Cytotoxicity of Oxysterols on Cultured Smooth Muscle Cells from Human Umbilical Arteries (43514)

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Abstract. **The lethal effect of 25- and 26-hydroxycholesterol on smooth muscle cells derived from human umbilical arteries was investigated. The extent of cellular death corresponded with increasing oxysterol concentrations and incubation times. Incubation of the cells with 0.5, 2.5, or 10 μg/ml of 25- or 26-hydroxycholesterol revealed a proportionality between the degree of cellular death and oxysterol concentration over the 5 days of the experiment. Correlation coefficients among the degree of cellular death, the exposing period, and oxysterol concentration were significantly different ($P < 0.05$). However, none of these changes were noted in the smooth muscle cells cultured for 5 days in a medium containing the same concentration of cholesterol or 0.5% ethanol. An increase in the concentration of either serum or cholesterol in the culture medium did not significantly reduce the cytotoxicity of 2.5 μg/ml of 26-hydroxycholesterol. The results of this study suggest that oxysterols have an injurious effect on arterial cells, and that the injurious effect could not be altered by cholesterol, which was present at a serum concentration 12 times higher than that of oxysterols.** [P.S.E.B.M. 1993, Vol 202]

Oxysterols are considered important to the development of atherosclerosis (1–3). The hypothesis that oxysterols are responsible for arterial injury (4) is now one of the major ideas explaining the development of atherosclerosis at the cellular level. Imai *et al.* (5) have demonstrated that aortas of rabbits fed concentrates of impure cholesterol that had been oxidized in an unsealed drum for several years exhibited diffuse fibrous lesions in the intima. Oxysterols have been shown to be toxic to a variety of cells, including vascular endothelial cells (6–8), smooth muscle cells (2), fibroblasts (9–10), and P-815 mastocytoma cells (11). Cholesterol derivatives such as 25-hydroxycholesterol, an auto-oxidative product of cholesterol, and 26-hydroxycholesterol, an enzymatic oxidative product of cholesterol, are potent effectors of cells (6, 12–13). A suppression of both the viable cell density and cytotoxic changes induced by 25- and 26-hydroxycholesterol in bovine pulmonary arterial and human umbilical vein endothelial cells and in bovine and rabbit arterial smooth muscle cells has been observed in our laboratory (14) and elsewhere (15). Previous studies have shown the effect of 25-hydroxycholesterol on the human aortic smooth muscle cell (16), a cell of the kind that plays a major role in the development of atherosclerotic lesions (17). We are interested in whether 26-hydroxycholesterol has similar cytotoxicity as 25-hydroxycholesterol in cultured smooth muscle cells from human umbilical arteries. Our model provides a better system to examine possible adverse effects of oxysterols in relation to human atherosclerosis than the rabbit or bovine smooth muscle cells, which have been used frequently in the past.

Materials and Methods

Preparation of Sterol. Cholesterol and 25-hydroxycholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). 26-Hydroxycholesterol was synthesized in our lab (14). The purity of all lipids, identified by gas chromatography, was more than 99.9%. Stock solutions of cholesterol and 25- and 26-hydroxycholesterol in absolute ethanol at concentrations of 0.1, 0.5, and 2 mg/ml were stored at −20°C under N_2 and diluted immediately before use to the experimental concentrations (0.5, 2.5, and 10 μg/ml) in Eagle's minimum essential medium ([MEM] Gibco, Grand Island, NY)

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supplemented with 20% fetal bovine serum ([FBS] Sigma). The final concentration of ethanol in the incubation medium was 0.5%.

Preparation of Human Umbilical Arterial Smooth Muscle Cells. The arteries were isolated from umbilical cords collected under aseptic conditions. The adventitia and the outer portion of connective tissue from these arteries were carefully separated from the medial layer. The lumenal surface was scraped with a sterile surgical blade to remove the endothelial cells and was washed thoroughly with MEM. The medium was cut, using a surgical blade, into pieces approximately 1 mm^2 while immersed in MEM and then transferred into 75-cm^2 tissue culture flasks (Corning Medical and Scientific Co., East Walpol, MA). Five milliliters of MEM containing 20% FBS were added carefully to avoid the detachment of the explants from the flasks. The flasks were incubated in a CO_2 incubator at 37°C for 4 to 5 weeks. Smooth muscle cells migrating from the arterial pieces after 3–4 weeks of cultivation were trypsinized and subcultured. The identification of smooth muscle cells was performed by the method of Easton *et al.* (18). The medium was changed every 3 days, and passage numbers of the cultured smooth muscle cells used in this study ranged from three to eight.

Determination of the Effect of Sterols on the Cell Growth, Cell Viability, and Protein and DNA Content. The confluent cells in 75-cm^2 tissue culture flasks were trypsinized and plated in 24-well plates (Corning) at a concentration of 1.0×10^4 cells/well in 1 ml of MEM containing 10% FBS. Amounts of 0.5, 2.5, and 10 μg of either cholesterol or 25- or 26-hydroxycholesterol were added to the wells after the cells had grown to subconfluence (counted as Day 0). The media containing only 10% FBS or both 10% FBS and 0.5% ethanol were used as controls. On Days 1, 3, and 5, smooth muscle cells were first washed gently twice with ice-cold MEM and trypsinized. The cell numbers were counted electronically with a Coulter counter and the viable cells were defined by their ability to exclude trypan blue. DNA concentration was determined by a fluorometric method (19), and protein content in smooth muscle cells was assayed by Bio-Rad protein assay (Bio-Rad, Laboratories, Richmond, CA) in a parallel experiment. Duplicate wells were measured for each determination.

Analysis of the Effect of the Normal Content of 26-Hydroxycholesterol in Human Serum on Cell Growth. One milliliter of smooth muscle cells at a concentration of 1×10^4 cells/ml was transferred into each well of 24-well plates and the cells were incubated for 24 hr with MEM containing 10% FBS. Then 0.1 or 0.2 μg of 26-hydroxycholesterol was added to the medium. Only 0.5% ethanol was added to control cultures. The cultured cells were incubated for another 1, 3, 5, 7, and 10 days at 37°C. The cell numbers from each incubation were then counted with the Coulter counter.

Determination of the Influence of FBS Concentration on Oxysterol's Injurious Effect on the Cells. The cells incubated in 24-well plates for 24 hr, as mentioned above, were used in this study. The cells were exposed to 1 ml of 2.5 μg/ml of 26-hydroxycholesterol and FBS at concentrations of 5%, 10%, or 20% with or without 10 μg of cholesterol for 1, 3, or 5 days, and then the cell numbers were counted.

Statistical Analysis. Significance of differences was analyzed by multiple analysis of variance and Dunnet tests. Comparisons between MEM 10% FBS with and without 0.5% ethanol were made by Student's *t* test.

Results

Effect of Ethanol on the Cell Growth. The control cultures with 0.5% ethanol vehicle in the absence of added sterols gave consistent results of no difference when compared with the MEM 10% FBS group during 5 days of culture (Table I). The smooth muscle cells grew normally in the medium containing 0.5% ethanol during 5 days of the experimental period.

Effect of Sterols on Cell Growth, Cell Viability, and Protein and DNA Content. After the cultured smooth muscle cells were incubated in the medium containing cholesterol or 25- or 26-hydroxycholesterol at 37°C for 5 days, changes in the cell number were determined. As shown in Table II, the administration of 10 μg/ml of 25-hydroxycholesterol resulted in a significantly rapid decrease ($P < 0.05$) of the cell number after 1 day of incubation. Lower concentrations of the oxysterol caused slower decreases in the cell number, with 2.5 μg/ml and 0.5 μg/ml of 25-hydroxycholesterol showing significant ($P < 0.01$ and 0.05, respectively) reduction in the cell number in 3 days of incubation. The decrease in the cell number resulting from 26-hydroxycholesterol exposure was similar to that of 25-hydroxycholesterol, except that the appearance of a significant decrease ($P < 0.05$) in the cell number with 10 μg/ml of 26-hydroxycholesterol was 1 day later than that for 25-hydroxycholesterol at the same concentration (data not shown). The number of smooth muscle cells was not affected by 10 μg/ml of cholesterol during 5 days of exposure. Correlation coefficients among the degree of cellular death, the exposing period, and ox-

Table I. Effect of 0.5% Ethanol on the Viable Number of Human Umbilical Artery Smooth Muscle Cells[a]

Mode of treatment	Cell no. (×10^4/well)		
	1 Day	3 Days	5 Days
MEM-20% FBS	1.88 ± 0.1	2.26 ± 0.2	2.34 ± 0.2
MEM-20% FBS + 0.5% ethanol	1.89 ± 0.2	2.13 ± 0.3	2.29 ± 0.3

[a] The data are expressed as mean ± SE of duplicate for each independent determination in six cultures.

Table II. Effect of Cholesterol and 25- and 26-Hydroxycholesterol on Cell Growth and Cell Viability of Human Umbilical Artery Smooth Muscle Cells[a]

Mode of treatment	1 Day	3 Days	5 Days
Number ($\times 10^4$)			
0.5% Ethanol	1.9 ± 0.2	2.1 ± 0.3	2.3 ± 0.3
CHOL (μg)			
10	1.9 ± 0.2	1.9 ± 0.1	2.3 ± 0.2
2.5	1.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.2
0.5	1.9 ± 0.2	2.2 ± 0.1	2.4 ± 0.2
25-OHC (μg)			
10	1.4 ± 0.2[b]	1.1 ± 0.1[c]	0.6 ± 0.1[c]
2.5	1.7 ± 0.2	1.3 ± 0.1[c]	1.0 ± 0.1[c]
0.5	1.7 ± 0.2	1.7 ± 0.1[b]	1.6 ± 0.1[c]
26-OHC (μg)			
10	1.5 ± 0.1	1.3 ± 0.2[c]	0.9 ± 0.1[c]
2.5	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.1[b]
0.5	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.1
Viability (%)			
0.5% Ethanol	95 ± 3.2	94 ± 3.6	93 ± 4.8
CHOL (μg)			
10	99 ± 2.4	97 ± 4.6	98 ± 5.1
2.5	94 ± 6.5	99 ± 3.5	98 ± 8.9
0.5	98 ± 2.3	98 ± 3.7	94 ± 6.9
25-OHC (μg)			
10	81 ± 3.6[b]	61 ± 8.8[c]	31 ± 7.6[c]
2.5	91 ± 5.7	75 ± 5.7[b]	44 ± 7.9[c]
0.5	95 ± 5.1	92 ± 3.9	85 ± 8.7
26-OHC (μg)			
10	91 ± 2.9	71 ± 6.1[c]	35 ± 4.8[c]
2.5	91 ± 6.6	72 ± 6.1[b]	41 ± 6.1[c]
0.5	94 ± 6.8	93 ± 3.7	88 ± 8.2

[a] The data are expressed as cell number ($\times 10^4$) and cell viability (%) per culture well. These values are mean ± SE of duplicate for each independent determination in six cultures. CHOL, cholesterol; 25-OHC, 25-hydroxycholesterol; 26-OHC, 26-hydroxycholesterol.
[b] $P < 0.05$ compared with 0.5% ethanol group in the same column.
[c] $P < 0.01$ compared with 0.5% ethanol group in the same column.

ysterol concentration (multiple regression) were significantly different ($P < 0.05$). Figure 1 showed that at the concentration of 10 μg/ml, the correlation coefficients between the degree of cellular death and the exposing periods were significantly different ($P < 0.05$).

No significant numbers of detached cells were observed in the cholesterol or ethanol groups. There was significant cell detachment for the groups incubated with oxysterols, and the number of detached smooth muscle cells increased with increased oxysterol concentrations and incubation periods. These detached cells did not grow when they were collected and cultured, indicating that they were dead or dying cells.

The viable cells decreased to 81% ($P < 0.05$) by Day 1, then continuously declined to 31% at 5 days for the cells incubated with 10 μg/ml of 25-hydroxycholesterol. The effect of 2.5 μg/ml of 25-hydroxycholesterol on cell viability was later and weaker than that for 10 μg/ml. The regularity of the decrease of cell viability

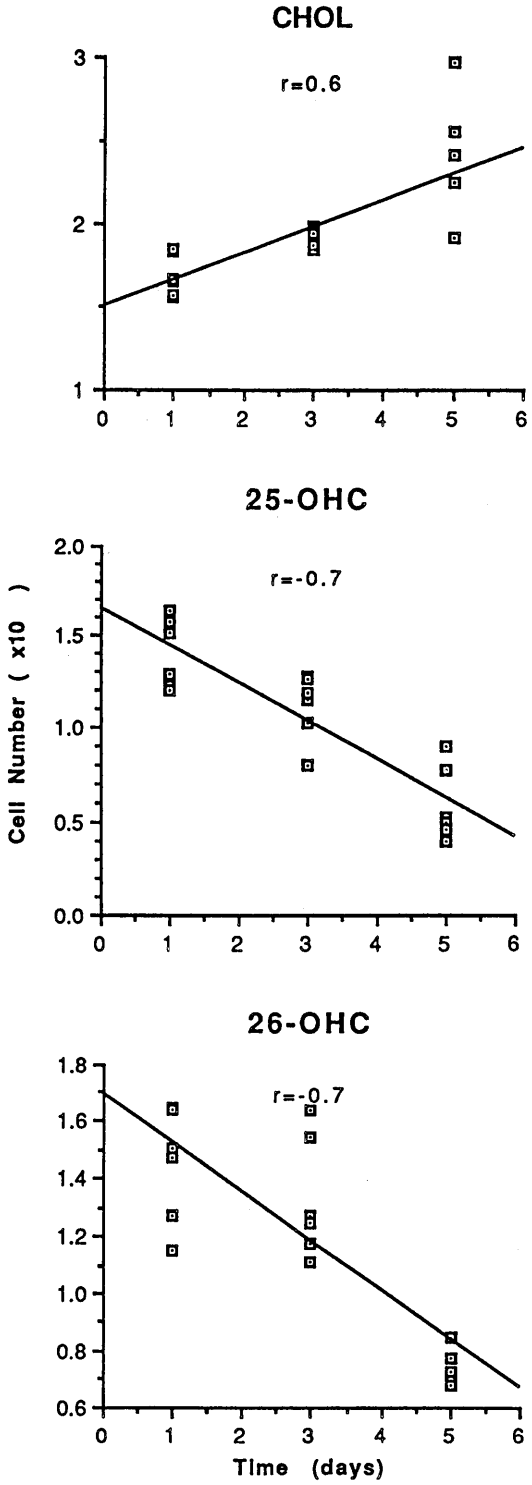

Figure 1. Relationship between the degree of cellular death and the exposing periods at the same concentration (10 μg) of cholesterol (CHOL) and 25- and 26-hydroxycholesterol (OHC). Correlation coefficients obtained from linear regression analysis were significantly different ($P < 0.05$). These data are from Table II.

caused by 26-hydroxycholesterol was similar to that caused by 25-hydroxycholesterol. Ethanol, cholesterol, and 0.5 μg/ml of oxidized cholesterol had no effect on cell viability.

The changes in DNA and protein content from

cholesterol and 25- and 26-hydroxycholesterol incubation at different concentrations were parallel to the changes in the viable cell number (Table III). The ratio of the protein and DNA content, however, was raised by 25- and 26-hydroxycholesterol with the time of exposure.

Effect of Normal Serum Concentrations of 26-Hydroxycholesterol on Cell Number. 26-Hydroxycholesterol at a concentration of 0.1 μg/ml did not significantly affect the viable cell number, as shown in Table IV. However, a trend of inhibitory effect was observed with smooth muscle cells incubated with 0.2 μg/ml of 26-hydroxycholesterol for 10 days of treatment.

Influence of FBS Concentration on Oxysterol's Lethal Effects on the Cells. Increasing FBS concentration from 5% to 20% did not diminish the cytotoxic effect of 2.5 μg/ml of 26-hydroxycholesterol on smooth muscle cells (Table V). The cell count when exposed to 2.5 μg/ml of 26-hydroxycholesterol with 5%, 10%, or 20% FBS was not significantly different, although there was a trend toward a decrease in cell number at the lower concentration of FBS. Nor did 10 μg/ml of cholesterol in the culture medium have an effect on the cytotoxicity of 2.5 μg/ml of 26-hydroxycholesterol.

Discussion

Exposure of human smooth muscle cells to 0.5% ethanol had no cytotoxic effect, as was shown previously with animal smooth muscle cells (6, 17). Cholesterol, up to 10 μg/ml, did not affect cell growth over 5 days of treatment. In contrast, however, oxysterols at a concentration of 2.5 μg/ml caused a decrease in the cell number, cell viability, and DNA and protein content within 2 days of exposure. Reducing the oxysterol concentration to 0.5 μg/ml still resulted in a significant decrease in the cell number in 3 days of treatment. Our results suggested that oxysterols, not cholesterol, were responsible for injury to cultured human arterial smooth muscle cells.

Cox *et al.* (15) have shown that the number of rabbit smooth muscle cells obtained from a medium containing 25-hydroxycholesterol is decreased whereas the number obtained from a cholesterol containing medium is not changed. Peng *et al.* (2) have demonstrated the effect of 25-hydroxycholesterol on morphologic changes in cultured aortic smooth muscle cells, and the more recent study by Jimi *et al.* (14) has shown that the oxysterol concentration and time of exposure to oxysterol were critical for bovine ciliac arterial smooth muscle cell growth. Unlike the reports cited above, our study was performed with human umbilical arterial smooth muscle cells. Our results suggest that the sensitivity to oxysterols of animal and human cells is different. In the experiment by Cox *et al.* (15), 10^{-6} *M* 25-hydroxycholesterol in the medium supplemented with 10% FBS showed no effect on both confluent and sparse rabbit smooth muscle cell growth after 88 hr of the treatment. Jimi *et al.* (14) found that during the 10 days of experiment, 0.5 μg/ml (equal to 1.25×10^{-6} *M*) of 26-hydroxycholesterol did not reduce the number of cultured smooth muscle cells. In our experiment, a significant increase ($P < 0.05$) of dead or dying cells was observed in response to 0.5 μg/ml of 25- and 26-hydroxycholesterol in only 3 and 5 days of incubation. A comparison between our study and other studies indicates that human smooth muscle cells are much

Table III. Effect of Cholesterol and 25- and 26-Hydroxycholesterol on the Protein and DNA Content of Human Umbilical Artery Smooth Muscle Cells[a]

Mode of treatment	1 Day			3 Days			5 Days		
	P (μg)	D (μg)	P/D (μg)	P (μg)	D (μg)	P/D (μg)	P (μg)	D (μg)	P/D (μg)
0.5% Ethanol	25 ± 2.6	2.2 ± 0.5	11.4	35 ± 5.1	2.3 ± 0.3	15.2	39 ± 5.4	2.4 ± 0.4	16.3
CHOL (μg)									
10	23 ± 3.0	2.5 ± 0.6	9.2	34 ± 5.0	2.3 ± 0.3	14.8	35 ± 5.1	2.3 ± 0.6	15.2
2.5	24 ± 2.3	2.4 ± 0.6	10.0	33 ± 5.0	2.5 ± 0.3	13.2	34 ± 5.2	2.6 ± 0.6	13.1
0.5	24 ± 2.1	2.2 ± 0.3	10.9	34 ± 4.0	2.5 ± 0.3	13.6	36 ± 5.5	2.4 ± 0.5	15.0
25-OHC (μg)									
10	15 ± 3.2[b]	1.5 ± 0.3	10.0	17 ± 2.8[c]	0.3 ± 0.1[c]	56.7	4.5 ± 1.0[b]	0.1 ± 0.1[b]	45.0
2.5	22 ± 2.4	2.4 ± 0.4	9.2	20 ± 3.7[c]	0.9 ± 0.2	22.2	6.1 ± 1.5[b]	0.2 ± 0.1[b]	30.5
0.5	23 ± 3.2	2.5 ± 0.4	9.2	29 ± 4.6	1.8 ± 0.3	16.1	29 ± 5.9	2.5 ± 0.5	11.6
26-OHC (μg)									
10	21 ± 1.1	1.9 ± 0.3	11.1	14 ± 3.5[b]	0.5 ± 0.1[c]	28.0	3.8 ± 1.5[b]	0.1 ± 0.1[b]	38.0
2.5	23 ± 2.1	2.3 ± 0.3	10.0	21 ± 2.7[c]	1.2 ± 0.2	17.5	7.1 ± 1.6[b]	0.5 ± 0.2[b]	14.2
0.5	23 ± 2.4	2.5 ± 0.5	9.2	33 ± 5.8	2.4 ± 0.4	13.8	31 ± 5.9	2.4 ± 0.5	12.9

[a] The data are expressed as protein (μg) and DNA (μg) per culture well. These values are mean ± SE of duplicate for each independent determination in six cultures. CHOL, cholesterol; 25-OHC, 25-hydroxycholesterol; 26-OHC, 26-hydroxycholesterol; P, protein content; D, DNA content.
[b] $P < 0.01$ compared with 0.5% ethanol in the same column.
[c] $P < 0.05$ compared with 0.5% ethanol in the same column.

Table IV. Effect of a Normal Concentration of 26-Hydroxycholesterol in Human Serum on the Viable Number of Human Umbilical Artery Smooth Muscle Cells[a]

Mode of treatment	Cell no. ($\times 10^4$/well)				
	1 Day	3 Days	5 Days	7 Days	10 Days
0.5% Ethanol	0.99 ± 0.0	1.49 ± 0.0	1.52 ± 0.2	1.54 ± 0.2	1.57 ± 0.1
26-OHC 0.1 μg/ml	0.98 ± 0.2	1.46 ± 0.1	1.46 ± 0.2	1.40 ± 0.1	1.56 ± 0.1
26-OHC 0.2 μg/ml	0.99 ± 0.1	1.23 ± 0.2	1.22 ± 0.0	1.24 ± 0.1	1.23 ± 0.2

[a] The data are expressed as mean ± SE of duplicate for each independent determination in six cultures. 26-OHC, 26-hydroxycholesterol.

Table V. Protecting Effect of Serum or Cholesterol Concentration on Cell Number in Human Umbilical Artery Smooth Muscle Cells[a]

Mode of treatment	Cell no. ($\times 10^4$/well)		
	1 Day	3 Days	5 Days
2.5 μg/ml 26-Hydroxycholesterol			
+20% FBS	0.99 ± 0.1	0.71 ± 0.1	0.43 ± 0.0
+20% FBS & 10 μg CHOL	0.94 ± 0.0	0.68 ± 0.1	0.47 ± 0.1
+10% FBS	0.95 ± 0.0	0.51 ± 0.1	0.27 ± 0.0
+10% FBS & 10 μg CHOL	0.93 ± 0.1	0.57 ± 0.1	0.24 ± 0.1
+5% FBS	0.98 ± 0.1	0.49 ± 0.0	0.29 ± 0.1
+5% FBS % 10 μg CHOL	0.97 ± 0.0	0.42 ± 0.1	0.25 ± 0.1

[a] The data are expressed as mean ± SE of duplicate for each independent determination in six cultures. CHOL, cholesterol.

more sensitive to oxysterols than animal smooth muscle cells. Given the position, the cytotoxic degree of oxysterols is related to, among other things, the type of cell lines. Moreover, the comparison between our own experiments also indicated that 25-hydroxycholesterol was a more toxic sterol than 26-hydroxycholesterol. The observation that 10 μg/ml of 25-hydroxycholesterol in the culture medium, which is approximately equal to 1/200th of the concentration of cholesterol in the usual human serum, could induce the death of the smooth muscle cells in only 1 day suggests that even relatively small amounts of 25-hydroxycholesterol accumulation *in vivo* could injure arterial walls within a very limited time.

26-Hydroxycholesterol is synthesized by a mitochondriol P-450 enzyme *in vivo* that is widely distributed in tissues (20). The level of 26-hydroxycholesterol in the serum of normal adults, identified by isotope dilution mass spectrometry, is from 9.2 to 25.6 μg/100 ml (21). The concentration of 26-hydroxycholesterol used in incubation with the smooth muscle cells, 0.2 μg/ml, is within the normal level in human serum. We found that it could reduce the cell growth during the first 3 days, then stop the growth for the remaining 7 days of a 10-day experiment. These results show that 26-hydroxycholesterol can inhibit cell growth even when 26-hydroxycholesterol is at the normal level of human serum. In a pathologic study, Smith and Van Lier (22) reported that the content of 26-hydroxycholesterol in stage III areas of atherosclerotic lesions was higher than that in noninvolved areas of the aorta. The accumulated amount of 26-hydroxycholesterol in human aorta correlated positively with the severity of atherosclerosis (23).

The mechanism with which oxysterols cause cell death is still unknown, although there are currently two principle hypotheses on the cytotoxicity of oxysterols. One is that oxysterols depress cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (16) and consequently cause membrane dysfunction. 25-Hydroxycholesterol has been found to be one of the most potent inhibitors of cholesterol biosynthesis. It is possible that 26-hydroxycholesterol has the same role in inhibiting HMG-CoA as 25-hydroxycholesterol. One of the reasons why the addition of 10 μg/ml of cholesterol to the culture medium failed to diminish the cytotoxicity of 2.5 μg/ml of 26-hydroxycholesterol might be explained by the oxysterols' inhibition of exogenous cholesterol uptake by the cells (24). It is suggested that the membrane structure and function altered by oxysterols could result in cell death. The second explanation is that oxysterol molecules insert into the lipid bilayer (25). The incorporation of 0.5 mol% of 25-hydroxycholesterol into liposomes substantially increases the permeability of liposomes to Ca^{2+} (26), whereas cholesterol at 10 mol% had no effect on liposome permeability to Ca^{2+} (26). We found that calcium accumulated in the bovine arterial smooth muscle cells incubated in the presence of 25-hydroxycholesterol containing liposomes in an

amount proportional to the time of incubation (27). In view of these studies, we suggest that the stronger polar groups in 25- and 26-hydroxycholesterol perturb the biomembrane in arterial smooth muscle cells to the extent that the membrane becomes "leaky" to Ca^{2+}. When membrane permeability to Ca^{2+} is altered and the intracellular Ca^{2+} rises to an abnormally high level, cell death occurs.

Although we believe that the decrease of total protein and DNA content caused by oxysterols resulted from the reduction of cell numbers, the changes in the protein to DNA ratio suggest that additional, uneven, inhibitory effects were occuring. Determination of which, or whether both of these processes were affected is beyond the scope of the experiments reported here.

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