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Original article

Stabilization of atherosclerotic plaque by pitavastatin in Watanabe heritable hyperlipidemic rabbits: A serial tissue-characterizing intravascular ultrasound study

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ABSTRACT

Background: To examine the effects of pitavastatin on atherosclerotic plaque in Watanabe heritable hyperlipidemic (WHHL) rabbits using serial in vivo tissue-characterizing intravascular ultrasound.
Methods: A total of 11 WHHL rabbits of 10–12 weeks of age were divided into two groups, control and pitavastatin-administered groups. A total of 29 atherosclerotic plaque segments from control group and 43 plaque segments from the pitavastatin group were serially imaged by 40 MHz intravascular ultrasound in vivo with a tissue characterization software (iMAP™, Boston Scientific, Natick, MA, USA) at the baseline and the follow-up (16th week).
Results: The level of low-density lipoprotein cholesterol was significantly decreased in pitavastatin group. During the follow-up period, plaque area was significantly increased in the control group, whereas it was not significantly changed in the pitavastatin group. The fibrotic, necrotic, and necrotic plus lipidic areas were significantly increased in the control group, while no significant change was revealed for tissue profile in pitavastatin group. The change in the percent areas of fibrotic and lipidic plus necrotic tissues were significantly different between the two groups especially in the superficial half portion of plaque.
Conclusions: These data indicate that pitavastatin could attenuate atherosclerotic plaque formation and that it could stabilize the plaque in WHHL rabbits. Considering the fact that these were observed even with a high follow-up level of cholesterol, these data might come from the pleiotropic effects of pitavastatin.

Introduction

It has been established that lowering levels of low-density lipoprotein cholesterol (LDL-C) with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) inhibitors (statins) is effective against the development of cardiovascular events [1–3]. As the mechanism for the preventive effects, various phenomena have been reported using postmortem as well as experimental studies using animals [4,5]. Previous clinical multicenter studies using intravascular ultrasound (IVUS) have revealed that statins can inhibit the atherosclerotic forming process of plaque, and that it can even reduce the plaque volume [5–8]. Furthermore, it has been proved that statins can change tissue composition of plaques as a stabilization from their vulnerability to rupture [9–13].

Evidence was obtained as a consequence of innovation in intravascular imaging, including not only IVUS, but also coronary angioscopy, optical coherence tomography, and so on. However, human studies usually have a lot of confounding factors in the statistics, and more genuine evidence would be necessary. Therefore, experimental animal study is also important to prove the net effect of statins on plaque characteristics. Previous studies with animals included a qualitative postmortem histologic demonstration [14,15], or serial examinations using magnet resonance imaging [16], positron-emission tomography [17], or gray-scale

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IVUS [18]. However, no animal studies have been performed serially with color IVUS. We have successfully developed a way of experimental study to be able to observe plaque change by serial color IVUS in Watanabe heritable hyperlipidemic (WHHL) rabbits [19], a model of human familial hypercholesterolemia, over 16 weeks.

Pitavastatin is a unique HMG-CoA reductase inhibitor with powerful lipid-lowering effects. Its ability to lower LDL-C is comparable to that of atorvastatin and it also enhances high-density lipoprotein cholesterol (HDL-C) [20]. Previous human studies have reported that pitavastatin can regress plaque volume [5,12,21] as well as stabilize plaque tissue composition [12,21]. Therefore, the purpose of the present study was to investigate the effect of pitavastatin on tissue characteristics of atherosclerotic plaque in WHHL rabbits using serial in vivo IVUS studies, which included volume measurement of gray-scale images and quantitative tissue characterization with color images from a commercially available application (iMAP™, Boston Scientific, Natick, MA, USA).

Methods

Animal preparation and medication

Male and female homozygous WHHL-myocardial infarction (WHHL-MI) rabbits (developed at Kobe University, Kobe, Japan) were housed in a room at 22 ± 2 °C temperature and $65 \pm 5\%$ humidity, illuminated from 08:00 h to 20:00 h. A total of 11 WHHL rabbits were divided into 2 groups, control and pitavastatin groups. These rabbits were all fed on 100 g/day of standard chow (CR-3; CEAR Japan, Inc., Tokyo, Japan). Pitavastatin was synthesized and provided by Kowa Co. Ltd, Aichi, Japan. Pitavastatin was orally administered at concentration of 0.0006% (w/v) in the pitavastatin group. The duration of treatment was 16 weeks beginning at 10–12 months of age. The dosage of pitavastatin was determined according to the previous studies that used it in WHHL rabbits [14]. At the IVUS examination, the rabbits were anesthetized with 3% sevoflurane. To endure this relatively long observation period for the rabbits after the first IVUS examination, meticulous monitorings about water balance as well as infection were performed. Cefazolin sodium of 0.03 g/kg was administered intramuscularly at the first IVUS and 24 h later. In addition, a drip infusion of extracellular saline solution of 200 mL/day over the first 24 h was also performed from the first IVUS. All animal experiments were performed with the approval of the Animal Experiment Committee of Nihon University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No, 85-23, revised 2011).

Measurement of plasma lipid

Blood samples were collected from a sheath into femoral artery after overnight fasting at the baseline and at the follow-up period. Plasma concentration of total cholesterol level was determined using a L-type Wako CHO-H (Wako Pure Chemical Industries, Osaka, Japan), and that of triglyceride level was measured using a L-type Wako TG-H (Wako Pure Chemical Industries). Plasma lipoprotein fractions were separated and determined for low-density lipoprotein (LDL-C) level using Cholestest®LDL (SEKISUI MEDICAL, Tokyo, Japan) and HDL-C level using Cholestest®N HDL.

IVUS procedure

IVUS was used to examine plaque volume, lumen volume, vessel volume, and plaque tissue characteristics within the brachiocephalic arterial segments at the baseline and at the follow-up period of 16th week of treatment. IVUS imaging was performed with a 2.5 F, 40 MHz imaging catheter (Atlantis SR Pro2™ and iLab™ Ultrasound Imaging System, Boston Scientific). The imaging catheter was inserted from femoral artery, and was advanced into right common carotid artery. The transducer was positioned to the brachiocephalic artery that was target vessel. The transducer was withdrawn by a motorized pullback device at a speed of 0.5 mm/s. The imagings used the same imaging system with the same type of IVUS catheter for both the baseline and follow-up examinations.

IVUS measurement

The target plaque was assessed by volumetric and tissue characteristic analysis using QIvus-iMap™ Full Edition 2.0 (Medis Associated BV, Leiden, Netherlands). A series of cross-sectional images for every 0.5 mm-apart vessel slice was measured by manual on-screen planimetry, which was used to trace the leading edges of the luminal and external elastic membrane borders. The tissue characterization was performed with the iLab™ system. Radiofrequency (RF) signals from region of interest (ROI) were first obtained, and then an algorithm installed in iMAP™ system characterized tissue components within the ROI using spectrum of the RF signal. This tissue characterization is based on a neural network-based learning algorithm for determination of RF spectrum shape by comparing with a library of RF spectrum which was obtained from pre-determined tissues [22]. In this system, a database of 12 000 image ROIs of histologically established types was used to design a pattern recognition algorithm to predict the tissue of a given ROI by examining similarity of the RF-spectrum against the database using 40 tissue detector arrays, in which four types of tissue components were identified, such as calcified, fibrotic, lipidic, and necrotic areas. In the iMAP system, a tissue was defined as fibrous when it had no distinct lipid core but had a fibrocellular matrix with dense collagen bands. A lipid core was defined as a contiguous area of lipid-containing foam cells, extracellular lipids, cholesterol crystals, and lipid pool. A necrotic core was defined as necrotizing material [23]. The accuracy of the iMAP system for these tissue characterizations was described in a previous paper [22]. For each vessel cross-section, the area of each of these tissue components was measured within total plaque area, and within the inner half (superficial half) and the outer half (deep half) of the plaque, which were divided by a centerline of plaque thickness. The software of QIvus-iMap™ Full Edition 2.0 can draw the centerline by a special algorithm of mathematical dot-to-dot interpolation, in which each dot is determined by visual inspection. The centerline was made by connection among the middle points which were determined along the radial axes from the gravity center of the lumen between lumen-intima and media-adventitia borders. In this serial study, the same cross-section was imaged referring to the positions of the inlet and outlet of the brachiocephalic artery. In order to precisely match the portion and strictly compare the area between the baseline and the follow-up, IVUS image acquirement was performed meticulously by equal longitudinal dividing of the vessel between the inlet and the outlet cross sections with a distinct image of bifurcation of the branching.

Statistical analysis

Data are presented as the mean ± SD. The statistical analysis was carried out using paired or unpaired *t*-tests. Difference with *p*-values less than 0.05 were regarded as statistically significant. The software used in these analyses was JMP (SAS Institute Inc., Cary, NC, USA).

Results

Lipid profile

Table 1 shows laboratory data both at the baseline and the follow-up period. There was no difference in lipid profile between the two groups at the baseline. After treatment with pitavastatin, plasma total cholesterol (T-CHO) and LDL-C were significantly reduced by 28.6% and 21.2%, respectively, while these values were not significantly changed in the control group. HDL-C and triglyceride (TG) were not significantly changed during the follow-up period between the two groups.

Gray-scale IVUS data

Table 2 shows IVUS-derived vessel area, lumen area, and plaque area for the two groups at the baseline and the follow-up period. There were no significant differences in these values between the two groups at the baseline. Plaque area was significantly increased in the control group, however, it was not significantly changed in the pitavastatin group. Vessel area was not significantly changed in the control group, while it was significantly decreased in the pitavastatin group. As a consequence, lumen area was significantly decreased in both groups.

Table 1
Lipid profile of the two groups (±SD).

	Control group ($n=5$)	Pitavastatin group ($n=6$)	p value (Control group vs. pitavastatin group)
T-CHO (mg/dl)			
Baseline	871.0 ± 225.6	1151.8 ± 247.5	0.112
Follow-up	780.0 ± 105.7	801.8 ± 112.6	0.773
p value (baseline vs. follow-up)	0.418	0.014	
LDL-C (mg/dl)			
Baseline	684.0 ± 181.2	850.0 ± 132.2	0.147
Follow-up	650.6 ± 51.6	663.2 ± 68.0	0.766
p value (baseline vs. follow-up)	0.696	0.003	
HDL-C (mg/dl)			
Baseline	7.6 ± 1.4	10.0 ± 4.7	0.313
Follow-up	7.6 ± 1.7	5.7 ± 1.1	0.154
p value (baseline vs. follow-up)	1.000	0.074	
TG (mg/dl)			
Baseline	252.8 ± 78.8	164.2 ± 72.1	0.112
Follow-up	171.8 ± 110.6	158.8 ± 51.0	0.773
p value (baseline vs. follow-up)	0.292	0.878	

T-CHO, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride. Values are mean ± SD.

Table 2
Serial changes in gray-scale IVUS parameters (±SD).

	Baseline	Follow-up	p value (Baseline vs. follow-up)
Control group			
Vessel area (mm^2)	16.35 ± 4.50	15.90 ± 3.49	0.618
Lumen area (mm^2)	9.44 ± 3.29	7.66 ± 2.06	0.021
Plaque area (mm^2)	6.91 ± 2.04	8.25 ± 1.99	0.007
Pitavastatin group			
Vessel area (mm^2)	16.09 ± 3.35	14.56 ± 2.34	<0.001
Lumen area (mm^2)	8.21 ± 1.80	6.97 ± 1.97	<0.001
Plaque area (mm^2)	7.88 ± 2.07	7.59 ± 2.00	0.185

IVUS, intravascular ultrasound. Values are mean ± SD.

Table 3
Serial changes in color IVUS parameters (±SD).

	Baseline (mm^2)	Follow-up (mm^2)	p value (Baseline vs. follow-up)
Control group			
Fibrotic	4.24 ± 0.88	4.79 ± 1.00	0.013
Lipidic	0.50 ± 0.31	0.75 ± 0.43	0.053
Necrotic	2.05 ± 1.41	2.86 ± 1.43	0.018
Lipidic + Necrotic	2.55 ± 1.65	3.62 ± 1.73	0.017
Calcified	0.19 ± 0.17	0.17 ± 0.15	0.763
Pitavastatin group			
Fibrotic	4.75 ± 1.04	4.47 ± 0.87	0.056
Lipidic	0.73 ± 0.38	0.74 ± 0.38	0.795
Necrotic	2.18 ± 1.27	2.21 ± 1.30	0.856
Lipidic + Necrotic	2.91 ± 1.59	2.95 ± 1.58	0.827
Calcified	0.18 ± 0.14	0.13 ± 0.10	0.117

IVUS, intravascular ultrasound. Values are mean ± SD.

Color IVUS data

Fig. 1 shows images of iMAP™ at the baseline and the follow-up period for the two groups. In particular, necrotic area shown as pinkish area was apparently increased in controls while it was decreased in the pitavastatin group.

Table 3 shows the absolute area of each tissue component in the two groups at the baseline and the follow-up period in the entire layer of plaques. During the follow-up period, fibrotic area, necrotic area, and necrotic plus lipidic area were significantly increased in

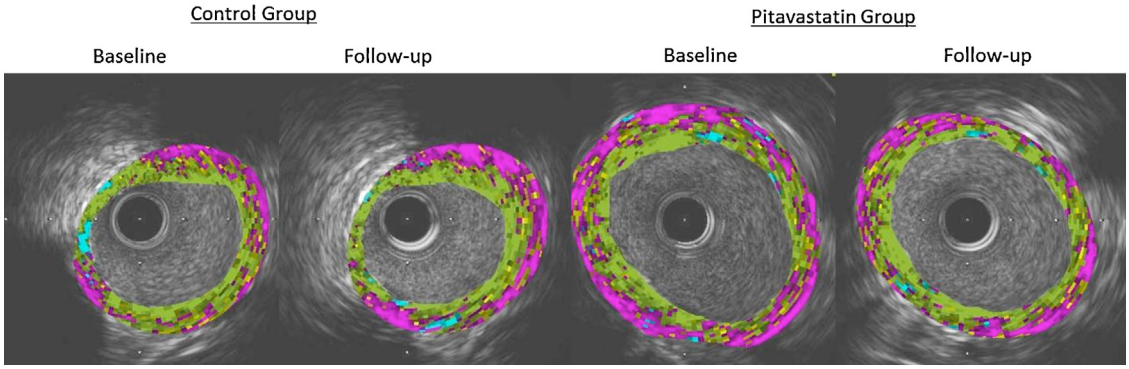

Fig. 1. Example iMAP™ images of the control group and the pitavastatin group. In these examples, plaque area was increased in an example of the control group, while it was decreased in an example of the pitavastatin group. The percent content of necrotic area was decreased and fibrotic area was increased in the example of the pitavastatin group, whereas necrotic area was increased and fibrous area was decreased in the example of the control group.

the control group, whereas no significant change was revealed for tissue profile in the pitavastatin group. In this study, we compared nominal change in the percent area of each tissue component in total plaque area as well as in superficial half of plaque area during the follow-up period (Figs. 2 and 3). There were no significant differences in the nominal change of the percent area for all types of tissue component between control and pitavastatin groups in the entire layer of the plaques (Fig. 2). However, in the superficial half portion of plaque, there were significant differences in nominal change of percent area of fibrotic, lipidic, and lipidic plus necrotic tissues (Fig. 3). In the control group, fibrotic tissue tended to decrease, whereas lipidic, lipidic plus necrotic, and calcified

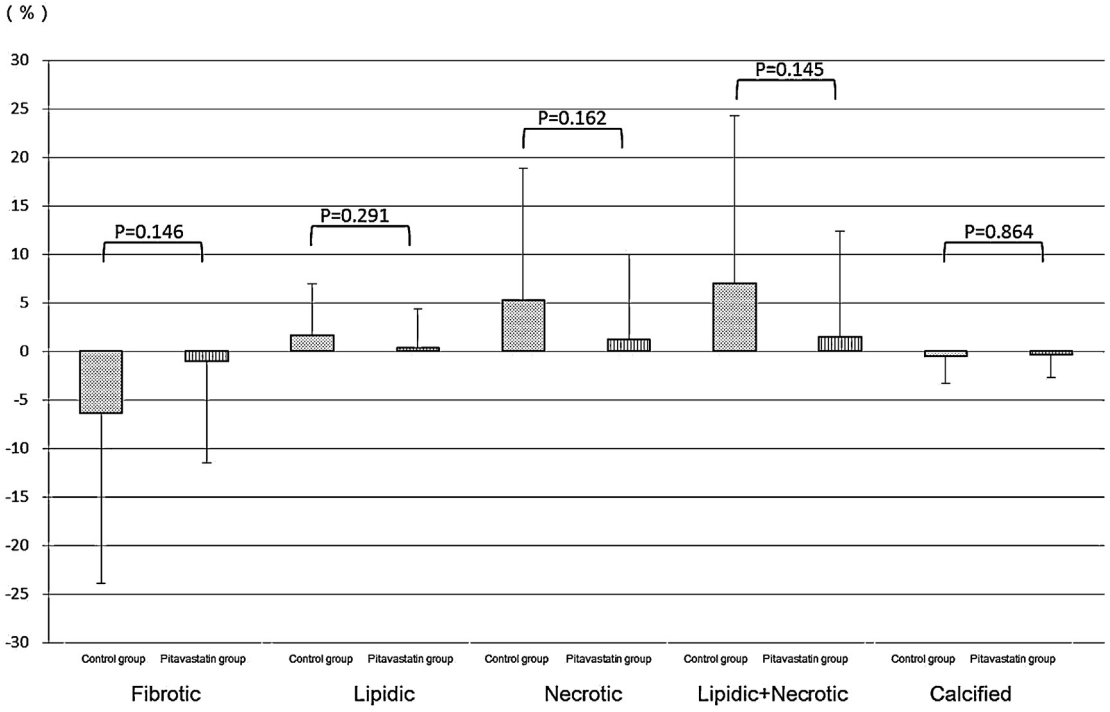

Fig. 2. Nominal change of % content of each tissue component within total plaque. There was no difference in its nominal change for all types of tissue component between the control and pitavastatin groups. Data are expressed as mean ± SD.

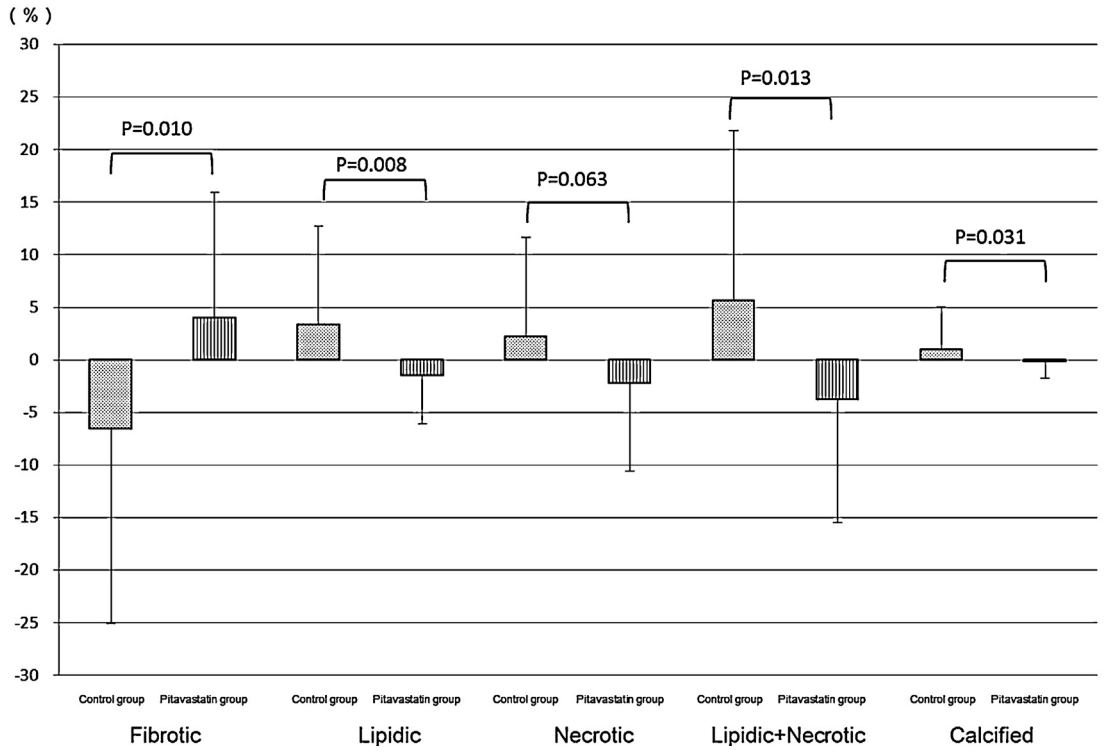

Fig. 3. Nominal change of % content of each tissue component within the superficial half of the plaque. There were significant differences in nominal change of the percentage for fibrotic, lipidic, lipidic plus necrotic, and calcified areas between the two groups. Data are expressed as mean ± SD.

areas tended to increase. On the contrary, in the pitavastatin group, fibrotic tissue tended to increase, and lipidic, lipidic plus necrotic, and calcified areas tended to decrease. There were no significant correlations between the change in LDL-C level from baseline to follow-up and the change in lipidic plus necrotic area in the pitavastatin group both for the entire layer of plaque and for the superficial half of plaque (Fig. 4).

Discussion

The major findings of the present study using WHHL-MI rabbits with a serial IVUS examination were that administration of pitavastatin inhibited the progression of atherosclerotic plaque area as well as necrotic and/or lipidic areas during the follow-up period of 16 weeks, even with a high level of LDL-C. These features were found particularly in the superficial half of the plaque. Therefore, it was suggested that pitavastatin not only attenuated progression of atherosclerosis but also induced plaque stabilization in WHHL rabbits. We believe that this would be the first report evaluating statin-induced changes in plaque amount and in its tissue composition serially by IVUS in an experimental animal model, which has less confounding factors compared to human studies.

It has been well established from multicenter human studies that statins can induce regression of plaque volume [5,7,8]. In this study, the plaque area did not decrease during the follow-up period. This might be because the level of LDL-C was high at around 600–800 mg/dl, although the level was significantly decreased by pitavastatin. In human studies, plaque progression was observed usually when the level of LDL-C was decreased to less than 100 mg/dl by statin. It might rather be noteworthy that pitavastatin could even attenuate the process of atherosclerosis at such a high level of LDL-C.

In the present study, this remarkable feature of pitavastatin even at high levels of LDL-C could be observed especially in its stabilizing effect on plaque. In the pitavastatin group, the increase in lipidic and/or necrotic area was significantly inhibited especially within the superficial half of the plaque, while the fibrotic area significantly increased around the superficial area compared to the control group. Furthermore, vessel area was significantly decreased only in the pitavastatin group during the observation

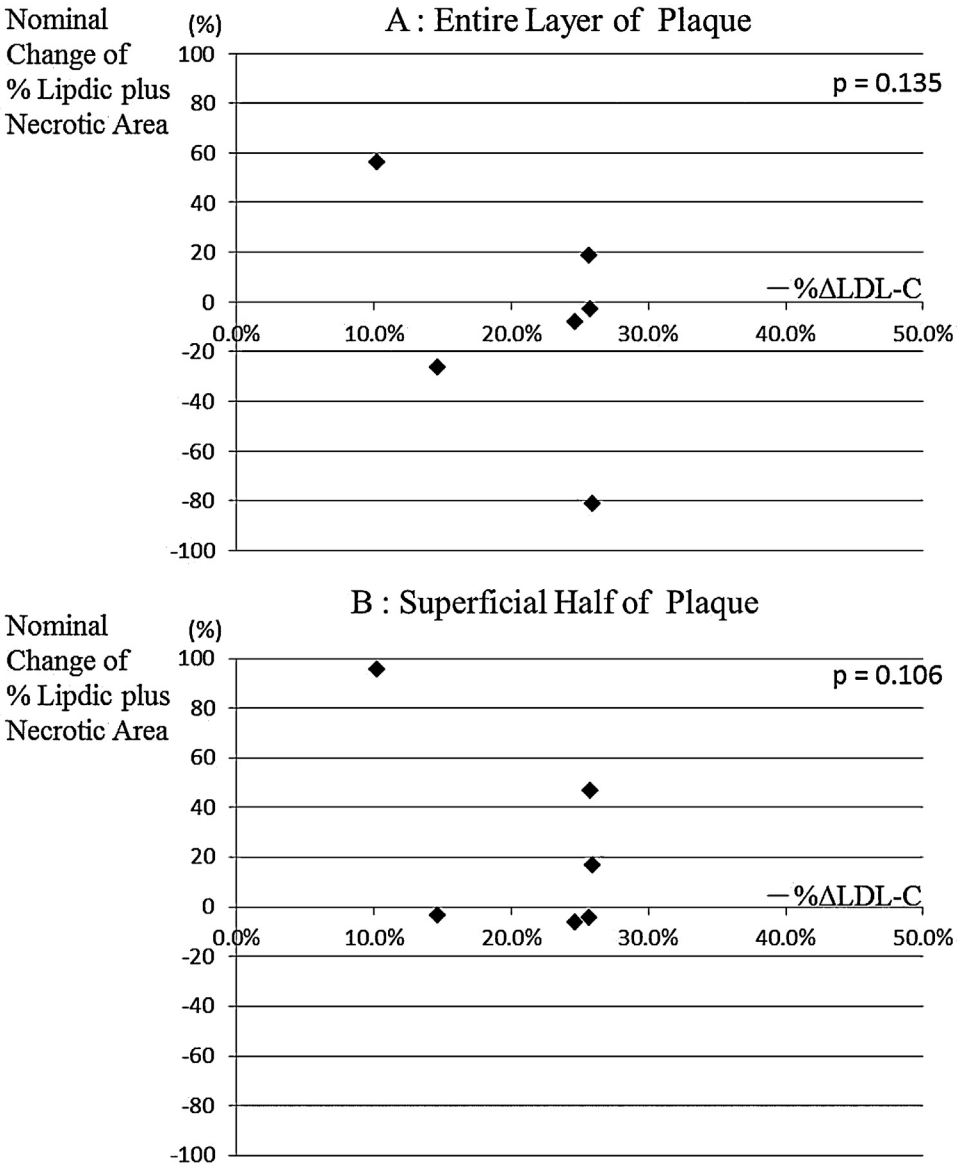

Fig. 4. Relationship between the change in low-density lipoprotein cholesterol (LDL-C) level from the baseline and the change in lipidic + necrotic area. There were no significant correlations between these two values in the pitavastatin group both for the entire layer of plaque (A) and for the superficial half of plaque (B).

period. It can be speculated that these effects corresponded to plaque stabilization. These data support the previous human studies on plaque stabilizing effects of statins [9,10,13,21,24].

Hattori et al. reported using integrated backscatter IVUS (IB-IVUS) and optical coherence tomography that pitavastatin induces an increase in fibrous cap thickness and a decrease in lipid volume within human coronary plaque [21]. Otagiri et al. reported with IB-IVUS that use of rosuvastatin in patients with acute coronary syndrome significantly decreased lipid component of the plaque [13]. Kawasaki et al. revealed with three-dimensional IB-IVUS that lipid mass diminished from coronary plaque by administration of statin [24]. Although we used iMAP^{TM} which was different from IB-IVUS in terms of imaging mechanism, similar findings were obtained, because lipidic plus necrotic area in iMAP^{TM} corresponds to lipidic area in IB-IVUS according to the definition of each tissue component in each system [25]. Hirayama et al. [9] as well as Kodama et al. [10] reported similar results using coronary angioscopy that yellow grade of plaque surface color was decreased by statin. Therefore, our study would reconfirm these facts that lipidic and/or necrotic area within coronary plaque tends to decrease as an effect of statin especially at the superficial layer of the plaque. On the other hand, the fibrous area within total plaque area was increased in the control group, and was not changed in the pitavastatin group during the follow-up period in our study, which seemed to be inconsistent with previous studies that fibrous cap thickness was increased. However, fibrous components can be found not only in the superficial cap zone in the plaque, but also in total plaque area, because fibrous tissue forms fibrous cap as well as the whole matrix of plaque. Therefore, the change in fibrous area is determined by which change is predominant. In our study, measurement of fibrous area was also performed in the superficial half of the plaque, suggesting that fibrous cap thickness might be increased in the pitavastatin group, but that it might be decreased in the control group. Therefore, from these viewpoints our data on the change in fibrous area were not inconsistent with the previous data. Considering the high level of LDL-C even at the follow-up period, our data might show the pleiotropic effects of pitavastatin, since there were no significant correlations between the change in LDL-C level and the change in lipidic + necrotic area during the observation period within plaque.

It has been shown that pitavastatin has various pleiotropic effects; it reduces the inflammatory response [26], improves endothelial function [27], and increases thrombomodulin expression [28]. These features might be involved in the plaque stabilization of our study, although we did not directly measure these markers. Previous experimental animal studies have suggested that various processes are involved in the plaque attenuation or stabilization. HDL, the major endogenous mediator of reverse lipid transport, enters plaque and takes cellular as well as extracellular lipids [29]. It is estimated that some lipoprotein-derived lipids become scarce enough to enable monocytes to leave the plaque [30]. The emigration cells take with them their intracellular lipids, and their potential for secretion of unhelpful lipases, proteases, and tissue factor [31]. In addition, removal of necrotic, debris, calcifications and fibrosis also occurs [32–35], facilitated by new, normally functioning macrophages. Furthermore, some acceptor particles might play a role in the mobilization of cholesterol [36]. In addition to these various mechanisms, it has been demonstrated that renin-angiotensin system might also be involved in the plaque regression [37].

Study limitations

First, the number of the rabbits examined was relatively small, although the WHHL rabbits were raised with genetic uniformity. Second, despite careful observation of water balance, the rabbits tended to have dehydration especially in the follow-up period, so that the areas of lumen as well as vessel area might be underestimated. Especially in the statistics for the relationship between the change in LDL-C level and the change of tissue components, the limitation of under-powered statistics should be considered.

Conclusions

The present study using serial IVUS and histological examination with WHHL-MI rabbits revealed that pitavastatin attenuated atherosclerotic plaque area, and that it induced plaque stabilization as well as its anti-inflammatory change. These data would provide more genuine evidence with a less-confounding animal model regarding the mechanism of statin in its preventive and/or regressive effect on atherosclerotic plaque.

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Disclosure of conflict of interest

Kowa Pharmaceutical gave a bulk powder of pitavastatin. However, this company was not involved in designing and achieving this study at all. Investigators independently made the decision on the study design and database maintenance, wrote the manuscript, and decided to submit the article. Dr Hiro and Dr Hirayama received honoraria for lectures from Kowa Pharmaceutical. Dr Hiro and Dr Li also worked at the endowed chair donated by Boston-Scientific Japan Co. Ltd at Nihon University School of Medicine. The other authors declare that there is no conflict of interest.

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