Oxysterol-Induced Apoptosis of Vascular Smooth Muscle Cells is Reduced by HMG-CoA Reductase Inhibitor, Pravastatin

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We investigated the mechanism by which 7-ketocholesterol damages vascular smooth muscle cells and the protective effect of the hydroxymethyl glutaryl CoA reductase inhibitor, pravastatin on it. When 7-ketocholesterol (50 μmol/L) was added to cultured human vascular smooth muscle cells, the extent of cell detachment increased and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling was positive. DNA extracted from the smooth muscle cells exposed to 7-ketocholesterol showed a ladder pattern on agarose electrophoresis. The fragmented DNA also increased in smooth muscle cells incubated with 7-ketocholesterol dose-dependently. In the presence of pravastatin, the cell detachment induced by 7-ketocholesterol was inhibited and the amount of fragmented DNA decreased significantly. These effects of pravastatin were inhibited by mevalone. The results suggest that 7-ketocholesterol-induced apoptosis of vascular smooth muscle cells is inhibited by pravastatin, and mevalonate acts as a trigger of the apoptosis. J Atheroscler Thromb, 2002; 9: 65-71.

Key words: 7-ketocholesterol, Pravastatin, Apoptosis, Smooth muscle cell

Introduction

The cholesterol-lowering agent pravastatin, an inhibitor of hydroxymethyl glutaryl CoA (HMG-CoA) reductase, is known to prevent coronary heart disease (1-4). In the West of Scotland Coronary Prevention Study (WOSCOPS), pravastatin administration for 5 years reduced by 33% the number of definite nonfatal case of myocardial infarction, as compared with placebo (1). Interestingly, this apparent benefit was not seen in non-statin trials. Therefore, Gaw hypothesized that statin has important anti-atherogenic effects potentially unrelated to its lipid-lowering action (5). These ancillary mechanisms include effects on smooth muscle cell proliferation (6), platelet thrombus generation (7), oxidizability of lipoproteins (8) and natural killerT cells (9). But, the precise mechanisms are unknown.

Myocardial infarction is mainly due to not a narrowing of the vessel rumen caused by intimal thickening of the artery, but to a rupture of the capsular portion of a plaque (10). The mechanism of this rupture is not fully understood. Recent studies mentioned that vascular smooth muscle cells decreased in number in advanced atherosclerotic arteries (11,12), and also that cell death in the lesions is by apoptosis (13). In atherosclerotic lesion, therefore, there may exist factors inducing apoptosis of smooth muscle cells. A candidate for such a factor is 7-ketocholesterol, one of the cholesterol oxidation products, which plays an important role as a cytotoxic agent (14-17).

We examined the mechanism of the cytotoxic effect of 7-ketocholesterol in vascular smooth muscle cells, and the effect of pravastatin on 7-ketocholesterol-induced cytotoxicity.
Materials and Methods

Materials
7-ketocholesterol (5-cholesten-3β-ol-7-one), cholesterol (5-cholesten-3β-ol) and mevalonate were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from GIBCO. Pravastatin was provided by Sankyo Co. Ltd.

Isolation and passage of human vascular smooth muscle cells
Tips of human coronary arteries were obtained from a cadaver at autopsy, and were subjected to explant. Briefly, 1-2 mm² pieces of gastric arterial media were placed in T-25 flasks. Complete DMEM with 10% FBS and 1% gentamycin was added. The medium was changed two or three times per week, and after approximately 4 weeks, cells were harvested from the flasks with trypsin-EDTA (GIBCO) followed by centrifugation (1,000 r.p.m. for 5 min). The cell pellet was resuspended in DMEM with 10% FBS and plated into T-75 flasks. Cells in the fifth or sixth passage were used in all experiments.

Evaluation of the cytotoxicity of 7-ketocholesterol
Cells in the fifth or sixth passage were seeded into 12-well Falcon plates, grown in DMEM containing 10% FBS for 24 hours, and then washed and placed in DMEM with 10% FBS. At this time, cholesterol, 7-ketocholesterol or pravastatin was added. These agents were dissolved in ethanol. The final concentration of ethanol in the culture medium was 1%. After incubation for a specified period, the supernatant was carefully removed from the dish, and residual detached cells were rinsed with 0.5 ml of DMEM. The cells in the supernatant and rinsed medium were enumerated with a cell counter (Sysmex F 150). The volume of the detached cells was over 80 μm³. Adherent cells were removed from the flasks by trypsinization and then counted. Cell detachment as defined in this study represents as a percentage, the number of cells in supernatants (detached cells) over the number of adherent cells plus the number of detached cells.

TUNEL method
Cells in the fifth or sixth passage were seeded into chamber slide (NUNC), grown in DMEM with 10% FBS for 24 hours, and then incubated with cholesterol and 7-ketocholesterol in 10% FBS. After incubation for 24 hours, DNA nicks in cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) [18] using an ApopTagTM in situ apoptosis detection kit (s7100-kit from Oncor Gaithersburg, MD,20877).

Agarose electrophoresis
DNA fragmentation was detected by agarose electro-

phoresis. Cells in the fifth or sixth passage were seeded into T-75 flasks, grown in DMEM with 10% FBS for 24 hours, and then incubated with cholesterol and 7-ketocholesterol in 10% FBS. After incubation for 3 days, cells in supernatants and adhered to flasks (total of about 4 x 10⁶ cells) were harvested by centrifugation at 200 g for 10 min. Cell pellets were washed with DMEM and disrupted with a high molecular weight buffer, pH 7.5 (150 μmol/L NaCl, 10 μmol/L Tris-HCl, 10 μmol/L EDTA), containing 0.1% sodium dodecyl sulfate and 0.1% proteinase K. After incubation for 90 min. at 55°C, samples were extract-
ed twice with phenol/chloroform (1:1, v/v), and digested with ribonuclease A (1 mg/ml) at 37°C for 60 min. The DNA was then precipitated with ethanol. The samples were kept at −70°C for 20 min. They were then centrifuged at 15,000 g for 20 min, rinsed with 70% eth-
anal and dried under vacuum. The DNA was dissolved in Tris-EDTA buffer, pH 7.4 (10 μmol/L Tris, 1 μmol/L EDTA) at 4°C for 24 hours. Loading buffer containing 50% glycerol, 5 x TAE (1 x TAE=40 mM Tris acetate, pH 8.5, 2 mM EDTA) and 0.1% bromophenol blue was added to the samples at a ratio of 1:4 (v/v). Samples were run on a 1.5% agarose gel at 10 V/cm in TAE buffer. DNA was visualized on an ultraviolet transilluminator after staining with ethidium bromide staining (0.5 mg/ml).

Quantitative assay of fragmented DNA
The DNA of cells in culture flasks was labeled with 5-bromo-2-deoxy-uridine (Brdu) for 6 hours. Thereafter, 7-ketocholesterol and pravastatin were added at varying concentrations. After incubation for specific periods of time, the amount of Brdu-labeled DNA released into the supernatant by dead cells was quantified by the quantita-
tive sandwich enzyme immunoassay (Cellular DNA Frag-
mentation ELISA, Boehringer Mannhein, Cat. No. 1586045). Data was corrected using the quantity of DNA in the well.

Results
Effect of cholesterol or 7-ketocholesterol on cell detach-
ment
Cell detachment (= the percentage of detached cells) is shown in Fig.1. Cholesterol did not increase cell detach-
ment, but 7-ketocholesterol significantly increased the number of detached cells with time from day 3 to day 5. These results indicated that 7-ketocholesterol was cytotoxic to smooth muscle cells.

DNA nicks in smooth muscle cells incubated with choles-
sterol and 7-ketocholesterol
DNA nicks were detected by the TUNEL method. Cells incubated with 7-ketocholesterol stained positive but cells incubated with cholesterol showed negative staining by TUNEL (Fig.2).
Fig. 1. Time course of cell detachment induced by cholesterol or 7-ketocholesterol. Cholesterol (open circles) or 7-ketocholesterol (closed circles) was added at 50 μmol/L to vascular smooth muscle cells incubated with DMEM containing 10% FBS. The final concentration of ethanol in the medium was 1%. At the times indicated, the adherent cells and detached cells were counted. Results are the mean ± SD of two experiments performed in triplicate.

DNA ladder

Fig. 3. Agarose electrophoresis of DNA extracted from smooth muscle cells incubated with 7-ketocholesterol. Smooth muscle cells were incubated with cholesterol (lane 1) or 7-ketocholesterol (lane 2) at 50 μmol/L for 3 days. The DNA was isolated and fractionated on 1.8% agarose gel. The DNA from cells incubated with 7-ketocholesterol showed a ladder pattern as a monomer of 180 base pairs.

Fig. 2. Microphotographs of TUNEL staining before the addition of 7-ketocholesterol (A), and after incubation with 7-ketocholesterol at 50 μmol/L for 24 hours (B). Nuclei in (B) show positive staining compared to those in (A). (×600)

DNA laddering

DNA from smooth muscle cells incubated with cholesterol or 7-ketocholesterol at 50 μmol/L was extracted and subjected to agarose electrophoresis (Fig. 3). The DNA from smooth muscle cells incubated with 7-ketocholesterol showed a ladder pattern as a monomer of 180 base pairs. On addition of cholesterol, a ladder pattern was not recognized.

Fig. 4. Time course and dose dependent effect of 7-ketocholesterol on the amount of fragmentation of DNA. Smooth muscle cells were incubated with 7-ketocholesterol at varying concentrations for 1 or 2 days. Fragmentation of DNA was assayed quantitatively by ELISA. Results are the mean ± SD of two experiments performed in triplicate.
Fig. 5. Time course of the effect of pravastatin on cell detachment induced by 7-ketocholesterol. Pravastatin (open circles) significantly reduced the extent to which 7-ketocholesterol at 50 μmol/L (closed circles) induced cell detachment. Pravastatin was added at 10^-4 M to vascular smooth muscle cells incubated with DMEM containing 10% FBS. The final concentration of ethanol in the medium was 1%. When indicated, the adherent cells and detached cells were counted. Results are the mean±SD of two experiments performed in triplicate.

Fig. 6. Dose-dependent effect of pravastatin on cell detachment induced by 7-ketocholesterol. Cell detachment induced by 7-ketocholesterol (50 μmol/L) decreased significantly on the addition of pravastatin. Pravastatin was added at 10^-3 M to 10^-4 M to vascular smooth muscle cells incubated with DMEM containing 10% FBS. The final concentration of ethanol in the medium was 1%. When indicated, the adherent cells and detached cells were counted. Results are the mean±SD of two experiments performed in triplicate.

Time course and dose-dependency of the effect of 7-ketocholesterol on the amount of fragmented DNA

The time course and dose dependent change of DNA fragmentation are shown in Fig. 4. After the addition of 7-ketocholesterol, the amount of fragmented DNA significantly increased with time and dose.

**Effect of pravastatin on cell detachment induced by 7-ketocholesterol**

In the presence of pravastatin (1×10^-4 M), cell detachment induced by 7-ketocholesterol decreased significantly with time (Fig. 5). The effect of the dose of pravastatin on the detachment was examined (Fig. 6). After incubation with pravastatin for 3 days, the cell detachment induced by 7-ketocholesterol decreased significantly as the dose rose from 10^-7 M to 10^-5 M. These results...
suggested that the cytotoxicity of 7-ketocholesterol might be suppressed by pravastatin.

**Effect of pravastatin on 7-ketocholesterol-induced fragmentation of DNA**

In the presence of pravastatin (10^{-4} - 10^{-7} M), the amount of 7-ketocholesterol-induced DNA fragmentation significantly decreased (Fig. 7). This decrease exhibited a dose-dependent and time-course effect.

**The effect of mevalonate on 7-ketocholesterol-induced fragmentation of DNA**

Mevalonate (10^{-6}M) alone did not promote 7-ketocholesterol-induced apoptosis (Fig. 8). In the presence of pravastatin, and on the addition of mevalonate, the fragmentation of DNA was inhibited. The inhibitory effect of pravastatin, however, was significantly diminished. This canceling effect by mevalonate was dependent on both dose and time.

**Discussion**

In the present study, the number of detached cells increased significantly in the presence of 7-ketocholesterol. DNA of vascular smooth muscle cells incubated with 7-ketocholesterol was TUNEL-positive and showed a ladder pattern on agarose electrophoresis. Furthermore, the amount of fragmented DNA increased. These results indicated that 7-ketocholesterol was cytotoxic to vascular smooth muscle cells, and this toxicity was mediated by apoptosis. Apoptosis is a physiologic process to maintain homeostasis in multicellular organisms both during embryogenesis and in adult hood (19, 20). Recently, it was reported that apoptosis is involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune disease and viral infection (21, 22). In atherosclerotic lesions, apoptotic smooth muscle cells are observed (23, 24). From our study, 7-ketocholesterol, which accumulated in advanced atherosclerotic lesions might induce apoptosis of vascular smooth muscle cells (25), and might cause the thinning of the capsular portion of the core lesion.

In this study, pravastatin suppressed the cell detachment and DNA fragmentation induced by 7-ketocholesterol in vascular smooth muscle cells, indicating that it suppressed the apoptosis caused by 7-ketocholesterol. This finding in vitro indicates a direct action by pravastatin on the artery in addition to LDL-cholesterol lowering effects. These results suggest that the mechanism by which pravastatin reduced myocardial infarction involves not only a LDL-cholesterol lowering effect, but also the prevention of a decrease in smooth muscle cell numbers in unstable atherosclerotic lesion.

Mevalonate is produced by an enzymatic reaction of HMG CoA reductase. We investigate whether mevalonate suppressed the inhibitory effect of on 7-ketocholesterol-induced apoptosis because the most important effect of pravastatin is inhibition of HMG CoA reductase. In the presence of mevalonate, the inhibitory effect of pravastatin on 7-ketocholesterol-induced apoptosis was suppressed significantly. From these results, it is suggested that pravastatin suppressed a decrease in cell numbers in atherosclerosis, which led the plaques to become stable.

Among inhibitors of HMG CoA reductase, simvastatin and lovastatin were reported to induce apoptosis of vascular smooth muscle cells in culture (26, 27) and mevalonate to inhibit apoptosis (28, 29). This difference in the effect of the inhibitors is probably due to the form (fat-soluble or water-soluble of statin), the species of cells cultured, and the concentration of statin. The most important difference is the conditions used for the culture. Cells in our study were incubated with oysterols. It is reported that oysterol-induced apoptosis depends on the activation of interleukin-1β converting enzyme (ICE), and bcl-2 inhibits this activation (30). We found that oysterol enhanced the expression of c-myc in smooth muscle cells (data not shown). It is possible that pravastatin had a direct effect on the expression of bcl-2 or c-myc in smooth muscle cells.

Pravastatin is known to decrease levels of farnesyl pyrophosphate, which is derived from hydroxymethyl glutaryl CoA, and a cell proliferator (31). Generally, HMG CoA reductase inhibited the cell proliferation, although the rates differed a little (32). Apoptosis is also known to be induced by cell proliferators such as platelet-derived growth factor (33, 34). So, the inhibition of cell proliferation by farnesyl pyrophosphate may suppress the oysterol-induced apoptosis of vascular smooth muscle cells. We are now studying the precise mechanism involved.

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