ARTICLES

Characterization of Simple and Reproducible Vascular Stenosis Model in Hypercholesterolemic Hamsters

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ABSTRACT: The importance of low-density lipoprotein (LDL) in the etiology of atherosclerosis is well recognized. We have established a reproducible stenosis model in hypercholesterolemic hamsters, and the process of arterial stenosis by thrombus or neointima was studied and compared with that in normal hamsters. The level of plasma LDL was 4.6 times higher in hamsters fed a high-cholesterol diet than in hamsters fed normal food. Endothelial injury in right common carotid arteries was induced using a modified catheter. Arterial blood flow was monitored continuously using a Doppler flow probe. Arterial patency after the initiation of injury in high-cholesterol hamsters was significantly changed as compared with that of normal hamsters. Neointima was observed 2 wk after the vascular injury. The neointimal area of high-cholesterol hamsters was significantly larger than that of normal hamsters. To characterize the stenosis in hypercholesterolemic hamsters, we measured platelet aggregation, thrombin time, activated partial thromboplastin time, and proliferating smooth muscle cells (SMC) *in vitro* and *in vivo*. The half-maximal inhibitory concentration value for platelet aggregation induced by thrombin or collagen, the DNA synthesis stimulated by platelet-derived growth factor (PDGF)-BB, and 5-bromo-2-deoxy-uridine labeling indices (proliferating index of SMC *in vivo*) in high-cholesterol hamsters were each significantly higher than the comparable value from normal hamsters. However, specific binding of PDGF-BB in SMC was not different between the two types of hamsters. Furthermore, we investigated the inhibitory effects of probucol or losartan on neointima formation using this model. Probucol, but not losartan, significantly reduced the neointimal area in hypercholesterolemic hamsters. These findings indicated that high levels of plasma LDL strongly contributed to the development of thrombus and neointima formation *via* both up-regulation of platelet aggregation and the enhancement of SMC proliferation. This stenosis model may be useful for the investigation of hypercholesterolemia-associated cardiovascular diseases.

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Hypercholesterolemia increases the risk of developing cardiovascular diseases, and low density lipoprotein (LDL) plays an important role in atherosclerosis (1). Recent studies have shown that LDL stimulates endothelial cells to produce a variety of growth factors that induce the migration and proliferation of granulocytes, macrophages, and endothelial cells (2,3). These studies raise the possibility that increases in LDL content stimulate the production of factors that play an important role in the proliferation of cells and matrix elements found in both atherosclerosis and vascular neointima formation.

On the other hand, both platelets and LDL are intimately involved in the pathogenesis of atherosclerosis (2). Platelet function is directly influenced by lipoproteins, and platelets from patients with hypercholesterolemia display enhanced platelet reactivity (4). Each platelet processes specific high-affinity binding sites for LDL, ranging from 1000 to 8000 copies per platelet with a dissociation constant (K_D) value between 40 and 100 nmol/L (5,6). Moreover, the LDL-induced sensitization is accompanied by decreased angular movement in platelet membranes, possibly caused by increased cholesterol transfer (7), and platelets enriched with cholesterol show increased arachidonic acid release and thromboxane B_2 formation (8).

Until now, rabbits fed a high-cholesterol diet or Watanabe rabbits (9) have been the main animals used for the investigation of hypercholesterolemia or atherosclerotic lesions of vascular diseases. Other small animals that can be used for this type of study are exogenously hypercholesterolemic rats and hamsters. In rats, a few months are required to establish hypercholesterolemia after the start of a high-cholesterol diet (10,11). On the contrary, only a few weeks are necessary for hamsters to be hypercholesterolemic (11). Recently, we established a simple and reproducible vascular stenosis model in hamsters (12). We investigated the inhibitory effects of antiplatelet compounds (13–16), angiotensin-converting enzyme inhibitors (17), and an angiotensin II receptor antagonist (18) using this model. Vascular stenosis often accompanies injury to endothelial tissues. Progression of stenosis occurs by thrombus formation during the acute phase or neointima formation during the chronic phase after endothelial injury. Our previous observations clearly indicated that the inhibition of both platelet activation and smooth muscle cell (SMC) proliferation or migration strongly reduced the development of vascular stenosis after endothelial injury (13, 18). On the other hand, the likelihood of vascular occlusion in patients is enhanced by the additive effect of other risk factors, such as diabetes and hypercholesterolemia. Therefore, in the present study, this model was applied to examine the effect of plasma LDL levels on platelets and SMC in order to

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Abbreviations: aPTT, activated partial thromboplastin time; BrdU, 5-bromo-2-deoxy-uridine; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HDL, high density lipoprotein; IA, intimal area; IC_{50}, half-maximally inhibitory concentration; IELA, internal elastic lamina area; LA, lumen area; LDL, low density lipoprotein; PDGF, platelet-derived growth factor; PRP, platelet rich plasma; SEM, scanning electron microscopy; SMC, smooth muscle cell; TEM, transmission electron microscopy; TT, thrombin time.

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define easily determined, clinically significant measures of vascular stenosis progression.

MATERIALS AND METHODS

Animals. Male syrian hamsters (SLC, Sizuoka, Japan) weighing 60–80 g were selected and fed either a standard chow (RC4; Oriental Yeast Co., Ltd., Japan) or chow supplemented with 0.5% (w/w) cholesterol. An operation for vascular injury using a modified catheter was carried out 4 wk after the start of the diet regimen. The body weight of hamsters fed a high-cholesterol diet was not statistically significantly different from that of hamsters fed a normal diet (body weight: 120–130 g). All experiments were performed in accordance with institutional guidelines governing animal experimentation.

Reagents. Collagen for platelet aggregation was obtained from Nycomed Arzneimittela GmbH (Munich, Germany). [Methyl-^{3}H] thymidine and [^{125}I] platelet-derived growth factor (PDGF)-BB were purchased from Amersham Japan (Tokyo, Japan). Probucol and losartan are a kind gift from Otuka Co. Ltd. (Tokushima, Japan) and Banyu Co. Ltd. (Tokyo, Japan), respectively. The other chemicals were obtained from Sigma (St. Louis, MO).

Induction of vascular stenosis and quantitation of neointima formation. Four weeks after the start of either the regular or the high-cholesterol dietary regimen, hamsters were anesthetized by intraperitonial injection of 50 mg/kg sodium pentobarbital. In brief, the right common carotid artery and the right femoral artery were exposed, and an arterial injury to the right common carotid artery using a 2FG catheter (Portex Ltd., Kent, United Kingdom) with a roughened tip was performed according to the previously described technique (12). Another catheter (i.d. = 0.5 mm, o.d. = 0.8 mm, polyethylene sp3, Natume Co. Ltd., Tokyo, Japan) was connected to the right femoral artery for monitoring blood pressure and pulse rate using a pressure transducer (AP601G; Nihon Koden, Tokyo, Japan) during experiments on day 0 (initiation of vascular injury). Blood flow in the carotid artery was continuously monitored for 90 min on day 0 using a Doppler flow probe (model PDV-20; Crystal Biotech Co. Ltd., Tokyo, Japan) positioned proximally to the injured area of the carotid artery. Our previous histological observations revealed that a platelet-rich thrombus was obviously established when the blood flow was zero (12). After the recovery from anesthesia, animals were kept in individual cages. Three, 5, 7, and 14 d after vascular injury, hamsters (n = 7 each) in each group were anesthetized and perfused transcardially with saline. The common carotid artery was excised, divided into several sections, and frozen. The tissue samples were cross-sectioned, and stained with hematoxylin (Sigma Chemical Co.) to determine the intimal area. Part of these samples were used for the stain of lipids using oil red. The total areas of the internal elastic lamina (IELA) and lumen (LA) were measured using a computerized image graphic analysis system. The measurement was performed in triplicate for each sample. The average of three intimal area (IA = IELA – LA) determinations was then expressed as a percentage of IELA.

Measurement of serum cholesterol. Blood samples (0.3 mL) from hamsters fed either a regular or a high-cholesterol diet were collected in each time period *via* the jugular vein after the animals had been anesthetized with ether. The samples were treated with 3.15% sodium citrate. High density lipoprotein cholesterol (HDL) and LDL cholesterol were determined by enzymatic assays of blood samples taken at 0 (before the start of diet), 2, 4, and 6 wk after the start of feeding regimens These measurement were performed with Nipro cholesterol determination kits (Nipro Co. Ltd., Osaka, Japan).

Platelet aggregation. At the end of the experiments, 4.0 mL of blood was collected from every hamster in each group by heart puncture into sodium citrate (3.15%, final concentration) and centrifuged for 10 min at 155 × g to obtain platelet-rich plasma (PRP). Platelets were counted and adjusted to 4×10^8 cells/mL (final concentration), and then platelet aggregation was induced by collagen (1.0–10.0 μg/mL) or ADP (0.5–5.0 μM) using PRP. Washed hamster platelets were prepared as described previously (19). Washed platelet aggregation was induced by thrombin (0.0001– 0.3 unit/mL). Platelet aggregation was followed using an aggregometer (Aggrecorder II, DA-3220; Kyotodaiichi-Chemical, Kyoto, Japan) at 37°C with a stirring speed of 800 rpm. All measurements were performed in triplicate.

Ex vivo *anticoagulant studies.* After the separation of PRP for platelet aggregation, the remaining blood samples were further centrifuged for 10 min at 1550 × g to obtain platelet-poor plasma (PPP). The activated partial thromboplastin time (aPTT) and thrombin time (TT) were determined using standard clinical laboratory procedures.

Electron microscopic observation. In separate experiments, several samples taken from hamsters fed a high-cholesterol or normal diet were used for histological observations by means of scanning or transmission electron microscopy. Hamsters were anesthetized and perfused transcardially with saline 2 wk after the initiation of vascular injury. The common carotid artery was excised and fixed with 2.0% glutaraldehyde in 50 mM sodium phosphate buffer for 30 min. Each segment was cut open longitudinally to allow visual inspection for scanning electron microscopy (SEM) or cross-sectioned for transmission electron microscopy (TEM) as described (12).

Proliferation index of SMC in vivo. Proliferating SMC were identified by *in vivo* DNA labeling with the thymidine analog 5-bromo-2-deoxy-uridine (BrdU) (12). BrdU (50 mg/kg) was injected subcutaneously 1, 8, 16, and 24 h prior to removal of the carotid artery. Following carotid artery removal 1, 3, 5, 7, or 14 d after injury from both control and cholesterol-supplemented hamsters (n = 4, each time point), frozen cross sections were prepared from these arteries. BrdU-positive cells were detected with a murine monoclonal antibody (Sigma), followed by treatment with goat antimouse immunoglobulin-antibodies conjugated to peroxidase. The complexes were then stained with diaminobenzidine (DAB). The BrdU labeling index was calculated using the following formula: (nuclei stained positive by DAB)/(total nuclei stained by hematoxylin) × 100. Animals were sacrificed by an overdose of sodium pentobarbital at the end of the experiment.

Cell culture and measurement of DNA synthesis. Vascular SMC were isolated from the thoracic aorta of hamsters fed either a normal or supplemented diet. The cells were cultured over several passages using the method of Ross (20). SMC were grown to confluency in 7500-mm^2 culture flasks in Dulbecco's modified Eagle's medium [DMEM; Gibco BRL, Grand Island, NY; 5% fetal calf serum (FCS), 100 μg/mL streptomycin, 100 U/mL penicillin, 4 μmol/L glutamine] at 37°C under humidified 5% CO_2/95% air. The cells were then treated with 0.25% trypsin in phosphate-buffered saline (pH = 7.4), washed, and counted. A total of 8×10^4 isolated cells were cultured as above. Cell culture medium was replaced every 2 d. On day 8, cells were detached with trypsin and counted. The cultured cells were stimulated with 5% FCS or various doses of angiotensin II (10^{-6}–10^{-8} M), thrombin (0.001–0.03 units/mL), vasopressin (10^{-6}–10^{-8} M), endothelin I (10^{-6}–10^{-8} M), or PDGF-BB (1.0–100 ng/mL) in 1 mL of DMEM at 37°C for 24 h. Six hours before harvest, the cells were pulse-labeled with [methyl-^{3}H]thymidine (0.5 μCi/dish). Incubation was terminated by adding 1 mL of 10% trichoroacetic acid, and radioactivity in the acid-insoluble materials was determined by using a Beckman LS-6000IC liquid scintillation spectrometer.

[^{125}I]PDGF binding. Cultured cells were subjected to a binding assay that was essentially as described (21). Briefly, the cells were treated at 37ºC in 10 mM HEPES-buffered Dulbecco's Eagle's medium (1.0 mL, pH = 7.4; Gibco BRL). The cells were incubated with or without a 1,000-fold molar excess of nonradioactive PDGF-BB for 20 min at 37ºC. At the end of the incubation, the cells were thoroughly washed with cold phosphate-buffered saline and solubilized using 0.1% sodium dodecyl sulfate (1.0 mL). The radioactivity of the lysate was then determined using a Wallac 1480 WIZARD 3″ automatic gamma counter (Turku, Finland).

The effect of probucol or losartan on neointima. To further define this stenosis model in pharmacological experiments, we investigated the effect of probucol or losartan using this model. Hamsters fed a high-cholesterol diet were divided into seven groups, a control group (n = 10), three groups treated with probucol (twice a day, p.o.) at doses of 30.0, 60.0, or 120.0 mg/kg per day (n = 6 each), and three groups treated with losartan (twice a day, p.o.) at doses of 2.0, 6.0, or 20.0 mg/kg per day (n = 6 each). Oral administration of each compound was started 2 h before the initiation of endothelial injury by a modified catheter and continued for the next 2 wk. Fourteen days after vascular injury, the common carotid artery of hamsters in each group was treated as mentioned in the paragraph on quantitation of neointima formation. At the end of observation period, blood pressure was monitored for 10 min. A catheter (i.d. = 0.5 mm, o.d. = 0.8 mm, polyethylene sp3, Natsume Co. Ltd.) connected to a pressure transducer (AP601G; Nihon Koden) was inserted into the left femoral artery for 10 min. After the measurement of blood pressure, a blood sample was taken for platelet aggregation and measurements of coagulation factors (TT and aPTT).

Statistics. All data are presented as the mean ± SEM. The statistical significance of the data was determined by analysis of variance followed by the Student-Newman-Keuls test.

RESULTS

Alteration of plasma cholesterol levels. The mean plasma LDL level in all animals before beginning a specific dietary regimen was <0.5 g/L. After 4 wk of a high-cholesterol diet, the LDL level had increased to ~2 to 2.5 g/L and remained elevated to the end of the observation period. In contrast, there was no significant difference in plasma HDL levels between hamsters fed normal and cholesterol-supplemented diets (Table 1).

Acute thrombus formation and vascular patency. Vascular patency is shown in Table 2. Blood flow in the carotid artery in normal hamsters (n = 28) was interrupted in 6.4 ± 0.6 min after the initiation of vascular injury by a catheter. After the blood flow was zero, spontaneous cyclic reflow and reocclusion were observed in 21 hamsters during the observation period of 90 min. The other arteries were not reperfused during the observation period. In the group of high-cholesterol hamsters, the time to occlusion was significantly shortened to 4.8 ± 0.4 min and only four arteries showed spontaneous cyclic reocclusion and reflow during the observation period (n = 28).

Neointima formation in response to endothelial injury. All hamsters developed concentric intimal lesions in response to endothelial denudation by a catheter. The ratios of time-dependent vascular stenosis by neointima formation are shown in Figure 1. In hamsters fed a high-cholesterol diet, the extent of vascular stenosis was significantly greater compared with that of hamsters fed a normal diet.

Platelet aggregation and hemostasis analysis. The half-maximally inhibitory concentration (IC_{50}) values for platelets induced to aggregate by collagen, ADP, and thrombin in ham-

TABLE 1
Plasma Cholesterol Levels (g/L) in Hamsters[a]

	Diet group		Normal group	
	LDL	HDL	LDL	HDL
Pretreatment	0.50 ± 0.07	0.49 ± 0.08	0.47 ± 0.08	0.50 ± 0.06
2 wk	1.12 ± 0.41	0.46 ± 0.09	0.49 ± 0.06	0.48 ± 0.05
3 wk	1.92 ± 0.31*	0.45 ± 0.06	0.49 ± 0.06	0.50 ± 0.07
4 wk	2.34 ± 0.22*	0.50 ± 0.07	0.49 ± 0.02	0.49 ± 0.09
6 wk	2.33 ± 0.29*	0.55 ± 0.07	0.52 ± 0.07	0.48 ± 0.07

[a]Hamsters (4 wk old) were allotted to a high-cholesterol diet group or to the normal diet group. Pretreatment means before beginning a dietary regimen; each week means the time after the start of a particular regimen. Vascular injury in each hamster occurred at 4 wk. *$P < 0.05$ vs. control (normal hamsters in each time course).

TABLE 2
Time to Occlusion and Vascular Patency Status in Hamsters[a]

	Normal hamsters	High-cholesterol hamsters[b]
Time to occlusion (min)	6.4 ± 0.8	4.8 ± 0.4*
PP	0	0
CR	21	4**
PO	7	24**

[a]Vascular patency was judged at the end of the observation period. Carotid arterial patency was expressed as persistent occlusion (PO) when no reperfusion was observed at all, as cyclic flow reduction (CR) when the arterial reflow alternately showed stops and flows, and persistent patency (PP) when the arterial flow was maintained until the end of observation period. Data correspond to the number of arteries in each group (n = 28 animals in each group).
[b]*$P < 0.05$; **$P < 0.01$. Time to occlusion data represented mean ± SEM.

TABLE 3
Platelet Aggregation and Hemostatic Analysis of Hamsters[a]

	Normal hamster	High-cholesterol hamsters
ADP	6.6 ± 0.9 μM	4.9 ± 1.9 μM
Collagen	7.8 ± 1.1 μg/mL	3.8 ± 0.2 μg/mL*
Thrombin	0.061 ± 0.1 unit/mL	0.002 ± 0.0004 unit/mL**
TT	15.4 ± 1.1 s	13.6 ± 2.3 s
aPTT	58.2 ± 6.8 s	52.2 ± 4.8 s

[a]The values of the half-maximally inhibitory concentration for platelet aggregation induced by ADP, collagen (PRP), or thrombin (washed platelets) in hamsters fed a normal diet or a high-cholesterol diet. aPTT, activated partial thromboplastin time; TT, thrombin time. Data represent mean ± SEM. *$P < 0.05$; **$P < 0.01$.

sters fed a normal or a high-cholesterol diet are shown in Table 3. When platelets were induced by thrombin or collagen, the IC_{50} values in high-cholesterol hamsters decreased compared with the values for hamsters fed a normal diet. In terms of hemostatic values, no significant difference was observed between normal and high-cholesterol hamsters. However, these values in high-cholesterol hamsters were slightly shortened as compared with those from normal ones.

Histological observation. According to observations obtained through TEM, foam cells and extracellular lipids in neointima were clearly observed in hamsters fed a high-cholesterol diet (Fig. 2). When neointima formation was established 2 wk after vascular injury, the vascular surface in injured area was not smooth; however, repaired endothelial cells were completely covered with neointima. Blood elements were not observed on vascular surfaces in hamsters fed a normal diet (Fig. 3A). On the other hand, platelets were locally adhered on neointimal vascular surfaces in hamsters fed a high-cholesterol diet 2 wk after vascular injury even if endothelial cells were recovered with the vascular surface in the injured area (Figs. 3B,3C).

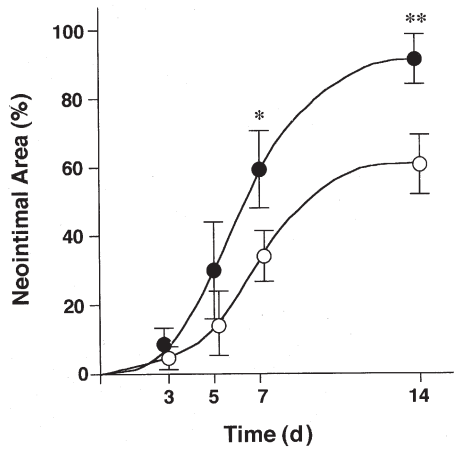


FIG. 1. The development of neointima in hamsters fed a normal or a high-cholesterol diet shown as percentage of luminal stenosis. Vascular injury occurred 4 wk after the start of regular (open circles) or high-cholesterol diet (closed circles), and neointima was measured 3, 5, 7, or 14 d after the vascular injury. *$P < 0.05$; **$P < 0.01$. The error bars represent SEM.

Proliferation of SMC in vivo. Figure 4 shows the percentage of proliferating SMC on days 1, 3, 5, 7, and 14 after vascular injury. The increased level of plasma cholesterol caused a significant increase in SMC proliferation, measured on days 1, 3, 5, and 7. These differences represented increases of 21.4, 27.3, 11.1, and 14.8% in proliferation index, respectively.

Proliferation index in vitro. The amount of DNA synthesis induced by various agonists is shown in Figure 5A. DNA synthesis is markedly increased in high-cholesterol hamsters when SMC are stimulated by FCS or PDGF. Dose-dependent alterations of DNA synthesis induced by PDGF are shown in Figure 5B. DNA synthesis in response to PDGF-BB stimulation of SMC in hamsters fed a high-cholesterol diet is significantly higher than in hamsters fed a normal diet. When SMC were stimulated by the other stimulation factors (thrombin, vasopressin, endothelin I, or angiotensin II), DNA synthesis was not changed in SMC from either group of hamsters.

[^{125}I]PDGF binding in SMC. The binding affinities in both groups of hamsters were unchanged (Fig. 6).

Effects of probucol or losartan on neointima. Figure 7 shows the inhibitory effects of probucol or losartan on neoin-

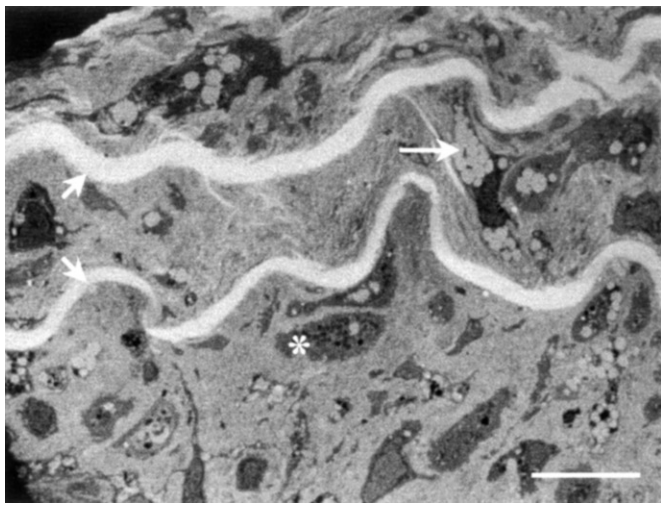
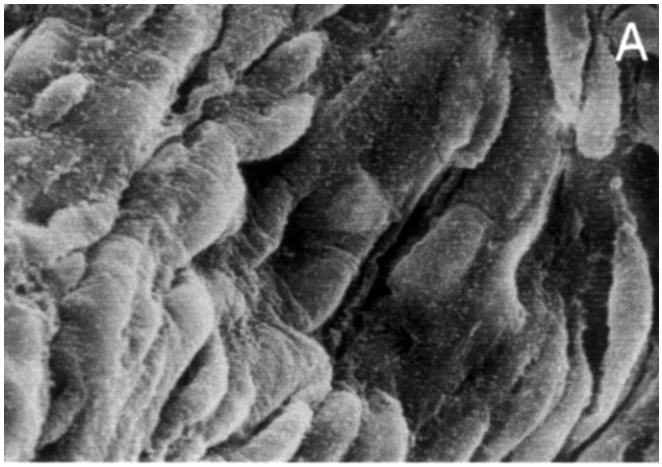
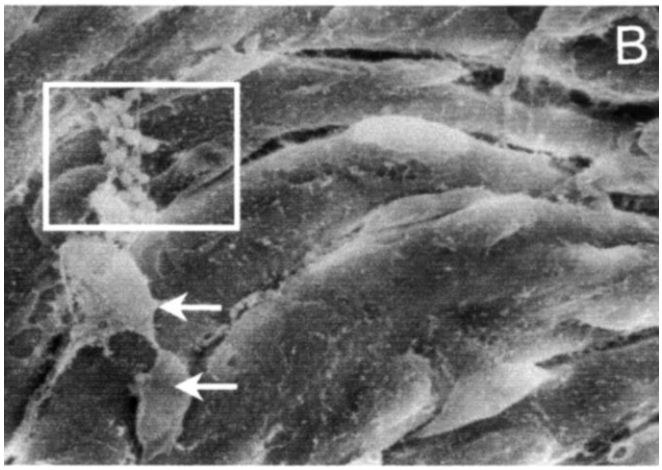


FIG. 2. A transmission electron micrograph of neointima formation 14 d after vascular injury in hamsters fed a high-cholesterol diet. Internal elastic lamina (IEL) can be clearly observed (small arrows) and lipid particles (large arrow) and foam cells (asterisk) are observed in media (during IEL) and newly formed intima. The scale bar indicates 200 μm.





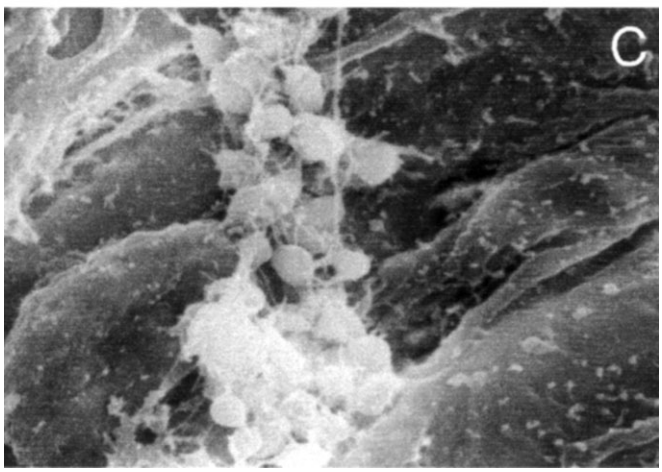


FIG. 3. Scanning electron micrographs from hamsters fed a normal or a high-cholesterol diet 14 d after the initiation of vascular injury. (A) Neointima formation on injured area. The luminal surface is entirely covered with newly formed endothelial cells, which are morphologically different from native ones and irregularly oriented. Blood elements are not observed in this area. (B) Locally activated platelets (a window) and monocytes (arrows) are adhered on irregularly oriented vascular surface. (C) A high magnification of a window of panel B. Adherent platelets consist of microthrombus formation.

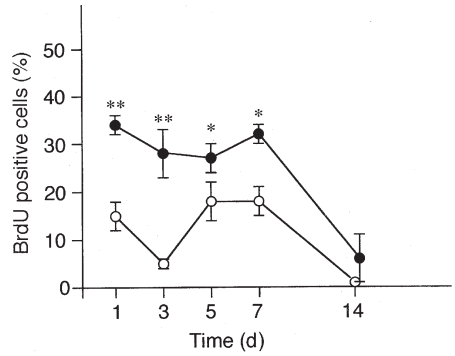
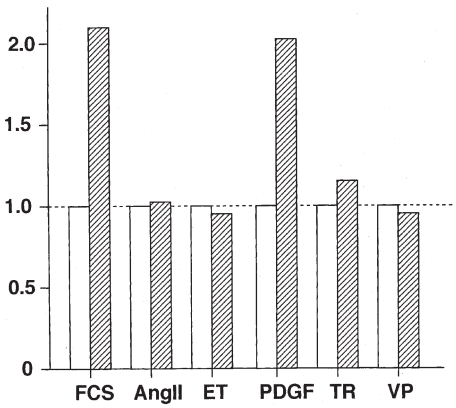


FIG. 4. Smooth muscle cell proliferation ($n = 4$, each time point) measured as the 5-bromo-2-deoxy-uridine (BrdU) index (%) following vascular injury in hamsters fed a normal (○) or a high-cholesterol diet (●). $^*P < 0.05$; $^{**}P < 0.01$ vs. control (normal hamsters). The error bars represent SEM.



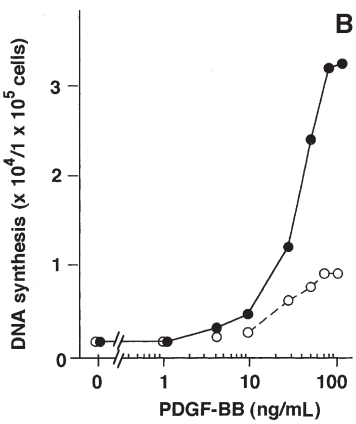


FIG. 5. The amount of DNA synthesis induced by various agonists (A) and dose-response line plot of DNA synthesis induced by platelet-derived growth factor (PDGF)-BB (B). (A) The cultured cells from either normally fed hamsters (open bars) or high-cholesterol-fed hamsters (hatched bars) were stimulated with 5% fetal calf serum (FCS), 10^{-6} M angiotensin II (AngII), 0.03 unit/mL thrombin (TR), 10^{-6} M vasopressin (VP), 10^{-6} M endothelin I (ET), or 30 ng/mL PDGF-BB (PDGF) in 1 mL of Dulbecco's modified Eagle's medium. Alteration of DNA synthesis in high-cholesterol hamsters shows a ratio vs. normal hamsters. (B) Dose-response line plot: DNA synthesis induced by PDGF-BB. Each plot presents data from hamsters with normal (open circle) or high-cholesterol diets (closed circles). Each point represents the mean of duplicate cultures.

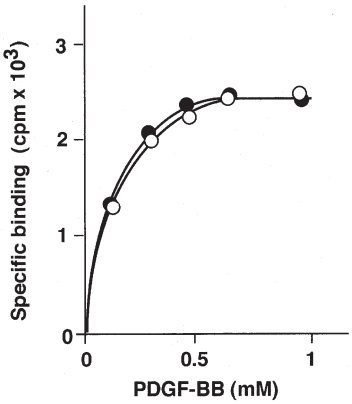


FIG. 6. Specific binding of PDGF-BB using explanted vascular smooth muscle cells from hamsters fed a normal or a high-cholesterol diet. Each plot presents data from hamsters with normal (open circle) or high-cholesterol diets (closed circles). Each point represents the mean of duplicate cultures. For abbreviation see Figure 5.

tima formation. The treatment with probucol at a dose of 120 mg/kg/d significantly reduced the neointimal area in hypercholesterolemic hamsters. However, losartan slightly decreased the neointimal area at the highest dose. Photomicrographs of typical neointima formation 2 wk after arterial injury of hamsters either treated with probucol at a dose of 120 mg/kg/d or not, are shown in Figure 8. Formation of neointima including a lot of lipids was observed in hamsters not treated with probucol. On the contrary, neointimal areas and points of lipid accumulation were clearly reduced by treatment with probucol. Platelet aggregation and coagulation factors were not different in hamsters fed a high-cholesterol diet compared with those fed a normal diet. Blood pressure was slightly decreased when the highest dose of losartan was used to treat hamsters.

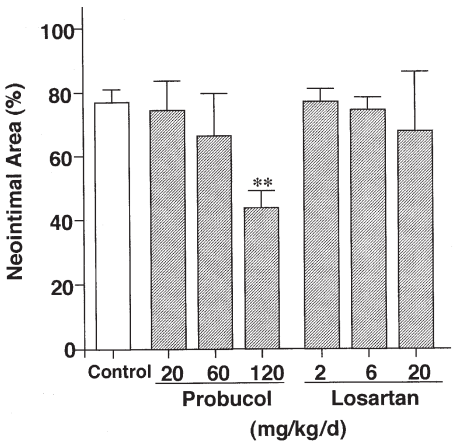


FIG. 7. This graph shows dose-dependent inhibition of neointima formation in hamsters fed a high-cholesterol diet by treatment with probucol or losartan. The compound was administered orally twice a day from 2 h before until 14 d after carotid artery injury; control (n = 12) and treated animals (n = 6) were analyzed at day 14. The ratio between neointimal area and the area within the internal elastic lamina was determined on three cross sections of carotid arteries. $^{**}P < 0.01$ vs. control. The error bars represent SEM.

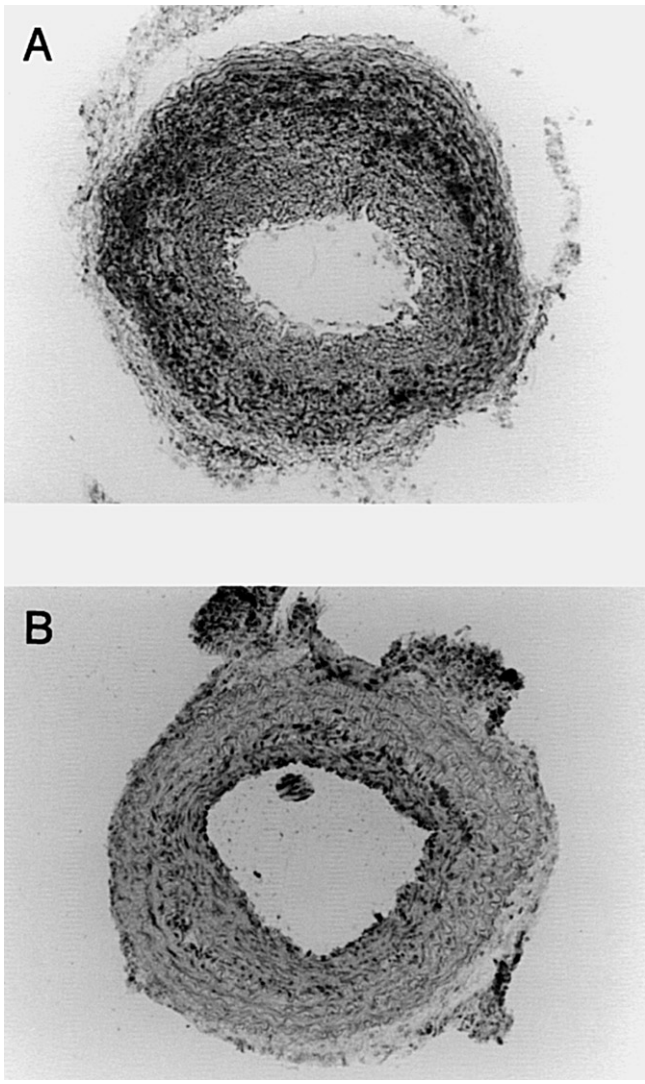


FIG. 8. Photomicrographs show neointima formation in damaged carotid arteries of hypercholesterolemic hamsters either treated with 120.0 mg/kg/d probucol (B) or not treated (A). Lipids were stained bright red. The tissue was counterstained with hematoxylin. A packed neointima was observed in both samples, however, the stenosis area in tissue from animals treated with probucol was diminished and lipids in stenosis clearly decreased.

DISCUSSION

This study demonstrates that high plasma LDL levels in hamsters fed a high-cholesterol diet are associated with marked increases in the development of thrombus and neointima formation, as compared to normally fed hamsters, in injured carotid arteries, which we have established and used to demonstrate the inhibitory effects of several kinds of compounds on vascular stenosis (12–18). Furthermore, these phenomena are mainly characterized by the enhancement of platelet activation induced by thrombin and of SMC proliferation induced by PDGF-BB.

A high arterial LDL level is an important factor in the development of vascular stenosis. Several molecular mechanisms have been suggested to account for the correlation of elevated LDL serum levels with the development of chronic vascular

diseases (22,23). In our experiments, the plasma LDL level of hamsters fed a high cholesterol diet (2.24 ± 0.32 g/L) 2 wk after the start of the diet regime was 4.6 times higher than that of hamsters fed a normal diet (0.49 ± 0.02 g/L), but the HDL plasma level was not significantly different. These levels are similar to those found in pathologic conditions, such as familial combined hyperlipidemia (24), nephrotic syndrome (25) (LDL = 1.6 to 2.0 g/L), and especially homozygous familial hypercholesterolemia (LDL = 3.0 to 5.5 g/L) (26,27).

Vascular stenosis was related mainly to thrombus development in the acute phase or to neointima formation in the chronic phase after endothelial injury. In the present experiments, the development of thrombus formation of high-cholesterol hamsters *in vivo* was clearly accelerated, because the time to occlusion and vascular patency in high-cholesterol hamsters were significantly changed as compared with those of normal hamsters. This phenomenon was mainly attributable to up-regulation of platelet activation since IC_{50} platelet aggregation values significantly decreased in high-cholesterol hamsters, but coagulation factors were not different in both hamsters. Our previous data indicated that platelets play a major role in the development of thombus formation since activated platelets adhere to injured vascular surfaces in the days immediately after injury (13,14). Moreover, LDL may induce a state of hypersensitivity in the platelets that contributes to the high rate of thrombosis formation observed in patients (7,28).

Our findings also showed that neointima formation in injured carotid arteries of hamsters fed a high cholesterol diet was significantly greater than in hamsters fed a normal diet. The number of proliferating SMC *in vivo* measured at each time point after vascular injury in hamsters fed a high-cholesterol diet was also significantly higher compared with hamsters fed a normal diet. LDL particles, trapped in the extracellular matrix of the vessel wall, are well known to undergo substantial structural and chemical modification in response to many kinds of stimuli (29). In our histological observations using TEM, lipid particles can be found in vascular neointima formation. Furthermore, to define the findings of increased neointima lesion in high-cholesterol hamsters, we studied the DNA synthesis using explanted SMC induced by various agonists. These results indicated that PDGF plays a key role in arterial stenosis by neointima after endothelial injury. When PDGF-BB were applied to SMC from hamsters fed a high-cholesterol diet, DNA synthesis increased markedly compared with synthesis in hamsters fed a normal diet. LDL is capable of increasing PDGF production and expression of PDGF receptors in human vascular SMC (30). Therefore, we performed binding experiments using ^{135}I-labeled PDGF-BB on SMC from hamsters fed a high-cholesterol diet or a normal diet. The results indicated there were no differences in binding assay in SMC between hamster populations. According to these findings, we speculated that the responsibility of post-PDGF receptors, such as intracellular signaling, could play an important role in the enhancement of neointima formation in hypercholesterolemia. Moreover, PDGF is an α granule component (31) that is released during the platelet reaction induced by collagen or ADP (32), or when platelets adhere to sites of injured blood vessels, as seen in our findings using SEM. Therefore, up-regulation of sensitivity in platelets in high-cholesterol hamsters also plays a role in the enhancement of neointima formation. Indeed, our previous findings indicated that platelets play a significant role in the development of neointima formation since antiplatelet compounds improved SMC proliferation during the acute phase of vascular injury (13,14).

To define the utility of this model in pharmacological experiments, we used losartan and probucol to treat hamsters fed a high-cholesterol diet, because probucol inhibited aortic cholesterol accumulation (33) and reduced neointima formation (34) and losartan also reduced neointima formation (35). In these experiments, probucol significantly reduced the neointimal area and attenuated lipid formation in vascular SMC. Indeed, foam cell lesions in hypercholesterolemic hamsters were decreased by treatment with probucol (33). On the contrary, losartan did not show significant effects on neointima formation even though our previous study showed that losartan significantly reduced neointima formation in normal hamsters (18). These findings indicated that probucol greatly affects the development of neointima formation promoted by hypercholesterolemia, but not the inhibition of angiotensin II by losartan.

Finally, we speculate that the physiological importance of such responses to the development of neointima formation in hypercholesterolemia may be modeled as follows: First, after endothelial injury, activated platelets adhere to and aggregate at damaged endothelial surfaces. Next, these platelets are sensitized by high levels of plasma LDL. The production of locally secreted PDGF by activated platelets increases proliferation of SMC in the region of the injury. The continuous production of PDGF may play a very significant role in the amplified development of neointima in hypercholesterolemia. This is especially relevant since the other known stimulants (angiotensin II, vasopressin, or endothelin) did not increase DNA synthesis in SMC from high-cholesterol hamsters. This model is a sensitive one for pharmacological experiments.

In conclusion, high levels of plasma LDL lead to intimal hyperplasia *via* up-regulation of platelet aggregation and enhancement of proliferating SMC. Further research is necessary to determine whether this LDL-induced hyperplasia occurs in humans with elevated LDL levels. If so, antiplatelet and antithrombin drugs could be part of a new supportive therapeutic concept in the treatment of hypercholesterolemia-associated cardiovascular diseases. This model could be a useful tool for investigation of this field since hamsters are smaller and permit a reduction in the amount of drug required.

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