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Original Article

Decrease in circulating myeloid dendritic cell precursors in patients with intracranial large artery atherosclerosis

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Abstract: Intracranial large artery atherosclerosis (ILAA) is a major cause of ischemic cerebrovascular disease. The aim of this study was to investigate whether the levels of circulating dendritic cell precursors (DCP) could reflect the severity of intracranial large artery atherosclerosis (ILAA). For this purpose, a series of angiography were taken to determine the severity and extent of coronary artery and intracranial large artery stenosis, and flow cytometry were taken to determine the levels of circulating mDC precursors and pDC precursors in patients with severe intracranial large artery atherosclerosis (ILAA) (n = 101) and mild intracranial large artery atherosclerosis (ILAA) (n = 123) according to the angiography. Circulating mDC precursors were lower in patients with severe intracranial large artery atherosclerosis (ILAA) than in mild intracranial large artery atherosclerosis (ILAA) ($P < 0.05$), but circulating pDC precursors were not significant differences ($P > 0.05$). According to these data, circulating mDC precursors could predict the severity of ILAA, which also could be able to reflect the severity of ILAA.

Keywords: Intracranial large-artery atherosclerosis (ILAA), dendritic cell (DC), coronary atherosclerosis, angiography

Introduction

Atherosclerosis (AS) is the most common pathological basis of coronary artery and cerebrovascular disease, which are one of the leading cause of disability and death in developed country and china [1, 2]. Recent epidemiological studies shown that coronary atherosclerosis is strongly associated with cerebral atherosclerosis, and have strongly prognostic significance for each other [3, 4]. Intracranial large artery atherosclerosis (ILAA), the main cause of ischemic cerebrovascular disease, is an independent predictor of coronary heart disease [5]. However, Dionesia *et al.* [6] showed that significant atherosclerotic disease in the carotid arteries could not predict significant atherosclerotic disease in the coronary arteries, vertebral arteries, or aorta in patients with acute ischemic stroke.

Inflammation plays a pivotal role in the development and progression of atherosclerosis. Recently emerging evidences have suggested that the immune system integrated with inflammation are involved in mediating all stages of atherosclerosis and its complications, including myocardial infarction and stroke [7, 8]. Dendritic cells (DCs) are highly potent professional antigen-presenting cells (APCs) uniquely able to initiate primary immune responses to various antigens by activation naive T cells, and central to the regulation of inflammation. Available evidence suggests that DCs play an important role in the pathogenesis of atherosclerotic process [9]. Moreover, Bobryshev et al. [10] found vascular dendritic cells (VDCs) are involved in the maintenance of homeostasis in normal arterial intima, and may be important in the development of atherosclerotic lesions, possibly through an immune mechanism. Previous laboratory animal studies have shown that dendritic cells (DCs) accumulate preferentially in regions predisposed to atherosclerosis in the normal murine aortic intima [11] and initiate nascent foam cell lesion formation at very early stages when the atherosclerotic plaque starts to develop in knockout mouse model [12]. In human atherosclerotic plaques, dendritic cells

(DCs) are present and accumulate preferentially within the vulnerable plaque shoulder by colocalizing with T cells [13-15]. Additionally, DCs accumulate in parallel to plaque complexity and inflammation in human atherosclerotic lesions [15], and with statin treatment, the number of DCs in atherosclerotic plaques was lowered. Correspondingly, several previous study have demonstrated that in patients with acute coronary syndrome, the number of myeloid DCs was increased in atherosclerotic plaques, but the number of circulating myeloid DC precursors was significantly decreased [15, 16]. The decreased circulating myeloid DC precursors may be recruited from blood into atherosclerotic lesions and subsequently develop into myeloid DCs which play a role in plaque progression and destabilization [17].

Since atherosclerosis is a systemic disease, the degree of atherosclerosis in different vascular systems may be consistent. However, recent study showed that the presence and degree of atherosclerosis in different types of arteries are not completely simultaneous and consistent [6]. It has been demonstrated that the levels of circulating DC precursors may reflect the coronary atherosclerotic burden and plaque destabilization [17]. The purpose of the present study was to assess whether the levels of circulating DC precursors could reflect the severity of intracranial large artery atherosclerosis (ILAA). For this purpose, digital subtraction angiography protocol was used to evaluate the stenosis of intracranial large artery atherosclerosis (ILAA), and coronary artery by angiography simultaneously, 4-color flow cytometry assay was used to determine the levels of the circulating DC precursors (mDC precursors and pDC precursors) in peripheral blood mononuclear cells.

Materials and methods

Patients and controls

The study protocol conforms to the principles of the Declaration of Helsinki and was performed with approval of the Ethics Committee of South Medical University. Subjects were selected from individuals who simultaneous underwent angiography for coronary artery, cerebral artery and another artery to investigate ischemic heart disease and ischemic cerebrovascular disease based on clinical indications (typical and atypical chest or head discomfort) and exclude another artery stenosis from December 2006 to October 2010. All subjects are Han Chinese, which were gave informed consent both verbally and in writing for participation in the study, and underwent angiography at Zhujiang Hospital of South Medical University before entering the study. Based on coronary artery angiography, the severity of coronary stenosis was evaluated by Genisi score. Based on cerebral angiography, the severity of intracranial large-artery atherosclerotic stenosis was characterized by measuring the degree of maximal diameter stenosis. According to the criteria of Warfarin-Asprin Symptomatic Intracranial Disease Study for Stroke, the severe stenosis were defined as at least one stenosis > 50% in a major intracranial artery (carotid artery, middle cerebral artery, vertebral artery, and basilar artery). Patients who had angiographic check were further divided into the severe intracranial large artery atherosclerosis (ILAA) group (with different degrees of coronary artery stenosis) and the mild intracranial large artery atherosclerosis (ILAA) group (with different degrees of coronary artery stenosis). Finally, 224 subjects (162 men and 62 women, age range from 32 to 84 years with mean age of 63.5 ± 8.84) were included in the present study.

In addition, patients with extracranial artery stenosis, other artery stenosis (such as kidney artery, aorta) and cerebral hemorrhage were exclude in the present study. Patients with autoimmune, neoplastic, liver, hematological or renal diseases, diabetes mellitus, surgery or trauma within the present, valvular heart disease, nonischemic cardiomyopathy, and chronic inflammatory conditions were also excluded from the study. In addition, patients who took medications, such as immunosuppressive agent, statins, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers before enrollment were also excluded. The study was approved by the local ethics committee. Each participant gave informed written consent.

Analysis of the DC precursors' percentage in peripheral blood mononuclear cells by fluorescence-activated cell sorting (FACS)

Fasting blood samples for flow cytometric analysis was obtained in the early morning after subjects' admission before coronary angiography. Immediately after blood collection in tubes containing EDTA, samples were analyzed by

Table 1. The clinical characteristics and laboratory data of severe ILAA group and the mild ILAA group

	Mild ILAA group (*n* = 123)	Severe ILAA group (*n* = 101)	*P*
Age (years)	63.51 ± 9.35	63.47 ± 8.21	0.969
Male gender, n (%)	87 (70.7)	75 (74.3)	0.558
Risk factors, n (%)			
Hypertension	39 (31.7)	23 (22.8)	0.138
Current smoking	30 (24.4)	26 (25.7)	0.816
Diabetes mellitus	22 (17.9)	29 (28.7)	0.055
HbA1c (%)	5.51 ± 1.04	5.63 ± 0.96	0.375
TC (mmol/L)	4.87 ± 1.25	5.19 ± 1.20	0.056
LDL-C (mmol/L)	2.73 ± 0.80	2.92 ± 0.78	0.069
TG (mmol/L)	1.38 ± 0.90	1.49 ± 0.93	0.384
Leukocytes (G/L)	7.42 ± 2.15	7.72 ± 3.08	0.401
Creatinine (μmol/L)	102.54 ± 66.92	105.60 ± 60.65	0.723
Uric acid (μmol/L)	320.63 ±137.86	318.25 ± 134.96	0.897
Genisi score	41.00 ± 37.02	48.59 ± 38.99	0.137
AMI history (%)	8 (6.5)	7 (6.9)	0.899
Stroke history (%)	7 (5.7)	8 (7.9)	0.507
mDC-p (%)	0.81 ± 0.37	0.62 ± 0.38	0.01
pDC-p (%)	0.17 ± 0.07	0.15 ± 0.07	0.113

Values are expressed as percentages or mean ± SD. AMI, acute myocardial infarction; HbA1c, haemoglobin A1C; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; mDC-p, myeloid dendritic cell precursors; pDC-p, plasmacytoid dendritic cell precursors.

flow cytometry (FACS-CALIBUR, CellQuest software, BD, USA). The four-color Dendritic Value Bundle Kit (BD Biosciences San Jose, California, USA) was used for dendritic cells (DCs) analysis according to the manufacturer's instructions. The four-color Dendritic Value Bundle Kit includes FITC-conjugated anti-lineage 1 (lin1) cocktail antibodies, anti-human leukocyte antigen (HLA)-DR-PerCP, anti-CD11c-APC, anti-CD123-PE, and isotype control mouse IgG2a-APC and mouse IgG1-PE antibodies. DCs were defined as cells positive for PerCP-conjugated anti-HLA-DR, negative for FITC-conjugated anti-lin1 and positive for either PE-conjugated anti-CD11c (myeloid DCs precursor or mDC precursors) or APC-conjugated anti-CD123 (plasmacytoid DCs precursor or pDC precursors) mAb (**Figure 1**). According to the clinic standards, routine blood analyses were performed in our hospital clinical laboratory.

Determination the severity of coronary artery lesions by Gensini score

Selective coronary and cerebrovascular angiography was conducted by two experienced interventional doctor blinded to the patients' clinical characteristics and biochemical results. The extent of coronary artery stenosis was assessed by quantitative coronary angiography. Gensini score was used to assess the severity and extent of coronary artery lesions. According to the degree of luminal narrowing and its location, the Gensini score was calculated by assigning a value to each coronary stenosis. Details of Gensini score as follows: 1-25%, 26-50%, 51-75%, 76-90%, 91-99%, and 100% of coronary luminal narrowing were given scores of 1, 2, 4, 8, 16, and 32 respectively, which then are multiplied by a factor that represents the importance of the lesion's position in the coronary arterial system: 5 for the left main coronary artery, 2.5 for proximal segment of the left anterior descending coronary artery (LAD) or the circumflex artery (LCX), 1.5 for mid-segment of LAD, 1 for distal segment of the CHD or mid-distal of LCX or right coronary artery, and 0.5 for others.

Statistical analysis

Statistical analysis was performed by SPSS software. Continuous variables were expressed as mean ± SD and categorical were expressed as counts and percentages. Differences between the severe intracranial artery stenosis and the mild intracranial artery stenosis groups were evaluated with the independent *t*-test for continuous variables and the nonparametric Mann-Whitney *U* method for categorical variables except for the levels of mDC precursors and pDC precursors, which were analyzed with analysis of covariance. Binary logistic regression analysis was conducted to identify variables independently associated with the severity of intracranial artery stenosis in all patients. A *P*-value < 0.05 was considered statistically significant. According to the criteria of Warfarin-Asprin Symptomatic Intracranial Disease Study for Stroke, the severe stenosis were defined as at least one stenosis > 50% in a major intracranial artery (carotid artery, middle cerebral artery, vertebral artery, and basilar artery).

Table 2. Predictors of the severity of ILAA in peripheral blood mononuclear cells at linear multivariate regression analysis: all patients

	B	SE	Exp (B)	*P*-value
Age	0.011	0.017	1.011	0.529
Male gender	-0.052	0.346	0.949	0.881
Risk factors, n (%)				
Hypertension	0.416	0.338	1.515	0.218
Current smoking	0.208	0.365	1.231	0.568
Diabetes mellitus	-0.846	0.389	0.429	0.030
HbA1c (%)	0.110	0.150	1.117	0.464
TC (mmol/L)	0.186	0.173	1.205	0.282
LDL-C (mmol/L)	0.079	0.253	1.082	0.754
TG (mmol/L)	0.080	0.241	1.083	0.740
Leukocytes (G/L)	0.014	0.059	1.014	0.812
Creatinine (μmol/L)	0.000	0.003	0.999	0.794
Uric acid (μmol/L)	0.000	0.001	1.000	0.810
Genisi score	0.000	0.006	1.000	0.952
AMI history (%)	-0.141	0.584	0.869	0.810
Stroke history (%)	-0.146	0.600	0.865	0.809
mDC-p (%)	-1.553	0.568	0.212	0.006
pDC-p (%)	-2.535	2.166	0.079	0.242

HbA1c, haemoglobin A1C; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; mDC-p, myeloid dendritic cell precursors; pDC-p, plasmacytoid dendritic cell precursors.

Results

Baseline characteristics

The clinical characteristics and laboratory data of subjects are summarized in **Table 1**. In our study, 123 patients with severe ILAA were compared with 101 mild ILAA group, we found no significant differences patients with major clinical (age, hypertension, current smoking, diabetes mellitus, AMI history, stroke history, HbA1c, TC, LDL-c, TG, leukocytes, creatinine, uric acid) and coronary artery angiographic data (Genisi score).

Immunohistochemical analysis

The clinical characteristics and laboratory data of both groups of ILAA patients had no significant differences (**Table 2**). In the present study, the occurrence of dendritic cells was analyzed by four-color flow cytometry. For analysis of mDCs, the number of HLA-DR$^+$CD123$^+$ cell expressed by pDC precursors and HLA-DR$^+$CD-11c$^+$ cell expressed by mDC precursors was evaluated. Immunostaining with these markers showed a significantly higher cells number of immature as well as mature mDCs in both groups of ILAA patients (**Figure 1**).

Additionally, immunostaining with HLA-DR, a functional marker expressed by activated antigen-presenting cells, revealed a significantly higher cells number in femoral plaques of ILAA patients (P = 0.01) (**Table 1**).

Decrease in circulating mDCPs and pDCPs in ILAA

The levels of mDC precursors were significantly lower in severe ILAA group than in mild ILAA group [0.375% (0.60-0.695) vs. 0.75% (0.61-0.695)] (**Table 1**), but the levels of pDC precursors were similar between in severe intracranial large artery atherosclerosis (ILAA) group and in mild intracranial large artery atherosclerosis (ILAA) group [0.16% (0.13-0.21) vs. 0.14% (0.12-0.20)] (**Table 1**).

Comparison of circulating DCPs in ILAA

In our data, we found no significant differences patients with major clinical (age, hypertension, current smoking, diabetes mellitus, AMI history, stroke history, HbA1c, TC, LDL-c, TG, leukocytes, creatinine, uric acid) and coronary artery angiographic data (Genisi score). At binary logistic regression analysis, we also found that the levels of mDC precursors and diabetes mellitus were the independent predictor of the severity of intracranial artery stenosis (**Table 2**).

Discussion

In this study, we demonstrate that the levels of circulating mDC precursors are lower in severe intracranial large artery atherosclerosis group than in mild intracranial large artery atherosclerosis group. Decreased circulating mDC precursors could predict the severity of ILAA, suggesting a new contributory mechanism to ILAA.

Previous clinical studies have demonstrated mDC precursors decrease in peripheral circulation in ACS [17, 18] and increase in vulnerable carotid plaques [15]. The decreased circulating mDCs precursor may be recruited into the atherosclerotic lesion. Several chemokines, such as monocyte chemoattractant protein-1 (MCP-

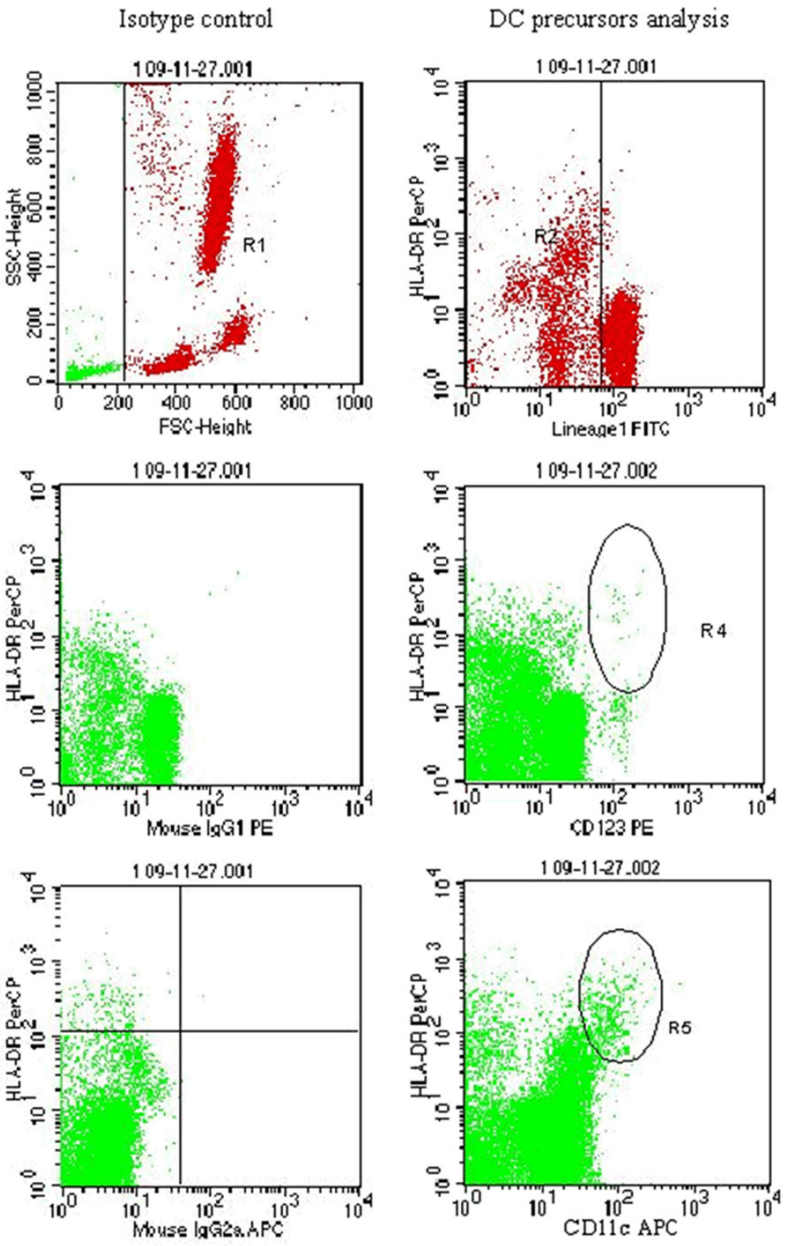

Figure 1. Detection of dendritic cell precursors (mDC precursors and pDC precursors) in peripheral blood by four-color flow cytometry. R1: region based on forward and side light scatter properties to exclude debris. R2: region containing DC, defined as HLA-DR$^+$ and lineage cells. R4 and R5: regions containing cells gated on R1 and R2. R4 identifies HLA-DR$^+$CD123$^+$ cell (pDC precursors), R5 identifies HLA-DR$^+$CD11c$^+$ cell (mDC precursors).

1) and fractalkine, which could be induced by several atherogenic factors, typically oxidized LDL-cholesterol [19], may contribute to recruitment of circulating mDC precursors into the atherosclerotic lesion. Monocyte chemoattractant protein-1 (MCP-1), a member of the chemokine family, was found to be highly expressed in human atherosclerotic lesions [20]. Deletion of MCP-1 or its corresponding receptor CCR2 could attenuate atherosclerosis in experimental mouse models [21-23]. Clinical evidence also has shown that the plasma levels of MCP-1 have independent prognostic value in the acute and chronic phases after ACS [24, 25]. Additionally, the role of MCP-1/CCR2 in DCs biology is classically seen as being critical for cell migration and maturation [26]. Deletion of the fractalkine receptor CX3CR1 resulted in decreased atherosclerosis and a decreased number of DC in atheromas in ApoE$^{-/-}$ mouse [27].

Atherosclerosis is a systemic disease that affects arteries at different sites with peculiar characteristics and differing degrees of progression, and as a common pathology for the cerebral artery stenosis and the coronary artery stenosis. Clinical studies demonstrated that the severity and extent of cerebral artery stenosis and coronary artery stenosis are not completely coexisting. Clinical pathology and animal experiments studies found that DC accumulated in the brain ischemic infarct area [28-30]. A previous study by Yilmaz *et al.* [31] showed that the numbers of circulating mDCPs, pDCPs and DCPs were significantly reduced, and a dense infiltration of mDCs co-localized with T-cells, single pDCs and high HLA-DR expression were observed in brain infract area. However, the distributions of circulating DC subsets in ILAA are still unknown and require further investigation.

Intracranial large-artery atherosclerosis is a major cause of ischemic stroke worldwide [32, 33], especially in Asians [33]. It is noteworthy that ILAA may be associated with coronary atherosclerosis and another atherosclerosis [34-36]. To exclude the effect of the coronary atherosclerosis and another atherosclerosis on the distribution of circulating DC precursor subsets, angiography, the gold standard for artery stenosis detection, was used to determine the extent and severity of the artery stenosis. We found that the levels of circulating mDC precursors were decreased in severe ILAA, but the levels of circulating pDC precursors were not significant changed. This result along with previous studies may indicate that the percentage of mDCs precursors reflects the total atherosclerotic burden, the decreased circulating mDCs precursors are recruited from blood into the atherosclerotic lesions.

We also found that diabetes mellitus was the predictor of the severity of ILAA at binary logistic regression analysis. It has been shown that diabetes mellitus is a more important determinant for intracranial atherosclerosis related stroke than extracranial atherosclerosis or non-atherosclerosis in a multi-ethnic community-based cohort [37] Diabetes mellitus was also considered as a significant risk factors for intracranial artery stenosis in asymptomatic populations [38]. Seifarth *et al.* [39] found that the levels of circulating mDCs and pDCs are decreased in patients with type 2 diabetes mellitus, especially for the levels of circulating mDC in these patients with poor blood glucose control. However, whether circulating mDC implicated in the pathogenesis of intracranial atherosclerosis in diabetes mellitus are still unclear.

There are some limitations in our study. First, because of abiding by the necessarily stringent inclusion and exclusion criteria, the relatively small sample size is the main limitation of our study. Second, we did not quantitative evaluate the severity and extent of intracranial atherosclerotic lesions based on present study, it could help to better understand the relation of circulating DC subset and the severity and extent of ILAA.

In conclusion, we found that a significant decrease in circulating mDCs in patients with ILAA. In addition, we also demonstrated markers indicative for mDCs are negatively correlated with the severity and extent of ILAA. Therefore, further studies are required to demonstrate whether regulation of the percentage of circulating mDC precursors in ILAA might yield new therapies, and the association of inflammation and DCs in ILAA.

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Disclosure of conflict of interest

None.

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