anti-FKHR antibody[30], or with control IgG, and rotated overnight at 4 °C. Immune complexes were collected with salmon-sperm DNA/protein G–sepharose slurry for 4 h with rotation, washed and then incubated at 65 °C for 6 h for reverse crosslinking. Chromatin DNA was extracted with phenol-chloroform, precipitated with ethanol, resuspended in water and subjected to PCR analysis. To amplify the IRS-2 promoter regions containing SRE, the following primer sets were used: 5'-TCCAGCAAACAGATGCTGAC-3' and 5'-CATTTAACTTGACTCTCCAGTGAA CT-3'. After amplification, PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Regular paper

Oxidized but not acetylated low-density lipoprotein reduces preproinsulin mRNA expression and secretion of insulin from HIT-T15 cells

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Abstract

We examined the effect of oxidized low-density lipoprotein (oxLDL) on the insulin secretion in the culture of HIT-T15 cell line, an islet β-cell line derived from a hamster pancreatic tumor. In order to check the uptake of modified LDL by HIT-T15 cells, we prepared DiI-labeled native LDL (nLDL), acetylated LDL (AcLDL), and oxLDL. After the addition of each LDL into the cultures of HIT-T15 cells, fluorescence microscopic study was done. It was suggested that AcLDL and oxLDL were taken up by HIT-T15 cells, as well as nLDL. mRNA expression of the LDL receptor, CD36, and SR-B1 was detected in HIT-T15 by RT-PCR. The medium insulin level was measured in the culture of HIT-T15 cells with each LDL. oxLDL significantly reduced the insulin secretion stimulated by various concentrations of glucose, the intracellular content of insulin, and the expression of preproinsulin mRNA compared to the control cultures without LDL addition. In contrast, nLDL and AcLDL had no effect on the insulin secretion, the intracellular insulin level, or the expression of preproinsulin mRNA. MTT assay findings (reflecting cell numbers) were not different between cultures with and without LDLs. These results indicated that oxLDL disturbed the insulin metabolism of HIT-T15 cells.

Keywords: Oxidized LDL; Scavenger receptor; Pancreatic beta cell; HIT-T15 cell; Lipid peroxidation; Oxidative stress

1. Introduction

Low-density lipoprotein (LDL) is considered to play a major role in atherogenesis [1–4]. Oxidative modification of LDL is the first step in foam cell formation. Oxidized LDL (oxLDL) is produced in the subendothelial space [5,6] and taken up by resident macrophages via scavenger receptors [7,8], leading to their transformation into foam cells. oxLDL can induce the expression of adhesion molecules, and the synthesis of cytokines, oxygen radical species, and certain growth factors in endothelial cells, macrophages, and smooth muscle cells [9–12]. To date, the physiological effects of oxLDL have been discussed primarily with respect to vascular wall cells. oxLDL is detected in the blood stream [13,14], and it thus seems likely that oxLDL might induce dysfunction of other cell types, in addition to arterial wall cells.

The pathogenesis of diabetes mellitus seems to involve two main mechanisms, (1) insulin resistance and (2) β-cell dysfunction. The progression of β-cell dysfunction induces insulin deficiency. Various studies have attempted to clarify the genetic and environmental factors involved in β-cell dysfunction; however, to date, these factors remain obscure. Oxidative stress has been shown to induce β-cell dysfunction

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; KRBB, Krebs–Ringer bicarbonate buffer; LDL, low-density lipoprotein; nLDL, native low-density lipoproteins; oxLDL, oxidized low-density lipoprotein; AcLDL, acetylated LDL; ROS, reactive oxygen species

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under hyperglycemic conditions [15–18]. Previous studies suggested that levels of antioxidants and the enzymatic activity of β-cells were much less than in other tissues [19,20], suggesting that pancreatic β-cells may be susceptible to oxidative stress.

In the present study, we hypothesized that lipid peroxides, as circulating oxidants, might cause pancreatic β-cell dysfunction. Accordingly, we challenged the insulin secretory cell line, HIT-T15, which is a pancreatic tumor cell line derived from hamster, in an attempt to evaluate the effect of oxLDL on insulin secretion, in comparison with the effects of native LDL (nLDL) and acetylated LDL (AcLDL).

2. Materials and methods

2.1. Materials

The β-cell-derived cell line HIT-T15 (no. 1777; American Type Culture Collection) was obtained from Dainippon Pharmaceutical Co. (Tokyo, Japan). The cell-counting assay kit was purchased from Wako Chemicals (Osaka, Japan). The insulin assay kit (Revis insulin kit rat-T) was from Shibayagi (Tokyo). Tissue culture media, antibiotics, and the First-strand Amplification System for the RT-PCR Kit were from Invitrogen (San Diego, CA). Culture dishes (12-well multiplates) were from Costar (Cambridge, MA), and bovine serum albumin (fraction V) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were from Sigma Chemical Co. (St. Louis, MO). ISOGEN and RNase- and DNase-free water were purchased from Nippon Gene Co. (Tokyo). TaqMan Universal PCR Mix and 96-well optical plate for real-time PCR were from Applied Biosystems Inc. (Foster City, CA). The DC protein assay kit was from Bio-Rad (Richmond, CA). 2-^{3}H-deoxyglucose (2-DG) was from Amersham Pharmacia Biotech (Buckinghamshire, UK), and Aquasol-2 solution was from Packard (USA). Rabbit polyclonal anti-CD36 antibody, goat polyclonal anti-scavenger receptor class B type 1 (SR-B1) antibody, and goat polyclonal anti-LDL receptor antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-insulin antibody was from Biogenesis (USA). Fluorescein-conjugated anti-rabbit IgG antibody, fluorescein-conjugated anti-goat IgG antibody, and Texas Red conjugated anti-mouse IgG antibody were from Vector Laboratories (Burlingame, CA).

2.2. Preparation and modification of low-density lipoprotein (LDL)

Human LDL (d=1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from human plasma of healthy volunteers after overnight fasting as described previously [21]. To prepare oxLDL, LDL was dialyzed against phosphate-buffered saline (PBS) to remove ethylenediaminetetraacetic acid (EDTA). Diluted LDL (1 mg/ml) with PBS was then incubated for 10 h at 37 °C with 5 μM $CuSO_4$ [22]. Acetylated LDL (AcLDL) was prepared by chemical modification of LDL with acetic anhydride as described previously [23]. Modification of LDL was analyzed by agarose gel electrophoresis, and fast mobility of bands were identified (data not shown). The oxidization of LDL was confirmed by measurement of thiobarbituric acid-reactive substances (TBARS). The mean values of the TBARS levels of each LDL used in the present study were 11.4±4.6, 1.8±1.2, and 160.6±37.4 (nmol/ml) in nLDL, AcLDL, and oxLDL, respectively.

2.3. Uptake of LDLs by HIT-T15 cells

DiI (1,1′-dioctadecyl-3,3′-tetramethylindocarbocyanine perchlorate; 0.5 μg/ml) solution (1 ml) was added to 100 mg of each lipoprotein and incubated at 37 °C for 12 h as described previously [24]. HIT-T15 cells were grown in Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in 5% CO_2 under 95% humidity. The cells were trypsinized and seeded in 12-well plates (2×10^5 cells/well) with Ham's F12 medium supplemented with 10% FBS at 37 °C for 24 h. After incubation, the cells were washed twice with Ham's F12 medium supplemented with 0.1% bovine serum albumin (BSA). DiI-labeled LDL (50 μg) was added to the cells. After 6 h of incubation at 37 °C, the cells were washed twice with PBS and fluorescence microscopy was performed at 530 nm.

2.4. Expression of lipoprotein receptors in β-cells

In order to check the expression of lipoprotein receptors, reverse transcription (RT)-PCR was performed on HIT-T15 cells and pancreatic islets that isolated from a 10-week-old Wistar rat by collagenase digestion, as described previously [25]. Total RNA was extracted by ISOGEN (Nippon Gene, Japan) according to the instructions provided by the manufacturer. First-strand cDNA was synthesized with oligo dT primer using the protocol provided by the manufacturer. PCR was performed by routine methods using the following primers: LDL receptor forward primer 5′-TAG ATA TTC CCA GTG GCC GC-3′, LDL receptor backward primer 5′-CCT CAT AGA TGG CCA AGG AGA-3′, CD36 forward primer 5′-TCA CTG TCT GTT GGA ACA GAG-3′, CD36 backward primer 5′-CTG TGC CAT TAA TCA TGT CGC A-3′, SR-B1 forward primer 5′-CAC TAC GCG CAG TAT GTG CT-3′, and SR-B1 backward primer 5′-TGG CAC TGG CGG GTT GTC-3′. PCR products were purified with RECOCHIP (TaKaRa, Kyoto, Japan) and then sequenced using the Big Dye Terminator Cycle Sequencing System (Applied Biosystems).

2.5. Measurement of insulin

HIT-T15 cells were seeded in 12-well plates as described above. After incubation at 37 °C for 24 h, the cells were washed twice with Ham's F12 medium supplemented with 0.1% BSA. In the duplicate culture with LDLs, 25 or 50 μg of each LDL was added to 1 ml of Ham's F12 medium supplemented with 0.1% BSA. These experiments were independently repeated 6 times (Fig. 3). After 24 and 48 h incubation, the cells were washed twice with Krebs–Ringer bicarbonate buffer (KRBB; 118 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2$–$2H_2O$, 1.19 mM $MgSO_4$–$7H_2O$, 25 mM $NaHCO_3$, 10 mM HEPES, and 0.1% BSA, pH 7.4). Then, 1 ml of KRBB containing various concentrations of glucose (0, 5.5, 16.6, or 27.7 mM) was added and incubated at 37 °C for 2 h. The medium was collected and prepared for the measurement of insulin by enzyme-linked immunosorbent assay (ELISA, Revis insulin kit Rat-T). These experiments in the culture with each LDL (50 μg/ml for 48 h) were duplicately performed 12 times, all in addition of each lipoprotein (Fig. 4A).

Intracellular insulin was extracted using the acid–ethanol method from the cells seeded only for this assay. Briefly, after the removal of medium and lipoprotein, cells were washed twice with PBS and extraction buffer was added (70% ethanol, 50 mM HCl). The cells were obtained by scraping and then were homogenized. The homogenized samples were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was transferred to a fresh tube. Protein concentrations were determined as described above. The experiments were independently repeated in the duplicate culture with no LDLs addition, nLDL, AcLDL, and oxLDL for 36, 24, 24, and 24 times, respectively.

2.6. Quantitative analysis of gene expression

Quantitative analysis of gene expression was performed by real-time PCR. HIT-T15 cells were seeded and treated with LDLs in 12-well plates as described above for the insulin secretion assays. After 48 h of incubation with 48 μg/ml of each LDLs, total RNA was extracted and first strand cDNA was synthesized as previously described.

Real-time PCR was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). All data (four independent experiments) were corrected for β-actin and normalized to the value of the non-treated group. Primers and probes were as follows: hamster preproinsulin forward primer, 5′ -CGT GGC TTC TTC TAC ACA CCC-3′ ; hamster preproinsulin backward primer, 5′ -AGC TCC AGT TGT GCC ACT TGT-3′ ; hamster neuroendocrine L-type calcium channel forward primer, 5′ -TCC ATC ACC TTT TTC CGT CTT T-3′ ; hamster neuroendocrine L-type calcium channel backward primer, 5′ -CGT GGC TTC TTC TAC ACA CCC-3′ ; rat β-actin forward primer, 5′ -TCC TGG CCT CAC TGT CCAC-3′ ; rat β-actin backward primer, 5′ -GGG CCG GAC TCA TCG TAC T-3′ ; hamster preproinsulin probe, 5′ -TCC CGT CGT GGA GTG GAG GAC C-3′ ; hamster neuroendocrine L-type calcium channel probe, 5′ -CCG AGT GAT GCG GTT GGT GA-3′ ; rat β-actin probe, 5′ -TTC CAG CAG ATG TGG ATC AGC AAG CA-3′ . PC products were 68 bp for hamster preproinsulin and 67 bp for rat β-actin. The preproinsulin expression assay of each lipoprotein was performed 8 times.

2.7. Determination of cell viability

The cytotoxic effect of oxLDL was determined using a Cell-counting Assay Kit (Wako, Osaka, Japan), which is a modified method of the MTT assay. After incubation, in addition of 50 μg/ml of each LDLs for 48 h, the cells were incubated with 500 ml of Ham's F12 medium containing WST-1 reagents at 37 °C for 3 h. After incubation, the absorbance of each medium was measured at 450 nm. MTT assay was performed 24 times, all in addition of each lipoprotein.

2.8. 2-^{3}H-deoxyglucose (2-DG) uptake

HIT-T15 cells were seeded and treated with LDLs in 12-well plates as described above for the insulin secretion assays. After incubation at 37 °C for 48 h, cells were washed twice with KRBB in the absence of glucose, and 1 ml of KRBB containing 2-^{3}H-DG (final concentration, 8 μmol/l) was added. After incubation at 37 °C for 20 min, cells were washed twice with KRBB and solubilized with 500 ml of 0.1 N NaOH. After the cells were homogenized, 250 μl of cell extracts was added to 4 μl of Aquasol-2, and the radioactivity was measured by scintillation counting. The experiments of 2-DG uptake were independently repeated 4 times.

2.9. Statistical analysis

Results were expressed as mean±S.E. Statistical analysis was performed using the un-paired Student's *t*-test. When comparing multiple groups, two-way ANOVA was used. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Fluorescence microscopic study

The photographs of direct light (left panel) and fluorescence microscopy (right panel) at 530 nm are shown in Fig. 1. No LDLs were added in Fig. 1A. Fig. 1B shows the uptake of nLDL, which is agreement with the results reported previously [26,27], and Fig. 1C and D show the uptake of AcLDL and oxLDL, respectively. The results suggested that LDLs were taken up by HIT-T15 cells.

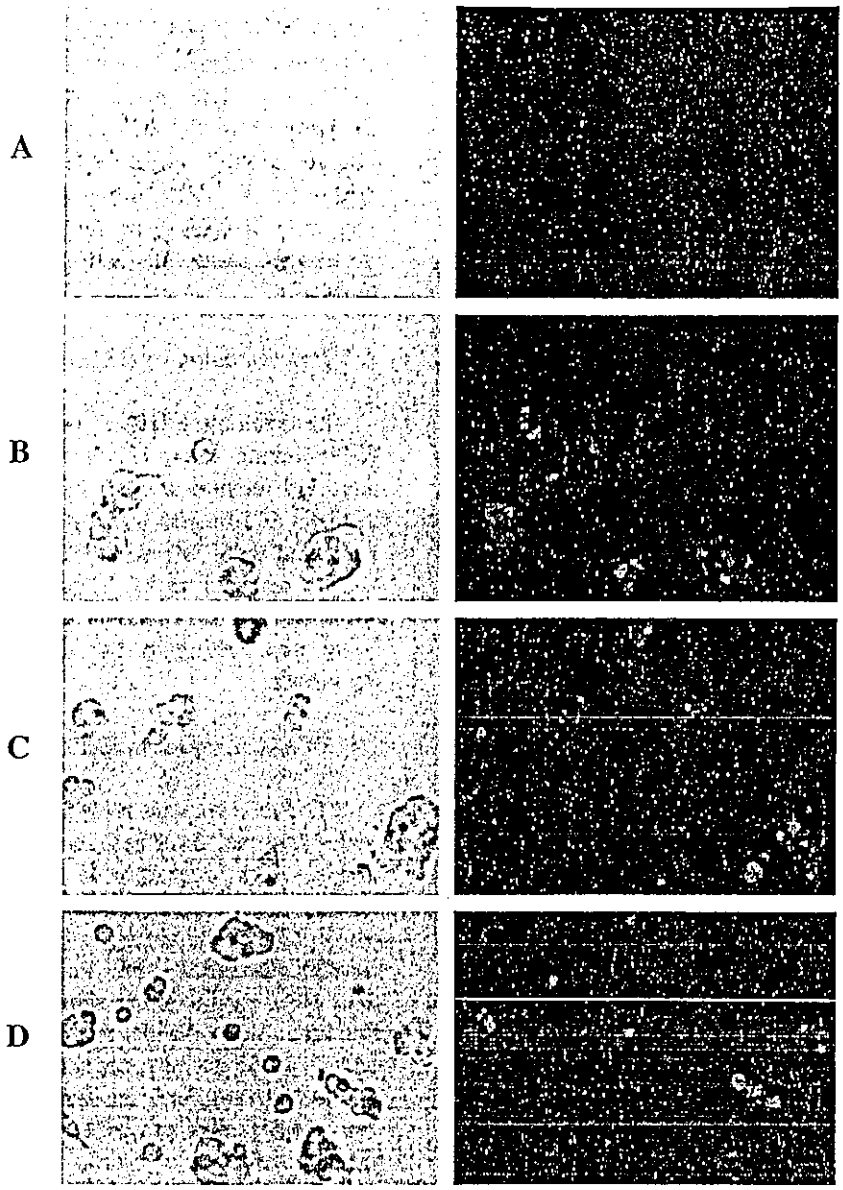

Fig. 1. Photomicrographs of HIT-T15 cells cultured without LDLs (A), with native LDL (B), AcLDL (C), and oxLDL (D). Each LDL (50 μg protein/ml) was added and incubated at 37 °C for 6 h. The preparations are shown in direct light (left) and in fluorescence (530 nm, right).

3.2. Expression of scavenger receptors in insulin-producing cells

RT-PCR for receptor genes, LDL receptor, SR-B1, and CD36 was performed using mRNA from HIT-T15 cells and isolated rat islets (Fig. 2). All of lipoprotein receptor mRNA expression were detected in both HIT-T15 cells and rat islets. PCR was also performed using a without-RT sample and only an-RT without mRNA sample as negative control. The fragment for each receptor was not expressed in the negative control (data not shown).

3.3. Effects of each LDL on HIT-T15 function

As shown in Fig. 3, insulin secretion by static incubation for 1 h with 5.5 mM glucose after exposure of oxLDL significantly decreased in a time- and dose-dependent manner compared with control culture (no LDL addition). The same tendency was observed in insulin secretion stimulated by 16.6 mM and 27.7 mM glucose. After 48 h of incubation with each LDL (50 μg/ml), insulin secretion, intracellular content, and MTT assays were performed, and the results are shown in Fig. 4. The addition of nLDL had no effect on insulin secretion and intracellular insulin content compared with the control culture (Fig. 4A,B). The expression of preproinsulin mRNA was quantified by real-time PCR, but no significant change was observed (Fig. 4C). Cell viability, measured by MTT assay, did not differ among the control and nLDL addition cultures (Fig. 4D). AcLDL also had no effect on insulin secretion, cellular insulin content, expression of preproinsulin mRNA, and cell viability, similar to nLDL. On the other hand, insulin

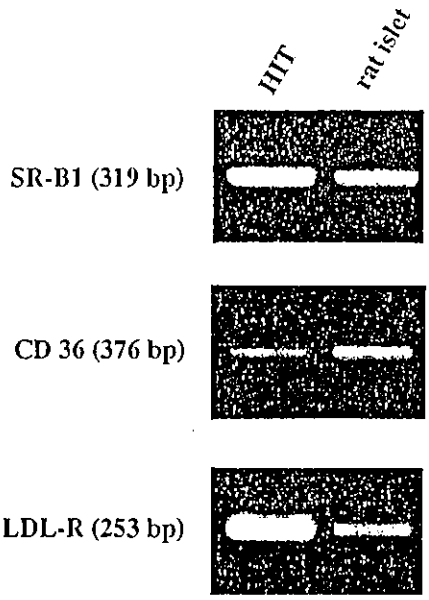

Fig. 2. Lipoprotein receptor gene expression in HIT-T15 cells and rat pancreatic islets. RT-PCR of SR-B1, CD36, and LDL receptor was performed using total RNA extracted from HIT-T15 cells and pancreatic islets of rat.

secretion by oxLDL-treated HIT-T15 cells was decreased to 54±6% of the control in 5.5 mM glucose, 51±6% in 16.6 mM glucose, and 52±12% in 27.7 mM glucose but not decreased in 0 mM glucose (P<0.05 vs. control, each, Fig. 4A). Intracellular insulin content also decreased (64± 4% of control, P<0.05) (Fig. 4B). Moreover, preproinsulin and neuroendocrine L-type calcium channel gene expression was decreased (77±4% and 83±7% of control, respectively, P<0.05) (Fig. 4C). However, no effect was found in cell viability (Fig. 4D).

Glucose uptake by HIT-T15 cells was examined using 2-^{3}H-DG in the cultures with each LDL. The glucose uptake ability of HIT-T15 cells was not altered by the addition of oxLDL, AcLDL, or nLDL compared with the control culture (data not shown).

4. Discussion

The present study revealed that oxLDL reduced the expression of the insulin gene with a consequent decrease in the intracellular insulin content and the insulin secretion of HIT-T15 cells.

The HIT-T15 cell line is a clonal cell line of Syrian hamster β-cells and a suitable model for insulin secretion studies [28,29]. The cells express glucose transporter genes, particularly GLUT2, which is responsible for glucose sensing, and they retain insulin secretory responsiveness to glucose. Insulin secretion by 5.5 mM glucose in HIT-T15 was significantly increased compared to basal insulin secretion without glucose [28–31]. In the present study, insulin responsiveness to glucose was also observed.

The uptake of DiI-nLDL, -oxLDL, and -AcLDL was observed, and mRNA expressions of LDL receptor, SR-B1, and CD36 were detected in HIT-T15 cells. These results suggest that HIT-T15 cell functions might be affected by not only nLDL, but also by modified LDLs (oxLDL and AcLDL), taken up via each receptor. The present study demonstrated the presence of LDL receptor in HIT-T15 and pancreatic islet cells of rat. As in previous reports [26,27,32], the expression of LDL receptor has previously been demonstrated in the pancreatic beta cell of the rat, human, and mice. Using immunostaining, Roehrich et al. [32] demonstrated the presence of SR-B1 in mice islets as a receptor of high-density lipoprotein. Our study confirmed the mRNA expression of scavenger receptors, not only SR-B1 but also CD36, in HIT-T15 cell and rat islets by RT-PCR method. PCR products were also confirmed to correspond to lipoprotein receptors by direct sequencing (data not shown). We performed the immunostaining of scavenger receptors for rat pancreatic tissue, and it was also clarified that SR-B1 and CD36 was co-expressed in insulin positive cells in rat pancreatic islets (data not shown). SR-B1 and CD36 are known as scavenger receptors [33] and have been shown to be a binding site of modified lipoproteins such as oxLDL and AcLDL. These data suggested that there occurred an interaction between each LDL and β-cells. Previous studies [32,34] indicated that LDL induced β-cell toxicity to cause apoptosis, but there has been no study that investigated insulin secretory function or preproinsulin gene expression. In our study, nLDL had no effect on insulin secretion, the mRNA expression of preproinsulin, or the cell viability, as

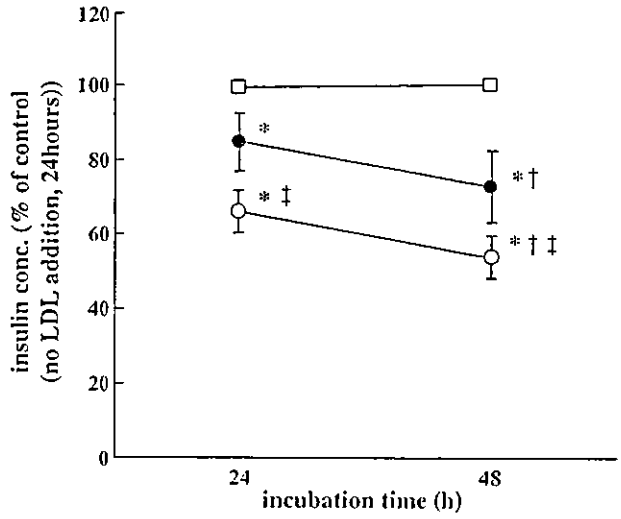

Fig. 3. Effects of oxLDL on insulin secretion in HIT cells. HIT cells (2×10^5 cells/well/ml) were treated with oxLDL (25 (•) or 50 μg/ml (○), n=6) or without oxLDL (□) at 37 °C for 24 h and 48 h. NaHEPES buffer was used as the control. After treatment with oxLDL, the cells were incubated with KRBB containing 5.5 mM glucose at 37 °C for 2 h. Data are mean±S.E. *P<0.05 vs. control of each incubation time. †P<0.05 vs. experiment with each dose of concentration of oxLDL for 24 h. ‡P<0.05 vs. experiment with 25 μg/ml oxLDL for each incubation time.

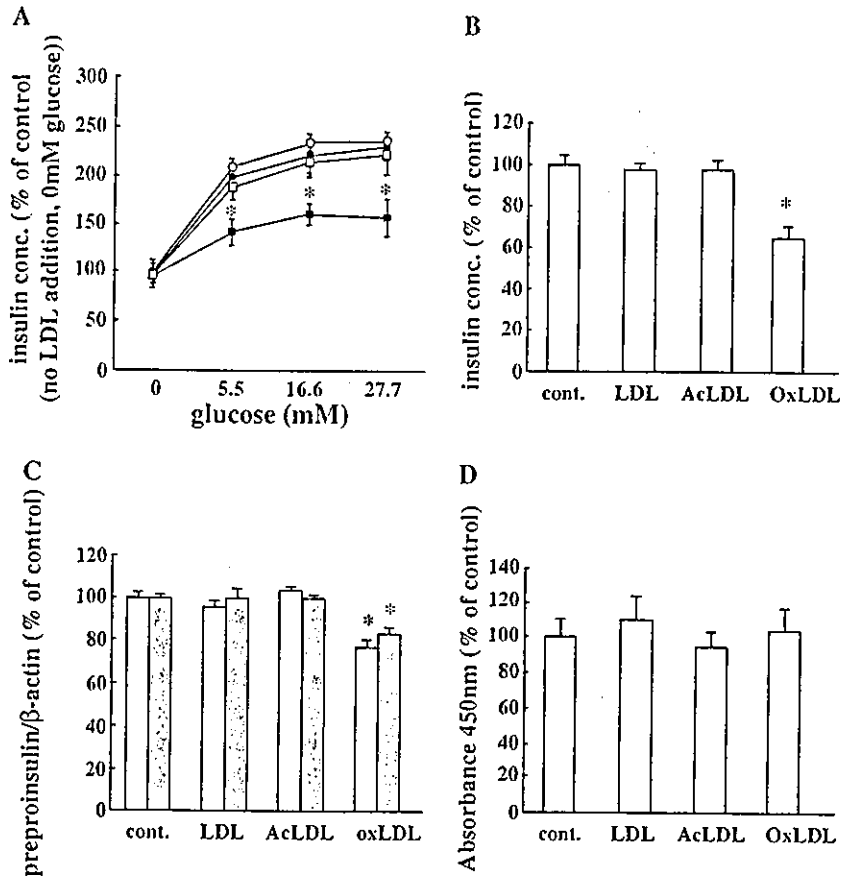

Fig. 4. Effects of each lipoprotein on insulin metabolism of HIT-T15 cells. After incubation, in addition of 50 μg/ml of each LDLs for 48 h, each experiment was performed. (A) Glucose-stimulated insulin secretion. Insulin in the culture medium was studied in cultures containing various concentrations of glucose, 0 mM, 5.5 mM, 16.6 mM, and 27.7 mM at 37 °C for 2 h. Data are mean±S.E. (n=12 for control (no LDL addition; ○), nLDL (•), AcLDL (□), and oxLDL (■)). *P<0.05 vs. the experiment with each dose of concentration of glucose in culture without LDLs. (B) Intracellular insulin level. Intracellular insulin was extracted by the acid–ethanol method and measured by ELISA. Data are mean±S.E. (n=36 for control (no LDL addition), n=24 for nLDL, AcLDL, and oxLDL) *P<0.05 vs. control. (C) Expression of preproinsulin and neuroendocrine L-type calcium channel mRNA. mRNA was quantified by real-time PCR method in the culture with each LDL. Data are mean±S.E. (n=8) *P<0.05 vs. control. (D) Cell viability in the culture with each LDL. Cell viability was assayed by the modified MTT method (cell-counting assay kit). Data are mean±S.E. (n=24). No significant differences were found.

shown by MTT assay. M.E. Roehrich et al. [32] showed that 6.2 mM LDL caused apoptosis to isolated pancreatic islet of mice, but lower than 3.2 mM of LDL (LDL-cholesterol concentration, about 10, holds higher compared with present study, 0.25 to 0.3 mM in the present study) did not cause apoptosis. On the other hand, M. Cnop et al. [34] demonstrated that apoptosis was caused on pancreatic β-cell of rat by 25 μg/ml LDL after incubation for 48 h, but not by 25 μg/ml oxLDL. The discrepant results among the studies might reflect differences in the grade of LDL modification and/or β-cell origin.

In this study, we demonstrated that the insulin secretion, the intracellular insulin content, and the preproinsulin mRNA were decreased in the culture with oxLDL, but not in the cultures with AcLDL (Fig. 4A, B–C). And insulin secretion stimulated by glucose was disturbed in the culture with oxLDL, but insulin secretion in the medium containing no glucose was not disturbed (Fig. 4A). These data suggested that oxLDL interfered with glucose stimulated insulin secretion. The present study also demonstrated the decrease of neuroendocrine L-type calcium channel gene expression. Therefore, oxidative stress on HIT-T15 cell will decrease the gene expressions which were related to insulin synthesis and secretion. These data suggested that the gene expression of HIT-T15 would be generally disturbed.

It is known that AcLDL is taken up via macrophage scavenger receptors, similar to oxLDL, to induce foam cell formation [4,7,35]. HIT-T15 cells took up both DiI-AcLDL and DiI-oxLDL, but AcLDL had no effect on gene expression, intracellular content, and secretion of insulin. We confirmed negatively charged oxLDL by agarose gel electrophoresis and the TBARS level of oxLDL. The TBARS value of oxLDL was strikingly elevated compared to other LDLs. These results suggested that the oxidization of LDL had an important role in disturbing β-cell function. When the excessively oxidized LDL (TBARS level 280 to 368.8 nmol/ml) was introduced in the culture, it induced cell death. Therefore, the excessively oxidized LDL was toxic and not suitable to assess the effect on β-cell function.

The previous studies indicated that β-cell dysfunction was induced in hyperglycemic states. Such a phenomenon has been called "glucose toxicity". Previous studies showed

that high glucose concentrations induced intracellular reactive oxygen species (ROS) production [15–18] and that ROS decreased the content of transcriptional factors, such as PDX-1, in the nuclei of β-cells [36–38]. Such conditions decreased insulin synthesis and prolonged exposure to high glucose levels induced apoptosis of β-cells. In vitro, glucose toxicity was rescued by the addition of antioxidant agents and overexpression of antioxidative enzymes [39–41]. We also investigated the effect of oxLDL to HIT-T15 under the co-incubation of antioxidants (*N*-acetylcycteine and glutathion). However, both antioxidants could not rescue from the reduction of insulin synthesis and secretion by oxLDL (data not shown). Several in vivo investigations also reported that the progression of β-cell dysfunction in diabetic rats, such as the Goto-Kakizaki rat and Zucker diabetic fatty rat, was protected by antioxidants [39,41–43]. Consistent with previous reports, our study using oxLDL suggested that oxidative stress was important in β-cell function. The mechanism of dysfunction induced by oxLDL remains obscure. Mitochondrial function was apparently not disturbed, as the MTT assay findings did not indicate cell toxicity. Similarly, the dysfunction was apparently not related to glucose uptake, as there was no change of 2-^{3}H-DG uptake in the culture with each LDL.

We conclude that oxidized stress through oxLDL caused β-cell dysfunction due to disturbed gene expression with a consequent decrease of insulin secretion; however, our study was limited to HIT-T15 cells. Further investigations are required to clarify the mechanism of the effect of oxLDL on β-cell dysfunction.

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A new HMG-CoA reductase inhibitor, pitavastatin remarkably retards the progression of high cholesterol induced atherosclerosis in rabbits

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Abstract

Background: The remarkable anti-atherosclerotic effects of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor have not been demonstrated in diet induced severe hyperlipidemia in rabbit model. *Objective:* We have investigated the effect of pitavastatin, a newly developed statin, on atherosclerosis in rabbits. *Methods and results:* Oophorectomized female NZW rabbits were fed 0.3% cholesterol chow for 12 weeks with or without pitavastatin (0.1 mg/kg per day) (Gp.NK and HCD). The level of serum cholesterol was decreased in Gp.NK compared with Gp.HCD (772.8 ± 70.2 versus 1056.9 ± 108.3 mg/dl), whereas no significant alterations were observed in triglyceride and HDL-cholesterol. NO dependent response stimulated by acetylcholine and calcium ionophore A23187 and tone related basal NO response induced by N^G-monomethyl-L-arginine acetate were all improved by pitavastatin treatment. Pitavastatin treatment increased the level of cyclic GMP in the aorta of cholesterol fed rabbits. In the aorta, the expression of eNOS mRNA was significantly up regulated and O_2^- production was slightly reduced in Gp.NK animals. Atherosclerotic area was significantly decreased in aortic arch and thoracic aorta from Gp.NK compared with those from Gp.HCD (15.1 ± 5.3 versus 41.9 ± 10.2%, 3.1 ± 1.1 versus 7.9 ± 1.2% in Gp.NK and Gp.HCD aortic arch and thoracic aorta). Anti-macrophage staining area, the MMP1 or 2 and the nitrotyrosine positive area were decreased in Gp.NK. *Conclusion:* Pitavastatin retards the progression of atherosclerosis formation and it improves NO bioavailability by eNOS up-regulation and decrease of O_2^-.

Keywords: Nitric oxide; Endothelial nitric oxide synthase; Superoxide anion; HMG-CoA reductase inhibitor; Atherosclerosis

1. Introduction

3-Hydroxymethyl-3-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis in the liver by blocking the conversion of HMG-CoA to mevalonate [1]. They were widely used for the treatment of hyperlipidemia and used for the prevention of coronary artery disease. Landmark clinical trials with pravastatin (WOSCOPS) and simvastatin (4S) demonstrate that these statins decrease the serum cholesterol level and reduce the incidence of myocardial infarction and also cardiovascular mortality [2,3]. Additionally, several large statin trials such as AFCAPS/TEXCAPS and LIPID showed the beneficial effect of other statins [4,5].

Pitavastatin is a newly developed statin whose cholesterol reducing effect is stronger than the other new statins such as atorvastatin or lovastatin and its side effects such as liver dysfunction, were reported less when compared to the other statins [6]. However, the anti-atherosclerotic effect of pitavastatin on high cholesterol diet induced atherosclerosis was unknown in the rabbit model. High cholesterol diet itself inhibits HMG-CoA reductase activity of cells throughout the body, especially in the liver. In addition, application of statin inhibits HMG-CoA reductase absolutely, and the complete suppression of HMG-CoA reductase inhibitor may result in it being impossible of produce cell membrane composed of cholesterol, a life-threatening condition. It is thus very important to evaluate the anti-atherosclerotic effect of

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; e-NOS, endothelial nitric oxide synthase; $ONOO^-$, peroxynitrite

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strong statin administration using high cholesterol diet induced atherosclerosis animal models.

HMG-CoA reductase inhibitors were shown to improve the endothelial function in a short time period [7]. Superoxide anion (O_2^-) production was increased in vessels of hyperlipidemic rabbits, and the release of peroxynitrite; $ONOO^-$ (formed from the reaction of NO and O_2^-) release was also increased in atherosclerosis [8]. These studies demonstrated that atherosclerosis was closely related to the level of NO production and reactive oxygen species (ROS). Hence, the present study was decided to determine whether the anti-atherosclerotic effects of pitavastatin is observed, and whether it is mediated by its lipid lowering effect and/or nitric oxide or superoxide mediated system in high cholesterol diet induced atherosclerosis in oophorectomized female rabbits. We used rabbits because they are herbivorous and easy to make atherosclerotic and it is also easy to damage liver function by high cholesterol diet or statins. Further, as sex steroids are known to affect on atherosclerosis formation via NO and antioxidant action, we used oophorectomized female rabbits in this study [9].

2. Materials and methods

2.1. Chemicals and solutions

Acetylcholine chloride (ACh), prostaglandin F2α (PGF2α), indomethacin and L-monomethyl-arginine (L-NMMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Nitroglycerin (NTG) was from Nihon Kayaku Co. (Tokyo, Japan). Krebs'–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.002 mM EDTA; disodium ethylendiamine-tetraacetic acid, pH7.4) was saturated with 95% O_2/5% CO_2. All concentrations are those in the final bath.

2.2. Animals

A total of 28 female New Zealand white rabbits, 3–4 months aged, weighing about 2.0 to 2.4 kg were obtained from Kitayama Rabbis (Ina, Japan). The rabbits were housed individually at 20 ± 3 °C with free access to water. Twenty rabbits were bilaterally oophorectomized and 8 were left non-oophorectomized. Four weeks after oophorectomy, the rabbits were divided into two groups (n—each = 10) and treated for 12 weeks. Gp.HCD was fed HCD (regular diet plus 0.3% cholesterol; Gp.NK was fed HCD with pitavastatin (0.1 mg/kg per day). Separately, 10 oophorectomized female rabbits were fed with regular diet with or without pitavastatin (0.1 mg/kg per day)(Gp.R, Gp.R-NK). Feeding was restricted to 120 g per day. Blood was withdrawn 24 h after the last feeding. All the rabbits were appeared to be healthy throughout the course of study. All the experiments were conducted in accordance with institutional guidelines for animal research.

2.3. Determination of plasma lipids

Plasma lipids levels were measured by enzymatic assays as described previously [10].

2.4. Isometric tension measurements

After twelve weeks of treatment, the rabbits were sacrificed by exanguination after being anesthetized with pentobarbital (50 mg/kg i.v.). The thoracic aorta was carefully taken from the portion of the orifice of left first costal artery down to the portion enclosed by the diaphragm, and cut into 2-mm wide transverse rings. Isometric tension measurement was performed as described before [11]. The rings were stretched to their optimal force, which was predetermined as the contractile response to 122 mM KCl, mounted in organ chambers and bathed in Krebs' Henseleit solution at 37 °C. Prostaglandin F2α induced sub-maximal force (2.6×10^{-6} M). Endothelium-dependent relaxation induced by ACh and endothelium-independent relaxation by NTG were determined. To investigate tone-related basal NO release assessed by responses to L-NMMA from aortic rings, moderate vascular tone (35–50% of the contraction obtained with 122 mM KCl) was induced by low prostaglandin F2α concentrations (0.8×10^{-6} M). In some experiments, indomethacin (5×10^{-6} M) was added for 60 min before the experiment to rule out contribution of prostanoids.

2.5. Histological evaluation of atherosclerosis and assays for tissue cholesterol content

Cross-sections of the aorta adjacent to segments of vascular responses were examined [12]. Briefly, the contours of the lumen and the internal elastic lamina (IEL) were traced. The mean surface involvement by atherosclerotic lesion per vessel (extent) was calculated after dividing the lesion circumference by the circumference of the internal elastic lamina. The circumferences of the lesion and the healthy region were defined as the circumferences of the respective parts of the internal elastic lamina. The area occupied by atherosclerotic lesions (total lesion burden: size/thickness) was defined as the % area bounded by the lumen and internal elastic lamina for luminal area (n = 6 for one vessel). The intima/media ratio was also measured. A 0.8 cm-long segment was homogenized and lipids were extracted and resuspended, then cholesterol levels were measured [13].

2.6. Immunocytochemical analyses

Cross-sections of the thoracic aorta were analyzed as described previously [14]. They were incubated with primary monoclonal antibody [for anti-macrophages (RAM11), smooth muscle cells (HHF35), MMP-1 and -2, nitrotyrosine or iNOS] for 60 min at room temperature. Negative controls

included substitution of primary antiserum for either PBS or irrelevant antibodies. Each field was scored for number of each antibody positive cells on slides and analyzed statistically as described by previous report [14]. Five samples were prepared from each rabbit.

2.7. Determination of cyclic GMP (cGMP)

The aortic cGMP concentration was determined by a specific radioimmunoassay (RPN226, Amersham, Buckinghamshire, England) [15]. Four aortic rings (each wet weight is 10 ± 1 mg) per rabbit were investigated.

2.8. Measurement of endothelial NO synthase (eNOS) mRNA

The expression of eNOS mRNA in the arterial wall was measured by RT-PCR methods [16]. Briefly, to make a DNA competitor, we designed and synthesized two primers [5′-ATTTAGGTGAC-ACTATAGAATACCAGTGTCCAA-CATGCTGCTGGAAATTGGTACGGTCATCATC-TGAC-AC-3′ (sense primer), 5′-TAAAGGTCTTCTTCCTGGTGA-TGCCAATACATC-AAACGCCGCGAC-3′ (anti-sense primer)] based on the sequences of human eNOS cDNA. We used a competitive RNA transcription kit (Takara Shuzo, Otsu, Japan). eNOS cDNA primers amplify a product with a predicted length of 486 bp, and the competitor was produced at a length of 558 bp. The same amount of mRNA was corrected using a β-actin.

2.9. Detection of aortic superoxide anion (O_2^-) generation

Formation of O_2^- from vessel was assayed by measuring the intensity of chemiluminescence probes in the presence of one of the Cypridina luciferin analogs, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazine-3-one (MCLA) [17]. In brief, the O_2^- generation signal from the 2 mm length of vessel with or without endothelium was detected by a luminescence reader (BLR-201, Aloka Co., Tokyo). To ensure the specificity of MCLA to detect O_2^- increasing concentrations of SOD (1–50 U/ml) were added to the tissues.

2.10. Data analysis

Results were expressed as mean±S.E.M. Data were compared by analysis of variance with repeated measurements. A level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma lipid concentration

Plasma lipid levels were measured before oophorectomy and after 0, 4, 8 and 12 weeks of oophorectomy. The addition of 0.3% cholesterol to the diet increased the total cholesterol level significantly compared with the baseline value. Plasma cholesterol levels were decreased in the pitavastatin group at 4, 8 and 12 weeks after oophorectomy compared with that of the HCD group. There were no significant differences in other lipid components such as HDL-C observed between the control and treated group animals (Table 1). Pitavastatin treatment in the regular diet group did not show any change of lipid profile (data not shown).

3.2. Histological examination of atherosclerosis

Histological examination of the thoracic aortae revealed more atheromatous lesions, as indicated by the mean percentage of luminal encroachment and the mean lesion area, in the hypercholesterolemic (Gp.HCD) than in NK104 treated groups (Gp.NK) (Fig. 1). Regular diet group showed no atherosclerotic lesion w/wo pitavastatin treatment.

3.3. Endothelium-dependent and -independent relaxation

In all groups, ACh produced endothelium dependent relaxations (EDRs) of the aortic rings with an intact en-

Table 1
Profile of plasma biochemistry

	BeO	0 week	4 weeks	8 weeks	12 weeks
T.Chol. (mg/dl)					
HCD	58.2±4.9	62.2±5.5	884.2±110.5	1082.2±123.3	1056.9±110.4
NK104	56.2±5.5	59.1±4.9	582.2*±95.8	752.4*±91.7	772.8*±79.1
Control	59.2±5.9	58.1±5.9	54.2±4.9	60.4±6.3	56.2±5.3
T.G. (mg/dl)					
HCD	48.5±6.3	51.2±8.3	54.2±10.1	57.4±13.7	52.9±24.0
NK104	46.2±6.5	49.1±5.1	51.2±10.3	52.1±11.7	40.8±6.8
Control	49.6±5.1	48.6±7.1	47.4±5.9	50.8±8.9	53.1±15.3
HDL-C (mg/dl)					
HCD	34.4±4.8	31.2±4.3	34.1±2.1	32.2±3.1	34.4±4.3
NK104	33.2±5.1	31.4±3.4	37.2±4.6	36.2±4.6	34.4±3.2
Control	34.6±5.1	31.5±2.1	31.4±5.2	30.8±4.9	30.1±5.1

* $P < 0.05$ vs. control.

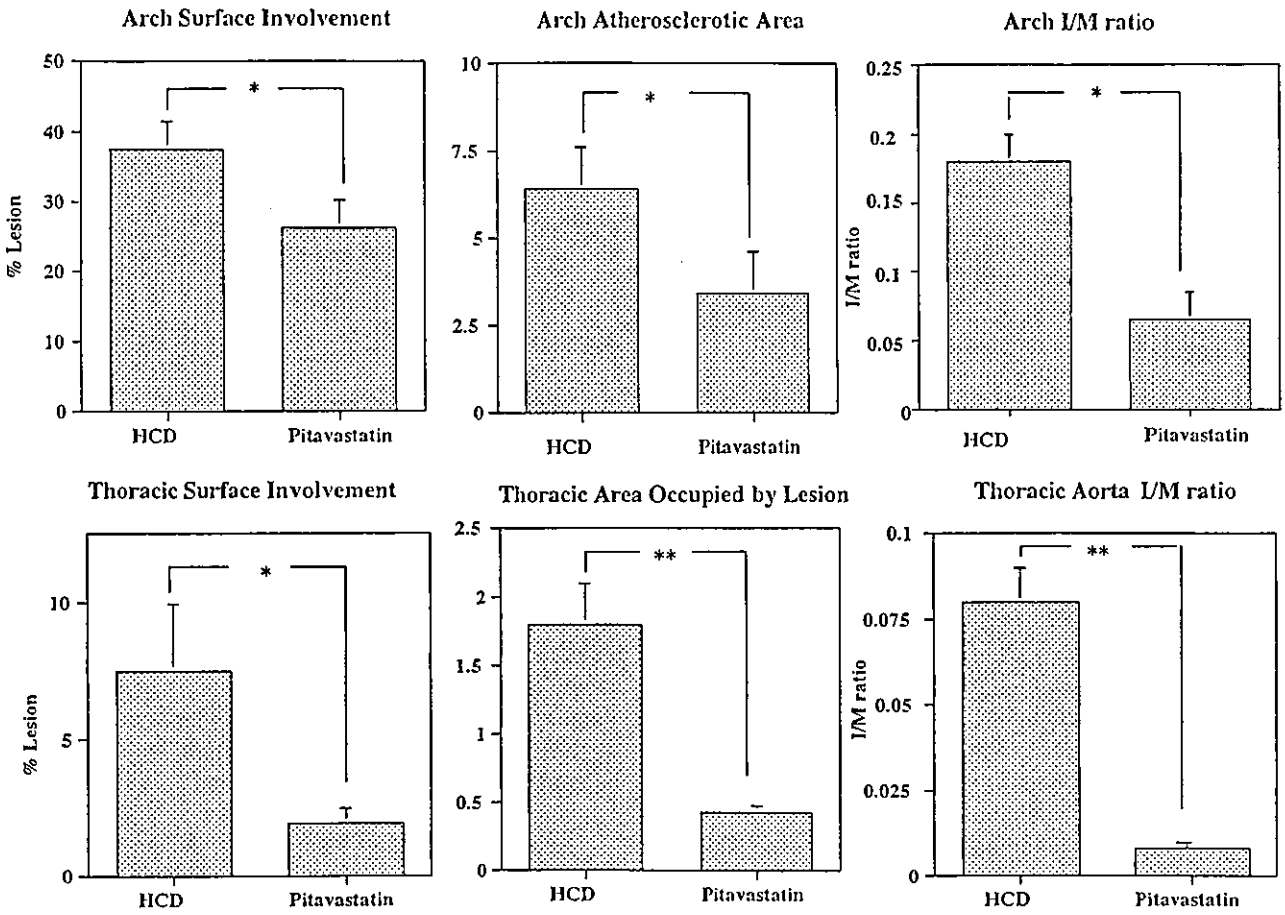

Fig. 1. Histological evaluation of atherosclerotic area (upper) of the thoracic aortae (lower). Left: The surface involvement of atherosclerotic area in the aortic arch and the thoracic aorta from of rabbits (Gp.HCD: high cholesterol diet [HCD, standard diet plus 0.3% cholesterol], Gp.NK, pitavastatin: HCD plus pitavastatin (1 mg/kg per day) $^{*}P < 0.05$, $^{**}P < 0.01$. Center: The area occupied by atherosclerotic areas of the aortic arch and the thoracic aorta from four groups of rabbits. Right: The Intima/Media ratio of the aortic arch and the thoracic aorta from four groups of rabbits.

dothelium (Fig. 2, left). The magnitude of the relaxation of aorta from the hypercholesterolemic animals (Gp.HCD) was diminished compared to those from regular diet group w/wo pitavastatin. However, EDRs in arteries from Gp.NK was remarkably larger than that from Gp.HCD. There was no significant difference in the response of vessels from regular diet group (Gp.R) and pitavastatin treated hypercholesterolemic animals (Gp.R-NK, data not shown). The non-receptor mediated vasorelaxation by calcium ionophore, A23187 showed the same tendency as ACh induced relaxation (data not shown). The endothelium-independent vasodilator, NTG, produced concentration-dependent relaxation in the thoracic aortic rings. No significant difference in relaxation was observed in aortic rings of all groups (Fig. 2, center). The inhibition of NOS by L-NMMA led to a contractile response in the aortic rings. The L-NMMA contractile response was higher in pitavastatin treatment (Gp.NK) (Fig. 2, right). Preincubation of indomethacin did not affect EDRs (data not shown).

3.4. Tissue cyclic GMP concentration

NO activates soluble guanylate cyclase in smooth muscle cell and led to produce cGMP. We examined cGMP concentration in homogenate samples of rabbit aorta. Pitavastatin treatment showed a significant increase of cGMP level Gp.NK as compared with HCD group (3.11 + 0.42 versus 2.24 + 0.34 pmol/wet g in Gp.NK versus Gp.HCD, $P < 0.05$).

3.5. Detection of mRNA for endothelial NO synthase

The ethidium bromide-stained bands were quantified by densitometry from a photograph of the gel. The signal for eNOS increased about 50% in samples from aortae of hypercholesterolemic rabbits (Gp.HCD), as compared to those from control (Gp.R)(data not shown). The amount of eNOS mRNA was increased in Gp.NK compared with that in Gp.HCD.

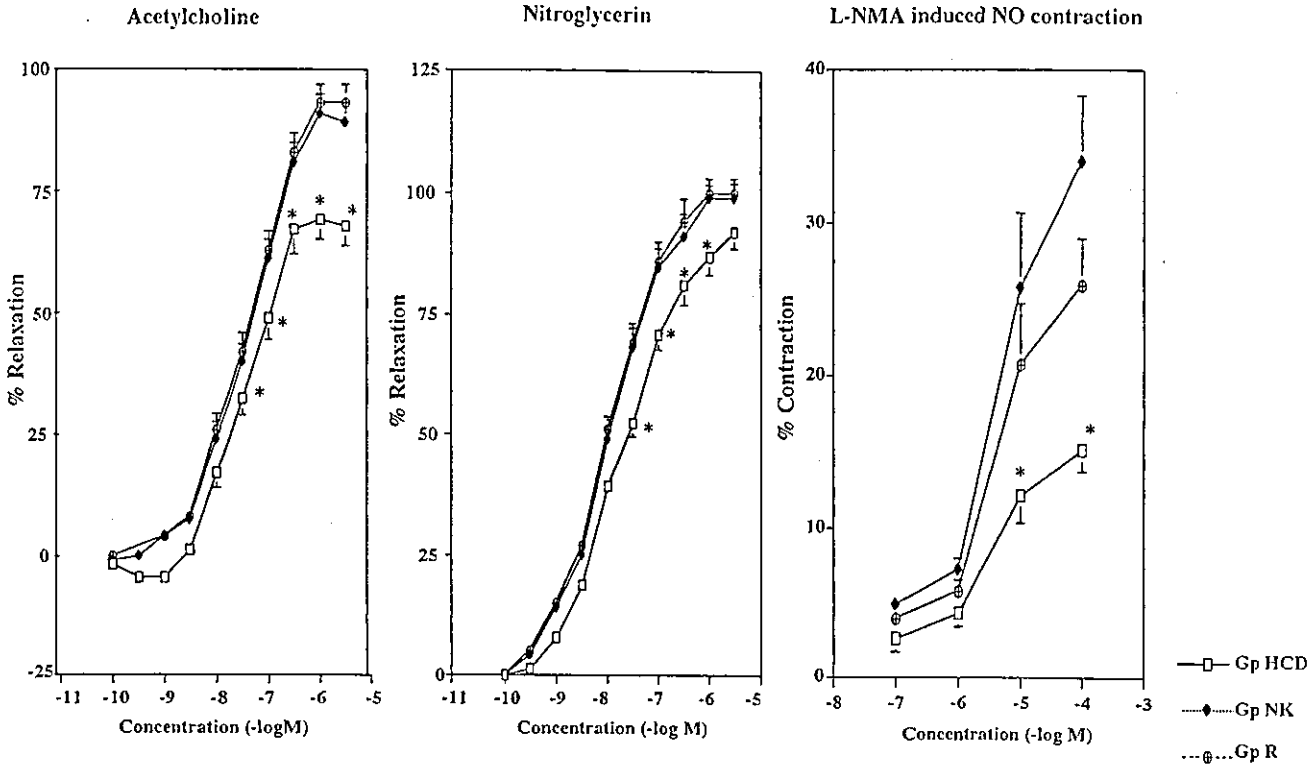

Fig. 2. Left: Cumulative concentration-response curves to acetylcholine (ACh) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas of rabbits fed with a high-cholesterol diet (HCD), HCD plus pitavastatin (NK), or a regular rabbit chow (R). Significant difference ($^*P < 0.05$) vs. HCD. Data are shown as means ± S.E.M. Center: Cumulative concentration-response curves to L-NMMA during contraction evoked by prostaglandin F2α (0.8×10^{-6} M). Right: Cumulative concentration-response curves to nitroglycerin (NTG) during contraction evoked by prostaglandin F2α (2.6×10^{-6} M) in the thoracic aortas. There is no significant difference between three groups.

3.6. An aortic superoxide anion production

We measured superoxide anion production from arterial wall with lucigenin analogue (MCLA). The chemiluminescence signals (CL signals) as superoxide anion production increased in aorta from cholesterol fed rabbits (Gp.HCD) as compared with regular diet group of rabbits (Gp.R) (Fig. 3 right). CL signals from vascular tissue with endothelium showed a decrease in Gp.NK as compared with HCD group. It means that the amount of O_2^- released is greater in aorta from HCD group than in those from pitavastatin group. In aorta without endothelium, CL signals were decreased in pitavastatin treated rabbits as compared to cholesterol fed rabbits (Fig. 3 right). The endothelium dependent chemiluminescence was drastically decreased in pitavastatin treated group. In other words, the relative difference of aortic O_2^- generation between HCD group and pitavastatin group was higher in the part of endothelium, and pitavastatin treatment decreased O_2^- generation more in endothelium than that in components of vessels other than endothelium. The amount of O_2^- released decreased in Gp.NK compared with in Gp.HCD.

3.7. Immunohistochemical study

Immunohistochemical analyses demonstrated a significant decrease in the number of macrophage derived cells in the atherosclerotic lesions in pitavastatin treated rabbits as compared to those from HCD group (Fig. 4). At the same time, the number of smooth muscle derived cells in atherosclerotic lesions of pitavastatin treated rabbit aortae tended to be decreased without statistical significance (data not shown). Pitavastatin treatment not only reduced the area of atherosclerosis, but also decreased the area stained by the macrophage antibody, the area stained by the iNOS antibody, and the areas positive for $ONOO^-$ established by nitrotyrosine staining. MMP-1 (interstitial collagenase), a matrix metalloproteinase that initiates collagen degradation, was localized predominantly in macrophages. The expression of MMP-1 and MMP-2 decreased in the pitavastatin treated group compared with that of Gp.HCD.

4. Discussion

Epidemiological studies have shown that lipid lowering therapy with statins such as simvastatin leads to a significant reduction in cardiac mortality and morbidity [2–5]. Atorvastatin was also shown to reduce the progression of coronary atherosclerosis through its strong lipid lowering action. In this experiment, we tried to investigate the anti-atherosclerotic effect of pitavastatin, a newly developed

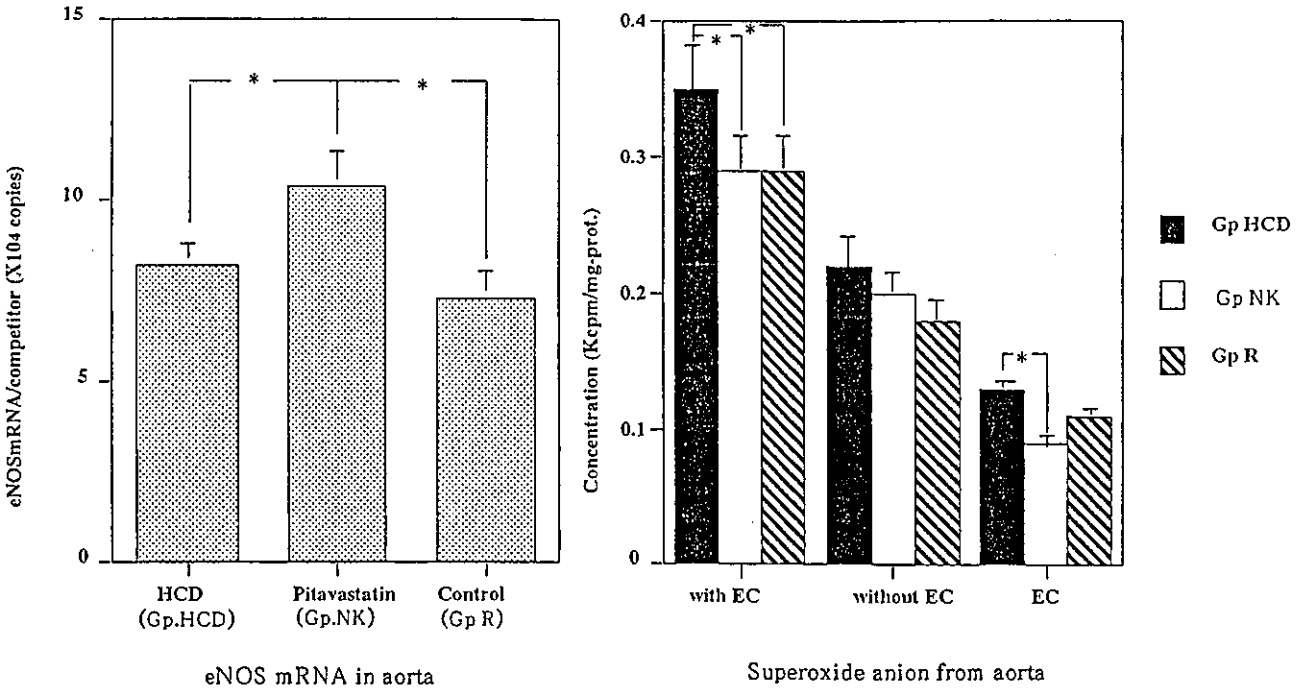

Fig. 3. Left: Quantification of eNOS mRNA in aortic arch using competitive RT-PCR. Ethidium bromide-staining gel after electrophoretic resolution of the competitor (558 bp) and eNOS target bands (486 bp) were determined. Relative amounts of eNOS mRNA to competitors using densitometry was shown. Data are shown as means ± S.E.M. Significant difference (*$P < 0.05$). Right: Effects of Pitavastatin on superoxide production from rabbit aortae. 'Endothelium+' means the data of vessel with endothelium. 'Endothelium−' means the data of vessel without endothelium.

statin, on aorta by selecting dose of pitavastatin which was reported to be comparable to the dose used in humans [6]. Attention has recently been focused on the molecular mechanisms responsible for these effects of statins, as well as their lipid lowering action. The present study therefore focuses on the status of endothelial functions, especially NO related, as determined by vascular responses. We measured nitric oxide metabolites, cGMP concentration, and eNOS mRNA expression in oophorectomized rabbits with or without pitavastatin treatment. In addition, we examined the O_2^- generation in the vessels with or without endothelium, immunohistochemistry related to peoxynitrite, matrix metalloprotease and apoptosis, and atherosclerotic lesions of hypercholesterolemic rabbits with or without pitavastatin treatment.

The HMG-CoA reductase inhibitors are potent inhibitors of cholesterol biosynthesis [1], decreasing serum cholesterol level by blocking the hepatic conversion of HMG-CoA to L-mevalonate in cholesterol biosynthetic pathway [1]. In the present study, serum cholesterol level was significantly decreased whereas no difference was observed in TG and HDL cholesterol (Table 1). This lipid lowering effect of pitavastatin in high cholesterol induced atherosclerotic rabbit model may be stronger than that of fluvastatin or simvastatin which we did previously, and the data is compatible with clinical data reported in Japan [18–20]. On the other hand, there are few reports concerning the effect of atorvastatin or lovastatin where the lipid lowering effects in rabbits were reported to be stronger than simvastatin or fluvastatin in hyperlipidemic patients. As these statins have inhibited HMG CoA reductase strongly, it may cause liver damage in rabbit. Pitavastatin was reported to have stronger LDL receptor induction in liver, however weaker HMG-CoA reductase inhibition than atorvastatin or simvastatin [21].

The EDRs were impaired in animals with experimentally induced atherosclerosis, which has been correlated to the decreased biological activity of endothelium derived NO [9,13]. The present investigation shows that endothelium dependent nitric oxide mediated relaxation in response to acetylcholine and calcium ionophore. A23187 and tone-related basal NO release evaluated by L-NMMA contraction were improved significantly by pitavastatin treatment (Fig. 2). The improvement of endothelial function by statin is often attributed to the reduction in serum cholesterol concentration. Indeed, a study demonstrated that a single treatment of LDL apheresis is sufficient to significantly improve EDRs in hypercholesterolemic humans [22]. Further, tissue cGMP concentration in aorta was also increased by pitavastatin treatment (Fig. 3). NO activates vascular smooth muscle soluble guanylate cyclase, thereby increasing cGMP in turn responsible for decreased intracellular Ca^{2+} concentration. The increased cGMP concentration clearly indicates that the increased production and bioavailabity of NO. In other words, increase of cGMP and greater contraction of aorta in response to L-NMMA shows increase of the basal

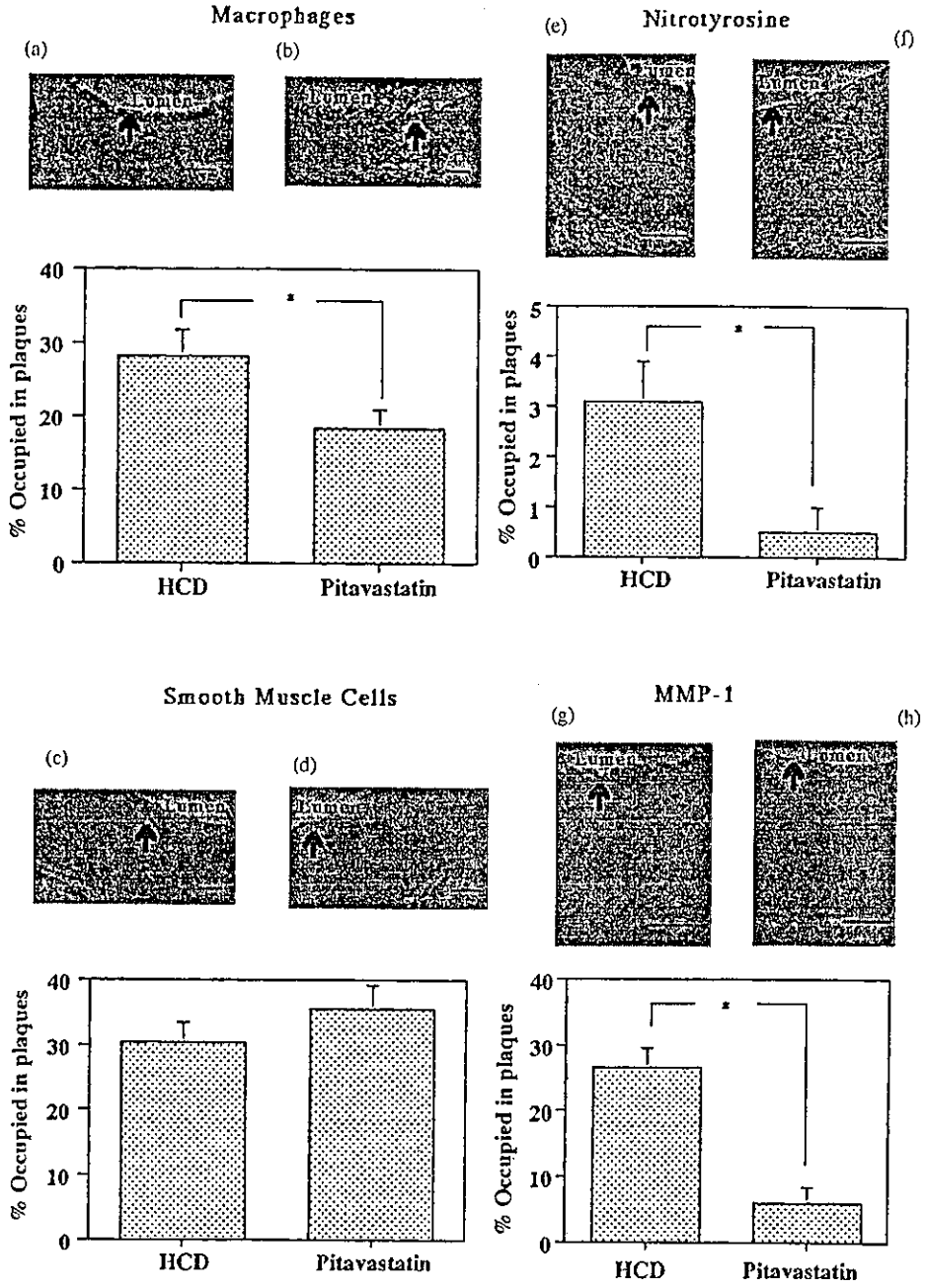

Fig. 4. Photograph: Immunohistochemical analysis of the thoracic aortae of rabbits from the HCD group and NK group (pitavastatin) using macrophages (a, b), smooth muscle cells (c, d), nitrotyrosine, a marker of peroxynitrite (e, f) and MMP-1 (g, h). Original magnification: 100× (a, b, c, d); 200× (e, f, g, h). Bar is 50 μM. Graph: % occupied area in atherosclerotic plaque by macrophages, smooth muscle cells, nitrotyrosine and MMP-1 evaluated by immuno-histochemistry.

release of NO. We hypothesized two mechanism of this improved NO bioavailability.

The eNOS mRNA expression was increased significantly in the aorta of pitavastatin treated rabbits (Fig. 3). This result is compatible with the observation that eNOS mRNA expression was increased in simvastatin treated cultured endothelial cells without changing lipid sub-fraction in the medium [23], and that the eNOS mRNA expression was increased by the stabilization of mRNA, not by the stimulation of transcription [23]. eNOS upregulation and inhibition of iNOS induction by statin were also reported [24]. We have also observed the increased expression of eNOS mRNA and protein in pitavastatin treated cultured bovine aortic endothelial cells and that it was also mediated by the stabilization of eNOS mRNA (data not shown). The increased expression of eNOS mRNA attributes increased NO synthesis. In endothelial cells, eNOS protein is translocated to the caveolae for myristoylation and palmitoylation. Our preliminary experi-

ment based on immunohistochemical study showed that the majority of eNOS protein exists in cytoplasm of endothelial cells in atherosclerotic lesions of cholesterol diet fed rabbits whereas almost all of eNOS exist in membranous part of aortic endothelial cells of regular diet fed rabbits (data not shown). The eNOS mRNA was increased by cholesterol diet in this study and recent other studies [16]. Taken together, we speculated the possibility that eNOS activity was regulated by both mRNA level and location of protein in cells.

The other mechanism of improved NO bioavailability is the decrease of O_2^- production. The oxidative inactivation of NO is regarded as an important cause of its decreased biological activity. O_2^- reacts with NO faster than SOD and forms peroxynitrite anion [25]. The peroxynitrite anion oxidizes sulfhydryls groups and yields products indicative for hydroxyl radical reaction with deoxyribose and dimethyl sulfoxide. These reactions induce membrane lipid peroxidation, to stimulate progression of vascular atherosclerosis. The presence of peroxynitrite-derived nitrotyrosines in atherosclerotic lesions has been demonstrated in our previous study in rabbit models [26]. The vascular release of superoxide was increased significantly in hypercholesterolemia and atherosclerosis [9]. This study shows that O_2^- production was decreased in arteries by pitavastatin treatment, especially in endothelial cells. Among several oxidases, as O_2^- producing enzymes, three are possible candidates in the release of O_2^- from endothelial cells. In the hypercholesterolemic rabbit, increasing serum activity of xanthine oxidase release increased amounts of O_2^- [27]. Recently, NO was reported to inhibit, in vitro [28], xanthine oxidase and xanthine dehydrogenase, which are present in endothelial cells. NADPH oxidase exists in culture endothelial cells and smooth muscle cells activated by TNF-α, and its activity is increased in hypercholesterolemia [29]. eNOS was also one of the candidates, because it was reported to release O_2^- in diabetic vessels [30]. Preliminarily, our data have shown that pitavastatin decreases O_2^- from NADPH oxidase in endothelial cells, and eNOS did not release O_2^- in high-cholesterol diet induced atherosclerosis (data not shown). However, we have to consider that statins may have a potential effect on superoxide production by mitochondria, considering the potential effects of statins on the metabolism of CoQ10 [31]. Coenzyme Q can undergo oxidation/reduction reactions in other cell membranes such as lysosomes, plasma membranes; deficiency of coenzyme Q has been described based on failure of biosynthesis by statins [31].

Nitroglycerin mediated endothelium independent relaxation is also improved by pitavastatin treatment. We speculated that it was due to the retardation of atherosclerosis formation by pitavastatin. However it is possible that pitavastatin has some effect on smooth muscle cell sensitivity to NO. It maybe necessary to elucidate more to understand the underlying mechanism.

Accumulation of macrophages in the vascular wall might be responsible for a variety of pathological events, such as generation of superoxide radicals, oxidation of LDL, subsequent foam cell formation, and release of cytokines, resulting in smooth muscle cell proliferation, and migration. The present investigation depicts the decreased number of macrophages in the intima following pitavastatin treatment. It may be due to the prevention of macrophages adhesions and migration by increasing NO bioavailability.

To determine other mechanisms of the anti-atherosclerotic effect of pitavastatin, we investigated the proportion of MMP-1 and 2 positive areas. The interstitial collagenase (matrix metalloproteinase-1, MMP-1) and MMP-2 expression in the lesion were measured by quantitative image analysis [32]. MMP-1 is localized predominantly in the macrophages and that plays a key role in initiating collagen degradation. The baseline lesions in the HCD group expressed high levels of MMP-1. Macrophage-related proteolysis might contribute to weakness of the protective fibrous cap of the plaque (Fig. 4). A reduction of both the macrophage content and the expression of immunoreactive MMP-1 were observed in aortae from pitavastatin treated rabbits. Immunoreactive MMP-2 showed the same tendency. This suggests that pitavastatin treatment plays a major role in plaque stabilization. Conclusively, the present study demonstrates that pitavastatin safely reduces plasma cholesterol level in high-cholesterol diet induced atherosclerosis, and that the anti-atherosclerotic effect of pitavastatin is mediated at least partly by increasing endothelium dependent vascular responses, eNOS mRNA expression, cGMP level and decreasing superoxide anion production. The antiatherosclerotic property of pitavastatin is due to two major pathways: one is due to its pleiotropic effect, such as improvement of endothelial function; the second stems from its lipid lowering effect. Although suggest the experiment was carried out in rabbits, the results suggest new possibilities of the usefulness of pitavastatin in cases of atherosclerosis, due to its NO bioavailability.

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Circ J 2005; **69:** 1–6

Relation of Serum Total Cholesterol and Other Factors to Risk of Cerebral Infarction in Japanese Men With Hypercholesterolemia

— The Kyushu Lipid Intervention Study —

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for the Kyushu Lipid Intervention Study (KLIS) Group

Background Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT), and few studies have examined the risk factors in the elderly.

Methods and Results Clinical and behavioral risk factors for cerebral infarction were examined in 4,349 Japanese men aged 45–74 years with a serum total cholesterol (TC) concentration of 220 mg/dl or greater who participated in the Kyushu Lipid Intervention Study. A total of 81 men developed definite cerebral infarction in a 5-year follow-up period. The Cox proportional hazards model was used with serum TC at baseline and during the follow-up, serum high-density lipoprotein-cholesterol (HDL-C), HT, diabetes mellitus (DM), and other factors as covariates. Serum TC during the follow-up, not at baseline, was positively associated with cerebral infarction, showing a stronger association in the elderly (≥65 years old) than in the middle-aged (<65 years old). Statin use was related to a moderate decrease in the risk of cerebral infarction when follow-up TC was not considered, but the decrease was almost nullified after adjustment for follow-up TC. A low concentration of serum HDL-C, diabetes mellitus, hypertension, and angina pectoris were each related to an increased risk. No clear association was observed for body mass index, smoking or alcohol use.

Conclusions Lowering cholesterol is important in the prevention of cerebral infarction in men with moderate hypercholesterolemia. A low concentration of HDL-C, DM, and HT are independent predictors of cerebral infarction. (*Circ J* 2005; **69:** 1–6)

Key Words: Cerebral infarction; Diabetes mellitus; High-density lipoprotein-cholesterol; Hypercholesterolemia; Japanese men

Stroke is a leading cause of death and disability in industrialized countries and of the 2 major types of stroke, cerebral infarction predominates, although hemorrhagic stroke remains common in Asian populations[1] Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT)[2] Findings regarding the relation between serum total cholesterol (TC) or low-density lipoprotein-cholesterol (LDL-C) and cerebral infarction are inconsistent in observational studies[3–16] whereas cholesterol-lowering trials have shown a decrease in the risk of cerebral infarction among patients assigned to statin treatment[17,18] The role of serum high-density lipoprotein-cholesterol (HDL-C) is receiving particular interest in the epidemiology of cerebral infarction. Low concentrations of serum HDL-C have been fairly consistently associated with an increased risk of cerebral infarction[4–9,14,19] Several[5,7,8,12,15,19] but not all[3,6] prospective studies reported that diabetes mellitus (DM) was associated with an increased risk of cerebral infarction. In the study reported here, we examined the relation of serum TC and HDL-C and other factors to the risk of cerebral infarction using data from the Kyushu Lipid Intervention Study (KLIS), a primary prevention trial of coronary heart disease (CHD) events and cerebral infarction in Japanese men with moderately elevated concentrations of serum TC[20–23] Furthermore, because few studies have investigated the risk factors for cerebral infarction in elderly persons[6,15,19] we examined the association with these factors in middle-aged and elderly men separately.

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Methods

Details of the study design, patient characteristics at baseline, and primary results of the KLIS have been described previously[20–23] In brief, a total of 5,640 men aged 45–74 years with serum TC concentration of 220 mg/dl or greater were enrolled by 902 physicians in Kyushu District during the period between May 1990 and September 1993.

Table 1 Characteristics of the Study Subjects by Statin Use

	Mean (SD) or proportion		p-value for difference*
	Statin (−)	Statin (+)	
No. of subjects	1,637	2,712	–
Age (years)	58.1 (8.2)	58.0 (7.9)	0.78
Baseline total cholesterol (mg/dl)	244 (17.9)	258 (25.8)	<0.0001
Follow-up total cholesterol (mg/dl)	225 (25.1)	219 (26.7)	<0.0001
Baseline HDL-cholesterol (mg/dl)	50 (12.0)	49 (12.0)	0.05
Body mass index (kg/m²)	23.9 (2.8)	24.2 (2.7)	0.008
Angina pectoris (%)	8.2	10.5	0.01
Hypertension (%)	42.6	44.5	0.24
Diabetes mellitus (%)	24.9	22.8	0.11
Prior use of hypolipidemics (%)	7.6	14.3	<0.0001
Current smoking (%)	40.0	37.5	0.10
Daily alcohol use (%)†	41.7	39.6	0.16

Values are mean (SD) unless otherwise specified.
*Comparison of means was based on t-test, and the chi-square test was used for proportions.
†Drinking alcohol 5 days per week or more frequently.

All the patients gave consent to participate in the study. Ineligible for the study were a history of myocardial infarction, coronary bypass surgery, coronary angioplasty, cerebral hemorrhage, or cerebral infarction; serum HDL-C concentration of 80 mg/dl or greater; and a life-limiting morbid condition such as severe renal or hepatic disease. Each physician was instructed to randomly allocate patients to either pravastatin treatment or conventional treatment as specified in a sealed envelope, but participating physicians did not necessarily follow that instruction[20] The patients were followed up until the end of 1997 for the occurrence of coronary events and cerebral infarction. The study was approved by the ethical committee of the principal investigator's affiliated institution.

Subjects

The present analysis included 4,349 of the 5,640 enrolled men; 1,291 were excluded for the following reasons: (1) withdrawal of consent (n=147), (2) no institutional contract (n=616; this category represented the lack of a written agreement between a participating hospital or clinic and a sponsoring pharmaceutical company, which became necessary in the course of the study because of the introduction of a new regulation for clinical trials in Japan), (3) found to be ineligible during follow-up (n=97), and (4) missing data (n=431).

Laboratory and Clinical Data

Serum concentrations of TC, HDL-C, triglycerides (TG), and other clinical and biochemical variables were determined at baseline and subsequently in the follow-up. Laboratory measurements were done at different laboratories, but each physician was requested to use the same laboratory throughout the study period. Average serum TC concentrations during the follow-up were determined on the basis of periodical follow-up measurements at 3 months, 6 months, and every year thereafter; the number of measurements ranged from 1 to 10 with a median of 6. Serum LDL-C was not used in the present study because the Friedewald method[24] was not applicable for 7% of the men who even at baseline had serum TG concentrations of 400 mg/dl or greater (n=282) or who had missing data (n=5). HDL-C was not measured in 46 men during the follow-up.

Hypertension was defined as present if a patient had systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or if was under medication for HT. Subjects were defined as having DM if they had either fasting plasma glucose ≥140 mg/dl or hemoglobin A1c ≥6.5% or if they were under medication for DM. The presence of angina pectoris (AP) and prior use of hypolipidemic drugs were based on the report of the study physicians. Statin use was defined if any statin drugs were prescribed during the follow-up period. Height and body weight were recorded, and body mass index (kg/m²) was calculated as an index of obesity. Current habits of smoking and alcohol drinking were ascertained, together with the number of cigarettes smoked per day and frequency of alcohol drinking per week.

Endpoints

Cerebral infarction was the secondary endpoint and coronary events were the primary endpoint. Definite cerebral infarction was diagnosed when typical symptoms and signs were accompanied with diagnostic findings on brain imaging or cerebral angiography. Diagnosis based only on clinical signs and symptoms was regarded as a suspected case[20] These endpoints were determined by the Endpoint and Adverse Effect Committee on the basis of periodical reports from the study physicians and, if necessary, by supplementary inquiry. From January to May 1998, an ad hoc survey was carried out to ascertain the occurrence of coronary events and cerebral infarction up to the end of 1997. Vital status was unknown for 36 men, and cerebral infarction and coronary events were not ascertained for 97 men. A total of 81 definite cases and 10 suspected cases of cerebral infarction were identified in an average observation period of 5.05 years. One definite case and 2 suspected cases of cerebral infarction occurred subsequent to a coronary event. None developed coronary events after cerebral infarction. Only definite cases of cerebral infarction were used in the present study, and the follow-up period continued until the event of a definite cerebral infarction regardless of coronary events.

Statistical Analysis

The Cox proportional hazards model was used to examine the relation of clinical and behavioral factors to the risk of cerebral infarction. The principal model included indicator variables for age (5-year class), baseline serum TC (<240, 240–259, ≥260 mg/dl), follow-up TC (<220, 220–239, ≥240 mg/dl), serum HDL-C (<40, 40–59, ≥ 60 mg/dl), BMI (<22.5, 22.5–24.9, ≥25.0 kg/m²), AP, HT, DM, prior