

Statin Treatment, Carotid Atherosclerotic Plaque Macrophage Infiltration and Circulating Inflammatory Markers

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Abstract: *Background:* Statin treatment is considered as first line therapy in patients with atherosclerotic disease. We evaluated the effect of pre-treatment with statins on carotid plaque infiltration by macrophages and on the circulating levels of proinflammatory cytokines in patients who underwent carotid endarterectomy.

Patients and Methods: One hundred fourteen patients were enrolled; 89 men and 25 women (mean age 67±8 years; range 42-83 years). Fifty three patients (46%) were on statin treatment at least 3 months before endarterectomy and 61 (54%) had never received statin treatment. The serum levels of high sensitivity C reactive protein (hsCRP), serum amyloid A (SAA), tumor necrosis factor α (TNF α), interleukin (IL)-1 β and IL-6 were evaluated preoperatively. The intensity of macrophage infiltration was evaluated by immunohistochemistry, using the monoclonal antibody CD 68. The area of the plaque covered by macrophages was measured as a proportion of the whole plaque area, using a custom designed image tool analysis.

Results: Patients on statins had lower serum total cholesterol levels (172±50 vs 194±35 mg/dl, p= 0.014), lower low density cholesterol levels (103±44 vs 123±31 mg/dl, p= 0.010) and lower serum hsCRP levels (1.8 [1.1-3.4] vs 3.4 [1.3-4.9] mg/l, p= 0.03), while SAA, TNF α , IL-6 and IL-1 β levels did not differ between the 2 groups. The infiltration of atherosclerotic plaque by macrophages was similar in statin treated patients and in controls (0.55±0.15% vs 0.49±0.19%, p= 0.21).

Conclusion: Patients on statins have similar macrophage accumulation in their carotid atherosclerotic plaques compared with patients not on statins. Inflammatory markers were also similar in both groups except for hsCRP which was significantly lower in those taking statins.

Key Words: Statins, carotid endarterectomy, macrophages, high sensitivity C reactive protein, inflammation, cholesterol.

INTRODUCTION

The relationship between atherosclerosis and inflammation is well established [1-3]. Inflammation plays a role in plaque formation; even "early atherosclerosis", fatty streaks, consists of macrophage accumulation [4]. Inflammation also has a role in plaque destabilization and rupture [5,6] since macrophages and other inflammatory molecules are increased in symptomatic plaques [7].

Treatment with statins is considered as first line therapy in atherosclerotic disease [8-15]. Statins also exert anti-inflammatory effects [9-17].

The aim of this study was to evaluate the inflammatory burden in human carotid artery specimens as well as circulating inflammatory marker levels, in patients on statins and those not receiving these drugs.

PATIENTS AND METHODS

We evaluated patients who underwent carotid endarterectomy for significant (>70%) carotid bifurcation stenosis. The degree of stenosis was calculated using Digital Subtraction Angiography and the North America Symptomatic Carotid Endarterectomy Collaborators study [18] criteria of evaluation and measurement of carotid artery stenosis. The criteria for patient selection have been described [19].

All patients underwent a computerized tomographic brain study and were examined by the same neurologists. Based on data from history and clinical examination the patients were characterized as symptomatic or asymptomatic. Patients with amaurosis fugax, transient ischemic cerebral symptoms or ipsilateral ischemic stroke were characterized as symptomatic. The remaining patients were characterized as asymptomatic.

Initially, 119 patients scheduled to undergo carotid endarterectomy were evaluated. Five patients that began statin therapy less than 3 months before endarterectomy were excluded from the study. The remaining 114 consecutive pa-

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tients formed the study population. Fifty three (46%) of them were on statin therapy at least 3 months before endarterectomy. The other 61 (54%) patients had never received statins. The following statins were used: 26 (47%) patients were on atorvastatin (22 ± 11 mg/day), 16 (29%) on simvastatin (27 ± 10 mg/day), 8 (15%) on pravastatin (30 ± 12 mg/day), 2 (4%) on lovastatin (20 mg/day) and 1 (2%) was on fluvastatin (20 mg/day).

The study protocol was approved by the Red Cross Hospital Ethical Committee. Informed consent was obtained from all patients.

LABORATORY ANALYSIS

Blood samples were obtained from all patients the day before endarterectomy, after an overnight fast. The samples were centrifuged for 15 min at approximately 1000 g. Serum was removed, aliquoted immediately and stored at -70° C until tested. Part of the blood sample was used to measure common serum parameters and the lipid profile. Serum interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF α) concentrations were measured using quantitative sandwich enzyme immunoassay kits (Quantikine HS, R&D Systems, Minneapolis, USA). The lower limit of detection was <0.1 pg/ml for IL-1 β , 0.04 pg/ml for IL-6 and 0.12 pg/ml for TNF α ; high sensitivity C-reactive protein (hsCRP) and serum amyloid A (SAA) were assayed by high sensitivity particle enhanced immunonephelometry (N Latex, Date-Behring Marburg GmbH, Marburg, Germany). The lower limit of detection was 0.18 mg/l for hsCRP and 0.8 mg/l for SAA. Values below the lower detection limit were considered as equal with this limit in the statistical analysis; there were no missing values. The coefficient variations for the assays used were for IL-1 β , IL-6, TNF α , hsCRP and SAA 14%, 11%, 22%, 6% and 12%, respectively. Homocysteine levels were determined using an enzymatic assay for the Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN 46250, USA), as previously described [20].

CAROTID ENDARTERECTOMY

All carotid endarterectomy procedures were performed by 1 group of vascular surgeons at the Department of Vascular Surgery of the Red Cross Hospital in Athens, from June 2004 to May 2006. Immediately after carotid endarterectomy the specimen was transferred in 0.9% normal saline solution to the laboratory. Transverse sections 4 mm thick were prepared from each atherosclerotic lesion. The section containing the most stenotic part of the bifurcation was labelled 0, while the consecutive parts distally were labelled +1, +2, +3 etc and proximally -1, -2, -3 etc. Each part was embedded in a different paraffin block. Sequential transverse sections 4 μ m apart were obtained from each block and stained with haematoxylin and eosin. These sections were used for classification of the atherosclerotic plaque according to the American Heart Association (AHA) criteria [4].

IMMUNOHISTOCHEMISTRY

In order to measure macrophage infiltration of atherosclerotic plaques, we used monoclonal antibodies to a cell marker (CD68, Q-Bend Clone, Dako Corporation, Carpinteria, California, USA). Sequential sections 4 mm apart were evaluated from the atherosclerotic part of each specimen. The number of slides evaluated per patient was 4.1 ± 2.4

(mean \pm SD). The whole plaque area, excluding parts of the specimen without obvious atherosclerotic lesions, was evaluated at x400 magnification. Evaluation of the images was performed with the Image Tool analysis system (Image Pro Plus, Media Software, Cybernetics, Canada) by 2 independent examiners blinded to the clinical history, and the mean value was recorded. The results were expressed as the proportion of the whole plaque area stained with the monoclonal antibody CD 68.

STATISTICAL ANALYSIS

Continuous parameters are reported as mean \pm standard deviation, non parametric variables as median (interquartile interval) and categorical variables as percentages. The t-test or Mann-Whitney test was used for comparing continuous variables between 2 groups, as appropriate. Categorical variables were compared by the chi-square test or Fisher's exact test. The Spearman correlation test was used to assess relationships between variables. A $p < 0.05$ was considered significant.

RESULTS

Clinical and demographic characteristics of the study population are shown in Table 1. Statin treated patients had lower serum total cholesterol (176 ± 49 vs 202 ± 40 mg/dl, $p = 0.011$) and low density lipoprotein cholesterol (LDL-C 103 ± 46 vs 127 ± 34 mg/dl, $p < 0.001$) than patients not receiving these drugs. The 2 groups had no differences in AHA plaque classification and in procedural complications (data not shown).

hsCRP serum levels were significantly lower in patients on statins than in the controls (Table 2). The serum levels of SAA, TNF α , IL-1 β and IL-6 did not differ between the 2 study groups (Table 2).

Patients on statin treatment had no differences in the lesion area covered by macrophages compared with patients not receiving these drugs ($0.55 \pm 0.15\%$ vs $0.49 \pm 0.19\%$, $p = 0.21$, data not shown). The interobserver variability was moderate ($\kappa = 0.61 \pm 0.09$).

DISCUSSION

The main finding of this study is that patients on statins had the same degree of macrophage infiltration of their carotid atherosclerotic plaque with patients not receiving these drugs although serum hsCRP levels were significantly lower.

Several, but not all, studies favour the hypothesis that statins have beneficial effect on macrophage accumulation. Tahara *et al.* [21] using fluorodeoxyglucose positron emission tomography reported that simvastatin reduces the number of inflammation-rich atherosclerotic plaques compared with a placebo group. Crisby *et al.* [22] reported that patients pre-treated with pravastatin had less inflammation in their carotid arteries, compared with patients on placebo. Similar results were reported in monkeys, using simvastatin or pravastatin [23]. On the other hand, Verhoeven *et al.* [24] demonstrated that statin treated patients have increased amount of macrophages in their carotid endarterectomy specimens. However, in patients treated with atorvastatin, the increased amount of CD68 positive cells was not associated with increased protease activity [24]. Our findings are not consistent with either of these hypotheses, since we did

Table 1. Clinical and Demographic Characteristics of the Statin and Control Groups

	Statin Group	Control Group	p
n	53	61	
Age (years)	65.7 ± 9.0	67.0 ± 8.3	0.40
Duration of statin treatment (months)	20 ± 17		
Cardiovascular risk factors			
Male sex	42 (79%)	45 (74%)	0.64
Coronary artery disease	13 (25%)	6 (10%)	0.07
Arterial Hypertension	39 (74%)	49 (80%)	0.53
Diabetes mellitus	15 (28%)	14 (23%)	0.66
Current cigarette smoking	31 (58%)	38 (62%)	0.82
Medical treatment			
ACE inhibitors	22 (41%)	22 (36%)	0.69
ARBs	10 (19%)	13 (21%)	0.93
Beta-blockers	18 (38%)	14 (23%)	0.27
Calcium channel blockers	15 (34%)	27 (44%)	0.12
Diuretics	10 (19%)	21 (34%)	0.08
Clopidogrel	22 (42%)	24 (39%)	0.91
Aspirin	23 (43%)	29 (48%)	0.67
Diabetes mellitus (oral drugs)	12 (22%)	10 (16%)	0.61
Diabetes mellitus (insulin)	2 (4%)	3 (5%)	0.91
Presence/absence of symptoms			
Symptomatic	28 (53%)	31 (51%)	0.86
Amaurosis Fugax	1 (2%)	2 (3%)	0.93
Transient Ischemic Attacks	22 (42%)	23 (38%)	0.95
Ischemic Stroke	5 (9%)	6 (10%)	0.86
Asymptomatic	25 (47%)	32 (52%)	0.57
Serum parameters			
Total Cholesterol (mg/dl)	176 ± 49	202 ± 40	0.011
Low-density lipoprotein (mg/dl)	103 ± 46	127 ± 34	<0.001
High-density lipoprotein (mg/dl)	38 ± 6	42 ± 10	0.16
Triglyceride (mg/dl)	159 ± 109	154 ± 66	0.86
Apolipoprotein A (mg/dl)	111 ± 21	118 ± 24	0.23
Apolipoprotein B (mg/dl)	89 ± 30	96 ± 26	0.11
Lipoprotein (a) (mg/dl)	48 ± 52	41 ± 29	0.87
Homocysteine (µmol/l)	10.4 ± 3.7	13.0 ± 5.1	0.07
Creatinine (mg/dl)	1.00 ± 0.44	1.03 ± 0.47	0.58

ACE, Angiotensin converting enzyme; ARBs, Angiotensin receptor blockers.

not demonstrate any difference in macrophage infiltration between patients receiving statins and those not on these drugs.

hsCRP was lower in patients treated with statins, while the levels of SAA, TNF α , IL-1 β and IL-6 were not different between the 2 groups. Statin-induced reduction in hsCRP

Table 2. Serum Inflammatory Markers in Statin Treated and Control Patients with Carotid Bifurcation Disease

Parameter	Statin Treated	Controls	P
IL-6 (pg/ml)	2.5 [1.8-4.1]	2.9 [1.9-5.2]	0.24
TNF- α (pg/ml)	1.3 [1.0-2.5]	1.2 [0.7-2.0]	0.74
IL-1b (pg/ml)	0.11 [0.06-0.41]	0.12 [0.06-0.43]	0.73
SAA (mg/l)	10.3 [4.6-22.1]	11.2 [3.8-23.6]	0.48
hsCRP (mg/l)	1.8 [1.1-3.4]	3.4 [1.3-4.9]	0.03

IL-6, interleukin 6; TNF- α , tumor necrosis factor- α ; IL-1b, interleukin 1b; SAA, serum amyloid A; hsCRP, high sensitivity C-reactive protein.

levels have been demonstrated in many studies in different patient populations [25-28]. The same is also true for SAA [29] although we did not observe this effect.

Statins are potent anti-inflammatory agents [2, 30-34]. The effect of statins on levels of inflammatory markers was evaluated in a group of hypercholesterolemic patients (n=68) [35]. Compared with diet alone, an 8-week regimen with atorvastatin plus diet recommendation was associated with a significant reduction in plasma levels of TNF α (2.9% vs 21.4%, respectively; p<0.0001), IL-6 (2.0% vs 22.1%, respectively; p<0.0001) and IL-1 (2.7% vs 16.4%, respectively; p<0.0001) [35]. A similar association between statin use and reduction of TNF α and IL-10 plasma levels was recently reported in patients with calcific aortic valve stenosis [36].

STUDY LIMITATIONS

This was a prospective cross sectional study comparing patients receiving statins for more than 3 months with patients who never received these drugs. The different statins, with varying dosage and treatment duration is a limitation. The possible effect of other drugs (like angiotensin converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers and others) on macrophage accumulation also needs to be considered. The antibody CD68 used to validate macrophages is a non-selective macrophage marker and cannot express the complicated functional status of the macrophages [24]. We also do not know if more aggressive LDL-C lowering would change the results.

CONCLUSIONS

Patients on statins have no differences in macrophage accumulation in carotid atherosclerotic plaques compared with patients who never took these drugs. Inflammatory markers were similar in both groups except for hsCRP which was significantly lower in those individuals taking statins.

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