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Original Article

Complement activation in the arteries of patients with severe atherosclerosis

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Abstract: Background: Excessive complement activation plays an important role in the pathogenesis of atherosclerosis (AS). We therefore wanted to investigate whether complement is activated in areas of AS by detecting the deposition of C3b/iC3b and membrane attack complex (MAC). We also analyzed the relationships between C3b/iC3b and MAC levels and the clinicopathological features of patients with AS. Methods: The sample comprised 79 patients who had been diagnosed with AS. Their levels of C3b/iC3b and MAC deposition were evaluated by immunohistochemistry (IHC). The results were translated into scores, and the patients' clinical features were recorded. Results: Compared with normal arteries, significantly greater deposits of C3b/iC3b and MAC were found in AS arteries. In the group with more C3b/iC3b deposition, the ratio of patients with hypertension was higher. Moreover, in the group with more MAC deposition, the ratio of patients with hypertriglyceridemia was higher. Conclusions: The finding of C3b/iC3b and MAC deposition in atherosclerotic arteries points to the activation of complement. Greater amounts of C3b/iC3b and MAC deposition imply excessive complement activation, which can lead to the development of AS. Hypertension and hypertriglyceridemia may, respectively, contribute to the activation of complement C3 or the formation of MAC.

Keywords: Complement, atherosclerosis, C3b/iC3b, MAC, hypertension, hypertriglyceridemia

Introduction

Atherosclerosis (AS) is a major health concerns worldwide. It is a chronic progressive inflammatory state comprising thickened arterial blood vessels due to the excessive deposition of cholesterol, which leads to the invasion and accumulation of white blood cells and proliferation of intimal smooth muscle cells (SMCs) [1-3]. The complement system, an important mediator of inflammation and immune responses, can be activated by three (classic, alternative and lectin) pathways [4, 5]. All three converge at the level of C3 to form C5 convertases, eventually leading to the polymerization of C9 and assembly of membrane attack complexes (MACs) [4]. Accumulating evidence indicates that the abnormality of complement components and the resulting excessive complement activation are associated with atherogenesis [1, 6-8]. There was a high frequency of the complement C3F allotype in the patients with hypertensive disease and/or coronary AS [9]. C3 and C4 were strongly correlated with cardiovascular risk factors, including high blood pressure, an elevated body mass index (BMI), and lipids [10]. High levels of C3 and C4 account for the increased incidence of cardiovascular disease in human [10]. Studies have shown that, when fed a high-fat diet, C6-deficient rabbits developed less AS than their C6-sufficient controls [11]. Evidence from another study suggests that C5a plays a proatherogenic role and identifies the C5aR antagonist as a potential antiatherosclerotic drug [12]. It has also been found that MAC may play a critical role in plaque formation and the rupture of aneurysms [8, 13-16]; that is, it may promote the proliferation of vascular SMCs and formation of foam cells, thus inducing the release of monocyte chemotactic protein-1 [17, 18]. Reduced expression of CD59, a membrane inhibitor for MAC formation, has been identified in patients with arterial hypertension as compared with healthy con-

trols [19]. In animal studies, a deficiency of mouse CD59 accelerated the development of AS and aneurysms owing to increased complement activation [8, 16]. These various findings establish a connection between complement activation and atherogenesis. However; the underlying contributors to complement activation remain poorly understood.

Cigarette smoking, hypertension, hypercholesterolemia, and diabetes are implicated as risk factors for atherogenesis [20-22]. It is well established that cigarette smoke can activate the complement system through the alternative pathway, causing lung inflammation and injury [23-25]. Shear stress affecting the walls of blood vessels is critical for vascular remodeling; however, it also induces AS in hypertensive patients, especially in the carotid artery [26]. Complement has also been reported to be activated through the classic pathway by shear stress-stimulated platelets and endothelial cells [27, 28]; in response, the complement regulatory proteins of CD59 and clusterin are upregulated to protect the vasculature from complement-mediated injury [29, 30]. Hypercholesterolemia seems more relevant in complement activation. Murine and human hypercholesterolemia can not only induce an increased production of complement components but also potently activate complement, thus leading to endothelial dysfunction and the proliferation of cultured vascular smooth muscle cells (VSMCs) and macrophages through Giprotein dependent ERK1/2 activation [31, 32]. Although there is no evidence that high levels of glucose can directly induce the production and/or activation of complement, low expression levels of complement regulators and the resulting excessive complement activation are commonly observed in patients with diabetes and its complications [33-35]. However, the contribution of the mentioned risk factors to the development of AS by activating complement requires further investigation.

We collected arterial specimens from 79 AS patients who required curative artery surgery and detected the deposition of C3b/iC3b and membrane attack complex (MAC) in these samples. Further, we analyzed the relationship of these findings with the patients' clinical features, including smoking history, blood pressure, cholesterol, and blood sugar. Our aim was to identify the level of complement activation and its correlation with clinical features of AS.

Materials and methods

Patients and clinicopathological information collection

A total of 79 patients with AS who received curative surgery between 2011 and 2013 at the Zhongshan Hospital of Fudan University, Shanghai, China (Y2013-011, March 1, 2013) were recruited in this study. Prior written informed consent was obtained from all patients. The study protocol was approved by the ethics board of the Zhongshan Hospital. The diagnosis of AS was confirmed by histology in all cases. The following patient characteristics were collected: age, gender, blood pressure, blood sugar, cholesterol, and smoking history.

Tissue microarrays

Arteries from surgical specimens were fixed in 10% buffered formalin within 30 minutes after resection. Specimens were processed following routine procedures after 24 hours of fixation. Sections were stained with hematoxylin and eosin. Then the sections were reviewed by two pathologists to confirm the diagnosis of AS, after which the tissue microarrays (TMAs) were constructed. Briefly, the representative areas of interest in the AS lesions and areas of normal arteries were circled. The corresponding regions were marked on archival formalin-fixed paraffin-embedded (FFPE) tissue blocks. The chosen areas were extracted and then vertically planted into the recipient block one by one. Then the planting surface was aggregated on the aggregation instrument. Arrays with a maximum of 70 cores were constructed and adenocarcinoma cores were used as orientation markers. Sections of TMA were stained with hematoxylin and eosin (**Figure 1**).

Immunohistochemical (IHC) staining

Anti-C3b/iC3b/C3c rabbit monoclonal antibody (clone 2/11; Hycult, Plymouth Meeting, PA) and anti-MAC (C5b-9) rabbit polyclonal antibody (ab55811, Abcam, Cambridge, MA) were used to perform IHC assays with the iView DAB Detection Kit (Ventana, Tucson, AZ) on a BenchMark XT automated staining system (Ventana). In brief, the TMA sections were deparaffinized with EZ Prep (Ventana) at 75°C and heat pretreated in cell conditioning 1 (CC1; Ventana) for antigen retrieval at 95°C using

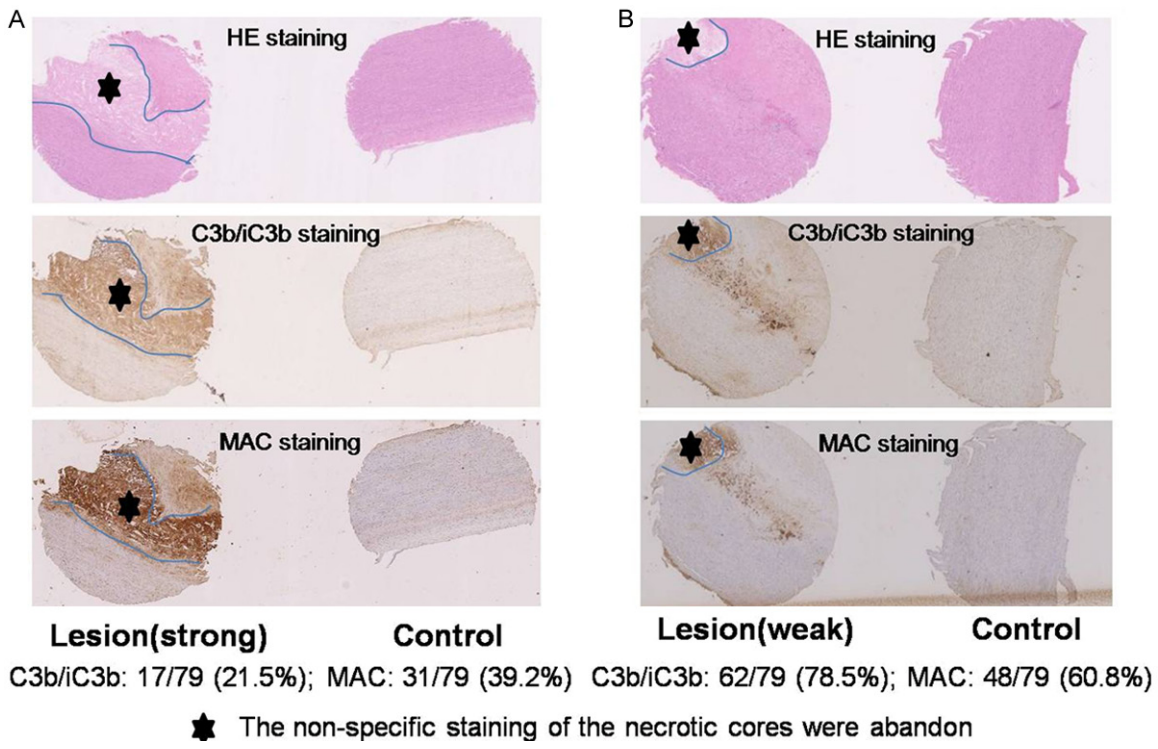

Figure 1. AS lesion and control area stained by hematoxylin and eosin (H&E) or IHC using tissue microarrays (TMAs). (A and B) Representative images of H&E and strong (A) or weak (B) C3b/iC3b or MAC IHC staining in AS areas. Images showing nonspecific staining of the necrotic cores were abandoned. The percentages of strong versus weak staining for C3b/iC3b or MAC deposition in 79 AS lesions are shown below the images. Strong staining: IHC score ≥1.00; Weak staining: IHC score <1.00. Original magnifications, 1.25×.

"standard cell conditioning". Then, after inactivation of the endogenous peroxidase by hydrogen peroxide for 4 minutes, TMA sections were incubated with anti-C3b/iC3b or MAC primary antibody at 37°C for 24 minutes. The TMA sections were blocked using the endogenous biotin blocking kit (Ventana) and incubated for 8 minutes with a biotinylated secondary antibody and then with a streptavidin-HRP conjugate at 37°C for 8 minutes. The slides were finally counterstained with hematoxylin II (Ventana) for 8 minutes and bluing reagent (Ventana) for 8 minutes. Isotype IgG was used as the negative control from the same species of primary antibody, which was diluted to match the concentration of the primary antibody.

Assessment of C3b/iC3b and MAC expression

C3b/iC3b and MAC expression status was assessed by two experienced, independent pathologists. If there was any discrepancy or disagreement, C3b/iC3b and MAC status was verified by a discussion panel of three pathologists. All observers were blinded with regard to the clinicopathologic patient characteristics. After abandoning samples showing nonspecific staining of the necrotic lipid core, C3b/iC3b and MAC deposition levels were translated into numerical scores. The percentage of the stained area was converted to a decimal value. For example, the intensity of the IHC signal "1+-3+" was converted to 1-3. The percentage of the stained area multiplied by the intensity of the IHC signal equaled the final score. In this study, we classified a score of equal to or greater than 1 as IHC-strong and a score of less than 1 as IHC-weak.

Statistical analysis

A chi-squared test was used for univariate analysis; cross-tabulations with qualitative variables were also analyzed with the Pearson chi-squared test. Differences in C3b/iC3b and MAC deposition levels between groups were analyzed using Student's t-test. A P value of<.05 was defined as statistically significant. No adjustments were made. All analyses were

Table 1. Clinical features of AS patients and their association with C3b/iC3b and MAC staining

		Clinical features	*P* value (C3b/iC3b scores in lesion area)	*P* value (MAC scores in lesion area)
Patients (n)		79		
Age (Mean ± SD, years)		63.95 ± 11.85		
Gender, n (%)	Men	67 (84.8)		
	Women	12 (15.2)	*0.007**	0.535
Smoking history, n (%)	Positive	24 (30.4)		
	Negative	55 (69.6)	0.917	0.870
Hypertension, n (%)	Positive	54 (68.4)		
	Negative	25 (31.6)	0.708	0.169
Diabetes, n (%)	Positive	33 (41.8)		
	Negative	46 (51.2)	0.194	0.649
Dyslipidemia, n (%)	Positive	46 (51.2)		
	Negative	33 (41.8)	0.530	0.314
Hypercholesteremia, n (%)	Positive	7 (8.9)		
	Negative	72 (91.1)	0.671	0.502
Hypertriglyceridemia, n (%)	Positive	12 (15.2)		
	Negative	67 (84.8)	0.337	0.559
Apolipoprotein low, n (%)	Positive	36 (45.6)		
	Negative	43 (54.4)	0.691	0.830
High-density lipoproteins low, n (%)	Positive	19 (24.1)		
	Negative	60 (75.9)	0.913	0.529
Low-density lipoprotein high, n (%)	Positive	8 (10.1)		
	Negative	71 (89.9)	0.296	0.175
Lipoproteinemia, n (%)	Positive	14 (17.7)		
	Negative	65 (82.3)	0.226	0.949

*: P<.05.

accomplished by the statistical package SPSS version 19.0 (SPSS Inc., Chicago, IL).

Results

Clinical characteristics of patients

Surgical arteries samples were obtained from 79 patients with AS who received curative artery surgery at the Zhongshan Hospital. The clinicopathologic characteristics of these patients are presented in **Table 1**. The average age of the patients was 63.95 ± 11.85 years, 67 (84.8%) patients were male and 12 (15.2%) were female, 24 (30.4%) had a smoking history and 55 (69.6%) did not. Of the total, 54 (68.4%) patients were diagnosed with hypertension and 25 (31.6%) were not; 46 patients (51.2%) displayed dyslipidemia and 33 (41.8%) did not. As for blood sugar, 20 (25.3%) patients were diagnosed with diabetes and 59 (74.7%) were not (**Table 1**).

Quantitative analysis of C3b/iC3b and MAC staining by IHC

To investigate the status of complement activation in the atherosclerotic arteries of AS patients, we stained the complement activation products C3b/iC3b and MAC of the TMA by IHC. The C3b/iC3b and MAC deposition levels were then transformed into numerical scores, as already noted. We observed that there was extensive staining of both C3b/iC3b and MAC in the lesion areas but not in the control areas of all specimens. The average scores of both C3b/iC3b and MAC staining in the lesion areas were significantly higher than those in the control areas (**Figure 2**). We classified score of equal to or greater than 1 as IHC-strong and a score of less than 1 as IHC-weak. As a result, 17 (21.5%) and 31 cases (39.2%) stained strongly for C3b/iC3b or MAC, respectively, in lesion areas; 62 (78.5%) and 48 cases (60.8%) stained weakly for C3b/iC3b or MAC, respec-

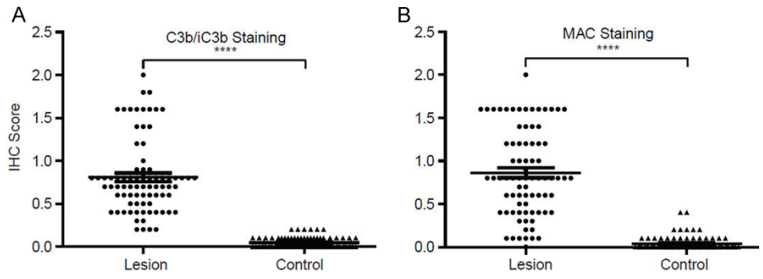

Figure 2. Complement activation in the resected arteries of patients with severe atherosclerosis (AS). (A, B) The immunohistochemical (IHC) scores of C3b/iC3b (A) and membrane attack complex (MAC) (B) staining in lesion areas were much higher than those in control areas. Data represent mean ± SEM; ****P<.0001; n=79.

tively (**Figure 1**). Furthermore, C3b/iC3b and MAC deposition colocalized in the lesion areas of AS arteries (**Figure 1**).

Correlation between C3b/iC3b and MAC deposition and clinicopathologic features

The relation of C3b/iC3b and MAC deposition status to clinicopathologic parameters is shown in **Table 1**. The scores of C3b/iC3b deposition status in the lesion areas of AS arteries was highly associated with gender (P=.007, **Table 1**). There were more male patients (67 [84.8%]) than female patients (12 [15.2%]). However, no statistically significant differences were observed for the association of C3b/iC3b staining with other clinical features, including blood pressure, smoking history, blood sugar, or lipid levels (P>.05, **Table 1**). In addition, there were statistically significant differences between MAC staining score and all the previously mentioned clinical features (P>.05, **Table 1**).

In the group with strong C3b/iC3b or MAC deposition, the ratio of patients with hypertension or hypertriglyceridemia was higher, respectively

To further identify the association between the previously described clinical features and degree of complement activation, we analyzed the correlation of different staining groups (strong and weak) of C3b/iC3b and MAC with the clinical features. As shown in **Table 2**, in the group of C3b/iC3b IHC-strong, the ratio of patients with hypertension was significantly higher (P=.040). Interestingly, only the feature of blood triglyceride level but not the levels of blood lipids, apolipoprotein, high-density lipoproteins, low-density lipoprotein, lipoprotein or other features were highly associated with the strong intensity of MAC staining (P=.038) in this study, which indicates that there were more patients with hypertriglyceridemia in the MAC IHC-strong group. These results indicate that complement activation at different stages may be affected by different clinical features.

Discussion

We collected AS artery specimens resected from 79 patients with severe AS who were enrolled in Zhongshan Hospital for curative surgery between 2011 and 2013 and stained the deposition of C3b/iC3b and MAC, two important complement activation products by IHC. We observed extensive complement activation characterized by the universal staining of C3b/iC3b and MAC in the lesion area of AS arteries (**Figure 1**). By analyzing the association between clinical features and complement activation, we found that there was more C3b/iC3b deposition in the AS arteries of men versus those in women (**Table 1**). Moreover, in the group with strong C3b/iC3b or MAC deposition, the ratio of patients with hypertension or hypertriglyceridemia was higher, respectively (**Table 2**). These results highlight the close correlation between complement activation and AS.

It is well known that young women have much less atherosclerosis and related disease than men, and epidemiologic studies have also shown that the onset of atherosclerosis occurs 10 years earlier in men than in premenopausal women, with complications such as myocardial infarction occurring about 20 years earlier [36]. Consistent with this, the total 79 severe AS patients who underwent artery surgery at Zhongshan Hospital between 2011 and 2013 displayed a similar gender difference, in that 67 patients (84.8%) were men and only 12 patients (15.2%) were women, or 5.6-fold more male than female patients. This suggests that the onset of severe AS requiring curative surgery occurs much later in women than in men. Estrogen is widely believed to be responsible for protecting premenopausal women from AS. However, hormone replacement therapy has

Table 2. Association of clinical features with degree of complement activation

	C3b/iC3b (IHC score)		*P* value	MAC (IHC score)		*P* value
	Strong	Weak		Strong	Weak	
Smoking history, n						
Positive	5	19		10	14	
Negative	12	43	0.059	21	34	0.481
Hypertension, n						
Positive	15	39		22	32	
Negative	2	23	*0.040**	9	16	0.442
Diabetes, n						
Positive	9	24		16	17	
Negative	8	38	0.218	15	31	0.117
Dyslipidemia, n						
Positive	11	35		20	26	
Negative	6	27	0.373	11	22	0.250
Hypercholesteremia, n						
Positive	2	5		5	2	
Negative	15	47	0.555	26	46	0.079
Hypertriglyceridemia, n						
Positive	2	10		8	4	
Negative	15	52	0.496	23	44	*0.038**
Apolipoprotein low, n						
Positive	9	18		15	21	
Negative	8	35	0.133	16	27	0.431
High-density lipoproteins low, n						
Positive	3	16		7	12	
Negative	14	46	0.365	24	36	0.514
Low-density lipoprotein high, n						
Positive	2	6		4	4	
Negative	15	56	0.550	27	44	0.384
Lipoproteinemia, n						
Positive	2	12		6	8	
Negative	15	50	0.373	25	40	0.493

Strong, score ≥1.00; Weak, score <1.0. *: P<.05.

failed to decrease atherosclerosis-related events in clinical trials [37, 38], pointing to the complexity of the association of AS not only with estrogen hormones but also with lipoprotein, Toll-like receptors, and leukocyte-platelet aggregation [39]. We found that the critical complement activation products of C3b/iC3b were strongly correlated with gender (i.e., there was much more C3b/iC3b deposition in men than in women). This finding indicates that in men, plasma may have higher complement activity than it does in women. Although there is a lack of evidence for a human gender difference in complement activity, the systemic complement activity of male C57BL/6J and BALB/cJ mice is remarkably higher than that of similar female mice at the terminal stage of complement activation [40].

In our study, in the group with strong C3b/iC3b deposition, the ratio of patients with hypertension was higher (**Table 2**). High blood pressure may induce strong wall shear stress, especially in the turning and furcate vessels such as the carotid, further activating complement activation [26-28]. Therefore, to protect the vasculature from complement-mediated injury, the complement regulatory proteins of CD59 and cluster in are upregulated [29, 30]. These findings may explain the strong association between hypertension and the intensity of C3b/iC3b but not MAC deposition. Triglyceride

is the most abundant constituent of body fat. Hypertriglyceridemia is highly relevant to AS, even in the absence of hypercholesterolemia, and predisposes to cardiovascular diseases (CVD). Thus hypertriglyceridemia is considered a major risk factor for CVD [41]. Patients who have hypertriglyceridemia may be at significant risk for CVD compared those who have elevated level of low-densitylipoprotein [42]. Although dyslipidemia was not correlated with the degree of complement activation in AS lesion areas in our study, the ratio of patients with hypertriglyceridemia was higher in the group with strong MAC deposition. Adipose tissue other than liver can also synthesize complement components and is a target of complement activation. Patients with hypertriglyceridemia showed a significantly high level of complement C3 [43]. Therefore the regulation of complement activation by triglyceride appears quite complicated and requires future investigation. In addition, other risk factors such as smoking history, diabetes, hypercholesteremia, and elevated levels of low-density lipoprotein are well established as contributors to AS. However, we failed to note that they were able to promote complement activation in this study (**Table 2**).

The complement system is widely recognized as a critical arsenal for immune surveillance and hemostasis in that it functions to eliminate invading pathogens and cell debris and to orchestrate immune responses. Thus dysregulation of complement contributes to various human disorders including atherogenesis [44]; however, it is considered to play a dual role in atherogenesis [45]. The protective action may result from C1q-mediated non-complement-related roles or complement receptors (CRs such as CR3, CR4, and CRIg) that mediate phagocytosis by enhancing the clearance of atherogenic lipoproteins or apoptotic cells [46-48]; while complement activation mainly plays a pro-atherogenic role [45, 49]. Targeted SMCs and endothelial cells by MAC may induce cell proliferation by activating MAPK and/or PI3K/Akt pathways. We determined the close association of complement activation with gender, hypertension, and hypertriglyceridemia in patients with severe AS requiring artery surgery. These findings may provide some approaches to preventing severe AS, such as by weight loss, regular exercise, dietary modification, and treatment with statins and antihypertensive drugs.

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Disclosure of conflict of interest

None.

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