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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)β_1 and sCTLA-4 in T2DM patients to determine the clinical association between TGFβ_1 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, TGFβ_1 and sCTLA-4 were higher in T2DM patients than in non-diabetic controls. The patients with high TGFβ_1 exhibited a significant increase in PDMP, MCP-1, sP-selectin, sE-selectin, sVCAM-1 and sCTLA-4 compared with those with low TGFβ_1. In contrast, anti-oxidized low-density lipoprotein immunoglobulin G (anti-oxLDL IgG) was significantly decreased in T2DM patients with high TGFβ_1. In addition, PDMP levels were positively correlated with sCTLA-4 and negatively correlated with anti-oxLDL IgG. These results suggest that PDMP, TGFβ_1 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, TGFβ_1, 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)

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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)β_1 and sCTLA-4 in T2DM patients to determine the clinical association of TGFβ_1 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].

Table 1. Demographics and clinical characteristics of the patients with and without type 2 diabetes.

	Non-diabetic	**T2DM**	***P*-value**
n	85	165	
Men/Women (n)	51/34	102/63	
Age (years)	64 ± 7	66 ± 10	NS
BMI (mg/m^2)	23.2 ± 5.1	27.4 ± 4.9	NS
FBG (mg/dL)	100 ± 22	241 ± 60	< 0.001
HbA1c (%)	5.1 ± 1.0	7.6 ± 1.7	< 0.01
TC (mg/dL)	228 ± 33	225 ± 39	NS
HDL-C (mg/dL)	44 ± 16	42 ± 15	NS
LDL-C (mg/dL)	139 ± 50	141 ± 47	NS
Complications, n (%)			
Angina pectoris	10 (11.8)	22 (13.3)	NS
Heart failure	7 (8.2)	16 (9.7)	NS
Cerebral infarction	6 (7.1)	18 (10.9)	NS
Medication, n (%)			
Statins	23 (27.1)	32 (19.4)	NS
ARBs	31 (36.5)	47 (28.5)	NS
Ca antagonist	22 (25.9)	33 (20.0)	NS
Aspirin	10 (11.8)	16 (9.7)	NS

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

Abbreviations: BMI, body mass index; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ARBs, angiotensin II receptor blockers; NS, not significant; SD, standard deviation.

Measurement of autoantibodies against oxidized LDL

Blood samples from patients and healthy controls were collected into tubes with sodium citrate or tubes without anticoagulant. Blood was allowed to clot at room temperature for a minimum of 1 hour. Serum or citrated plasma was isolated by centrifugation for 20 minutes at 1000 g at 4 °C and then stored at -30 °C until analysis. As positive controls, in each assay we used the recombinant products and standard solutions provided with the commercial kits. The ELISA kit for oxidized LDL was from Biodesign International, Inc. (Kennebunk, Maine, USA) [26].

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 for 20 minutes at 4 °C and stored at -30 °C until analysis. Plasma concentrations of sP-selectin, sE-selectin, sVCAM-1, monocyte chemoattractant protein-1 (MCP-1) and TGFβ_1 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, TGFβ_1 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, TGFβ_1 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 TGFβ_1. The levels of these markers in T2DM patients with or without elevated TGFβ_1 (High TGFβ_1: greater than the mean + two standard deviations of basal levels, low TGFβ_1: less than the mean - two standard deviations of basal levels) were used for the analysis. Forty-six patients had high TGFβ_1 levels, and 39 had low TGFβ_1 levels. The patients with high TGFβ_1 exhibited a significant increase in PDMP, MCP-1, sP-selectin, sE-selectin, sVCAM-1 and sCTLA-4 compared with those with low TGFβ_1. In contrast, anti-oxLDL IgG was significantly decreased in T2DM patients with high TGFβ_1.

Figure 1 shows the correlation of PDMP with other parameters in T2DM patients with high TGFβ_1 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, TGFβ_1 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

Table 2. Plasma or serum levels of soluble factors, chemokines and adiponectin in patients with type 2 diabetes and non-diabetic controls.

	Non-diabetic	**T2DM**	***P*-value**
n	85	165	
BUN (mg/dL)	18.1 ± 8.3	27.2 ± 13.4	< 0.05
S-CRTN (mg/dL)	0.65 ± 0.21	1.98 ± 1.02	< 0.05
AST (mg/dL)	33 ± 13	36 ± 16	NS
ALT (mg/dL)	27 ± 15	25 ± 18	NS
T-BIL (mg/dL)	0.73 ± 0.39	0.88 ± 0.59	NS
LD (U/L)	223 ± 703	234 ± 86	NS
CRP (mg/dL)	0.84 ± 0.77	1.26 ± 0.89	< 0.05
MCP-1 (pg/mL)	347 ± 89	575 ± 124	< 0.01
sP-selectin (ng/mL)	234 ± 79	289 ± 106	< 0.05
sE-selectin (ng/mL)	74 ± 42	98 ± 50	< 0.05
sVCAM-1 (ng/mL)	640 ± 142	951 ± 153	< 0.01
Adiponectin (μg/mL)	4.59 ± 1.07	2.48 ± 1.46	< 0.01
PDMP (U/mL)	11.4 ± 2.7	19.7 ± 3.1	< 0.01
Anti-oxLDL IgG (AcU/mL)	19.8 ± 5.6	23.8 ± 6.2	NS
$TGF\beta_1$ (pg/mL)	3,120 ± 1,345	3,650 ± 1,620	< 0.05
sCTLA-4 (pg/mL)	186 ± 22	241 ± 33	< 0.05

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

Abbreviations: BUN, blood urea nitrogen; S-CRTN, serum creatine; AST, aspartate aminotransferase; ALT, alanine transaminase; T-BIL, total bilirubin; LD, lactate dehydrogenase; CRP, C-reactive protein; MCP-1, monocyte chemoattractant protein-1; sP-selectin, soluble P-selectin; sE-selectin, soluble E-selectin; sVCAM-1, soluble vascular cell adhesion molecule-1; PDMP, platelet-derived microparticle; Anti-oxLDL IgG, anti-oxidized low-density lipoprotein immunoglobulin G; $TGF\beta_1$, transforming growth factor β_1; sCTLA-4, soluble cytotoxic T lymphocyte-associated antigen 4; NS, not significant; SD, standard deviation.

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 $TGF\beta_1$ 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 $TGF\beta_1$ 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, T regs, have been characterized as a negative regulator of immune effector cells [37]. Treg differentiation requires $TGF\beta_1$, and an environment with high levels of $TGF\beta_1$ selectively promotes Treg differentiation [39-41]. In the present study, the patients with high $TGF\beta_1$ levels exhibited a significant increase in PDMP and sCTLA-4

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

Analysis	Univariate		Multivariate	
	β	*P*-value	β	*P*-value
Age (years)	0.2159	0.08715		
Sex (men)	-0.0796	0.39445		
BMI (kg/m^2)	0.3952	0.01255*	0.2986	0.74331
Angina pectoris (%)	0.3561	0.0497*	0.2814	0.55127
Heart failure (%)	0.1995	0.27634		
Cerebral infarction (%)	0.2977	0.09631		
TC (mg/dL)	-0.0237	0.25114		
HDL-C (mg/dL)	-0.2615	0.00962*	-0.1834	0.11245
LDL-C (mg/dL)	0.4938	0.00127*	0.3347	0.05127
CRP (mg/dL)	0.2634	0.0617		
MCP-1 (pg/mL)	0.5769	0.00013*	0.4561	0.02344*
sP-selectin (ng/mL)	0.7689	< 0.00001*	0.6342	0.00124*
sE-selectin (ng/mL)	0.4635	0.00617*	0.3116	0.04128*
sVCAM-1 (ng/mL)	0.4993	0.00421*	0.3728	0.03619*
Adiponectin (μg/mL)	-0.6384	< 0.00001*	-0.5931	0.00182*
Anti-oxLDL IgG (AcU/mL)	-0.3255	0.00734*	-0.2961	0.07522
TGFβ$_1$ (pg/mL)	0.2637	0.02841*	0.1967	0.13446
sCTLA-4 (pg/mL)	0.3872	0.00629*	0.2997	0.05992

β indicates standardized regression coefficients. * indicates statistical significance.

Abbreviations: See table 1 and table 2.

compared with those with low TGFβ$_1$ 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 TGFβ$_1$ 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 TGFβ$_1$ levels exhibited not only a significant increase in sCTLA-4 but also a significant decrease in anti-oxLDL IgG compared with those with low TGFβ$_1$ 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 TGFβ$_1$, which may represent a mechanism by which PDMPs enhance peripheral tolerance.

Table 4. Comparison of various data for patients with type 2 diabetes according to the difference in TGFβ$_1$ levels.

	Low-TGFβ$_1$	**High-TGFβ$_1$**	***P*-value**
n	39	46	
HDL-C (mg/dL)	45 ± 19	40 ± 11	NS
LDL-C (mg/dL)	139 ± 43	145 ± 51	NS
PDMP (U/mL)	13.4 ± 4.9	25.4 ± 6.1	< 0.01
MCP-1 (pg/mL)	493 ± 117	641 ± 138	< 0.01
sP-selectin (ng/mL)	264 ± 91	315 ± 107	< 0.05
sE-selectin (ng/mL)	89 ± 45	114 ± 53	< 0.05
sVCAM-1 (ng/mL)	748 ± 123	1,125 ± 179	< 0.01
Adiponectin (µg/mL)	2.47 ± 1.11	2.56 ± 1.52	NS
Anti-oxLDL IgG (AcU/mL)	28.4 ± 7.1	15.2 ± 5.3	< 0.01
sCTLA-4 (pg/mL)	166 ± 27	382 ± 45	< 0.001

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

Abbreviations: See table 1 and table 2.

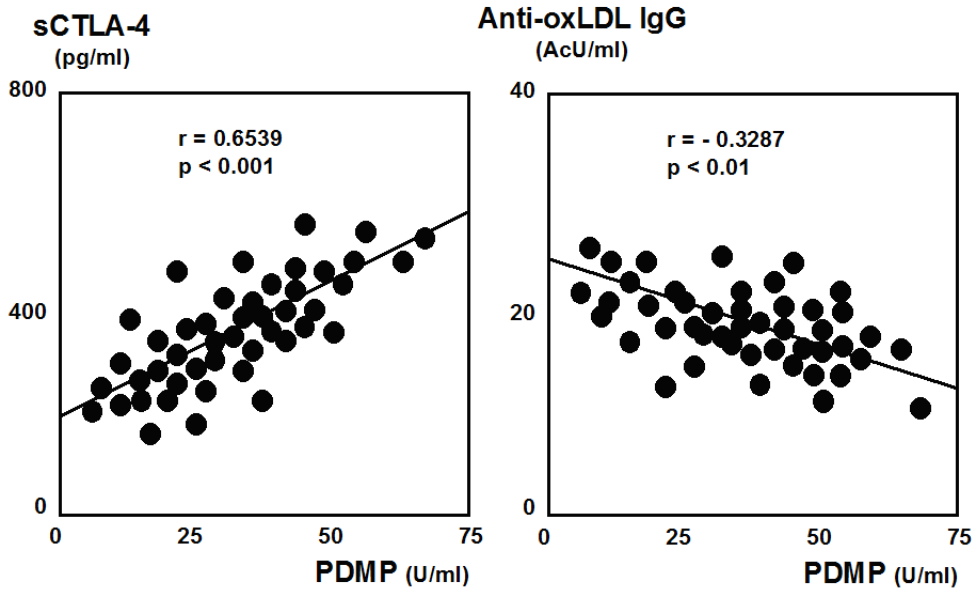

Figure 1. Correlation of platelet-derived microparticle levels with sCTLA-4 and anti-oxLDL IgG.

CONCLUSION

This study has two potential strengths. First, anti-oxLDL IgG was significantly decreased in T2DM patients with high levels of TGFβ$_1$. 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, TGFβ$_1$ and sCTLA-4 can partially modulate immune responses in T2DM patients, resulting in the decrease in

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 TGFβ_1 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.

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