Significance of T cell-related immune responses in atherosclerosis in patients with type 2 diabetes

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ABSTRACT
The presence of autoantibodies against oxidized low-density lipoprotein (oxLDL) in the lesions of patients with atherosclerosis provided the initial evidence of the involvement of adaptive immunity in the development of atherosclerosis. Patients with type 2 diabetes mellitus (T2DM) often have autoantibodies to oxLDL, and thus platelet-derived microparticles (PDMPs), macrophages, lymphocytes, and anti-oxLDL antibodies could all play important roles in the development of atherosclerosis in patients with T2DM. Soluble cytotoxic T-lymphocyte-associated antigen 4 (sCTLA-4) can modulate and terminate immune responses and is elevated in patients with some autoimmune disorders. However, sCTLA-4 levels have not previously been investigated in patients with T2DM. We investigated the levels of transforming growth factor (TGF)β₁ and sCTLA-4 in T2DM patients to determine the clinical association between TGFβ₁ and sCTLA-4. The levels of C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), soluble P-selectin (sP-selectin), soluble E-selectin (sE-selectin), soluble vascular cell adhesion molecule-1 (sVCAM-1), PDMP, TGFβ₁ and sCTLA-4 were higher in T2DM patients than in non-diabetic controls. The patients with high TGFβ₁ exhibited a significant increase in PDMP, MCP-1, sP-selectin, sE-selectin, sVCAM-1 and sCTLA-4 compared with those with low TGFβ₁. In contrast, anti-oxidized low-density lipoprotein immunoglobulin G (anti-oxLDL IgG) was significantly decreased in T2DM patients with high TGFβ₁. In addition, PDMP levels were positively correlated with sCTLA-4 and negatively correlated with anti-oxLDL IgG. These results suggest that PDMP, TGFβ₁ and sCTLA-4 can partially modulate immune responses in T2DM patients, resulting in the decrease in anti-oxLDL IgG and development of atherosclerosis.

KEYWORDS: platelet-derived microparticle, sCTLA-4, TGFβ₁, type 2 diabetes, atherosclerosis

INTRODUCTION
Patients with type 2 diabetes mellitus (T2DM) typically display hypercoagulability and platelet hyperaggregability, together with increased levels of platelet activation markers [1, 2]. These changes are associated with an increased risk of cardiovascular events [3, 4]. Platelet-derived microparticles (PDMPs)
are generated by platelet activation and play roles in normal hemostatic responses to vascular injury [5-7]. It is thought that PDMPs contribute to thrombin generation and thrombus formation by generating tissue factor [8, 9]. Therefore, PDMPs, with the participation of the blood coagulation system may ultimately cause atherosclerosis in T2DM patients. A high plasma level of low-density lipoprotein (LDL) cholesterol may also promote the development of atherosclerotic disease [10, 11]. Specifically, LDL that has been modified (e.g., by oxidation) is capable of loading macrophages with cholesterol, while unmodified LDL is not [12]. Notably, oxidized (ox)LDL is considered particularly atherogenic because of the hypercoagulability.

In contrast, atherosclerosis is classified as an inflammatory and immune-mediated disease [13, 14]. It has been suggested that both innate and adaptive immunity play a significant role in the development and progression of atherosclerosis [14-16]. The innate immune response initiates disease with the activation of monocytes/macrophages in the vessel wall, followed by more specific adaptive responses mediated by T and B cells [17]. Immune responses mediated by T cells and B cells could be the dominant factors in enhancing inflammation via induction of various cytokines and chemokines. In fact, the presence of autoantibodies against oxLDL in the lesions of patients with atherosclerosis and animal models provided initial evidence of the involvement of adaptive immunity in the development of atherosclerosis [18]. T2DM patients also often have autoantibodies to oxLDL [19, 20]. Thus, platelets, macrophages, lymphocytes, and anti-oxLDL antibodies could play important roles in the development of atherosclerosis in diabetes patients.

CD4+ regulatory T cells (Tregs) play a critical role in the maintenance of peripheral tolerance by suppressing the activation and proliferation of immune cells [21, 22]. They are divided into 2 subtypes, naturally occurring (nTreg) and induced (iTreg), based on their ontogeny and mode of action. Naturally occurring Tregs are generated in the thymus, and constitutively express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and the transcription factor forkhead-box p3 (Foxp3) [21]. Soluble CTLA-4 (sCTLA-4) can modulate and terminate immune responses [23]. Several reports have shown that sCTLA-4 levels are elevated in patients with some autoimmune disorders [23, 24]. However, to our knowledge, sCTLA-4 levels have not previously been investigated in patients with T2DM and atherosclerosis. We investigated the levels of transforming growth factor (TGF)β1 and sCTLA-4 in T2DM patients to determine the clinical association of TGFβ1 and sCTLA-4 with atherosclerosis.

MATERIALS AND METHODS

Patients

The study cohort included 85 non-diabetic and 165 T2DM patients (Table 1), selected from among those admitted to four hospitals (Saiseikai Izuo Hospital, Meisei Kinen Hospital, Daiwa Hospital, and Kansai Medical University) between April 2011 and June 2016 for the treatment of hypertension, hyperlipidemia, or diabetes. The study protocol was approved by the Institutional Review Board and written informed consent was obtained from each patient. Individuals were excluded if they had a history (within 3 months prior to enrollment) of inflammatory coronary artery or cerebrovascular disease, or if they had clinically detectable hepatic dysfunction (elevated transaminases), infection (fever or an elevated white blood cell count), or malignancy (detected on ultrasound or computed tomography). Of the included patients, 26 were taking aspirin owing to previous cerebral infarction or angina pectoris, 78 were taking angiotensin II receptor blockers (ARBs), 55 were taking Ca antagonists, and 55 were taking statins (Table 1). The doses of these drugs were not adjusted, and there were no other changes to drug therapy, during the present study.

Measurement of PDMP

PDMP levels were measured twice and the mean values were recorded. Furthermore, basic studies were carried out prior to this assessment using clinical specimens. The enzyme-linked immunosorbent assay (ELISA) kit used for PDMP quantifications was obtained from JIMRO Co. Ltd. (Tokyo, Japan) [25].
Measurement of soluble molecules and adiponectin

Blood samples from patients and controls under fasting conditions were collected into tubes with or without sodium citrate and allowed to clot at room temperature for a minimum of 1 hour. Citrated plasma or serum was isolated by centrifugation at 1000 g at 4 °C and then stored at -30 °C until analysis. Plasma concentrations of sP-selectin, sE-selectin, sVCAM-1, monocyte chemotactant protein-1 (MCP-1) and TGFβ3 were measured using monoclonal antibody-based ELISA kits (Invitrogen Inc, Camarillo, CA, USA), plasma adiponectin was measured with...
adiponectin ELISA kits (Otsuka Pharmaceuticals Co. Ltd., Tokyo, Japan), and sCTLA-4 was measured with an ELISA kit from BioLegend, Inc. (San Diego, CA, USA). The recombinant products and standard solutions provided with each kit were used as positive controls in each assay and all procedures were performed according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as the mean ± SD and were analyzed using multivariate regression analysis, as appropriate. Between-group comparisons were analyzed using the Newman-Keuls test and Scheffe’s test. The correlation between PDMP concentration and continuous variables was assessed using multivariate linear regression analysis. *P*-values less than 0.05 were considered statistically significant.

**RESULTS**

Patient demographics and clinical characteristics were similar in the T2DM and non-diabetic groups, except for fasting blood glucose and hemoglobin (Hb)A1c concentrations (Table 1). The levels of blood urea nitrogen (BUN), creatinine, C-reactive protein (CRP), MCP-1, sP-selectin, sE-selectin, sVCAM-1, PDMP, TGFβ1 and sCTLA-4 were higher in patients with T2DM than in non-diabetic controls (Table 2). However, adiponectin was lower in T2DM patients than in non-diabetic controls (Table 2).

Using univariate and multivariate regression analyses, we investigated the associations between the 18 variables and PDMP concentration in patients with T2DM (Table 3). Univariate analysis showed that BMI, angina pectoris, high-density lipoprotein cholesterol (HDL-C), LDL-C, MCP-1, sP-selectin, sE-selectin, sVCAM-1, adiponectin, anti-oxLDL IgG, TGFβ1, and sCTLA-4 were factors significantly associated with PDMP; whereas MCP-1, sP-selectin, sE-selectin, sVCAM-1, and adiponectin were significantly correlated with PDMP in multivariate analysis.

Table 4 shows the levels of various markers in T2DM patients according to the difference in TGFβ1. The levels of these markers in T2DM patients with or without elevated TGFβ1 (High TGFβ1: greater than the mean + two standard deviations of basal levels, low TGFβ1: less than the mean - two standard deviations of basal levels) were used for the analysis. Forty-six patients had high TGFβ1 levels, and 39 had low TGFβ1 levels. The patients with high TGFβ1 exhibited a significant increase in PDMP, MCP-1, sP-selectin, sE-selectin, sVCAM-1 and sCTLA-4 compared with those with low TGFβ1. In contrast, anti-oxLDL IgG was significantly decreased in T2DM patients with high TGFβ1.

Figure 1 shows the correlation of PDMP with other parameters in T2DM patients with high TGFβ1 levels. PDMP levels were positively correlated with sCTLA-4 (correlation coefficient *r* = 0.6539, *P* < 0.001). In contrast, PDMP levels were negatively correlated with anti-oxLDL IgG (*r* = -0.3287, *P* < 0.01).

**DISCUSSION**

The present study showed that levels of MCP-1, sP-selectin, sE-selectin, sVCAM-1, PDMP, TGFβ1 and sCTLA-4 were higher in patients with T2DM than in non-diabetic controls. In addition, PDMP was significantly correlated with MCP-1, sP-selectin, sE-selectin, sVCAM-1, and adiponectin in T2DM patients as shown by multivariate analysis. PDMPs play an important role in coagulation, and the increased levels of PDMPs may cause hypercoagulability [5-7]. Furthermore, we previously reported that PDMP levels are significantly higher in diabetic patients with elevated serum LDL levels than in similar patients with depressed serum LDL levels [27]. Therefore, the present results suggest the possibility that endothelial dysfunction owing to activated platelets and PDMP induced vascular damage in T2DM patients.

A high plasma level of LDL-C may promote the development of atherosclerotic disease [10, 11]. In particular, oxLDL is considered predominantly atherogenic [12], and the accumulation of oxLDL causes the generation of anti-oxLDL autoantibodies [28]. In fact, the immune response against oxLDL has been suggested by some studies to be associated with the severity of atherosclerosis [26, 29-31]. Regarding the significance of anti-oxLDL antibody, it is thought that anti-oxLDL antibodies
Recognition of the existence and function of immune cells in atherosclerotic lesions categorized atherosclerosis as an inflammatory disease [36]. Most atherosclerosis lesion-derived T cells are helper T (Th) cells [37, 38]. In contrast, Tregs, have been characterized as a negative regulator of immune effector cells [37]. Treg differentiation requires TGFβ1, and an environment with high levels of TGFβ1 selectively promotes Treg differentiation [39-41]. In the present study, the patients with high TGFβ1 levels exhibited a significant increase in PDMP and sCTLA-4 may be important for the clearance of oxLDL, and hyperimmunization with oxLDL results in high antibody titers and protection against atherosclerosis in hypercholesterolemic rabbits [32-34]. Interestingly, in the present study, anti-oxLDL IgG significantly decreased in T2DM patients with high TGFβ1 levels. In addition, PDMP levels were negatively correlated with anti-oxLDL IgG. These results suggest a relationship between PDMP and anti-oxLDL IgG in T2DM patients with high TGFβ1 levels.

Immune cells, both from the innate and adaptive arms of immunity, are present throughout all stages of atherosclerotic lesion development [35]. Recognition of the existence and function of immune cells in atherosclerotic lesions categorized atherosclerosis as an inflammatory disease [36]. Most atherosclerosis lesion-derived T cells are helper T (Th) cells [37, 38]. In contrast, Tregs, have been characterized as a negative regulator of immune effector cells [37]. Treg differentiation requires TGFβ1, and an environment with high levels of TGFβ1 selectively promotes Treg differentiation [39-41]. In the present study, the patients with high TGFβ1 levels exhibited a significant increase in PDMP and sCTLA-4.
Both TGFβ1 and CTLA-4 are key molecules in Treg-mediated immune responses and it has been reported that sCTLA-4 is increased in patients with autoimmune diseases [46, 47]. In most of these reports, the increase in sCTLA-4 is observed even in the active stage of the diseases [47]. In the present study, the patients with high TGFβ1 levels exhibited not only a significant increase in sCTLA-4 but also a significant decrease in anti-oxLDL IgG compared with those with low TGFβ1 levels. CTLA-4 plays a key role in the maintenance of peripheral tolerance as well as the termination of T-cell responses [42]. Native sCTLA-4 binds to costimulatory tracts such as CD80/CD86 [43]. Therefore, it has been suggested that sCTLA can act as a competitor of CD28 for binding to CD80 or CD86, thereby interfering with T-lymphocyte activation in the initiation of immune responses [44]. Similarly, Treg-mediated suppression is contact-dependent, and this suppression involves the interaction between CTLA-4 and costimulatory molecules on Tregs and B7 (CD80/86) [45]. sCTLA-4 derived from Tregs also inhibit inflammation via a similar mechanism. Both TGFβ1 and CTLA-4 are key molecules in Treg-mediated immune responses and it has been reported that sCTLA-4 is increased in patients with autoimmune diseases [46, 47]. In most of these reports, the increase in sCTLA-4 is observed even in the active stage of the diseases [47]. In the present study, the patients with high TGFβ1 levels exhibited not only a significant increase in sCTLA-4 but also a significant decrease in anti-oxLDL IgG compared with those with low TGFβ1 levels. In addition, PDMP levels were negatively correlated with anti-oxLDL IgG. Sadallah et al. [48] reported that PDMPs induce differentiation of CD4+ T cells towards functional Tregs dependent on TGFβ1, which may represent a mechanism by which PDMPs enhance peripheral tolerance.

Table 3. Multivariate regression analysis of platelet-derived microparticles in patients with type 2 diabetes.

<table>
<thead>
<tr>
<th>Analysis</th>
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<th>Multivariate</th>
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<td>P-value</td>
<td>β</td>
<td>P-value</td>
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<td>BMI (kg/m²)</td>
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<td>Angina pectoris (%)</td>
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<td>Heart failure (%)</td>
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<tr>
<td>Cerebral infarction (%)</td>
<td>0.2977</td>
<td>0.09631</td>
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<td>TC (mg/dL)</td>
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<td>HDL-C (mg/dL)</td>
<td>-0.2615</td>
<td>0.00962*</td>
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<td>LDL-C (mg/dL)</td>
<td>0.4938</td>
<td>0.00127*</td>
<td>0.3347</td>
<td>0.05127</td>
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<td>CRP (mg/dL)</td>
<td>0.2634</td>
<td>0.0617</td>
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<td>MCP-1 (pg/mL)</td>
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<td>0.00013*</td>
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<td>sP-selectin (ng/mL)</td>
<td>0.7689</td>
<td>&lt; 0.00001*</td>
<td>0.6342</td>
<td>0.00124*</td>
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<td>sE-selectin (ng/mL)</td>
<td>0.4635</td>
<td>0.00617*</td>
<td>0.3116</td>
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<td>sVCAM-1 (ng/mL)</td>
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<td>0.00421*</td>
<td>0.3728</td>
<td>0.03619*</td>
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<td>Adiponectin (µg/mL)</td>
<td>-0.6384</td>
<td>&lt; 0.00001*</td>
<td>-0.5931</td>
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<td>Anti-oxLDL IgG (AcU/mL)</td>
<td>-0.3255</td>
<td>0.00734*</td>
<td>-0.2961</td>
<td>0.07522</td>
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<tr>
<td>TGFβ1 (pg/mL)</td>
<td>0.2637</td>
<td>0.02841*</td>
<td>0.1967</td>
<td>0.13446</td>
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<td>sCTLA-4 (pg/mL)</td>
<td>0.3872</td>
<td>0.00629*</td>
<td>0.2997</td>
<td>0.05992</td>
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</table>

β indicates standardized regression coefficients. * indicates statistical significance.

Abbreviations: See table 1 and table 2.
sCTLA-4, and negatively correlated with anti-oxLDL IgG. No previous study had assessed these effects. We showed that PDMP, TGFβ1 and sCTLA-4 can partially modulate immune responses in T2DM patients, resulting in the decrease in

**CONCLUSION**

This study has two potential strengths. First, anti-oxLDL IgG was significantly decreased in T2DM patients with high levels of TGFβ1. Second, PDMP levels were positively correlated with sCTLA-4, and negatively correlated with anti-oxLDL IgG. No previous study had assessed these effects. We showed that PDMP, TGFβ1 and sCTLA-4 can partially modulate immune responses in T2DM patients, resulting in the decrease in

<table>
<thead>
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<th>Low-TGFβ1</th>
<th>High-TGFβ1</th>
<th>P-value</th>
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<tr>
<td>n</td>
<td>39</td>
<td>46</td>
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</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>45 ± 19</td>
<td>40 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>139 ± 43</td>
<td>145 ± 51</td>
<td>NS</td>
</tr>
<tr>
<td>PDMP (U/mL)</td>
<td>13.4 ± 4.9</td>
<td>25.4 ± 6.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>493 ± 117</td>
<td>641 ± 138</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>sP-selectin (ng/mL)</td>
<td>264 ± 91</td>
<td>315 ± 107</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>sE-selectin (ng/mL)</td>
<td>89 ± 45</td>
<td>114 ± 53</td>
<td>&lt; 0.05</td>
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<tr>
<td>sVCAM-1 (ng/mL)</td>
<td>748 ± 123</td>
<td>1,125 ± 179</td>
<td>&lt; 0.01</td>
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<tr>
<td>Adiponectin (µg/mL)</td>
<td>2.47 ± 1.11</td>
<td>2.56 ± 1.52</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-oxLDL IgG (AcU/mL)</td>
<td>28.4 ± 7.1</td>
<td>15.2 ± 5.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>sCTLA-4 (pg/mL)</td>
<td>166 ± 27</td>
<td>382 ± 45</td>
<td>&lt; 0.001</td>
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</table>

Data are shown as means ± SD. P-value, T2DM versus non-diabetic controls.

Abbreviations: See table 1 and table 2.

**Figure 1.** Correlation of platelet-derived microparticle levels with sCTLA-4 and anti-oxLDL IgG.
anti-oxLDL IgG and development of atherosclerosis. However, this study also had several limitations. First, the detection of Treg cells by flow cytometry was not performed. Second, we could not identify Th17 cells or the levels of interleukin-17, which are important effectors in patients with T2DM and atherosclerosis. Third, we could not clarify the significance of anti-oxLDL IgG relative to atherosclerosis with low TGFβ1 levels. Confirmation of these findings in larger and more detailed studies would be useful.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to report for this work.

REFERENCES