Apoptosis Induced by 7-Ketocholesterol is Enhanced in Smooth Muscle Cells Derived from OLETF Rats

Yoh Miyashita, Nobukiyo Koide, Tomokazu Oyama, Yoshiaki Itoh, and Kohji Shirai

Center of Diabetes, Endocrine and Metabolism, Sakura Hospital, Toho University School of Medicine, Chiba, Japan.

To clarify whether an increased proliferative potential of vascular smooth muscle cells (SMC) under diabetic conditions augments the susceptibility of the cells to 7-ketocholesterol-induced apoptosis, we investigated the difference in sensitivity to 7-ketocholesterol between SMC obtained from diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats and the control Long-Evans Tokushima Otsuka (LETO) rats. The outgrowth rate from aortic wall explants and cell proliferation were higher in SMC derived from OLETF rats (OLETF-derived SMC) compared to those from LETO rats (LETO-derived SMC). When 7-ketocholesterol was added to SMC, the amount of fragmented DNA increased significantly in OLETF-derived compared to LETO-derived SMC. The amount of fragmented DNA induced by 7-ketocholesterol decreased significantly in both OLETF- and LETO-derived SMC when they were incubated without fetal bovine serum. By adding PDGF-BB to LETO-derived SMC, the amount of fragmented DNA induced by 7-ketocholesterol increased significantly. These results suggest that apoptosis of SMC induced by 7-ketocholesterol may be accelerated when SMC acquire a high proliferative potential by prolonged exposure to diabetic condition. J Atheroscler Thromb, 2005; 12: 92–97.

Key words: 7-Ketocholesterol, Plaque rupture, Apoptosis, OLETF rat

Introduction

Myocardial infarction does not necessarily occur due to the narrowing of the vessel lumen by intimal thickening of the artery, but to rupture of the capsule of the plaque (1). The mechanism of this plaque rupture is not fully understood. Recent studies indicate a decrease in the number of vascular smooth muscle cells (SMC) in arteries with advanced atherosclerosis (2, 3), and implicate apoptosis as responsible for cell death in atherosclerotic lesions (4). Various factors existing in atherosclerotic lesions might induce apoptosis of SMC; for example, cytokines such as TNF-α and lipids. Among lipids, oxysterols have been reported to play an important role as an apoptosis-inducing factor (5–8). We have reported that 7-ketocholesterol, an oxysterol, induces apoptosis of human SMC (9). This finding suggests that accumulation of 7-ketocholesterol in atherosclerotic lesions may render atherosclerotic plaques unstable.

Diabetes mellitus is a very important risk factor for myocardial infarction, through accelerating atherosclerosis of the coronary artery (10, 11). The major mechanisms reported include accelerated glycation (12) and coagulation (13), increased protein kinase C activity (14), and increased oxidative stress in the body (15). However, these factors cannot fully explain the mechanism of plaque rupture. We hypothesized that SMC exposed to a prolonged diabetic condition might show increased sensitivity to 7-ketocholesterol.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a model of type 2 diabetes characterized by mild obesity and late spontaneous onset of hyperglycemia. The Long-Evans Tokushima Otsuka (LETO) rat can be used as a age-matched control for OLETF rat (16).
In this study, we examined the SMC outgrowth, proliferation and 7-ketocholesterol-induced (7-keto-induced) apoptosis in SMC derived from OLETF and LETO rats, and the effect of proliferative potential of SMC on 7-ketoinduced apoptosis.

Materials and Methods

Materials
7-ketocholesterol (5-cholesten-3β-ol-7-one), cholesterol and platelet-derived growth factor-BB (PDGF-BB) were purchased from Sigma Chemical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Gibco (Tokyo, Japan). OLETF and LETO rats were provided by Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan).

Cell culture
Twenty-week-old male OLETF rats (n = 3) and LETO rats (n = 3) were used. The serum biochemical profiles of OLETF and LETO rats are shown in Table 1. SMC were obtained from the thoracic aorta and explanted as described previously (9). In all experiments, cells were cultured in DMEM (100 mg/dl of glucose) containing 100 U/ml penicillin and 100 µg/ml streptomycin, and equilibrated in 95:5 (%) air: CO2. The purity and identity of the SMC cultures were verified using a monoclonal antibody against smooth muscle α-actin. Cells at passage 2 or 3 were used in experiments.

Observation of outgrowth
The vascular wall containing the medial and intimal layers was stripped from the thoracic aorta and cut into blocks of approximately 1 × 1 mm² and explanted in dishes 10 cm in diameter. About 17 explants were prepared for each rat, with a total of 50 explants. Outgrowth of smooth muscle cells was monitored daily by microscope for 14 days. The outgrowth rate at day 14 was calculated by dividing the number of explants with outgrowth by the total number of explants (n = 50). The medium was not changed until 5 days after explant.

Measurement of cell proliferation
Cells were plated at 6 × 10⁴ cells in 24-well dishes. After incubating for specified periods, the wells were washed with phosphate buffer saline (PBS), and then adherent cells were collected by trypsinin treatment. The numbers of adherent cells were counted with a cell counter (Sysmex F-150).

DNA fragmentation assay
The DNA of cells in culture flasks was labeled with 5-bromo-2′-deoxy-uridine (BrdU) for 24 hours. Thereafter, 7-ketocholesterol was added at varying concentrations. After incubation for specified periods of time, the amount of BrdU-labeled DNA released into the supernatant by dead cells was quantified by a quantitative sandwich enzyme immunoassay (Cellular DNA Fragmentation ELISA, Boehringer Mannhein, Catalog No. 1585045). Data was corrected using the quantity of total protein in the well.

Statistical analysis
Paired analysis between two groups was performed using Student’s t-test, where ANOVA indicated significant difference in multiple comparison. Data are expressed as the mean ± SEM. P values less than 0.05 were considered significant.

Results

Difference in proliferative potential of OLETF-derived and LETO-derived SMC
To clarify the difference in proliferative potential between SMC of OLETF rats and those of LETO rats, the outgrowth rate and the increase in cell number were studied. The outgrowth rates of SMC from aortic wall explant were 48% and 18% for OLETF and LETO rats, respectively. The SMC outgrowth rate was higher in OLETF rats compared to LETO rats (Table 2). Cell proliferation, indicated by cell number, was significantly higher in SMC derived from OLETF rats (OLETF-derived SMC) compared to SMC isolated from LETO rats (LETO-derived SMC) after culturing for 4 and 6 days (Fig. 1). These findings suggested that OLETF-derived SMC had a greater proliferative potential than LETO-derived SMC. However, the cell numbers of the two types of SMC were not significantly different on day 8.

Table 1. Biochemical profiles of OLETF and LETO rats.

<table>
<thead>
<tr>
<th></th>
<th>OLETF rats</th>
<th>LETO rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood sugar (mg/dl)</td>
<td>314 ± 25</td>
<td>188 ± 35</td>
</tr>
<tr>
<td>Basal immunoreactive insulin (pg/dl)</td>
<td>972 ± 65</td>
<td>318 ± 59</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>189 ± 21</td>
<td>86 ± 13</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>286 ± 69</td>
<td>36 ± 15</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>51 ± 8</td>
<td>42 ± 6</td>
</tr>
</tbody>
</table>

Table 2. Rates of SMC outgrowth from aorta explants of OLETF and LETO rats.

<table>
<thead>
<tr>
<th></th>
<th>OLETF rats</th>
<th>LETO rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of aorta explants</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Number of explants showing outgrowth</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>Outgrowth rate (%)</td>
<td>48%</td>
<td>18%</td>
</tr>
</tbody>
</table>
Comparison of 7-keto-induced apoptosis between SMC from OLETF and LETO

Next, 7-keto-induced fragmented DNA was assayed to examine whether there is a difference in sensitivity to 7-ketocholesterol between OLETF-derived SMC and LETO-derived SMC. By the addition of 7-ketocholesterol, the amount of fragmented DNA increased depending on the dose of 7-ketocholesterol in both OLETF- and LETO-derived SMC (Fig. 2). However, the increase in fragmented DNA was significantly higher in OLETF-derived SMC compared to LETO-derived SMC at 7-ketocholesterol concentrations of 50 and 100 µmol/l (Fig. 2). These results suggested that OLETF-derived SMC were more susceptible to 7-ketocholesterol-induced apoptosis than LETO-derived SMC.

Effect of proliferative potential on 7-keto-induced apoptosis

We hypothesized that the high sensitivity of OLETF-derived SMC to 7-ketocholesterol might be due to its high proliferative potential. Then, to clarify whether the proliferative potential of SMC affects 7-keto-induced apoptosis, we assayed 7-keto-induced fragmented DNA of SMC in the resting and proliferative phases. First, the effect on SMC in resting phase was examined by assaying 7-keto-induced fragmented DNA under incubation without FBS. Under incubation with 10% FBS, the amount of fragmented DNA was significantly high in OLETF-derived SMC compared to LETO-derived SMC. But, under incubation without FBS, the amount of fragmented DNA decreased significantly to the same magnitude in both OLETF- and LETO-derived SMC, and no significant difference was recognized between OLETF- and LETO-derived SMC (Fig. 3).

Next, to elucidate the effect of increased proliferation of SMC on the susceptibility to 7-keto-induced apoptosis, we used PDGF-BB to stimulate the proliferation of LETO-derived SMC. The amount of 7-keto-induced fragmented DNA was assayed after adding PDGF-BB to LETO-derived SMC. The amount of fragmented DNA in LETO-derived SMC increased significantly when incubated with PDGF-BB compared to without PDGF-BB (Fig. 4). The effect of PDGF-BB was dose-dependent. By the addition of PDGF-BB without 7-ketocholesterol, no significant increase of fragmented DNA was observed (Fig. 4). These results suggested that SMC with an enhanced proliferative potential may show increased sensitivity to 7-ketocholesterol and hence augmented apoptosis.

Discussion

In diabetes mellitus, it is known that atherosclerosis, especially in the coronary artery, frequently occurs. Augmented SMC proliferation has been proposed as a mechanism of increased atherogenesis in diabetes, based on experimental evidence obtained using OLETF
To elucidate the mechanism by which myocardial infarction occurs at high frequency in diabetes mellitus, we studied the difference in sensitivity to 7-ketocholesterol between OLETF-derived and LETO-derived SMC. As shown in Fig. 2, our results suggest that 7-keto-induced apoptosis is markedly enhanced in OLETF-derived SMC compared to LETO-derived SMC. The high sensitivity to 7-ketocholesterol in OLETF rats might be one cause of plaque fragility in diabetes mellitus.

In this study, SMC of OLETF rats showed high proliferative potential and high sensitivity to 7-ketocholesterol compared to SMC of LETO rats. From these findings, we hypothesized that 7-keto-induced apoptosis might be enhanced in cells in a high proliferative state. To clarify this hypothesis, we investigated the effect of proliferative potential on 7-keto-induced apoptosis. First, we investigated the sensitivity to 7-ketocholesterol of cells in the resting phase. Under incubation without FBS, both OLETF- and LETO-derived SMC showed little apoptosis induced by 7-ketocholesterol (Fig. 3). Next, we studied whether 7-keto-induced apoptosis in LETO-derived SMC was enhanced by increasing the proliferative potential of these cells, because LETO-derived SMC showed lower sensitivity to 7-ketocholesterol compared to OLETF-derived SMC. As a reason for this difference, the cell characteristics could be changed by long exposure to high glucose concentration. For example, Kanzaki et al. (18) reported that PDGF receptors were overexpressed in SMC of diabetic animals. On the other hand, the genetic difference between OLETF and LETO rats may also be considered. Recently, genetic abnormality of diabetes mellitus has been reported (19, 20). In the present study, the SMC of OLETF rats showed higher outgrowth rate and increase in cell number than those of LETO rats (Table 2, Fig. 1). From these results, we confirmed that the proliferative potential of SMC was higher in OLETF than in LETO rats. These results might explain one aspect of intimal thickening or high frequency of restenosis after percutaneous transluminal coronary angioplasty in diabetes mellitus patients (21). However, the major mechanism by which myocardial infarction occurs is not explained by cell proliferation, but by rupture of the capsular portion of the plaque (1).

7-Ketocholesterol is an oxysterol that exists in human atherosclerotic plaque (22) and induces apoptosis of SMC (9). The reduction of cell numbers by apoptosis in atherosclerotic plaque may make plaque fragile, and lead to plaque rupture.

---

**Fig. 3.** The effect of 7-ketocholesterol on fragmented DNA of SMC in resting phase.

Cells were plated at $6 \times 10^4$ cells in 24-well dishes, and incubated with DMEM containing 10% FBS for 2 days. After that, cells were incubated with DMEM containing 10% FBS (solid bar) or without FBS (open bar). Then 7-ketocholesterol was added to the wells at a final concentration of 50 $\mu$M, and incubated for 3 days. After that, DNA fragmentation of DNA was assayed quantitatively by ELISA. 7-Ketocholesterol was dissolved in ethanol. The final concentration of ethanol in the culture medium was 1%. Results are mean $\pm$ SEM. The experiment was performed in triplicate. * $p < 0.05$ vs LETO.

---

**Fig. 4.** Effect of PDGF-BB on fragmentation of DNA of LETO-derived SMC.

LETO-derived SMC were plated at $6 \times 10^4$ cells in 24-well dishes, and incubated with DMEM containing 10% FBS for 24 hours. Then, cells were incubated with 7-ketocholesterol (closed circle) or without 7-ketocholesterol (open circle). After incubation for 24 hours, PDGF-BB at varying concentrations was added to the cells. After incubation for 3 days, fragmentation of DNA was assayed quantitatively by ELISA. 7-Ketocholesterol was added at a final concentration of 50 $\mu$M. 7-Ketocholesterol was dissolved in ethanol. The final concentration of ethanol in the culture medium was 1%. Results are mean $\pm$ SEM. The experiment was performed in triplicate. * $p < 0.05$. 

---

7-Ketocholesterol is an oxysterol that exists in human atherosclerotic plaque (22) and induces apoptosis of SMC (9). The reduction of cell numbers by apoptosis in atherosclerotic plaque may make plaque fragile, and lead to plaque rupture.
rived SMC. By the addition of PDGF-BB that promotes proliferation, 7-keto-induced apoptosis was enhanced in SMC from LETO (Fig. 4). From these findings, we conclude that 7-keto-induced apoptosis may be enhanced with increasing proliferative potential in SMC. In atherosclerotic lesions under diabetic conditions, proliferation of SMC can be observed, but apoptosis of SMC may also occur with accumulation of 7-ketocholesterol.

Our study indicates that the proliferative potential of OLETF-derived SMC is not always higher than that of LETO-derived SMC (Fig. 1). In the early stage, OLETF-derived SMC showed high proliferative potential compared to LETO-derived SMC, but the cell numbers of both OLETF- and LETO-derived SMC reached similar levels after incubation for 8 days. Therefore, it is expected that 7-keto-induced apoptosis is not always remarkably enhanced in SMC of OLETF rats compared to LETO rats. Further study is necessary to clarify the mechanism by which the proliferative potential is regulated.

The OLETF rat is a well known model of insulin resistance that develops spontaneous persistent hyperglycemia, disorder of serum lipid metabolism, mild obesity and atherosclerosis after about 18 weeks old (16, 23). This characteristic is clinically consistent with metabolic syndrome (24, 25). Metabolic syndrome, which is an important disease associated with myocardial infarction (26), is considered to increase oxidative stress in a body (27, 28). Increase of oxidative stress might promote production of oxysterols such as 7-ketocholesterol. OLETF rats used in this study had already acquired the characteristics of metabolic syndrome (Table 1). In these models, oxidative stress might increase, producing 7-ketocholesterol in the body. If 7-ketocholesterol increases, myocardial infarction might easily occur because smooth muscle cells are more susceptible to 7-ketocholesterol-induced apoptosis. From these findings, we speculated that the high sensitivity of smooth muscle cells to 7-ketocholesterol, and the increase of 7-ketocholesterol in the body, might be one of the mechanisms of myocardial infarction in metabolic syndrome.

In summary, our study suggests that under diabetic conditions, smooth muscle cells in the aorta are more susceptible to 7-ketocholesterol-induced apoptosis, and one of mechanisms might be due to the high proliferative potential of the cells induced by the diabetic condition.

References


