Altered levels of circulating natural antibodies against VEGFR1-derived peptide in atherosclerosis

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Abstract

Background: Several lines of evidence have pointed to a protective role of natural antibodies in chronic diseases like atherosclerosis and cancer. Vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 are important regulators of angiogenesis and may be involved in the development of atherosclerosis. In this retrospective study, we developed an in-house enzyme-linked immunosorbent assay to assess whether natural IgG levels against VEGFR1 and the regulatory T cell markers CD25 and FOXP3 were associated with atherosclerosis.

Methods: A total of 218 patients with atherosclerosis and 200 healthy controls were enrolled. All patients had atherosclerotic carotid plaques. Carotid intima–media thickness was analyzed using a diagnostic ultrasound system.

Results: Plasma anti-VEGFR1 IgG levels were significantly lower in patients with atherosclerosis than control subjects. Decreased anti-VEGFR1 IgG levels were more obvious in male patients. Spearman correlation analysis showed no significant correlation between natural IgG levels and carotid intima–media thickness.

Conclusions: Decreased levels of anti-VEGFR1 IgG may be involved in development of atherosclerosis and related conditions.

Keywords
Atherosclerosis, natural antibody, vascular endothelial growth factor receptor 1, enzyme-linked immunosorbent assay, peptide, angiogenesis

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Introduction

Atherosclerosis is a multi-factorial, chronic inflammatory disorder that develops within the walls of large and medium-sized arteries and can cause several adverse clinical events including acute coronary syndrome and ischemic stroke. The disease is characterized by inflammatory infiltration, subendothelial accumulation of oxidized lipids, angiogenesis and fibrosis. Both innate immunity and adaptive immunity contribute to a proatherogenic inflammatory response at each stage of atherosclerosis development, from the beginning of the fatty streak to plaque rupture. The pathogenic events underlying atherogenesis are progressive and include endothelial dysfunction, inflammatory cell adhesion and oxidative stress. Vascular endothelial growth factor (VEGF) receptors 1 (VEGFR1) and 2 (VEGFR2) are important regulators of angiogenesis and may be involved in development of atherosclerosis. VEGFs function to stimulate angiogenesis via activation of VEGFR2 while VEGFR1 has been thought to modulate the function of VEGFR2.

Regulatory T (Treg) cells are CD4<sup>+</sup>CD25<sup>+</sup> T cells that can suppress excessive immune responses and maintain immune tolerance in the periphery. Treg cells specifically express fork-head box P3 (FOXP3), a master regulator that is critical for their development and immunosuppressive function. Treg cells can suppress atherosclerosis development or progression by down-regulating effector T cell-mediated inflammatory responses through multiple mechanisms, including secretion of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor beta (TGF-β), cell-contact dependent suppression, and depletion of IL-2. Several studies of experimental atherosclerosis showed that adoptive transfer of Treg cells prevented the development of atherosclerosis, while depletion of Treg cells using an anti-CD25 antibody promoted the formation of atherosclerotic plaques.

Natural antibodies are immunoglobulins generated by innate B cells such as B-1 lymphocytes without immunization and play an important role in maintaining immune homeostasis. Levels of natural antibodies decrease with advancing age. Thus, age-related decreases in the amount or efficacy of natural antibodies may increase the risk of developing several diseases such as atherosclerosis, type 2 diabetes, Alzheimer’s disease and malignancies. Natural antibodies have been shown to be involved in several chronic diseases such as amyloid protein-related neurodegeneration and cancer. In a recent study, we found that decreased levels of natural antibodies against CD25-derived peptides were associated with the development of lung cancer.

The present study was thus designed to assess the levels of natural antibodies against peptide antigens derived from CD25, FOXP3 and VEGFR1 in patients with atherosclerosis. We investigated whether these natural antibodies were associated with development of atherosclerosis.

Materials and methods

Subjects

Plasma samples were collected from patients with atherosclerosis who were admitted to the Department of Neurology, Second Hospital of Jilin University, Changchun between November 2015 and March 2017. All patients showed atherosclerotic carotid plaques and carotid intima–media thickness was analyzed using a diagnostic ultrasound system (iE Elite, Philips, Franklin, TN, USA). Participants who had suffered from any type of malignancy or autoimmune disorders such as autoimmune thyroid disease, pernicious anemia, type 1 diabetes, celiac disease, multiple sclerosis, systemic lupus
erythematous and inflammatory bowel disease were excluded. Because most patients with atherosclerosis were taking lipid-lowering drugs at the time of sampling, information regarding circulating lipid levels was not collected. This study was approved by the Ethics Committee of the Second Hospital of Jilin University and was performed in accordance with the ethical guidelines of the Declaration of Helsinki. All participants were of Chinese Han descent and all provided written informed consent for use of their blood samples in this study. All samples were anonymized prior to analysis, so the identities of participants could not be ascertained in any way.

**Detection of plasma IgG levels**

We designed an in-house enzyme-linked immunosorbent assay (ELISA) to assess whether levels of natural IgG against VEGFR1, CD25 and FOXP3 were associated with atherosclerosis. Seven linear peptide antigens, including three derived from CD25 (CD25a, CD25b and CD25c), two derived from FOXP3 (FOXP3a and FOXP3b) and two derived from VEGFR1 (VEGFR1a and VEGFR1b) were designed using computational epitope prediction software (http://www.iedb.org) and synthesized by solid-phase chemistry with >95% purity. Detailed information regarding the in-house ELISA for detection of plasma IgG levels against the above three targets was described previously.\(^\text{24,25}\) To minimize the effects of non-specific binding, the specific binding ratio (SBR) was used to assess relative levels of plasma IgG, and was calculated as follows:

\[
SBR = \frac{OD_{\text{sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}}
\]

where OD refers to optical density, NC refers to negative control plasma and PC refers to positive control plasma. Pooled plasma from >100 unrelated healthy individuals was used as a quality control sample on every plate. The reproducibility of the in-house ELISA was evaluated by assessing inter-assay deviation through the coefficient of variation (CV).

**Statistical analysis**

All antibody test data were expressed as means ± standard deviations (SDs). Statistical analysis was performed using IBM SPSS version 22.0 (IBM, Armonk, NY, USA). The Kolmogorov–Smirnov one-sample test was used to assess normality of the distribution of plasma IgG levels. The Mann–Whitney \(U\) test was used to assess differences in plasma IgG levels between patients and controls because of the skewed distribution of plasma antigen-specific IgG levels. Spearman correlation analysis was used to examine the relationships between levels of plasma IgG against CD25, FOXP3 or VEGFR1 and carotid intima–media thickness. Values of \(P < 0.017\) were considered statistically significant as differences in antibodies against three target molecules were independently tested.

**Results**

Of the 218 patients with atherosclerosis enrolled in the study, 127 were male and 91 were female and their mean age was 61.2 ± 11.4 years. A total of 200 healthy subjects aged 60.9 ± 11.7 years were simultaneously recruited from local communities, 109 of whom were male and 91 were female. Demographic information and clinical characteristics of study participants are shown in Table 1.

We assessed levels of natural IgG against five peptides derived from three target antigens (VEGFR1, CD25 and FOXP3). The sequences of these five peptide antigens are shown in Table 2. The normality of
the distribution of plasma IgG levels against these antigens was assessed using the Kolmogorov–Smirnov test and the distributions were found to be skewed (Table 3).

The in-house ELISA showed good reproducibility with CV values ranging from 7.4% to 21.2% (Table 4). As shown in Table 5, plasma IgG levels against VEGFR1b were significantly decreased in patients with atherosclerosis compared with control subjects ($Z = -2.45$, $P = 0.014$). Male patients appeared to contribute most significantly to the decreased anti-VEGFR1b IgG levels ($Z = -2.45$, $P = 0.014$). Plasma IgG levels against the other six peptide antigens showed no significant differences between the patient group and the control group.

There was no significant correlation between carotid intima-media thickness and plasma IgG levels against CD25, FOXP3 or VEGFR1 (Table 6).

### Discussion

A number of studies have confirmed that carotid ultrasound can predict the existence

| Antigen | Sequence (N–C) | NCBI Accession | Position (aa) |
|---------|---------------|----------------|---------------|
| CD25a   | kpghcreppweneateriyhfvgqmvvy | NP_000408 | 99–126 |
| CD25b   | iyhfvvgmvyqcvqgyralhrgpaesve | NP_000408 | 116–144 |
| CD25c   | khtsqfgeekpqspegpesetsch | NP_000408 | 167–187 |
| FOXP3a  | dmfaffrnhpatwknairhnlslhkcd | NP_001107849 | 335–359 |
| FOXP3b  | Kctfpnpsaprkdstlsavpqssyh | NP_001107849 | 134–156 |
| VEGFR1a | degvyhckatnqkgsvessaytvqgtsdk | NP_002010 | 725–754 |
| VEGFR1b | cqitwfknnhk iqqepgilg pgsstd | NP_002010 | 691–715 |

FOXP3, fork-head box P3; VEGFR1, vascular endothelial growth factor 1.
and severity of coronary artery disease. Carotid artery screening is of practical value in patients with coronary artery disease because of the strong correlation between carotid artery and coronary artery disease. Recent studies demonstrated the presence of natural autoantibodies in the blood of patients with atherosclerosis against lipoprotein lipase.

Stroke is a complex disease in which both genetic and environmental factors play vital roles. Revascularization is an important feature of severe atherosclerosis. Because dietary factors and genetic susceptibility vary, the degree of lipid deposition in the blood vessel wall can differ and atherosclerosis is often associated with hypercholesterolemia. About 50% of patients with ischemic stroke show hypercholesterolemia, leading to an increase in stroke-related mortality. Physiologically, VEGFs play important roles in endothelial integrity, survival and physiological function and play important roles in atherosclerosis and angiogenesis. Increased VEGF signaling exacerbates atherosclerosis through the formation of new blood vessels and heightened inflammation of atherosclerotic plaques.

The present study demonstrated that plasma IgG levels against the VEGFR1-derived peptide antigen VEGFR1b were significantly lower in patients with atherosclerosis compared with healthy controls. This difference was especially apparent in male patients (Table 5). This finding suggested that dysfunction of VEGFR1 is likely to contribute to the development of atherosclerosis, although we failed to detect a significant correlation between anti-VEGFR1b IgG levels and carotid intima-media thickness (Table 6). The VEGFR family consists of three transmembrane receptors with tyrosine kinase activity (VEGFR1, VEGFR2 and VEGFR3). VEGFR1 and VEGFR2 are highly expressed in vascular endothelial cells while VEGFR3 is mainly expressed in lymphatic endothelial cells. Because most VEGFR1 isoforms are soluble, they can block VEGF binding to VEGFR2 and influence the formation of blood vessels. It was reported that

### Table 3. Kolmogorov–Smirnov test for normal distribution of plasma IgG levels.

|        | Skewness | Kurtosis | P     |
|--------|----------|----------|-------|
| CD25a  |          |          |       |
| Patient| -0.081   | -0.398   | 0.261 |
| Control| -0.111   | -0.316   | 0.230 |
| CD25b  |          |          |       |
| Patient| 0.190    | 0.364    | 0.421 |
| Control| 1.062    | 3.684    | <0.001|
| CD25c  |          |          |       |
| Patient| -0.308   | -0.394   | 0.020 |
| Control| 0.558    | 0.104    | 0.003 |
| FOXP3a |          |          |       |
| Patient| 0.386    | -0.087   | 0.035 |
| Control| 0.339    | 0.277    | 0.111 |
| FOXP3b |          |          |       |
| Patient| 0.452    | 0.206    | 0.011 |
| Control| 0.419    | 0.627    | 0.056 |
| VEGFR1a|          |          |       |
| Patient| 1.134    | 1.937    | <0.001|
| Control| 0.617    | 0.315    | <0.001|
| VEGFR1b|          |          |       |
| Patient| 1.107    | 2.349    | <0.001|
| Control| 0.367    | -0.290   | 0.038 |

*Values of P > 0.05 were considered represent normal distribution.

FOXP3, fork-head box P3; VEGFR1, vascular endothelial growth factor 1.

### Table 4. Inter-assay deviation of the in-house ELISA for plasma IgG antibodies.

| Antibody | No. of plates | Coefficient of variation (%) |
|----------|---------------|------------------------------|
| CD25a    | 20            | 19.1                         |
| CD25b    | 20            | 12.2                         |
| CD25c    | 20            | 21.2                         |
| FOXP3a   | 20            | 12.0                         |
| FOXP3b   | 20            | 10.8                         |
| VEGFR1a  | 20            | 7.4                          |
| VEGFR1b  | 20            | 11.9                         |

ELISA, enzyme-linked immunosorbent assay; FOXP3, fork-head box P3; VEGFR1, vascular endothelial growth factor 1.
the anti-VEGF monoclonal antibody bevacizumab used to treat solid cancer could produce cardiovascular toxicity.\textsuperscript{32} Potentially, imbalances between VEGFR1 and VEGFR2 signaling could be involved in the development of atherosclerosis.

Several reports have demonstrated that oxidized low-density lipoprotein (oxLDL), a trigger of atherogenesis, may inhibit the functions of Treg cells.\textsuperscript{33} OxLDL can induce apoptosis of Treg cells and hamper their immunosuppressive functions through down-regulation of FOXP3 expression.\textsuperscript{34–36} Recent work has suggested that activated Treg cells suppress the progression of atherosclerosis and that FOXP3 genetically controls a transcriptional program that protects against development of atherosclerotic plaques.\textsuperscript{37} Although our study failed to detect a significant change in circulating IgG levels against CD25 and FOXP3, there was a trend toward decreasing anti-FOXP3b IgG levels in patients with atherosclerosis.

### Table 5. Levels of plasma IgG against CD25, FOXP3 and VEGFR1 in patients with atherosclerosis and control subjects.

| IgG     | Group     | Patient SBR (n) | Control SBR (n) | Z\textsuperscript{a} | P \textsuperscript{b} |
|---------|-----------|----------------|----------------|----------------------|----------------------|
| CD25a   | Male      | 0.73 ± 0.20 (127) | 0.72 ± 0.19 (109) | 0.40 | 0.686 |
|         | Female    | 0.75 ± 0.21 (91) | 0.70 ± 0.21 (91) | 1.41 | 0.158 |
|         | Both      | 0.73 ± 0.20 (218) | 0.71 ± 0.20 (200) | 1.20 | 0.231 |
| CD25b   | Male      | 0.81 ± 0.21 (127) | 0.80 ± 0.23 (109) | 0.43 | 0.669 |
|         | Female    | 0.84 ± 0.19 (91) | 0.82 ± 0.19 (91) | 0.99 | 0.323 |
|         | Both      | 0.82 ± 0.20 (218) | 0.81 ± 0.21 (200) | 0.92 | 0.359 |
| CD25c   | Male      | 1.32 ± 0.43 (127) | 1.31 ± 0.51 (109) | 0.49 | 0.622 |
|         | Female    | 1.37 ± 0.46 (91) | 1.29 ± 0.48 (91) | 1.26 | 0.208 |
|         | Both      | 1.34 ± 0.44 (218) | 1.30 ± 0.49 (200) | 1.22 | 0.222 |
| FOXP3a  | Male      | 0.93 ± 0.28 (127) | 0.92 ± 0.24 (109) | −0.15 | 0.878 |
|         | Female    | 0.97 ± 0.27 (91) | 0.92 ± 0.20 (91) | 1.09 | 0.274 |
|         | Both      | 0.94 ± 0.27 (218) | 0.93 ± 0.23 (200) | −0.53 | 0.594 |
| FOXP3b  | Male      | 0.85 ± 0.25 (127) | 0.92 ± 0.25 (109) | −2.17 | 0.03 |
|         | Female    | 0.91 ± 0.25 (91) | 0.93 ± 0.21 (91) | −0.74 | 0.457 |
|         | Both      | 0.87 ± 0.25 (218) | 0.92 ± 0.23 (200) | −2.224 | 0.025 |
| VEGFR1a | Male      | 1.56 ± 0.45 (127) | 1.65 ± 0.42 (109) | −1.83 | 0.067 |
|         | Female    | 1.67 ± 0.57 (91) | 1.67 ± 0.47 (91) | −0.53 | 0.595 |
|         | Both      | 1.60 ± 0.50 (218) | 1.66 ± 0.44 (200) | −1.82 | 0.069 |
| VEGFR1b | Male      | 1.44 ± 0.39 (127) | 1.50 ± 0.38 (109) | −2.45 | 0.014 |
|         | Female    | 1.59 ± 0.52 (91) | 1.60 ± 0.41 (91) | −0.81 | 0.416 |
|         | Both      | 1.50 ± 0.45 (218) | 1.58 ± 0.39 (200) | −2.46 | 0.014 |

Plasma IgG levels are expressed as means ± SDs of the SBR.\textsuperscript{a} Mann–Whitney U test; \textsuperscript{b} Values of P < 0.017 were considered statistically significant.

FOXP3, fork-head box P3; VEGFR1, vascular endothelial growth factor 1; SBR, specific binding ratio.

### Table 6. Spearman correlation analysis of carotid intima-media thickness and plasma IgG levels against CD25, FOXP3 and VEGFR1.

| Antibody | df | Coefficients of correlation (r) | P |
|----------|----|--------------------------------|---|
| CD25a    | 216 | −0.011                         | 0.870 |
| CD25b    | 216 | −0.057                         | 0.405 |
| CD25c    | 216 | −0.026                         | 0.698 |
| FOXP3a   | 216 | 0.015                          | 0.829 |
| FOXP3b   | 216 | −0.020                         | 0.765 |
| VEGFR1a  | 216 | 0.018                          | 0.788 |
| VEGFR1b  | 216 | −0.020                         | 0.765 |

FOXP3, fork-head box P3; VEGFR1, vascular endothelial growth factor 1.
(Table 5). Further investigation is needed to test circulating IgGs against a range of FOXP3-derived peptide antigens.

Gender differences in the pathophysiology of atherosclerosis have long been recognized. Gender differences in sex hormones and genetic background may be associated with increased susceptibility to atherosclerosis in men. The present study found a gender difference in circulating natural antibodies and a significant decrease in anti-VEGFR1b IgG levels was observed only in male patients (Table 5). This finding supports the hypothesis that men are more likely to develop atherosclerosis than women.

There were several limitations of this study. First, the sample size was quite small, and therefore power was insufficient to draw firm conclusions regarding negative results. Second, the in-house ELISA against individual peptide antigens has low sensitivity. Thus, antibody detection may not be suitable for screening of atherosclerotic patients in clinical settings.

**Conclusion**

Deficiency of plasma anti-VEGFR1 IgG may contribute to the development of atherosclerosis. Decreased anti-VEGFR1b IgG levels in the circulation may be a useful biomarker for identification of a subgroup of atherosclerosis-related conditions involving dysfunction of VEGFR1.

**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

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