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Galectin-3, Carotid Plaque Vulnerability, and Potential Effects of Statin Therapy

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WHAT THIS PAPER ADDS

This study aims to investigate the role of the novel biomarker galectin-3 in carotid vulnerability in patients with high grade carotid stenosis. Galectin-3 may help identifying carotid plaques prone to rupture and cerebral embolization. This study aims also to investigate the impact of statin therapy on galectin-3 levels. This relationship may provide additional information regarding the mechanism by which statins induce carotid plaque stability.

Objectives: Galectin-3, a member of galectines, a family of β-galactoside-specific lectins, has been reported to propagate vascular inflammation. The role of galectin-3 in carotid atherosclerosis is controversial. The aim of this study was to investigate the relationship of galectin-3 with plaque vulnerability in patients with high grade carotid stenosis.

Methods: This was a cross sectional study of patients undergoing carotid endarterectomy (CEA). Carotid plaques obtained from 78 consecutive patients (40 symptomatic [SG], 38 asymptomatic [AG]) undergoing CEA were histologically analyzed for galectin-3, macrophages (CD68) and laminin. Pre-operatively the biochemical profile and plaque echogenicity (gray-scale median, GSM) score were determined.

Results: There were no significant differences in clinical and demographic parameters between SG and AG ($p > .05$). The SG had a lower GSM score (44.21 $\pm$ 18.24 vs. 68.79 $\pm$ 28.79, $p < .001$) and a smaller positive stained area for galectin-3 (4.89 $\pm$ 1.60% vs. 12.01 $\pm$ 5.91%, $p < .001$) and laminin (0.88 $\pm$ 0.71% vs. 3.46 $\pm$ 2.12%, $p < .001$) than the AG. On the other hand, intra-plaque macrophage content was increased in SG ($p < .001$). For the whole cohort, symptomatic status was independently associated with intra-plaque contents of both galectin-3 (OR = 0.634, $p < .001$), and GSM score (OR = 0.750, $p < .001$). Notably, patients on long term statin treatment had elevated galectin-3 and lowered macrophage intra-plaque concentrations compared with those on short term treatment ($p < .05$).

Conclusions: A low galectin-3 intra-plaque concentration seems to correlate with clinically and ultrasonically defined unstable human carotid plaques. Long term statin treatment may induce increase of intra-plaque galectin-3 concentration mediating plaque stabilization.

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INTRODUCTION

Stroke is one of the leading causes of mortality and disability in the Western world. It is well established that carotid atherosclerosis significantly predisposes to cerebral ischemic events, and mounting evidence correlates carotid plaque texture with its stability.[1] Nowadays, a family of β-galactoside-specific lectins, known as galectins, is recognized as specific oligosaccharide structures or ligand glycoproteins or glycolipids on the membranes of neighboring cells or in the extracellular matrix (ECM).[2] Galectin-3, a prominent member of that family has been reported to propagate vascular inflammation by inducing the expression of pro-inflammatory mediators in macrophages and the migration of monocytes into vascular walls.[3] Despite the known interplay between atherosclerosis and inflammation, the contributory role of galectin-3 in atherogenesis progression is still controversial.[4,5] Moreover, limited evidence disputes the potential role of galectin-3 in plaque destabilization.[6,7]

The primary aim of this study was to investigate the relationship between galectin-3 and ipsilateral neurological symptoms and ultrasound index of carotid plaque

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vulnerability. The hypothesis that long-term statin therapy could favorably change concentrations of galectin-3 within atherosclerotic plaques excised from patients undergoing carotid endarterectomy (CEA) was also tested.

METHODS

Patients selection

Seventy eight consecutive patients with high grade carotid stenosis, undergone CEA in the Department of Vascular Surgery, "Attikon" University General Hospital, were enrolled from January 2012 to October 2013. Written informed consent from each patient was obtained before enrollment and all procedures were performed according to the principles of Helsinki Declaration and were approved by the hospital human ethics committee.

Enrolled patients were assigned into either the symptomatic group (SG) with at least intermediate stenosis (>50%) or the asymptomatic group (AG) who had high grade (>70%) stenosis of the internal carotid artery (ICA). Patients with cerebral hemorrhage, severe liver or renal impairment, heart failure (NYHA I-IV) and disorders commonly associated with probable sources of cardioembolism, such as atrial fibrillation and cardiac valve abnormalities were excluded. Patients with concurrent conditions/diseases interfering with the expression of inflammatory mediators, like major trauma, surgery, cardiovascular ischemic events within the recent 1 month, malignancies, chronic inflammatory, autoimmune diseases, and patients with acute infection at the time of enrolment were also ineligible.

Clinical examination

Clinical parameters, such as body mass index (BMI), waist hip ratio (WHR), and blood pressure (BP) were obtained. In particular, the BMI was calculated using the following equation: BMI = weight (kg)/height $(m)^2$. Waist circumference was assessed midway between the lower rib margin and the iliac crest. The hips were measured at the level of the greater trochanter. Thus, WHR expressed waist circumference divided by hip girth. BP was measured twice after 15 minutes rest in a sitting position, and the mean value was estimated. The percentage of body fat mass was also measured using the body composition analyzer (Bodystat 1500, Bodystat Ltd, Isle of Man, British Isles).

Moreover, current medications and co-morbidities, including hypertension (blood pressure ≥140/90 mmHg or use of antihypertensive agents), smoking status, diabetes (fasting plasma glucose [FPG] ≥126 mg/dL or use of antidiabetic medications) and established coronary artery disease (history of myocardial infarction or percutaneous coronary intervention or coronary artery bypass) were documented through a structured questionnaire.

Carotid ultrasound examination

Carotid ultrasound examinations were performed by two experienced operators, with the use of a linear array 12 MHz ultrasound transducer (General Electric LogiqE, Riverside, CA, USA). Peak systolic velocity (PSV) and ICA/common carotid artery PSV ratio were calculated and thereafter the percentage of arterial stenosis was graded according to the recommendations of the Society of Radiologists in Ultrasound.[8]

Ultrasound image driven characterization of carotid plaque vulnerability and gray scale median (GSM) evaluation has been in a spotlight of extensive investigation for decades.[9] In this study, the GSM score was estimated from digitized B-mode ultrasound image recordings of a longitudinal section of the carotid artery. Specifically, for each patient, images were recorded at a minimal depth (4 cm) without magnification (zoom). The recorded images were assessed to identify the one with the best quality, which was, then, transferred to a computer. A GSM measurement of the plaque area was automatically obtained using in house software developed in Matlab (version 2012b; MathWorks, Natick, USA),[10] which estimates the GSM score following a standardized approach proposed by Elatrozy et al.[11] and has been commonly used by the scientific community. The technicians doing the image analyses were unaware of the clinical status of participants. For symptomatic patients, the GSM score of the culprit lesion, ipsilateral to the brain infarct was recorded, whereas in asymptomatic patients the average value of GSM of all plaques causing stenosis greater than 50% in both carotids was considered.

Histopathological analysis of carotid plaques

Surgically removed carotid plaques were collected for histopathological analysis. Plaques were fixed in 10% neutral buffered formalin and were sectioned in 0.5 cm blocks. All sections of the plague were embedded in a single paraffin block in order for a single section to contain the whole length of the plague, including areas of maximum stenosis and sites at equal intervals across the whole plaque length. Three consecutive 3 μm sections were used for antibody staining.[12] Immunohistochemistry was performed on an automated sample carrier (BondMax, Leica Biosystems, Wetzlar, Germany). Primary antibodies against CD68 (clone PGM-1, 1:100, Thermo Scientific, MI, USA), galectin-3 (clone 9C4, 1:150, Leica Biosystems, Newcastle, UK), and laminin (clone 4C7, 1:30, Dako, Glostrup, Denmark) were incubated after citrate pre-treatment. A commercially available horseradish peroxidase/diaminobenzidine (DAB) detection system (Bond Polymere Refine, Leica Biosystems) was used.[13] Instead of subjectively selecting areas to measure staining, the entire slide was scanned using PathSight (Fairfield Imaging) on a Leica DMBL microscope. The positivity percentage of each marker was measured using ImagePro Plus as the percentage of positive stained area to the whole hematoxylin stained area. Selection of the stained area was based on automated color selection of brown for DAB positive cells, cyan blue for hematoxylin positive DAB negative cells, subtracting areas of background staining, and artifacts caused by folding of paraffin sections.

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Blood assays

Fasting blood samples for FPG were obtained between 8:00 and 10:00 a.m. and lipid parameters were all measured in an automatic enzymatic analyzer (Olympus AU560, Hamburg, Germany). For multiplex bead based assays, serum and plasma were collected after a centrifugation at 2,500 rpm for 10 minutes and frozen at −80 °C until analysis. Levels of galectin-3 were measured in serum, while levels of high sensitivity C-reactive protein (hsCRP) were measured in plasma. Bead assays were performed according to the manufacturer's protocol (MilliPore, Billerica, MA, USA) and were analyzed on a Luminex 200 platform (Luminex Corp, Austin, TX, USA).

Statistical analysis

Normality of distribution was assessed with Kolmogorov–Smirnov test. Results of normally distributed continuous variables are expressed as the mean value ± SD. Continuous and categorical variables were compared using the Student *t* test and chi-square test, respectively. A two tailed *p*-value <.05 was considered to be statistically significant. Pearson's correlation coefficient was calculated to determine the strength of the association between the continuous characteristics. Variables showing a significant correlation with galectin-3 were then entered in a standard multiple linear regression analysis after adjustment for age, to check for independent associations. Logistic multiple regression analysis was also performed to find the independent determinants of symptoms. Owing to the low number of patients for many comparisons, a type I error was avoided by selecting a lower significance level for multiple regression tests, for example, by rejecting the null hypothesis when $p < .01$ instead of $p < .05$. The computer software package SPSS (version 20.0; SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

RESULTS

Seventy eight patients with significant ICA stenosis were included in the study. Forty were symptomatic having suffered an ipsilateral ischemic stroke ($n = 17$), transient ischemic attack ($n = 20$) or amaurosis fugax ($n = 3$). The median time interval between the onset of neurological symptoms and surgery was 20 (interquartile range: 7–32) days. Patient demographics and co-morbidities are presented in Table 1. All participants were receiving statins for at least 1 week prior to CEA and were on an antiplatelet regimen (71 patients were on aspirin, 4 patients on clopidogrel due to aspirin intolerance and 3 patients on a dual antiplatelet regimen due to previous percutaneous coronary intervention). Symptomatic and asymptomatic patients did not significantly differ in the distribution of gender, diabetes, hypertension, and smoking habits ($p > .05$). Similarly, there was also no significant difference in age, BMI, fat mass, WHR, and the degree of carotid stenosis ($p > .05$). However, SG had a lower GSM score ($p < .001$) (Table 1) than AG.

Table 1. Clinical and carotid ultrasound parameters of symptomatic and asymptomatic patients undergoing carotid endarterectomy.

	Symptomatic patients ($n = 40$)	Asymptomatic patients ($n = 38$)	*p*
Men/women	27/13	28/10	.585
Age (years)	69 ± 9	72 ± 9	.738
Smokers, *n*	14 (35%)	16 (42.1%)	.320
Diabetes, *n*	12 (30%)	10 (26.3%)	.569
Hypertension, *n*	34 (87.5%)	32 (84.2%)	.701
CAD, *n*	16 (40%)	17 (44.7%)	.851
BMI (kg/m^2)	29.72 ± 4.65	29.05 ± 4.47	.712
Fat mass (%)	39.91 ± 5.89	38.45 ± 7.02	.398
WHR	0.941 ± 0.077	0.930 ± 0.071	.492
SBP (mmHg)	139 ± 16	135 ± 16	.501
DBP (mmHg)	85 ± 10	82 ± 7	.293
Carotid stenosis (%)	83.9 ± 9.8	87.2 ± 10.5	.789
GSM score	44.21 ± 18.24	68.79 ± 28.79	<.001

Note. Data are means ± SD. *n*, number of patients. BMI = body mass index; DBP, diastolic blood pressure; GSM = grayscale median; SBP = systolic blood pressure; WHR = waist hip ratio. A two tailed $p < .05$ was considered to be statistically significant.

As shown in Table 2, there were no significant differences in glycemic and lipid parameters between groups ($p > .05$). On the other hand, there were significantly higher levels of hsCRP in the SG than the AG group ($p = .002$). The intraplaque analysis showed a significantly higher content of macrophages ($p = .003$) within plaques obtained from symptomatic than asymptomatic patients. Conversely, SG had a smaller positive stained area for laminin ($p < .001$) and galectin-3 ($p < .001$) (Table 2 and Fig. 1).

Among symptomatic patients, subgroup analysis of patients already receiving long term statin therapy (>1 month) ($n = 24$) and those on short-term treatment (7–15 days preoperatively) ($n = 16$) was performed. Long term

Table 2. Values of biochemical and immunohistochemical variables in symptomatic and asymptomatic patients undergoing carotid endarterectomy.

	Symptomatic patients ($n = 40$)	Asymptomatic patients ($n = 38$)	*p*
TChol (mg/dL)	162.43 ± 35	166.13 ± 43.4	.936
HDL-C (mg/dL)	49.95 ± 14.63	41.13 ± 8.76	.138
LDL-C (mg/dL)	89.05 ± 29.73	103.40 ± 40.17	.919
TG (mg/dL)	109.95 ± 62.58	151.00 ± 76.16	.358
FPG (mg/dL)	142 ± 45	144 ± 47	.801
hsCRP (mg/L)	5.33 ± 1.23	3.96 ± 0.63	.002
WBC (cells/μL)	7705 ± 2399	7729 ± 1631	.069
Serum galectin-3 (ng/mL)	2.99 ± 2.47	4.43 ± 3.32	.601
Galectin-3 (%)	4.89 ± 1.60	12.01 ± 5.91	<.0001
Laminin (%)	0.88 ± 0.71	3.46 ± 2.12	<.0001
CD68 (%)	13.81 ± 8.03	9 ± 5.71	.003

Note. Data are means ± SD. TChol = total cholesterol; TG = triglycerides; hsCRP = high sensitivity C reactive protein; WBC = white blood cells count; %, positive stained area expressed as percentage of the total plaque size.

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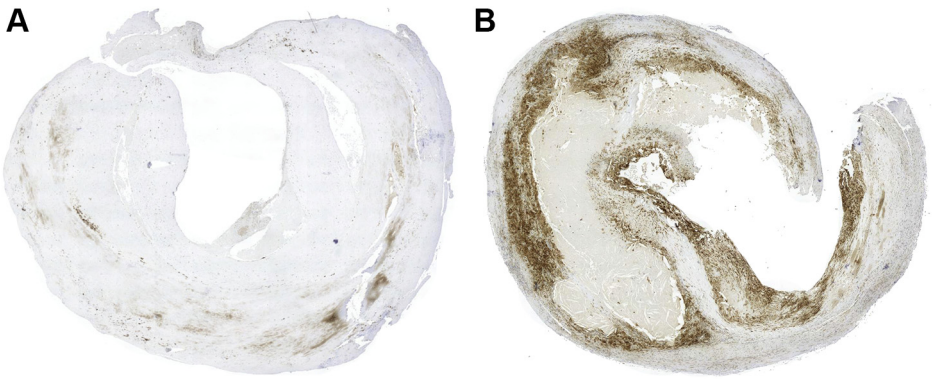

Figure 1. Representative histological sections of the human carotid plaques at the level of maximum stenosis of each lesion stained with galectin-3. (A) Symptomatic group; (B) asymptomatic group.

rather than short term statin therapy was associated with increased galectin-3 (5.85 ± 2.02% vs. 3.33 ± 1.12%, $p = .039$) and decreased relative macrophage (11.89 ± 5.15% vs. 17.22 ± 5.15%, $p < .001$) concentrations. Following similar analyses in the asymptomatic group, a significantly higher intra-plaque concentration of galectin-3 was observed in long-term (>1 month) statin-treated patients ($n = 26$) than in those receiving statins in the short term (7–15 days preoperatively) ($n = 12$) (13.1 ± 6.08% vs. 9.61 ± 4.28%, $p = .046$). With the exception of lower total cholesterol, low-density lipoprotein, and hsCRP circulating levels in the long term statin subgroups, the rest of variables did not differ between the above mentioned subgroups ($p > .05$) (data not shown).

Correlations

Correlations of both immunohistochemical and serum values of galectin-3, with the rest of the variables in human sample cohort were sought. In univariate analysis, carotid plaque galectin-3 content was significantly correlated with GSM score ($p < .001$), laminin ($p < .001$), and female gender ($p = .043$). In multivariate analysis, GSM score remained an independent determinant of galectin-3 concentrations (Table 3). Conversely serum galectin-3 levels significantly correlated with diabetes and hsCRP in univariate analysis. However, only diabetes showed independent correlation in multiple linear regression analysis (Table 4).

Finally, the authors investigated the independent determinants of ipsilateral cerebrovascular events. In single logistic regression analysis, age, history of CAD, serum hsCRP, low HDL, and GSM score, as well as intra-plaque contents of macrophages and galectin-3 were significantly associated with neurological symptoms in the whole study group ($p < .05$). After entering the latter variables into the multiple logistic regression analysis model, the independent association of ipsilateral cerebrovascular events with intra-plaque low content of galectin-3 (OR = 0.634, $p < .001$) and low GSM score (OR = 0.750, $p < .001$) was observed.

DISCUSSION

The current study demonstrated lower content of galectin-3 in symptomatic versus asymptomatic carotid plaques. Most importantly, the incidence of cerebrovascular events was independently determined by low levels of intra-plaque galectin-3 and a low GSM score. Long term, pre-operative therapy with statins was associated with higher intra-plaque galectin-3 levels than short term therapy.

The majority of animal studies have consistently indicated the correlation between galectin-3 and atherosclerosis development and its co-localization with inflammatory cells within atherosclerotic lesions.[14] Contrary to the proposed pro-atherogenic/pro-inflammatory action of galectin-3, Iacobini et al.[4] demonstrated the opposite effects of galectin-3, since in its genetically depleted mice there were more extensive and complex atherosclerotic lesions than in wild type mice. The potential contradictory actions of galectin-3 have been also outlined by two recent histopathological studies in human carotid plaques. The first one examined 21 carotid plaques

Table 3. Standard multiple regression analysis of carotid plaque galectin-3 content (dependent variable) and other independent variables, after adjustment for age.

	Carotid plaque galectin-3 content		
	β	95% CI	p
GSM score	0.620	0.487 to 0.761	<.001
Laminin	0.401	0.101 to 0.689	.069
Female gender	0.132	−0.011 to 0.291	.149
Age	0.339	−0.172 to 0.558	.455

Note. Because of the small number of patients statistical significance was defined as $p < .01$. CI = confidence interval; GSM = grayscale median score.

Table 4. Standard multiple regression analysis of serum galectin-3 levels (dependent variable) and other independent variables, after adjustment for age.

	Serum galectin-3 levels		
	β	95% CI	p
Diabetes	0.626	0.514 to 0.771	<.001
hsCRP	0.316	0.205 to 0.503	.041
Age	0.192	−0.023 to 0.306	.344

Note. Because of the small number of patients statistical significance was defined as $p < .01$. CI = confidence interval; GSM = gray-scale median score.

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and showed a remarkable upregulation of galectin-3 expression and protein levels in unstable rather than stable regions in the same patient.[7] In contrast, a second study questioned the positive interplay between galectin-3 and carotid plaque instability, implicating a dual role.[15] In particular, galectin-3 was not only expressed by inflammatory cells infiltrating unstable regions, but it was also highly expressed in macro-calcified, stable regions, predominantly occupied by VSMCs. Those authors hypothesized galectin-3 to modulate inflammation and osteogenesis in divergent ways.

To the authors' knowledge this is the first study evaluating galectin-3 in human carotid plaques in relation to clinical criteria of plaque vulnerability. The symptomatic group had lower average protein concentrations of galectin-3 than the asymptomatic group. Besides this, intra-plaque galectin-3 was positively associated with GSM score. In addition to macrophages, VSMCs may be important cellular sources of galectin-3 within atherosclerotic plaques.[16] Thus, a possible explanation may derive from the limited calcification and less fibrous tissue accumulation, which predominantly characterizes vulnerable atherosclerotic lesions.[17] Unfortunately, in the current study the magnitude of the intra-plaque calcification, ECM, and vascular smooth muscle cells (VSMCs) was not examined, so it was hypothesized that softer symptomatic plaques are poor in VSMC content, leading to less galectin-3 expression. Another plausible explanation may be attributed to the dual (pro-inflammatory and anti-inflammatory) role of galectin-3 within symptomatic plaques. Perhaps, large macrophage rich areas show increased galectin-3 production, while the compensatory anti-inflammatory action of galectin-3 may be excessively impaired in the rest of the non-inflamed regions of the same plaque.[18] Future studies searching underlying pathways are required to clarify the precise pro-atherogenic or anti-atherogenic actions of galectin-3 and their regulators.

It is still the subject of debate to assume that atherosclerosis related agents, like galectin-3, could enter the circulation and reflect ongoing activity within atherosclerotic plaques.[19] Accordingly, serum galectin-3 levels in this study did not correlate with its respective concentration within carotid plaques, implying that circulating galectin-3 may be not a marker of carotid plaque vulnerability. Limited data have previously documented the relationship between coronary plaque destabilization and plasma levels of galectin-3.[6] In the present study, all symptomatic patients underwent CEA at least 1 month after the acute cerebrovascular event and all of them were on statin treatment for adequate time. Those conditions might have blunted the prognostic power of circulating galectin-3.

Another important finding of the present study was the upregulation of galectin-3 in the small subgroup of symptomatic patients already treated with long term statins. This is the first study reporting pharmaceutical modification of galectin-3 in atherosclerotic conditions. It is well known that chronic statin treatment mediates carotid plaque stabilization through the induction of plaque calcification and the shrinkage of inflammatory cells infiltration.[20] Concerning the dual role of galectin-3 with a prevalent anti-inflammatory activity, this may corroborate the statin related galectin-3 upregulation. Perhaps, this is an emerging novel action within statins' spectrum. On the other hand, the opposite effect of statin administration on galectin-3 using atherosclerosis-prone mice was observed.[7,21] In particular, atorvastatin treatment markedly reduced intra-plaque concentrations of galectin-3 accompanied with plaque regression and plaque stabilization. Firstly, species differences may explain the divergent effects of statins on galectin-3. Secondly, the impact of statins on advanced atherosclerotic lesions was examined, and no firm conclusions about the action of galectin-3 (pro-inflammatory versus anti-inflammatory) during atherosclerosis evolution in rodents could be drawn. More studies, in larger cohorts, are required to unravel the response of galectin-3 to pharmaceutical modification.

Several drawbacks should be considered in the present study. Firstly the regional distribution of galectin-3 was not focused on, as previous researchers have reported. Although doing so might have shed more light on cellular sources, those histological indices with spatial distribution do not necessarily reflect plaque rupture or erosion. Perhaps the use of other indices of plaque instability, like fibrous cap thickness and ECM and VSMC content, would have provided more firm evidence. Representative segments of each whole plaque were examined. However, the concomitant histological assessment of plaque instability would have supported the findings. Another important limitation was the absence of assays of galectin-3 expression within plaques. That laboratory approach would provide a more precise estimation of its activity. Given the relatively small sample size, these results, and especially those derived from multiple regression analyses, should be viewed as preliminary. Moreover, this study did not prospectively address the potential clinical impact of modifying galectin-3. A future pharmacodynamic study will provide a rationale, along with results from other trials, for studying the effects of statins on plaque stability in larger trials of longer duration.

Despite the limitations discussed above, the findings support the hypothesis that galectin-3 is predominantly an anti-inflammatory mediator in advanced carotid plaques. Thereby, low intra-plaque concentrations of galectin-3 are more likely to represent suppressed anti-inflammatory mechanisms and may subsequently contribute to plaque vulnerability. The beneficial effect of galectin-3 could be enhanced by long term statin therapy. However, the latter remains to be verified by further studies.

CONFLICT OF INTEREST

None.

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