis

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ABSTRACT

Cholesterol undergoes spontaneous autoxidation, leading to the production of potentially atherogenic oxidation derivatives. When 25-hydroxycholesterol (25-OH) or cholestane-3β,5α,6β-triol (triol) was injected intravenously into rabbits, the aortic surfaces showed numerous balloon-like protrusions and crater-like defects indicative of endothelial damage. Alterations in membrane function caused by these cholesterol oxides could be the mechanism for their cytotoxic effect. Carrier-mediated hexose transport by cultured rabbit aortic smooth muscle cells, measured using 2-deoxyglucose, was reversibly inhibited by triol within one hour. A membrane-bound enzyme, 5'-nucleotidase, was inhibited after 24 to 48 hrs incubation with either 25-OH or triol. Endocytosis was also significantly inhibited by both 25-OH and triol. Depletion of membrane cholesterol content by the cholesterol oxides could account for the membrane functional alterations. Cholesterol biosynthesis is markedly inhibited by 25-OH. Triol has a lesser effect on cholesterol biosynthesis, but it is more potent in blocking uptake of cholesterol by arterial cells in culture.

Cholesterol oxides may also influence cholesteryl ester accumulation by arterial smooth muscle cells. Incubation of cells with 25-OH resulted in a four-fold increase in cholesteryl esterifying activity but no effect on cholesteryl ester hydrolytic activity.

The cholesterol oxides appear to be transported in the blood primarily by very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Oxidized LDL has cytotoxic effects and enhances macrophage lipid accumulation. These effects may be directly related to the cholesterol oxide content of these lipoproteins.

Introduction

Cholesterol now has a well established role in the pathogenesis of atherosclerosis. Cholesterol undergoes spontaneous autoxidation, leading to the production of oxidized derivatives which may be more injurious to the arterial wall than cholesterol itself. The evidence and mechanisms for this effect will be reviewed.

The initial event in atherogenesis is believed to be an injury to arterial endo-
thelium, followed by an inflammatory response in the arterial wall. The response includes increased endothelial turnover and permeability, intimal edema and infiltration of monocytes that ingest excessive intimal lipids and transform into foam cells. Endothelial denudation and replication may influence lesion progression, not only by allowing platelet adhesion and lipid penetration, but also by production of substances promoting smooth muscle proliferation.

These substances stimulate migration of smooth muscle cells to the intima and their modulation into a synthetic phase. Proliferating smooth muscle cells, which secrete collagen, elastin and proteoglycans to the surrounding matrix, and intimal lipid deposition eventually give rise to the typical atherosclerotic lesion.

While there are some suggestions that hypercholesterolemia may cause arterial injury and increased endothelial turnover, the mechanism is not well characterized. In one earlier study, hypercholesterolemia was endogenously induced in chickens on a cholesterol-free diet by the subcutaneous implantation of diethylstilbestrol. These were compared with chickens with comparable levels of hypercholesterolemia (about 400 mg per dl) induced by feeding a two percent USP grade cholesterol-containing diet. After six and one half months on these regimens, numerous large atherosclerotic lesions were observed in the thoracic aortas of the cholesterol-fed group of chickens, whereas the former group with endogenous hypercholesterolemia showed only minor degrees of atherosclerosis.

Cholesterol readily oxidizes in air at room temperature. In the preparation of cholesterol diets to induce experimental atherosclerosis, it is common practice to mix chow with a solution of cholesterol in organic solvent, then air dry it to remove the solvent, thereby exposing thin films of cholesterol to ideal conditions for autoxidation.

The more extensive atherosclerosis in the cholesterol-fed chicken may well have been due to cholesterol autoxidation products in the USP grade cholesterol.

Imai et al reported that purification of stock five-year-old USP grade cholesterol largely prevented its atherogenicity when fed to rabbits, whereas administration of the concentrated impurities present in USP cholesterol produced severe arterial damage, with smooth muscle cell death and focal intimal edema. These impurities have been shown to be largely composed of cholesterol oxidation products. The major components of cholesterol autoxidation were identified to be cholestan-3β, 5α, 6β-triol, 5α, 6α- and 5β, 6β-epoxycholesterol, 7-ketocholesterol, 7α- and 7β-hydroxycholesterol and 25-hydroxycholesterol. Rabbits fed a cholesterol-containing diet for two months were found to have two to five times the normal plasma of the α and β epoxides and cholestanetriol and five to eight times the normal liver levels of these oxy-sterols.

In our earlier in vitro studies, a concentrate of cholesterol oxidation products was prepared by recrystallizing five-year-old USP cholesterol from a methanol extract. Pathophysiological effects of these oxidation products were studied in rabbit aortic smooth muscle cell cultures. The concentrate of the cholesterol oxidation products showed remarkable in vitro cytotoxic effects, whereas purified cholesterol at the same concentration produced no toxic effects. The concentrate of cholesterol oxidation products was fractionated further by thin layer chromatography (TLC). The effects of each TLC fraction were tested and the results showed that 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol were most toxic and probably responsible for the biological toxicity of the contaminants of cholesterol. These two cho-
Lesterol oxidation products were then tested for their angiotoxicity after intravenous administration. Injections of five mg per kg of either compound caused damaging effects in rabbits, including fibromuscular thickening and cell death in the aorta and segmental thickening in both major and minor branches of the pulmonary artery. Of the identifiable compounds of cholesterol oxidation products used, the 25-hydroxycholesterol and cholestane-3β,5α,6β-triol were as potent as the concentrate of cholesterol contaminants in inducing these angiotoxic effects in rabbits. Both 5α,6α-epoxycholesterol and 7-ketocholesterol were less toxic. Intravenous injections of 25-hydroxycholesterol and cholestane-3β,5α,6β-triol in ethanol likewise caused aortic damage in rabbits. Purified cholesterol did not produce damaging effects.

In a subsequent study, three groups of New Zealand White male rabbits were given intravenously either 25 mg per kg of 25-hydroxycholesterol, cholestane-3β,5α,6β-triol or vehicle only. The luminal surfaces of aortas receiving 25-hydroxycholesterol were examined by scanning electron microscopy and showed numerous balloon-like protrusions and crater-like defects as well as circulating formed elements adhering on the luminal surfaces. The luminal surfaces of aortas of rabbits given cholestane-3β,5α,6β-triol had similar but more frequent lesions than the 25-hydroxycholesterol group (figure 1). Microthrombi were occasionally found. The aortas of the control group had significantly fewer lesions. Transmission electron microscopy studies showed intracytoplasmic vacuoles and diffuse subendothelial edema in the aortas of the two groups receiving the cholesterol oxidation products. The balloon-like protrusions and crater-like defects observed by scanning electron microscopy appeared to represent initial endothelial cell injury induced by cholesterol oxidation products.

There are at least two possible mechanisms by which cholesterol oxidation products can cause cell injury. Inhibition of cholesterol biosynthesis by cholesterol oxidation products could result in several consequences. If phospholipid concentrations are not altered significantly by cholesterol oxidation products, the molar ratio of membrane cholesterol to phospholipid would decline. Decreased cellular cholesterol content could eventually suppress membrane biogenesis and inhibit cell growth. Another mechanism could be that the cholesterol oxidation products, owing to their similar molecular structure, may be incorporated into cell membranes or displace cholesterol from cell membranes. Cholesterol in cell membranes imparts the optimal rigidity essential for membrane function. Decreased cellular cholesterol content or substitution of cholesterol oxidation products for cholesterol in these cell membranes would increase fluidity and alter a number of membrane-associated functions, resulting in cell death. Experiments were designed by the present authors to test both of these mechanisms, i.e., effects on cholesterol biosynthesis and on cholesterol uptake, and effects on membrane associated functions, including carrier mediated hexose transport, ion transport and membrane bound enzymes, large molecule uptake by endocytosis, and prostacyclin synthesis.

**Effects on Cholesterol Biosynthesis**

In cultured rabbit aortic smooth muscle cells, the activity of hydroxy methyl glutaryl coenzyme A (HMGCoA) reductase, the rate-limiting step in cholesterol biosynthesis, was reduced by 83 percent when cells were incubated with 3 μg per ml of 25-hydroxycholesterol and by 63...
percent when incubated with cholestane-3β,5α,6β-triol. In other studies, deoxyribonucleic acid (DNA) synthesis, as measured by methyl-3H thymidine incorporation per mg of cellular protein, declined in parallel with the HMGCoA reductase activity, and cell division was blocked in the G₁ phase of the cycle. This may be a mechanism for the cytotoxicity of the cholesterol oxidation products. Cholesterol biosynthesis as measured by 14C-acetate incorporation, was proportionately decreased by the above two cholesterol oxidation products.

Effects on Cholesterol Uptake

Labeled [4-14C]-cholesterol was added to culture media containing various concentrations (0 to 50 μg per ml) of cholesterol oxidation products. These media were transferred to culture flasks containing monolayers of aortic smooth muscle cells and then incubated for one hour at 37°C. Uptake of labeled cholesterol was measured after the cultures were washed four times with Hank’s solution. For removal of non-specific surface-bound sterol from the cells, the monolayers were incubated at 37°C for 45 minutes in media containing pronase. The non-specific surface-bound cholesterol amounted to 35 percent of total cholesterol uptake. The most potent inhibitor of cholesterol uptake was cholestane-3β,5α,6β-triol. When triol was present at 50 μg per ml of medium, uptake of cholesterol by the cultured...
cells decreased more than 70 percent (figure 2). The next most potent inhibitor of cholesterol uptake was 5α,6α,-epoxycholesterol, which reduced uptake of cholesterol by 60 percent. 25-Hydroxycholesterol also inhibited uptake of cholesterol up to 60 percent. In addition, 7α,- and 7β-hydroxycholesterol and 7-ketocholesterol inhibited cholesterol uptake to a similar extent of between 30 to 50 percent.

**Effects of Cholesterol Oxidation Products on Cellular Cholesterol Levels and Their Distribution in Subcellular Fractions**

As shown in figure 3, when cholestane-3β,5α,6β-triol was incubated with rabbit aortic smooth muscle cells for one to four days, cellular cholesterol concentrations showed an early decline at 24 hours and continued decreasing to 62 percent of control values at four days. In contrast, 25-hydroxycholesterol did not significantly affect cellular cholesterol levels at 24 hours, but further incubation with this sterol resulted in precipitous drops in cholesterol levels to 47 percent of control values by Day 4. To determine whether or not cholestane-3β,5α,6β-triol and 25-hydroxycholesterol were specifically localized in particular membrane fractions, these sterols were incubated with the cells for 24 hours prior to sterol analysis of specific membrane fractions. The amount of cholestane-3β,5α,6β-triol per unit protein was higher in the plasma membrane-enriched fraction than in the other subcellular fractions, including mitochondrial and microsomal fractions. In contrast, 25-hydroxycholesterol was more evenly distributed throughout the various subcellular fractions.

**Figure 2.** Uptake of cholesterol in presence of cholestane-3β,5α,6β-triol and 5α,6α-epoxycholesterol. Standard uptake assay conditions with 50 μg per ml of labelled cholesterol, and different concentrations of cholestane-3β,5α,6β-triol or 5α,6α-epoxycholesterol from 0-100 μg per ml. Data represent mean ± SEM of six experiments. (Artery 13:144–164, 1985.)

**Figure 3.** Effect of cholestane-3β,5α,6β-triol and 25-hydroxycholesterol on cellular cholesterol levels. Cells were incubated with either sterol at a final concentration of 10 μg per ml for the time periods indicated. (Proc. Soc. Exptl. Biol. Med. 180:126–132, 1985.)
Effects on Carrier Mediated Hexose Transport

Carrier mediated hexose transport is of critical importance in maintaining normal membrane function. Because D-glucose moves bidirectionally, a glucose analogue, 2-deoxy-D-glucose, is utilized for D-glucose transport studies. This compound is taken up by the same transport mechanism as D-glucose; once inside the cell, it is phosphorylated and not further metabolized, and this prevents reverse transport. The effects of various autoxidation derivatives on hexose transport in cultured rabbit aortic smooth muscle were studied. The most potent inhibitor of hexose transport was cholestane-3β,5α,6β-triol (table 1). The inhibitory effect of the triol appeared to be dose dependent, with a 58 percent decrease in hexose transport at the highest level (50 μg per ml) tested. The inhibitory effect was not the result of cell toxicity, as the total protein content of the cultured cells did not vary and dye exclusion studies showed the cultured cells to be viable during the one hour incubation period. Furthermore, the inhibitory effect of the triol on hexose transport was completely reversible. When duplicate cultures incubated with triol for 30 minutes were transferred to control nutrient media (triol-free), the rate of hexose transport returned to the control level by 1.5 hour.

Effects on Membrane-bound Enzymes

It has been suggested that slight perturbations in the microenvironment of intrinsic membrane-bound enzymes can result in large changes in the activity of these enzymes. Plasma membrane-bound enzyme activity was used as an index of the functional status of the plasma membranes of cultured aortic smooth muscle cells which were incubated for 24 to 48 hours with 10 μg per ml of either 25-hydroxycholesterol or cholestane-3β,5α,6β-triol. The cells were homogenized, after which the plasma membrane-enriched fraction was separated by ultracentrifugation using a discontinuous sucrose density gradient. Cholestane-3β,5α,6β-triol and 25-hydroxycholesterol both caused significant decreases in 5'-nucleotidase activity at 24 and 48 hours (table II). Only cholestane-3β,5α,6β-triol caused significant inhibition of Na+, K+-adenosine triphosphate activity after incubation of 48 hours.

Effects on Horseradish Peroxidase Uptake by Endocytosis

Rabbit vascular endothelial cells were incubated with cholestane-3β,5α,6β-triol, 25-hydroxycholesterol, 5α,6α-epoxycholesterol, or 7-ketocholesterol for 15 minutes to 48 hours. Subsequently, the cells were incubated with horseradish peroxidase (HRP) before finally lysing with 0.05 percent Triton X-100. The cell lysates were assayed for HRP by adding a substrate mixture of H₂O₂ and O-dianisidine and measuring

### Table I

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Percent Changes Compared to Control</th>
<th>Transport of 2-Deoxy-D-glucose (mmoles/10 min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle only)*</td>
<td></td>
<td>225.29 ± 45.78</td>
</tr>
<tr>
<td>25-Hydroxycholesterol †</td>
<td>-15.51</td>
<td>190.34 ± 47.30</td>
</tr>
<tr>
<td>20α-Hydroxycholesterol</td>
<td>-3.86</td>
<td>236.58 ± 29.07</td>
</tr>
<tr>
<td>5α,6α-Epoxycolesterol</td>
<td>-1.07</td>
<td>221.08 ± 21.80</td>
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<tr>
<td>Cholest-3β,5α,66-diol-6-one</td>
<td>-27.25§</td>
<td>163.89 ± 25.47</td>
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<tr>
<td>7-Ketocholesterol</td>
<td>-2.67</td>
<td>239.26 ± 26.50</td>
</tr>
<tr>
<td>4-Cholestane-3-one</td>
<td>+1.24</td>
<td>228.09 ± 25.82</td>
</tr>
<tr>
<td>Cholestane-3β,5α,6β-triol</td>
<td>-30.56ff</td>
<td>156.44 ± 38.00</td>
</tr>
</tbody>
</table>

*Vehicle was ethanol which was less than 0.4 percent of final volume of media.
† Sterols were dispensed in basal culture medium supplemented with 10 percent fetal bovine serum at a final concentration of 25 ug per ml. All cells were incubated with 2 ml of medium containing a test sterol for one hour prior to transport assay.
§ Values represent mean ± S.E.M. of at least two experiments.
ff p<0.05 compared to control value.
§ p<0.01 compared to control value.
TABLE II
Effect of Cholestane-3β,5α,6β-triol and 25-Hydroxycholesterol on 5'-Nucleotidase Activity in Cultured Aortic Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Time (Hours)</th>
<th>5'-Nucleotidase Activity (μM/min/mg x 10^-2)</th>
<th>Percent Change Compared to the Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>30.76±6.75*</td>
<td>---</td>
</tr>
<tr>
<td>(vehicle only)*</td>
<td>24</td>
<td>21.83±13.41†</td>
<td>-41.12</td>
</tr>
<tr>
<td>25-Hydroxycholesterol</td>
<td>48</td>
<td>22.60±3.47†</td>
<td>-26.52</td>
</tr>
<tr>
<td>Cholestane-3β,5α,6β-triol</td>
<td>24</td>
<td>18.82±5.89†</td>
<td>-38.81</td>
</tr>
<tr>
<td>5α,6α-epoxycholesterol</td>
<td>48</td>
<td>15.83±3.09†</td>
<td>-48.53</td>
</tr>
</tbody>
</table>

* Control values represent the average values obtained from zero time, 24 and 48 hour incubation with vehicle (ethanol).
† Values represent the mean ± S.E.M. of at least six experiments.
‡ p<0.01 compared to control value.


The potent inhibition of cholesterol biosynthesis by cholesterol oxidation products could result in several consequences. The decrease in cellular cholesterol levels as shown in our study and others would deplete the cholesterol available for membrane synthesis. The molar ratio of membrane cholesterol to phospholipid would decline, and membrane associated functions could be disturbed. Since the inhibitory effects on many membrane-associated functions, including carrier-mediated hexose transport and endocytosis of horseradish peroxidase induced by cholestane-3β,5α,6β-triol, occurred as early as 15 minutes, it is unlikely that this immediate effect was due to loss of membrane cholesterol secondary to inhibition of cholesterol biosynthesis. In fact, the inhibitory effect of the triol on membrane function occurred even in the presence of an exogenous source of cholesterol (fetal bovine serum).

Cholestane-3β,5α,6β-triol has also been shown to affect other membrane functions. Gordon et al have shown that several cholesterol oxidation products, including triol, inhibit leukocyte chemotaxis in vitro. Streuli et al have demonstrated that the triol inhibited lymphocyte-E rosette formation. In both of these studies, the inhibitory effects were observed after a short exposure to the sterols. Cholestane-3β,5α,6β-triol was found to increase in the plasma membrane fraction five-fold more than in other cell fractions when the arterial smooth muscle cells were incubated with the triol. It would appear, therefore, that triol may displace cholesterol from cell membranes and thereby disrupt their hydrophobic interior. In contrast to the triol, 25-hydroxycholesterol, the most potent inhibitor of cholesterol biosynthesis, had a delayed effect on membrane functions, and inhibited cell growth only after prolonged incubation of more than 24 hours. These effects could be reversed by adding mevalonate or cholesterol to the culture medium, substantiating that the delayed effects of 25-OH on membrane function may be due to membrane cholesterol depletion secondary to reduced synthesis.

Mechanisms of Cytotoxic Effects of Cholesterol Oxidation Products

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Prostaglandin Metabolism and Cholesterol Oxidation Products

Recently, the balance or imbalance of prostacyclin (PGI2) and thromboxane
production has been implicated in atherogenesis. Many lipid peroxides, particularly fatty acid peroxides, were shown to be potent inhibitors of PGI₂ synthesis and may play an important role in the development of atherosclerosis. Martinez-Sales et al demonstrated that the aortas of rats fed a diet enriched with two percent cholesterol autoxidation products for 24 hours prior to sacrifice showed a slight increase in both lipid peroxide and prostacyclin production. They believed the results suggested that the increase in the prostacyclin production might be a protective compensatory mechanism in response to vessel wall injury.

Our study using cultured rabbit aortic smooth muscle cells preincubated with cholesterol oxidation products for 24 hours showed that the effect varied depending on the type of cholesterol oxidation products. Although cholestane-3β,5α,6β-triol had an inhibitory effect on PGI₂ synthesis (11.1 ± 1.7 pg per μg protein vs. 16.2 ± 1.9 control), 25-hydroxycholesterol appeared to be somewhat stimulatory (35.5 ± 4.8). The explanation could be the prompt and direct membrane effect by cholestane-3β,5α,6β-triol, resulting in an immediate inhibition of PGI₂ production versus the somewhat delayed membrane effect of 25-hydroxycholesterol.

**Cholesteryl Ester Metabolism and Cholesterol Oxidation Products**

Another possible mode of action of cholesterol oxidation products in atherogenesis might be at the stage of cholesteryl ester accumulation and foam cell formation.

Lipoproteins from plasma or other sources enter cell lysosomes where cholesteryl esters are hydrolyzed and the free cholesterol subsequently resynthesized into lipid droplets of cholesteryl esters by the enzyme acyl CoA cholesterol acyltransferase (ACAT). Cholesteryl esters in lipid droplets are continuously being acted on by hydrolytic enzymes. Free cholesterol derived from this hydrolysis has the ability to migrate through the cell membrane and is available for transport out of the cell by high density lipoproteins (HDL).

In cultured mouse L fibroblast cells, incubation with 25-hydroxycholesterol or cholestane-3β,5α,6β-triol resulted in intracellular lipid accumulation. The cholesteryl ester content of cultured hepatocytes from rabbits fed oxidized cholesterol derivatives was greatly increased as compared to hepatocytes from control or pure cholesterol-fed rabbits. The effects have been examined by us of cholesterol oxidation products on enzymes that participate in accumulation and removal of cellular cholesteryl esters. Rabbit aortic smooth muscle cells in culture were pre-incubated for 24 hours in lipoprotein deficient media and then incubated for 15 minutes, one or five hours with 5 μg per ml media of either 25-hydroxycholesterol, cholestane-3β,5α,6β-triol cholesterol, or ethanol control. After one hour of incubation, ACAT activity showed a four-fold increase in the 25-hydroxycholesterol group (7.63 percent ± 2.24 percent esterified per mg protein per hour as compared to the control 1.96 percent ± 1.27 percent). Cholestane-3β,5α,6β-triol did not affect ACAT activity after any of the incubation time intervals. The activities of acid cholesteryl ester hydrolase and neutral cholesteryl ester hydrolase were not significantly affected by either 25-hydroxycholesterol or cholestane-3β,5α,6β-triol at any time interval. Enhancement of ACAT by 25-hydroxycholesterol without change in cholesteryl ester hydrolase may predispose to cholesteryl ester accumulation by arterial smooth muscle cells.
Absorption and Transport of Cholesterol Oxidation Products

Since cholesterol oxidation products are present in our diets, it is important to determine to what extent these compounds can be absorbed and whether or not they can be transported by lipoproteins which can bind and internalize into arterial cells. The absorption and transport of one of the most toxic cholesterol oxidation products, 25-hydroxycholesterol, was studied previously in squirrel monkeys. Using the dual-isotope plasma ratio technique, the absorption of 25-hydroxycholesterol was demonstrated to be approximately 30 percent. Its transport in plasma lipoproteins has also been studied. When squirrel monkeys were fed radioactive sterols (both purified cholesterol and 25-hydroxycholesterol), the distribution of labeled cholesterol in very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) was almost identical to that of unlabeled cholesterol. On the other hand, the majority of 25-hydroxycholesterol radioactivity was located in LDL and VLDL (55.7 percent ± 2.9 and 34.1 percent ± 2.9, respectively) and only 10.2 percent ± 0.5 was present in HDL. These results indicated that the majority of 25-hydroxycholesterol is transported to the peripheral tissues, such as the arterial wall, by VLDL and LDL. The high density lipoproteins carry only a relatively minor amount of 25-hydroxycholesterol.

Our recent studies have shown to what extent cholesterol oxidation products can be absorbed and transported when present together with a normal dietary cholesterol intake. Radiolabeled pure [4-14C]-cholesterol was kept at 60°C under air to autoxidize for five weeks, after which approximately 12 percent cholesterol oxidation products were formed. The mixture, suspended in gelatin, was given to rabbits by gastric gavage. Rabbits were sacrificed at four, 24, and 48 hours after treatment. Cholesterol and its autoxidation products were separated by thin layer chromatography into five fractions. Radioactivities of each fraction of cholesterol oxidation products and cholesterol in the original mixture before administration and in the rabbit sera after administration were similar, suggesting that the rates of absorption of cholesterol oxidation products are not significantly different from that of cholesterol.

Lipoproteins were fractionated by ultracentrifugation into VLDL, LDL, and HDL. Radioactivities of each lipoprotein sterol fraction separated by thin layer chromatography showed that fractions containing cholestane-3β,5α,6β-triol, 7α- and 7β-hydroxycholesterol, and 7-ketocholesterol were selectively transported in VLDL, whereas most of the 25-hydroxycholesterol was present in LDL. In addition, HDL contained only minute amounts of cholesterol oxidation products. Oxygenated sterols, including epoxycholesterol, 7-ketocholesterol, and 26-hydroxycholesterol, have previously been found in human plasma. These oxygenated sterols were also predominantly located in VLDL and LDL. The concentrations of α- and β-epoxycholesterol in VLDL were three times greater than in HDL, and the concentration of 7-ketocholesterol in both VLDL and LDL was also three times greater than in HDL. Chronically elevated levels of plasma VLDL and LDL have been associated with an increased incidence of atherosclerosis in numerous epidemiological studies. The selective transport of cholesterol oxidation products by VLDL and LDL may account in part for the atherogeneity of these lipoproteins. This hypothesis is strengthened by the demonstration that under certain condi-
tions LDL and VLDL are injurious or toxic to cultured endothelial cells and arterial smooth muscle cells.\textsuperscript{18,21}

It has also been shown that toxic LDL and VLDL can be formed by free radical-induced oxidation of a lipid component of the lipoprotein.\textsuperscript{20,40} The mechanism by which the oxidized lipid fraction of LDL and VLDL might damage cultured cells and the identity of the toxic agent are not yet established. It is possible that these toxic components are cholesterol oxidation products. When Krieger et al\textsuperscript{32} replaced the free and esterified cholesterol of plasma LDL with 25-hydroxycholesterol oleate, the resulting particle bound to LDL receptors on human fibroblasts and was hydrolyzed in lysosomes in a manner similar to that of native LDL. This oxysterol-containing LDL suppressed 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. When fibroblasts were incubated with the oxysterol-containing LDL without added cholesterol, the cells developed structural abnormalities, their growth was inhibited, and the cells died. The toxic effects of the oxysterol-containing LDL were prevented when the growth medium was supplemented with cholesterol or mevalonate. In another study, treatment of LDL with cholesterol oxidase, resulting in oxidation of cholesterol to cholestenone, resulted in the LDL becoming toxic to cultured bovine aortic endothelial cells.\textsuperscript{4}

The earliest cellular interaction that occurs in hypercholesterolemia has been shown to be adhesion of monocytes to dysfunctional endothelial cells.\textsuperscript{55} Monocytes have very few binding sites for native LDL but ingest large amounts of oxidized or charge-modified LDL via high-affinity scavenger receptors\textsuperscript{15,19} which are not down-regulated in the presence of excess cholesterol.\textsuperscript{7}

It therefore appears that the presence of lipoproteins containing cholesterol oxidation products form dietary sources or through oxidative modification by arterial cells, not only could cause arterial cell injury but could also enhance lipid accumulation and foam cell formation.

**Experimental Atherosclerosis Induced by Cholesterol Oxidation Products**

A few investigators have produced experimental atherosclerosis by feeding pure cholesterol oxidation products alone or in combination with pure cholesterol. Cook et al in 1968 were the first to use cholesterol oxidation products to induce experimental atherosclerosis.\textsuperscript{11} They had tested 103 compounds or organ cultures of chicken hearts\textsuperscript{5} and rabbit aorta.\textsuperscript{34} Cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol was found to be the most toxic. This compound, therefore, was chosen to be fed to the New Zealand white male rabbits in doses of 0.01 percent of a standard commercial diet, corresponding to about 30 mg per kg of body weight per day. The duration of feeding ranged from 27 to 350 days. Lesions were observed in the aorta, starting with deposition of sudanophilic material, progressing through various pathological changes in the intima and media, and terminating with the aorta showing considerable fibrosis and calcification.

Cook et al\textsuperscript{11} pointed out that lesions induced in the rabbit aorta by feeding a high cholesterol diet bear only a partial resemblance to human atherosclerosis. In the human type, not only are lipid deposits present, but there are other changes such as disruption of elastic tissue, alteration in ground substance, tissue necrosis, and calcification. These authors claimed that the changes induced by prolonged feeding of relatively small amounts of cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol produced lesions in rabbits with a closer resemblance to those of human atherosclerosis. More recently, cholestane-3\(\beta\),5\(\alpha\),6\(\beta\)-triol administered
orally to rats produced toxic damage to aortic endothelial and smooth muscle cells.\textsuperscript{36} The endothelium showed extensive areas of cellular degeneration and desquamation, with platelet and fibrin adherence. The smooth muscle cells showed nuclear damage, cytoplasmic vacuolar degeneration, and cellular necrosis in wide areas of the media. Jacobson et al\textsuperscript{28} have recently studied White Carneau pigeons gavage-fed either 0.05 percent pure cholesterol or 0.05 percent pure cholesterol with trace levels of cholestane-3β,5α,6β-triol for three months. These are amounts similar to the estimated U.S. dietary intake levels. Aortic lipids, aortic calcium and coronary artery histopathology were assessed. Aortic lipids showed no significant differences between the two groups, but aortic calcium accumulation in the cholesterol with triol group was 1.16 ± 0.35 mg per g, as compared to 0.82 ± 0.27 in the cholesterol-fed groups, an increase of 42 percent (P < 0.01). Coronary artery atherosclerosis, as measured by percent mean luminal stenosis, was 5.23±5.4 percent in the cholesterol with triol group as compared to 2.80 ± 1.4 percent in the cholesterol-fed group, a significant difference of 87 percent (P < 0.01). These results indicate that low level dietary intake of cholestane-triol is atherogenic to a greater degree than pure cholesterol alone. The data suggest that cholestane-triol is an atherogenic risk factor in doses approximating U.S. dietary exposure to cholesterol and its auto-oxides. Furthermore, the results call for reinterpretation of data from studies using animal models fed U.S.P. cholesterol and of human epidemiologic data not accounting for cholesterol oxide intake.

Two recent reports indicate that Indian immigrants to London and the West Indies have higher rates of atherosclerosis than would be expected from their lack of the common risk factors.\textsuperscript{3,36} The diet of this population is high in Ghee, a butter product containing 12 percent of its sterols as cholesterol oxides.\textsuperscript{27} Since cholesterol oxidation products are found in a number of other human foodstuffs, particularly dried eggs, dairy products, or deep-fat frying media,\textsuperscript{12,42,49} further experimental studies are imperative and should provide knowledge highly relevant to the prevention of human atherosclerotic cardiovascular disease.

Acknowledgments

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