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Evaluation of Regression of Advanced Carotid Atherosclerotic Plaques

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Objective Recent studies have determined evidences of effects of hypolipidemic drug on atherosclerotic plaque as well as marked change in composition that affects plaque stability. In particular, high dose statin treatment plays an important role in regression of advanced atherosclerotic plaque. In this study, we aimed to investigate the relationship between the disruption of atherosclerotic plaque and inflammatory molecules and propose the reliable guideline of therapy of advanced (calcified) carotid atheroma.

Methods The outpatients of 23 who had advanced carotid atherosclerotic plaque were selected from the neurology outpatient clinic and were treated statin, aspirin, and clopidogrel for secondary prevention for a year. All participants were measured carotid artery intima-media thickness, plaque morphology, and thickness of carotid plaque by an external B-mode ultrasound and were assessed express P-selectin expression (CD62p), platelet-leukocyte aggregation, CD11b and CD40L by flow cytometry and by the platelet function analyzer.

Results The outpatients of 23 were more than 60 years old (69.8±6.9, male=16, female=7). The average of carotid artery intima-media thickness was 0.93 mm at left and 0.91 at right. We found the significant fact that the number of total plaques in all subjects was 83. The distribution of plaques in different arterial segments was: common carotid artery, 21.7%; bulb, 54.2%; and internal carotid artery, 24.1. 77.1% (n=64) of total plaques were heterogeneous with hyperechoic morphology and 14.5% (n=12) of those were hypoechoic plaques in echogenicity. High sensitivity C-reactive protein of participants was 1.9±2.3. The difference of carotid atherosclerotic plaque thickness before and after intensive statin treatment was 2.6±0.8 and 2.4±0.7 (p=0.001). The level of CD11b and CD40L in monocyte decreased significantly after high dose statin therapy.

Conclusions These findings support the central role of statin to reduce plaque thickness and the level of inflammatory markers. Intensive statin therapy is crucial for patients with advanced atherosclerotic plaques in carotid artery. Thus, it is urgent to establish optimal dose of stain for each patient without undergoing adverse reactions. **Vascular Neurology 2011;3:45-50**

Key Words Atherosclerosis, Calcified carotid artery atheroma, Circulation inflammatory molecules, Stroke.

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Introduction

Although there has been a raising awareness of the importance of stroke as aging is increasing, mechanism of stroke is less clear. However, it has been assured that cerebrovascular accident due to rupture of a vulnerable atherosclerotic plaque is the most common cause of stroke.[1,2] Approximately 87% of stroke in the United States are ischemic, about 10% are intracerebral hemorrhages.[3] In Korea, from 70% to 80% of stroke are ischemic and most of the population aged 50 and over have atherosclerotic plaque.[4] Those facts of Korea and the ultrasound (US) sustained the assumption that vulnerable plaque rupture triggers stroke.

So far, stroke has been depended on the primary treatment[5] but lots of clinical trials supporting the efficacy of HMG-CoA reductase inhibitors (statins) have been conducted since its launch.[6-10] As a result, numerous findings reported that statin therapy plays a remarkable role to reduce the accumulation of atheroma[11-20] and the level of circulating inflammatory marker.[21-27] Nevertheless, there is little data and guideline on efficiency of statin treatment in patient with calcified or advanced plaques. Also, little is known about the natural course of an advanced plaque.

In reality, patients with calcified or hyperechoic carotid atheroma came to the tertiary hospital, so most clinical doctors are confused what is the best treatment between medication and operation. Korea Food and Drug Administration recommended

statin use when high sensitivity C-reactive protein (CRP) is more than 0.2 mg/dL in serum without approved dose. Food and Drug Administration (FDA) of the US has warned about high-dose statin because of side-effects such as severe myopathy, muscle pain, kidney damage, etc.[28] Thus, FDA strictly has approved statin of 10 to 20 mg as the general does.

Therefore, this study performed to evaluate the hypothesis that thickness of plaque and the level of circulating inflammatory molecules in patients with carotid atherosclerosis would highly decrease under receiving statin therapy and to propose the reliable guideline of treatment of calcified carotid atheroma. We also analyzed the regression of calcified carotid atheroma monthly under statin medication.

Materials and Methods

Study design and subjects

This study performed a prospective observational study and enrolled 27 patients who had carotid atherosclerotic plaque was selected from the neurology outpatient clinic of Yonsei University hospital and was treated with statin, aspirin, and clopidogrel for secondary prevention for a year. Participants were detected advanced or calcified plaques that ranged more than 3 mm in plaque thickness.

Exclusion criteria included operation or angioplasty for symptomatic stenosis performed within 3 months or planned for the future, known allergy against or previous treatment with aspirin, clopidogrel, and statin therapy, bleeding history, severe kidney or liver disease, cancer, acute myocardial infarct, and thyroid disease. Twenty-seven subjects were identified by clinical data record and were willing to participate. Of these, 4 patients were excluded because of terminal stage cancer (n=2) and end stage renal disease (n=2). Therefore, 23 subjects were entered into the study. Characteristics of the participants were shown in Table 1. All participants provided written informed consent and the study protocol was approved by the Institutional Review Board of the Yonsei University Hospital.

Table 1. Clinical and demographic characteristic of the study population

Variables	Case group (n=23)
Age (y)	69.8±6.9
Male % (n)	69.6 (16)
Female % (n)	30.4 (7)
Body mass index	24.3±2.7
Current smoking, % (n)	8.7 (2)
Current drinking, % (n)	17.3 (4)
History of diabetes mellitus, % (n)	8.3 (2)
History of hypertension, % (n)	16.7 (4)
History of stroke, % (n)	16.7 (4)
History of transient ischemic attack, % (n)	66.7 (16)
History of carotid artery atherosclerosis, % (n)	83.3 (20)
Total carotid plaques, (n)	83
Carotid artery plaques, (n)	3.8±1.3
Common carotid plaque, % (n)	21.7 (18)
Bifurcation (bulb), % (n)	54.2 (45)
Internal carotid artery, % (n)	24.1 (20)
Lt carotid artery ID, mm	8.1±0.8
Rt carotid artery ID, mm	8.2±1.0
Lt carotid artery IMT, mm	0.9±0.2
Rt carotid artery IMT, mm	0.9±0.2
Systolic blood pressure	126.3±10.9
Diastolic blood pressure	75.3±10.4
Fasting glucose, mg/dL	104.0±27.2
Total cholesterol, mg/dL	144.7±32.5
High density lipoprotein, mg/dL	46.4±11.6
Low density lipoprotein, mg/dL	74.4±28.1
Triglyceride, mg/dL	117.5±31.5
High sensitivity CRP, mg/dL	1.9±2.3

Data are shown as mean±SD or number of subjects, frequency (%). ID: inner diameter, IMT: intima-media thickness.

Basic investigation

All participants were measured carotid artery intima-media thickness (CIMT), plaque morphology, and thickness of carotid plaque by B-mode ultrasonography and were assessed the level of circulating inflammatory markers such as express P-selectin (CD62p), platelet-monocyte complexes, CD40 ligand (CD40L) and Mac1 every month by flow cytometry and by the platelet function analyzer. All subjects answered a questionnaire of their medical history, smoking habits and medication. Recordings of height and weight were performed and sitting brachial blood pressure (BP) was measured using a validated automatic BP device (JMW160KA, Seoul, Korea).

Serum glucose, total and high density lipoprotein cholesterol, triglycerides and low density lipoprotein cholesterol were measured with fasting. Subjects visited to the neurology outpatient clinic monthly and were inquired about potential reverse effects and received a physical examination.

Pre-study for determination the maximum therapeutic dosage of a statin

FDA of South Korea recommended statin use when high sensitivity CRP is more than 0.2 mg/dL in serum but there is no approved statin dose and no data for the maximum dose of that. Therefore, we had to perform an observational study to determine the maximum therapeutic dosage of a statin. We randomized outpatients with atherosclerosis who visited neurology outpatient. They provided written informed consent and the study protocol was approved by the Institutional Review Board of the Yonsei University Hospital. We prescribed randomized outpatients statin of 20 mg/day (mg/d) at first. They were asked about potential side effects and received a physical examination

including laboratory tests. If they had no side-effects or progressed the thickness of atheroma for 2 months, we increased gradually statin dose of 20 mg/d. We maintained statin of 60 mg/d in women and 80 mg/d in men as a maximum dose. They did not undergo any adverse effects and we decided to provide men statin of 80 mg/d and women statin of 60 mg as the maximum therapeutic dosage.

In this study, men in subjects took aspirin (100 mg/d), clopidogrel (75 mg/d), and statin (80 mg/d) and women took aspirin (100 mg/d), clopidogrel (75 mg/d), and statin (60 mg/d) for a year. Intake of study drugs was controlled by counting of delivered tablets.

B-mode ultrasound examination

Carotid artery plaque was assessed by high-resolution B-mode ultrasound (Accuvix V10, Seoul, Korea) using a system with a multi-frequency 5 to 10 MHz linear-array transducer. All measurements were performed by a technologist trained in ultrasound research according to a standard scanning and reading protocol. The CIMT was measured offline on the far wall of the common carotid artery (CCA) in a longitudinal view in a region free of plaque using a computerized system. The upper limit of normal for the intima-media thickness (IMT) was defined as 1.0 mm, and lesions with an IMT ≥1.1 mm were defined as atheromatous plaques, which were measured everywhere.

Plaque was also defined as normal with 1.0 mm ≥plaque thickness, mild to moderate with that of 1.0 mm to 3.0 mm, and moderate to severe with that more than 3 mm.

Maximum CIMT measures were obtained from walls of three arterial segments of both carotid arteries; the near and far wall of the proximal 10 mm of the internal carotid artery (ICA), the near and far wall of the carotid bifurcation beginning at the tip of the flow divider and extending 10 mm proximally, and the near and far wall of the arterial segment extending 10 to 20 mm proximally to the tip the flow divider into the CCA.[29] Thickness of calcified carotid atheroma was measured in a longitudinal and vertical view for screening accurate plaque. Color Doppler and pulsed Doppler were also used to assist in confirmation of possible plaques.

Plaque echogenicity was assessed by the gray scale median (GSM) using the histogram function. Plaque morphology was classified by GSM value, which was calculated from pixel analysis within in the range 0-256. Plaques were defined as hypoechoic if GSM value was lower than 70, as hyperechoic or calcified if GSM was higher than 70, and as heterogenous if echogenicity was mixed hypoechoic with hyperechoic.[30]

Assessment of platelet and leukocyte activation

A trained nurse collected blood samples twelve times throughout the study. Blood samples were collected into Vacutainer tubes containing 0.5 mL of 3.2% buffered sodium citrate. Immediately after that the citrated blood sample was added to a fixation solution (4% formaldehyde) to minimize *ex-vivo* platelet activation and then was prepared as below for flow cytometry.

The whole blood samples were used to assess platelet activity. Whole blood resuspended in Tyrode's solution was incubated with phycoerythrin (PE)-conjugated anti-CD41a to immunologically identify platelets. The samples were simultaneously incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD62P at saturating concentrations for 15 min at room temperature in the dark. Platelet-bound anti-CD62P was determined by analyzing 5,000 platelets for FITC fluorescence. Results were expressed as a percentage of antibody-positive platelets.

The red blood cell-lysed blood samples were used to determine Mac-1 expression in leukocytes and evaluate platelet-leukocyte interaction. Granulocytes were identified by forward and sideward scatter properties of PE-CD45 fluorescence positive leukocytes. Monocytes and lymphocytes were identified by strong expression of PE-CD14 and PE-CD162, respectively. To observe the Mac-1 expression in leukocyte subsets, the samples were simultaneously incubated with CD11b-FITC for 20 min at room temperature in the dark. Platelet-leukocyte subset aggregates (platelet-granulocyte, -monocyte, and -lymphocyte aggregates) were recorded with simultaneous detection of CD42b-FITC labeled platelets. As isotype control experiments, FITC-conjugated IgG antibody was used. A minimum of 5,000 cell events were analyzed in each assay. The results of Mac-1 were expressed as the mean fluorescence intensity of antibody-positive leukocytes for each leukocyte subgroup. The percentage of platelet-granulocyte, -monocyte, and -lymphocyte complexes were calculated.

The blood samples were analyzed on the flow cytometer within three hours of venepuncture. Flow cytometric specimens were kept before analysis at 0-4°C and analyzed using FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA).

Statistical analysis

Characteristics and plaque thickness of participants are presented as means±SD and results as means. The regression of carotid artery plaque was compared using the paired t-test or Wilcoxon signed rank test. Probability values of $p<0.05$ were considered statistically significant (Statistical Package for the Social Sciences, Windows version 15.0, Chicago, IL, USA).

Results

Twenty-three subjects were included in this study. We analyzed plaque regression monthly between Dec 2009 and Nov 2010. The population of subjects were over aged 60 (69.8±6.9, male=16, female=7). In terms of laboratory data, triglyceride and high sensitivity CRP was 117.5±31.5 and 1.9±2.3. The average of carotid artery IMT was 0.93 mm at left and 0.91 at right. The number of total plaques in all subjects was 83 (3.8±1.3). The distribution of plaques in different arterial segments was: CCA, 21.7%;

Table 2. Comparison of carotid plaque thickness and echogenicity before and after statin treated

Variables	Total plaques (n=83) % (n)	Maximum (mm)	Mean (mm)	*p* value
Before statin treatment (baseline)			2.6±0.8	0.001
Lt CCA	16.9 (14)	4.9	2.8±1.0	
Lt Bulb	27.7 (23)	3.9	2.9±0.7	
Lt ICA	15.7 (13)	2.8	2.1±0.6	
Rt CCA	4.8 (4)	3.1	2.4±0.5	
Rt Bulb	26.5 (22)	3.8	2.5±0.7	
Rt ICA	8.4 (7)	3.4	2.3±0.6	
Echogenicity				
Hypoechoic (echolucent)	14.5 (12)			
Hyperechoic	8.4 (7)			
Heterogenous, mixed	77.1 (64)			
After statin treatment (follow-up)			2.4±0.7	0.001
Lt CCA	16.9 (14)	4.5	2.6±1.0	
Lt Bulb	27.7 (23)	3.9	2.7±0.7	
Lt ICA	15.7 (13)	3.5	2.1±0.7	
Rt CCA	4.8 (4)	3.0	2.1±0.7	
Rt Bulb	26.5 (22)	3.4	2.3±0.5	
Rt ICA	8.4 (7)	3.3	2.1±0.6	
Echogenicity				
Hypoechoic (echolucent)	15.7 (13)			
Hyperechoic	12.0 (10)			
Heterogenous, mixed	72.3 (60)			

Data are shown as mean±SD or number of plaques, n (%); thickness of plaques, mm. $p<0.05$ by Wilcoxon signed rank test for all comparisons between before and after statin. CCA: common carotid artery, Bulb: bifurcation, ICA: internal carotid artery, ECA: external carotid artery.

Table 3. Difference of plaque thickness between pre and post-statin treatment

	t	Dif	Sig. (2-tailed)
Before statin-After statin	3.601	82	0.001

p values by paired t-test, *p* values<0.05 were considered as significant.

bulb, 54.2%; and ICA, 24.1. Subject characteristics are detailed in Table 1.

There were no significant differences between before and after statin treated in carotid plaque thickness and echogenicity. The difference of carotid atherosclerotic plaque thickness between before and after intensive statin therapy was 2.6±0.8 and 2.4±0.7 (p=0.001) (Table 2 and 3). The number of echogenicity was: echolucent, 14.5%; hyperechoic, 8.4%; and heterogeneous with hyperechoic morphology, 77.1 in baseline and echolucent, 15.7%; hyperechoic, 12.0%; and heterogeneous with hyperechoic morphology, 72.3 in statin use.

Table 3 showed that plaque thickness of pre-statin is contrasted with that of post-statin. The change of plaque thickness in pre and post-statin therapy was shown in Fig. 1. There was a considerable downward trend in plaque thickness between before and after statin treatment. Plaque thickness of all carotid artery segments except Lt ICA decreased significantly after statin therapy. The reduced width of plaque thickness in CCA and

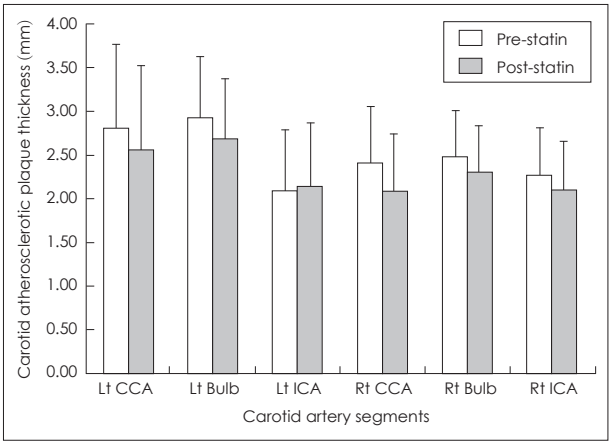

Figure 1. The change of plaque thickness before and after statin therapy. CCA: common carotid artery, Bulb: bifurcation, ICA: internal carotid artery, ECA: external carotid artery.

bulb is much higher than that in ICA.

The level of inflammatory molecules monthly was shown in Fig. 2. The level of CD11b-monocyte, CD40L-monocyte and CD40L-granulocyte fluctuated for early 9 months. From September to December, there were significant reductions in the level of CD11b-monocyte, CD40L-monocyte and CD40L-granulocyte. There was no considerable difference in the level of CD11b-granulocyte, platelet-leukocyte aggregates (PMA) from

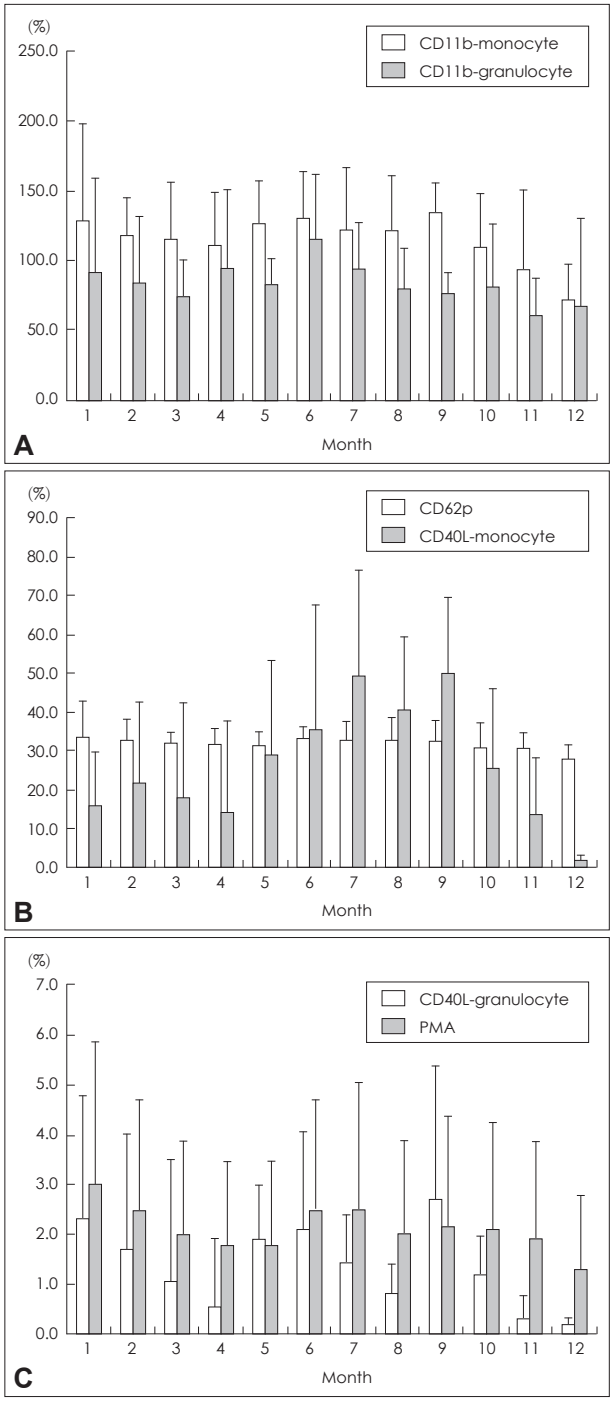

Figure 2. The level of CD11b-monocyte and CD11b-granulocyte (A). The level of CD62p and CD40L-monocyte (B). The level of CD40L-granulocyte and PMA (C). PMA: platelet-leukocyte aggregates. CD 62p: P-selectin expression.

January to December. The level of express P-selectin (CD62p) showed steady pattern during the experiment.

Discussion

There is a well recognized need for clinically applicable medication to monitor plaque thickness and circulation inflammatory markers in the management and prevention of stroke. The administration of statin was well tolerated and safe in our patients during the treatment period of one year. Serious adverse reactions were not occurred related to statin use. The main hypothesis of this study was that statin may reduce the level of circulating inflammatory biomarkers and plaque thickness in patients with calcified (advanced) atherosclerotic plaque.

We monitored the thickness of calcified plaque in pre-statin treatment and in post-statin treatment during one year (Fig. 1). There was a remarkable decrease in calcified plaque thickness unlike the previous studies,[21,22] although which is different from the subjects of our study, found no difference in Coronary artery Calcification progression in 80 subjects treated with 80 mg of simvastatin versus placebo and in 366 asymptomatic patients randomized to either 10 or 80 mg of atovastatin over 12 months. We need more follow time to affect the plaque thickness and the inflammatory biomarkers for support this study convincingly.

In addition, the levels of circulating inflammatory molecules, such as CD11b-monocyte, CD40L-monocyte and CD40L-granulocyte decreased significantly, which corresponded to earlier studies[23-25] that did not coincide with the subject of this study. So, further researches aimed at patients with calcified plaque need to support this study convincingly.

There was non-considerable reduction in the level of CD62p and PMA unlike previous studies which reported that statin therapy reduces the level of CD62p expression and platelet-leukocyte adhesion in hypercholesterolemia subjects.[26,27] There is no evidence to explain why CD62p expression and PMA did not decrease in our patients. We speculated that the difference between subjects with calcified plaque and without would exist in the mechanism of the circulating inflammatory molecules. Thus, further investigation to subjects with calcified plaque requires. The problem also remains how best to treat patients with calcified atherosclerotic plaque without adverse effects. The clinical trials mentioned in this study provide substantial support for the institution of intensive statin treatment in diverse clinical setting. Nevertheless, the FDA has warned safety and adverse effects of intensive statin therapy and has recommended to prescribe low dose or moderate dose. If new solutions to solve known problems of high dose statin therapy are reconfirmed by the majority of clinical trials, the management for patients with calcified atherosclerotic plaque will be established.

Conclusion

These findings support the central role of statin to reduce plaque thickness and the level of inflammatory markers. Intensive statin therapy is crucial for patients with advanced atherosclerotic plaques in carotid artery. Thus, it is urgent to establish optimal dose of stain for each patient without undergoing adverse reactions.

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