Hypercholesterolemia induces regression in neointimal thickening due to apoptosis of vascular smooth muscle cells in the hamster endothelial injury model

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Abstract

Objective: Hypercholesterolemia is a major risk factor in the development of atherosclerosis. Although matrix metalloproteinase may play a key role in plaque rupture, apoptosis of vascular smooth muscle cells (VSMCs), which is induced by cholesterol and its oxides may also contribute to instability and rupture of plaque. Thus, we investigated the roles of hypercholesterolemia in vascular remodeling following endothelial injury in the hamster femoral artery. Methods: The endothelium was injured by photochemical reaction between green light and the photosensitizer dye, Rose Bengal. Photochemical reaction is routinely used in our laboratory to produce endothelial injury without mechanical stretching in experimental animals. Results: In normocholesterolemic hamsters (NCH), neointimal thickening gradually progressed within a 3-week observation period after endothelial injury. In hypercholesterolemic hamsters (HCH), neointimal thickening gradually progressed until the second week after endothelial injury. In contrast, at the third week neointimal thickening regressed and was thinner than that at the second week. There was no significant difference in in vivo proliferation of VSMCs detected by in vivo BrdU uptake between HCH and NCH. Apoptotic cells in the neointima and the media were observed in HCH from 2 to 4 weeks after endothelial injury, but not in NCH. At 2 weeks after endothelial injury, the numbers of TUNEL-positive VSMCs in HCH were significantly higher than those in NCH (neointima: 1.2 ± 0.3 vs. 0.3 ± 0.1%, P < 0.05, media; 2.9 ± 0.6 vs. 0.6 ± 0.2%, P < 0.01). Cholesterol deposit, which is detected by oil red O staining was observed in a neointimal or medial area in HCH, but not in NCH. Conclusions: These findings suggest that hypercholesterolemia with endothelial injury may induce VSMC apoptosis, followed by the regression of neointimal thickening, further hypercholesteremia might play a role in inducing plaque rupture through apoptosis of VSMC.

Keywords: Apoptosis; Atherosclerosis; Cholesterol; Remodeling; Smooth muscle

1. Introduction

Acute coronary syndrome is caused by atherosclerotic plaque rupture. The fibrous cap of plaques consists of vascular smooth muscle cells (VSMCs) and extracellular matrix, and covers the surrounding area with necrotic core [1]. In atherosclerotic plaque, it was reported that the accumulation of macrophage foam cells and inflammatory cells were reported [2,3], and matrix metalloproteinase may contribute to plaque rupture [4,5]. Recent studies have focused attention on the development of atherosclerosis and plaque rupture, and reported that the decrease in VSMCs and extracellular matrix in the fibrous cap was observed [6]. VSMCs are major components in the fibrous cap and produce elastin, collagen and other matrix proteins, VSMCs may contribute to plaque stability to prevent hemodynamic stress such as a rapid change in blood pressure or flow disturbance directly or indirectly [7]. Furthermore, some studies have demonstrated that apoptosis (programmed cell death) of VSMCs also plays a key role in vascular wall remodeling following percutaneous transluminal coronary angioplasty (PTCA), and in early and advanced atherosclerotic lesions associated with plaque rupture [8,9]. Some studies on apoptosis in the

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development of atherosclerosis, have revealed that loss of VSMCs by apoptosis may be important for the mechanisms of instability and rupture of plaques [10]. Macrophage foam cells and inflammatory cells infiltrate into atherosclerotic plaque and reduce the density of VSMCs and extracellular matrix in fibrous cap [6]. However, it was evaluated that the ability of cholesterol and its oxides to induce apoptosis in VSMCs in vitro or in vivo atherogenic study [11,12].

We previously reported a simple animal model of vascular intimal thickening which occurs in response to photochemically induced vascular endothelial injury [13,14]. In our model, photochemical reaction between green light and systematically administered red dye, Rose Bengal, produces reactive oxygen species that then leads to non-mechanical and non-invasive vessel endothelial injury. We can adapt this photochemical model of endothelial injury to induce intimal thickening in guinea pigs, hamsters, rats and mice.

In the present study, we investigated the role of hypercholesterolemia in vascular remodeling using our model of endothelial injury in hamsters. Hamsters are known to be a useful species for investigating cholesterol metabolism and atherosclerosis, because they have a lipid metabolism comparable with humans due to the similar composition and metabolism of both lipoproteins and bile acids [15,16]. In the present study, we demonstrated apoptosis of neointimal VSMCs, followed by regression of neointimal thickening by endothelial injury in the femoral artery of hypercholesterolemic hamsters.

2. Methods

2.1. Endothelial injury

Male Syrian hamsters (SLC, Japan) weighing 70–85 g were fed a normal or high-cholesterol diet (10% coconut oil and 0.4% cholesterol) to induce hypercholesterolemia for 4 weeks before endothelial injury and were continually fed the diet until the end of each experiment. Endothelial injury in the hamster’s left femoral artery was photochemically induced as described previously [13,14]. In total, 202 (102 animals in normal diet and 100 in high-cholesterol diet) hamsters were anesthetized by intraperitoneal injection of 70 mg/kg sodium pentobarbital. Animal body temperature was maintained at 38°C with a heating pad throughout the experiment. A cannula was inserted into the jugular vein for Rose Bengal injection. The left femoral artery was carefully exposed, and the probe of a pulse Doppler (PDV-20, Crystal Biotech America, USA) was attached to the artery for monitoring blood flow. Green light (wavelength 540 nm) was achieved using a xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) with a heat-absorbing filter and green filter. The irradiation was directed by an optic fiber, positioned about 5 mm above the exposed femoral artery proximal to the probe for monitoring blood flow. The intensity of irradiation was 0.9 W/cm². At 5 min after baseline blood flow had stabilized, Rose Bengal solution (20 mg/kg, Wako, Tokyo) was administrated over a 2-min period. The femoral artery was considered to be occluded when the blood flow had completely stopped as indicated by the flow monitor. The time required to occlude the artery after administration of Rose Bengal was taken as the time to the occlusion. The irradiation was stopped for 20 min after the start of Rose Bengal injection. The irradiated segment of the femoral artery was occluded by platelet- and fibrin-rich thrombi. At 24 h after endothelial injury, the hamsters were reanesthetized and a spontaneous reflow of the occluded artery was observed in all hamsters. The investigation conforms with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Histological and morphological procedures

At the end of each experiment, hamsters were euthanized by intravenous pentobarbital overdose. Femoral arteries were in situ perfusion-fixed with 10% neutral-buffered formalin at physiological pressure (90–100 mmHg) for 15 min following washing with saline, and immersed in the same fixative for ≥24 h. Both sides of the femoral arteries were removed. Fixed femoral artery segments were then embedded in paraffin and cut consecutively into 3-μm thick sections. Sections were also taken for measurement of neointimal and medial areas at 0.3-mm intervals. The sections were stained with hematoxylin and eosin (HE) and the neointimal area was defined as the region between the lumen and the internal elastic lamina. The medial area was defined as the region between the internal and external elastic lamina.

We demonstrated that a neointima was formed from borders between uninjured and injured sites into the injured center [14]. The injured center had a significantly thickened neointima compared with neighboring positions. Therefore, in all cases, the section showing the greatest intima–media ratio was selected for planimetry. The cross-sectional areas of the neointima and media (one section per animal) and the numbers of intimal and medial VSMCs (five sections per animal) were measured using a computerized apparatus (Videoplane, Germany).

2.3. Lipid and atherosclerotic lesions analysis

Blood samples were taken from an orbital vein before cholesterol feeding, and thereafter, immediately before endothelial injury and at the end of each experiment. After the centrifugation, plasma samples were analyzed for total cholesterol (TC) level and were enzymatically measured using a Likitec TC kit (Roche Diagnostics, Switzerland). Lipid accumulation in the vascular vessels was assessed.
by oil red O staining of cross-sections. After washing by perfusing saline, the femoral artery was fixed with 10% neutral-buffered formalin at physiological pressure (90–100 mmHg) for 30 min. Arterial vessels were removed and stocked in the same fixative at 4°C until sectioning. Femoral artery segments were sectioned at a thickness of 10 μm thick using a cryostat at −25°C and the sections were stained with oil red O for neutral lipids and with hematoxylin for nucleic tissue.

2.4. Cell proliferation

Bromodeoxyuridine (BrdU) labeling was performed to clarify the numbers of VSMCs undergoing DNA synthesis. Both groups were injected intraperitoneally with BrdU (30 mg/kg, Sigma) and 5-fluoro-2'-deoxyuridine (3 mg/kg, Sigma) at 18, 6, and 1 h before sacrifice. Arterial segments labeled with BrdU were excised at 3 days and 1, and 2 weeks after the endothelial injury. After fixation with Carnoy’s fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), embedding, consecutive sectioning, and immunohistological staining (Cell Proliferating Kit, Amersham–Pharmacia Biotech, UK), the numbers of proliferating cells were determined in both intimal and medial areas of the arterial sections using light microscopy. Sections were also taken for measurement of neointimal and medial areas at 0.3-mm intervals. For each femoral artery segment, the cells of five consecutive sections in different places were counted twice in a double blind manner, and the observations were averaged. All sections were counterstained with Mayer’s hematoxylin.

2.5. Analysis of apoptosis

2.5.1. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-Biotin nick end labeling (TUNEL) assay

In situ detection of apoptosis was performed using TUNEL assay with the ApoTag in situ apoptosis detection kit (Intergen, USA) in formalin-fixed artery. Briefly, tissue sections were deparaffinized and rehydrated by transferring the slides through the following solutions: xylene three times for 5 min, 100% ethanol two times for 3 min, 95% ethanol two times for 3 min, 80% ethanol for 3 min, 70% ethanol for 3 min, and finally PBS for 5 min, and incubated with 0.3% H₂O₂ in PBS to quench endogenous peroxidase activity. Nuclei were stripped of proteins by incubation with proteinase K (Dako, Denmark) for 15 min. DNA fragments were labeled with TdT and digoxigenin-dUTP at 37°C for 1 h. The enzymatic labeling of the DNA fragments with digoxigenin-dUTP was detected with peroxidase-conjugated antibody against digoxigenin. Diaminobenzidine (DAB) was used as the substrate for peroxidase. Sections were also taken for measurement of neointimal and medial areas at 0.3-mm intervals. For each femoral artery segment, the cells of five consecutive sections in different places were counted twice in a double blind manner, and the observations were averaged. All sections were counterstained with Mayer’s hematoxylin.

2.5.2. DNA chromatin morphology

TUNEL stain is not specific for detection of apoptosis because of the lightly staining background. Thus, other techniques for detecting evidence of apoptosis should be performed. Han et al. reported that using a fluorescent DNA-binding dye, propidium iodide (PI, Sigma) staining combined with TUNEL staining in fixed tissue slices, could clearly detected apoptotic cells [17,18]. Thus, we performed not only TUNEL staining but also PI staining, in which morphologically condensed or fragmented nuclei were identified, at five consecutive slices of the irradiated site. Paraffin-embedded tissue was dewaxed, rehydrated and stained with PI (5 μg/ml) solution in minimum essential medium–Hepes containing 50 μg/ml DNAase-free RNAase A for 30 min at 37°C. Sections were examined under a fluorescent microscope. Only clearly condensed or fragmented nuclei were counted as apoptotic cells.

2.6. Immunocytochemistry

To identify macrophages and VSMCs in the femoral artery, immunohistological staining was performed with peroxidase-labeled primary monoclonal antibody (Enhanced Polymer One-step Staining, Dako, Denmark). Tissue samples for macrophages and VSMCs were fixed with 10% neutral-buffered formalin. The sections were rinsed for 2×5 min in PBS. After the blocking of endogenous peroxidases using 0.3% H₂O₂ in PBS, non-specific antibody binding was prevented by preincubating the sections with goat serum for 10 min at room temperature. Subsequently, tissues were incubated with the following primary antibodies; anti-human α-actin for VSMCs, anti-human CD68 for macrophages and anti-human von Willebrand factor (vWF) for endothelial cells (Dako) for 1 h at 37°C. Antibody visualization was established after 3 min exposure to DAB (Nichirei, Japan). All sections were counterstained with Mayer’s hematoxylin.

2.7. Statistical analysis

All values are represented as the mean±S.E.M. Differences between two unpaired groups were analyzed with Student’s t-test. A value of P<0.05 was considered significant.

3. Results

3.1. Plasma lipids

Immediately before endothelial injury, the mean plasma cholesterol level in HCH was significantly (P<0.01)
increased by approximately 3-fold more than that in NCH (338.4 ± 6.8 mg/dl in HCH vs. 148.3 ± 1.7 mg/dl in NCH). The plasma cholesterol level in HCH continued to be high during the experimental period. There was no significant difference in animal weights between HCH and NCH.

3.2. Vascular neointimal thickening

We investigated whether a high plasma cholesterol level may amplify neointimal formation with the model of a photochemically induced endothelial injury. The mean intimal and medial areas were quantified at 3 days and 1, 2, 3 and 4 weeks after endothelial injury, and neointima-media ratio (N/M ratio) was represented as the ratio of the neointimal to the medial areas.

Typical microphotographs of neointimal thickening at 1, 2 and 3 weeks after endothelial injury are shown in Fig. 1. Morphological measurement of a cross-section in each femoral artery revealed a marked distinction between HCH and NCH. Fig. 2 shows the neointimal and medial areas, and N/M ratio. Vascular smooth muscle cells in the neointima were migrated from the media and they gradually proliferated in the neointima in both NCH and HCH at 1 week after endothelial injury (Fig. 1A, D). In NCH, neointimal thickness continued to increase until 3 weeks after endothelial injury and reached maximally at the third week and then, continued to be thick until 4 weeks after endothelial injury. Neointima/media ratio at the third week was 0.53 ± 0.12 (Figs. 1C and 2C).

In HCH, neointimal thickness gradually increased until the second week after endothelial injury (Fig. 2A) and was similar to that in NCH at the second week (N/M ratio: 0.43 ± 0.12 in HCH and 0.39 ± 0.12 in NCH, Fig. 1B, E). In contrast, the neointimal area was regressed at the third week after endothelial injury by 48% compared with the second week (Figs. 1F and 2A) and continued to regress until the fourth week. At the fourth week, the N/M ratio was 0.18 ± 0.01 and the neointimal area was thinner by 32% compared with that in NCH (Fig. 2C). There was no significant difference in the medial area between NCH and HCH from 3 days to 4 weeks after endothelial injury (Fig. 2B). In the intact femoral artery of each hamster, neointimal thickness was not observed during the experimental period.

Lipid accumulation, which was detected by the oil red O staining was observed in the femoral artery in HCH from 3 days to 4 weeks after endothelial injury. Fig. 1G shows that fat slightly accumulated in VSMCs and the extracellular matrix of the neointimal and medial areas at 2 weeks after endothelial injury. In contrast, there was almost no lipid accumulation in the neointima and the media of NCH and the intact femoral artery of HCH during the experimental period (data not shown).

We investigated macrophage infiltration into endothelial injured arterial vessels by mouse anti-human CD68 antibody. There were no CD68-positive cells in either the neointima or media between NCH and HCH from 3 days to 4 weeks after endothelial injury (data not shown).

3.3. Endothelial cell injury

Typical microphotographs of vascular vessels with anti-human vWF staining at 3 days, and 2 and 4 weeks after endothelial injury are shown in Fig. 3. At 3 days after the injury, there were no endothelial cells staining anti-vWF antibody inside the internal elastic lamina of either NCH or HCH (Fig. 3A, D). Two weeks after injury, the neointimal thickness was observed between NCH and HCH. There was a weak staining by anti-vWF antibody between the neointimal lesion and lumen (Fig. 3B, E). The endothelial regrowth completely covered the site of endothelial injury within 4 weeks after endothelial injury and the time course of the endothelial regrowth was similar between NCH and HCH (Fig. 3C, E).

3.4. Cell numbers in the neointima

Table 1 shows the numbers and cell density of VSMCs in the neointimal area from 3 days to 4 weeks after endothelial injury. In NCH, the cell numbers gradually increased until the third week and continued to be high until the fourth week. The cell numbers in HCH increased by the same degree until the second week; however, they were reduced by 53% at the fourth week and were significantly (P < 0.05) lower than those in NCH. In contrast, there was no significant difference in the cell density of the neointimal area between NCH and HCH.

3.5. Cell proliferation

To verify whether hypercholesterolemia may contribute to in vivo VSMCs proliferation activity following endothelial injury, cell proliferation at the irradiated site of both NCH and HCH was examined by counting the numbers of positive BrdU staining cells from 3 days to 2 weeks after endothelial injury (Fig. 4). The ratio of BrdU-positive cells divided by all cells was maximal at the first week in both NCH and HCH (15.1 ± 4.8 and 12.5 ± 4.3% in the neointima). However, at the second week, cell proliferation activity returned to the same low level as that of the third day. There was no significant difference in cell proliferation activity of either the neointima or the media between NCH and HCH from 3 days to 2 weeks after endothelial injury.

3.6. In situ detection of apoptotic cells

To verify why neointimal thickening was regressed by hypercholesterolemia, we investigated whether hypercholesterolemia may induce apoptosis of VSMCs. In Fig. 5B, C, we
Fig. 1. Light photomicrographs of typical histological cross-sections of the femoral arteries in NCH and HCH after endothelial injury. (A)–(C) Sections in NCH at 1, 2 and 3 weeks after endothelial injury with hematoxylin and eosin staining. (D)–(F) Sections in HCH at 1, 2 and 3 weeks after endothelial injury with hematoxylin and eosin staining. (G) Sections in HCH at 2 weeks after endothelial injury with oil red O staining. Arrows indicate slight fat accumulation in medial VSMC as small red granules. Some artificially large particles of oil red O staining were observed. Internal elastic lamina is indicated by arrowheads in (A)–(G). Bar=40 μm (A)–(F), and 20 μm (G).
totic morphological feature of apoptosis. In HCH, the numbers of TUNEL-positive cells significantly \((P<0.05)\) increased in both intimal and medial areas. At 2 weeks after endothelial injury, VSMCs in the neointima \((1.2\pm0.3\%)\) and the media \((2.9\pm0.6\%)\) became apoptotic \((\text{Fig. } 5\text{C,D})\). Although the numbers of TUNEL-positive cells in HCH declined 3 weeks after endothelial injury, they were still higher than those in NCH up to 4 weeks after endothelial injury. There was no significant difference in the ratio of TUNEL-positive cells within 1 week after endothelial injury between HCH and NCH. In NCH, the ratio of TUNEL-positive cells was similar between 1 and 4 weeks after endothelial injury. The ratio of TUNEL-positive cells in non-endothelial injured vascular cells of both NCH and HCH were similar between 1 and 4 weeks after endothelial injury.

To confirm the presence of apoptotic nuclei, each slice of the irradiated artery was stained with PI. Morphological assessment revealed that the increase in apoptotic nuclei between HCH and NCH, which exhibited shrinkage and nuclear condensation, was similar compared with that in TUNEL staining.

![Fig. 1. (continued)](image)

![Fig. 2. Neointimal formation in NCH and HCH. (A) Neointimal area; (B) medial area; (C) the ratio of the neointimal area divided by the medial area (N/M ratio). Neointimal or medial area were measured at 3 days after endothelial injury, NCH \((n=7)\) and HCH \((n=7)\); at 1 week, NCH \((n=7)\) and HCH \((n=7)\); at 2 weeks, NCH \((n=12)\), HCH \((n=11)\); at 3 weeks, NCH \((n=13)\), HCH \((n=12)\); at 4 weeks, NCH \((n=13)\), HCH \((n=13)\). Values represent the means±S.E.M. values. \((\bigcirc)\) NCH, \((\bullet)\) HCH, *\(P<0.05\) and †\(P<0.03\) versus NCH group.)

![Graph A: Neointima](image)

![Graph B: Media](image)

![Graph C: N/M ratio](image)
Fig. 3. Light photomicrographs of typical immunohistochemical cross-sections of endothelial cell recovery in the femoral arteries of NCH and HCH after endothelial injury. (A)–(C) Sections in NCH at 3 days, and 2 and 4 weeks after endothelial injury stained by anti-human vWF antibody. (D)–(F) Sections in HCH at 3 days, and 2 and 4 weeks after endothelial injury stained by anti-human vWF antibody. Internal elastic lamina are indicated by arrowheads in (A)–(F). Bar=40 μm (A)–(F).
Table 1

<table>
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<td>4 weeks</td>
<td>113±17</td>
<td>171.4±36.0</td>
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<tr>
<td></td>
<td>HCHs</td>
<td></td>
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Values are presented as means±S.E.M.

*P<0.05 versus NCHS.

4. Discussion

We have shown that regression of neointimal thickness was observed in hypercholesterolemia with endothelial injury. The findings of the present study support the observation that hypercholesterolemia is responsible for the VSMCs loss at the development of atherosclerosis.

It was reported that smooth muscle cells migrate into the neointima from the media and then proliferate during vascular remodeling following PTCA in human and bal-
followed by arterial thrombus formation [13], which
induced both migration and proliferation of VSMCs due to
platelet-derived growth factor and thrombin release from
thrombi [18]. The time to thrombotic arterial occlusion
after endothelial injury was not significantly different
between NCH and HCH (12.5±1.1 min in NCH versus
13.5±0.5 min in HCH), and, thus, hypercholesterolemia in
the present study did not affect platelet- and fibrin-rich
thrombus formation.

It was previously reported that endothelial cells might
act as a modulator of VSMCs growth for either growth
factor or growth-inhibitory molecules. In the present study,
because the endothelial regrowth was completely covered,
and, therefore, the site of endothelial injury was not
different between NCH and HCH, there may be no
influence of the endothelial regrowth on neointimal thick-
ening between NCH and HCH. These findings suggest
that endothelial injury may stimulate cell proliferation of
the neointima in both HCH and NCH and the regression of
neointimal thickening in HCH may not be caused by
inhibition of cell proliferation.

In the present study, we could detect TUNEL-positive
cells in neointimal and medial areas, which were sig-
ificantly increased at the second week compared with
those in NCH. In addition, the VSMC numbers at the
neointimal area at the fourth week were decreased com-
pared with those in NCH, but the cell density was not. The
induction of VSMCs apoptosis in the development of
atherosclerosis has been reported in previous studies. This
suggests that the hypercholesterolemia may be responsible
for apoptosis of VSMCs, thus, the numbers of VSMCs,
which migrated to the intima, may be reduced, and,
therefore, neointimal thickening may regress.

VSMC apoptosis in the media of HCH was higher
compared with NCH, while the proliferation in the two
groups was comparable. In addition, the medial area and
the number of VSMCs in the HCH were comparable with
those in NCN. Proto-oncogenes including c-fos and hydro-
gen peroxide are known to be associated with not only
proliferation of VSMCs but also with apoptotic pathways
[19,20]. VSMCs which undergo proliferation and migration
could be more vulnerable to apoptosis, and this may
explain why there was no difference in the medial area

loon injuries in various animal models [18]. Since there
was no significant difference in in vivo cell proliferation
between HCH and NCH during the experimental period,
VSMCs proliferation in hypercholesterolemia may not be
responsible for the regression of the neointima in HCH.

In the model used in the present study, platelet adhesion
and aggregation were induced after endothelial injury,
between HCH and NCH, while the neointima in HCH regressed.

It was reported that immune cells including macrophages and lymphocytes play a key role in vascular remodeling or development of atherosclerosis including VSMCs apoptosis [6,21]. In the present study, we could not histologically detect macrophages and lymphocytes in the neointima in HCH (data not shown). This suggests that the proliferation and apoptosis of VSMCs in this present study, were not induced by macrophages or lymphocytes.

It was reported that cholesterol itself has no direct angiotoxicity; however, cholesterol oxides induce apoptosis in vitro in many types of cells, including endothelial cells and VSMCs [11,12]. 7-Ketocholesterol and 25-ketocholesterol, which are the major oxysterols in oxidized LDL, induced apoptosis by a rapid decrease of bcl-2 protein or increase of CPP32 protease activity in the cells [22,23]. In addition, reactive oxygen species including hydrogen peroxide which was produced by photochemical reaction [13], or these metabolites such as oxidized fatty acids exert the effect on VSMCs apoptosis [20,24]. In the present study, lipid deposits were observed in the injured vessel wall in HCH, in which VSMCs apoptosis was observed, but no lipid diposits or VSMC apoptosis was observed without endothelial injury. In line with this assertion, a high plasma cholesterol level is associated with increased oxidized LDL [25]. Therefore, a high plasma cholesterol level in the present study may increase the oxidized LDL level and the oxidized lipids may deposit in the vascular wall with endothelial injury and contribute to apoptosis of VSMCs.

It was reported that in endothelial injury by balloon injury, apoptosis of VSMCs induced by 40% at 9 days after balloon injury [18,26]. In contrast, in human atherosclerotic lesion, apoptosis is induced by only 3% [21,27]. Balloon injury causes mechanical damage to vessels, including stretching and disrupting vessels, which strongly induce apoptosis. However, in balloon injury models, the regression of neointimal thickening may be caused by a balance between cell proliferation and the induction of apoptosis. The reason why the regression of neointimal thickening cannot be detected in balloon injury models is that balloon injury stimulates cell proliferation greater than it induces apoptosis of VSMCs [28,29]. It was reported that the balloon-injury to the aortic vessel induces
VSMC proliferation rather than apoptosis during the early phase after the balloon injury (until 3 weeks after the injury) [28], furthermore, macrophages, which were infiltrated into the vessel wall in hypercholesterolemia, but not in normocholesterolemia enhanced VSMCs proliferation greater than apoptosis [29]. In the present study, we could detect the regression of neointimal thickening due to apoptosis. This may because the macrophages that infiltrated into the vessel wall were not observed and photochemical injury selectively damages the endothelium, but not the media, while balloon injury stretches and disrupts the vessel wall.

To verify if the effect of intra-arterial cholesterol in combination with the photochemical reaction might induce apoptosis, additional experiments with a high cholesterol diet (only after endothelial injury) are helpful for understanding mechanisms, which might contribute to apoptosis following endothelial injury attributed to hypercholesterolemia. However, in the present study, the high cholesterol diet started 4 weeks prior to endothelial injury because it takes 2–4 weeks for plasma cholesterol level to increase. Accordingly, if a high cholesterol diet is started just after endothelial injury, the level of plasma cholesterol may not be high enough at that time. Consequently, apoptosis may not be appreciable and the effect of intra-arterial cholesterol in combination with the photochemical reaction on apoptosis cannot be interpreted. Therefore, we decided not to carry out additional experiment with a high cholesterol diet.

In the present study, lipid deposits were observed in the vessel wall by using oil red O staining. In the vessel wall with endothelial injury in HCH, lipid deposits were observed while, in the vessel wall without endothelial injury, namely the contralateral femoral artery not receiving photoin irradiation, lipid deposits were not seen. Based on this observation, we think that lipids may infiltrate the vessel wall following endothelial injury. Therefore, it is not very likely that intra-arterial lipids and reactive oxygen species produced by photochemical reaction to cause apoptosis of VSMCs.

In conclusion, hypercholesterolemia with endothelial injury may induce VSMC apoptosis, followed by regression of neointimal thickening, further hypercholesteremia might play a role in inducing plaque rupture through apoptosis of VSMC.

References

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