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 −.

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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]. 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
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 is 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 F$_2$α (PGF$_2$α), indomethacin and l-1-monomethyl-arginine (L-NMMMA) 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$_2$PO$_4$, 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 EDTA; disodium ethylendiamine-tetraacetic acid, pH7.4)

#### 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 = 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). 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 intimal/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].

#### 2.3. 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 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 F$_2$α 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 F$_2$α 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 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 were conducted in accordance with institutional guidelines for animal research.

#### 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 were conducted in accordance with institutional guidelines for animal research.
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

\[5^{\prime}-\text{A TTTAGGTGAC-ACTA TAGAA TACCAGTGTCCAA-CA TGCTGCTGGAAA TTGGTACGGTCA TCA TC-TGAC-3^{\prime}}\]

(anti-sense primer), and

\[5^{\prime}-\text{TAAAGGTCTTCTTCCTGGTGCA-} \]

(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 \(H_9252\)-actin.

2.9. Detection of aortic superoxide anion (O\(_2^\cdot\)) generation

Formation of O\(_2^\cdot\) 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\(_2^\cdot\) 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\(_2^\cdot\) 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).

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<th>BeO</th>
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<tr>
<td>T.Chol. (mg/dl)</td>
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<td>62.2 ± 5.5</td>
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<td>1082.2 ± 123.3</td>
<td>1056.9 ± 110.4</td>
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<td>56.2 ± 5.5</td>
<td>59.1 ± 4.9</td>
<td>582.2 ± 95.8</td>
<td>752.4 ± 91.7</td>
<td>772.8 ± 79.1</td>
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<tr>
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<td>59.2 ± 5.9</td>
<td>58.1 ± 5.9</td>
<td>54.2 ± 4.9</td>
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<td>56.2 ± 5.3</td>
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<td>NK104</td>
<td>46.2 ± 5.5</td>
<td>49.1 ± 5.1</td>
<td>51.2 ± 10.3</td>
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<td>49.6 ± 5.1</td>
<td>48.6 ± 7.1</td>
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<td>50.8 ± 8.9</td>
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<tr>
<td>T.G. (mg/dl)</td>
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<td>51.2 ± 8.3</td>
<td>54.2 ± 10.1</td>
<td>57.4 ± 13.7</td>
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<tr>
<td>HCD</td>
<td>34.6 ± 4.8</td>
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<td>34.1 ± 2.1</td>
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<tr>
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<td>HDL-C (mg/dl)</td>
<td>34.6 ± 5.1</td>
<td>31.5 ± 2.1</td>
<td>31.4 ± 5.2</td>
<td>30.8 ± 4.9</td>
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* \(P < 0.05\) 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 four groups of rabbits. 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.

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 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 aortas 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
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 peroxynitrite, 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 rabbits with or without pitavastatin treatment was 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 with 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 bioavailability of NO. In other words, increase of cGMP and greater contraction of aorta in response to l-NMMA shows increase of the basal
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 thiols/lysylglycine 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-$\alpha$, 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]. Preliminary, 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 proinflammatory cytokines 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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