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Demonstration of increased toll-like receptor 2 and toll-like receptor 4 expression in monocytes of type 1 diabetes mellitus patients with microvascular complications

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Abstract

Type 1 diabetes mellitus (T1DM) is associated with increased microvascular complications and is a proinflammatory state. The toll-like receptors (TLRs) are pattern recognition receptors on monocytes and important in atherosclerosis. We have shown increased TLR2 and TLR4 expression on monocytes of T1DM compared with controls. In this report, we tested the surface expression of TLR2 and TLR4 on monocytes of T1DM patients with microvascular complications (T1DM-MV) compared with those without (T1DM) and healthy controls. The study was performed at the University of California Davis. Healthy controls (n = 31), T1DM patients (n = 31), and T1DM-MV patients (n = 34) were included. The TLR2 and TLR4 surface expression was significantly increased in T1DM-MV monocytes compared with T1DM and controls ($P < .01$). In addition, nuclear factor κB activity and interleukin-1β release were significantly increased in monocytes from T1DM-MV compared with T1DM ($P < .005$). Thus, we make the novel observation that TLR2 and TLR4 expression and signaling are increased in T1DM-MV compared with T1DM and may contribute to the accentuated proinflammatory state and complications of T1DM.

1. Introduction

Type 1 diabetes mellitus (T1DM) is associated with an increased risk of vascular complications, and T1DM patients with proteinuria and/or retinopathy have a significantly increased risk of fatal coronary artery disease [1]. Inflammation plays a pivotal role in all stages of atherosclerosis. The monocyte-macrophage, a crucial cell in atherogenesis, is readily accessible for study. We and others have demonstrated that patients with T1DM exhibit increased inflammation as evidenced by increased plasma C-reactive protein (CRP) levels and increased monocyte activity, and these are more pronounced in T1DM with microvascular complications [2-5].

Members of the toll-like receptor (TLR) family play a critical role in the inflammatory components of atherosclerosis. Toll-like receptors are a family of pattern recognition receptors that are important in the regulation of immune function and inflammation [6-8]. Their activation by various ligands triggers a signaling cascade leading to cytokine production and initiation of an adaptive immune response [6-8]. Toll-like receptor 2 and TLR4 expression is up-regulated in atherosclerotic plaque macrophages and in animal models of atherosclerosis [6-8]. Knockout of TLR4 is associated with reduction in lesion size, lipid content, and macrophage infiltration in hypercholesterolemic apolipoprotein E¯/¯ mice [9]. In addition, TLR2/low-density lipoprotein receptor-deficient¯/¯, and in a recent article, TLR2/apolipoprotein E¯/¯, mice are protected from the development of atherosclerosis [10,11].

We have previously demonstrated that TLR2 and TLR 4 are up-regulated in monocytes of patients with T1DM [12]. This was associated with an increase in cytokines, chemokines, nuclear factor (NF) κB activity, MyD88, and Trif. However, although T1DM with microvascular complications

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(T1DM-MV) exhibit accentuated inflammation compared with T1DM [5], there is a paucity of data examining TLR2 and TLR4 surface expression in T1DM-MV compared with T1DM and healthy controls; and this was the aim of the present report.

2. Research design and methods

The T1DM patients (n = 31) and T1DM-MV patients (n = 34) (onset <20 years and on insulin therapy since diagnosis; present age 18 years or older with duration of diabetes 1 year or more) were recruited from the Diabetes and Pediatric Clinics at University of California Davis Medical Center and advertisements in the local newspaper. Microvascular complications included retinopathy and nephropathy. None of the patients were on Glucophage (Bristol-Myers Squibb, Princeton, NJ) and/or the thiazolidinediones, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or statins as described previously [5,12]. Other exclusion criteria were as follows: mean hemoglobin A_{1c} (HbA_{1c}) over the last year greater than 10%; inflammatory disorders, for example, rheumatoid arthritis; macrovascular complications such as strokes, myocardial infarction, etc; abnormal liver, renal, or thyroid function; malabsorption; steroid therapy; smoking; abnormal complete blood count; alcohol consumption greater than 1 oz/d; consumption of N-3 polyunsaturated fatty acid capsules (>1g/d); and chronic high-intensity exercisers. *Microvascular complications* were defined as retinopathy, nephropathy, and neuropathy and determined in T1DM patients.

Healthy controls (n = 31), age older than 18 years, were included if they had normal complete blood count; no family history of diabetes or other chronic diseases; normal kidney, liver, and thyroid function; and fasting plasma glucose less than 100 mg/dL. Healthy controls and T1DM and T1DM-MV patients were matched for age (within 10 years), sex, and race. Exclusion criteria were as described previously [5,12]. Informed consent was obtained from participants in the study, which was approved by the institutional review board at University of California Davis. After history and physical examination, fasting blood (30 mL) was obtained.

Mononuclear cells were isolated from fasting heparinized blood by Ficoll-Hypaque centrifugation, followed by magnetic separation of monocytes using the depletion technique (Miltenyi Biotech, Auburn, CA), as described previously [5,12]. Briefly, non-monocytes such as T cells, NK cells, B cells, dendritic cells, and basophils, are indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and CD235a (glycophorin A), as well as Anti-Biotin MicroBeads. Highly pure unlabeled monocytes are obtained by depletion of the magnetically labeled cells.

Monocytes from control and T1DM were incubated with antihuman TLR2 and TLR4 antibodies (InvivoGen, San Diego, CA) or isotype controls, and surface expression of TLR2 and TLR4 was analyzed using BD FACSArray (Franklin Lakes, NJ) [12]. Results were expressed as mean fluorescence intensity of 10 000 cells. Nuclear factor κB activity was examined as readout of TLR signaling as described previously [12] and expressed as nanograms of NFκB p65 per milligram cell protein. The release of interleukin (IL)-1β in the supernates of monocytes in resting and lipopolysaccharide-activated cells was also determined as a readout of up-regulated TLR expression and expressed as picograms per milligram of cell protein as described previously [5,12].

Statistical analyses were performed using SAS software (SAS Institute, Cary, NC). Data are expressed as mean ± SD for parametric data and as median and interquartile range for nonparametric data. After analysis of variance, parametric data were analyzed using paired, 2-tailed t tests and nonparametric data using Wilcoxon signed rank tests. Level

Table 1
Baseline characteristics

	Controls (n = 31)	T1DM (n = 31)	T1DM-MV (n = 34)
Age (y)	32 ± 13	32 ± 13	34 ± 11
BMI (kg/m^2)	25 ± 4	25 ± 4	26 ± 6
Male-female ratio	12:19	12:19	13:21
Glucose (mg/dL)	85 ± 11	131 ± 68*	153 ± 80*
HbA_{1c}(%)	5.4 ± 0.3	7.8 ± 1.1*	8.4 ± 1.5*
Free fatty acids (mmol/L)	0.28 ± 0.1	0.39 ± 0.23 *	0.44 ± 0.26*
Total cholesterol (mg/dL)	175 ± 26	180 ± 37	182 ± 37
Triglycerides (mg/dL)	80 ± 39	81 ± 47	71 ± 57
LDL cholesterol (mg/dL)	112 ± 20	111 ± 28	112 ± 34
HDL cholesterol (mg/dL)	47 ± 15	52 ± 17	51 ± 19
Hs-CRP (mg/L)	1.1 (0.6,1.7)	1.7 (0.9,2.1)*	2.5 (0.7,2.1)†
IL-1β (pg/mL)	10.3 (4.1,7.8)	25.3 (5.3,44.2)*	46.4 (14, 38)†

Data are expressed as mean ± SD and median and interquartile range for CRP. BMI indicates body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Hs-CRP, high-sensitivity C-reactive protein.

* $P < .05$ compared with controls.

† $P < .05$ compared with controls and T1DM.

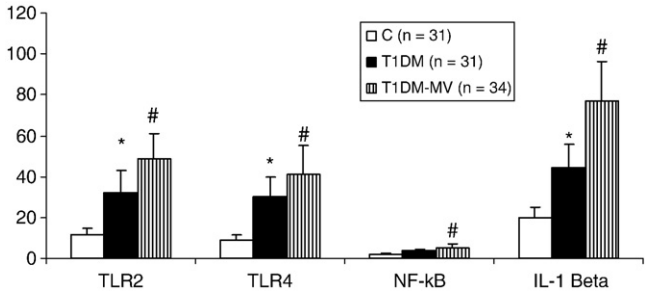

Fig. 1. Increased TLR2/TLR 4 surface expression, mononuclear NFκB binding activity, and IL-1β release in T1DM-MV. Monocytes were obtained from controls (C; n = 31), T1DM (n = 31), and T1DM-MV (n = 34); and TLR2 and TLR4 surface expression (mean fluorescence intensity), nuclear NFκB activity(nanograms per milligram protein), and IL-1β release (picograms per milligram protein) were examined as described in "Research design and methods." $^{*}P < .001$ compared with C, and $^{\#}P < .005$ compared with T1DM and C.

of significance was set at $P < 0.05$. Spearman rank correlation was computed to assess association between variables.

3. Results

Baseline subject characteristics have been provided previously [5,12]. There were no significant differences in age, body mass index, and male to female ratio between control, T1DM, and T1DM-MV groups. In addition, there were no significant differences in the lipid profile. As expected, levels of glucose, HbA_{1c}, and free fatty acids were significantly higher in T1DM and T1DM-MV compared with controls [5]. Furthermore, levels of high-sensitivity CRP and circulating IL-1β were significantly increased in T1DM and T1DM-MV compared with controls; and this was more pronounced in the T1DM-MV group (Table 1).

Monocyte surface expression of TLR2 and TLR4 was significantly up-regulated in T1DM-MV compared with T1DM and controls (Fig. 1). Downstream signaling of TLR, that is, NFκB activity and release of IL-1β, was significantly increased in resting and activated monocytes from T1DM-MV compared with T1DM patients and controls ($P < .005$) (data for NFκB and IL-1β are shown for resting monocytes). In addition, there was a significant correlation between TLR2 expression and NFκB activity ($r = 0.57$, $P < .005$) and IL-1β release ($r = 0.67$, $P < .01$), and TLR4 expression and NFκB activity ($r = 0.7$, $P < .005$) and IL-1β release ($r = 0.79$, $P < .01$), respectively.

4. Discussion

Type 1 diabetes mellitus is a proinflammatory state characterized by increased levels of circulating biomarkers of inflammation and monocyte activity [3-5]. Toll-like receptor 2 and TLR4 play a critical role in atherosclerosis [6-10]. We have previously shown increased inflammation in T1DM-MV compared with T1DM [5,12]. The increased inflammation in T1DM-MV may be mediated in part via activation of the innate immune pathway by the TLRs. However, there are no studies examining TLR expression in T1DM-MV compared with age- and sex-matched T1DM and their contribution to the accentuated proinflammatory state of T1DM. In this report, we provide novel data on up-regulated TLR2 and TLR4 expression and signaling in monocytes of T1DM-MV compared with T1DM and controls.

Toll-like receptors are characterized by an extracellular ligand binding domain, single transmembrane domain, and intracellular domain [6-11]. Upon ligand binding, the TLR subunits associate, leading to the formation of a complex of toll-interacting region domain containing adaptor proteins of the MyD88 family. Subsequent downstream signal transduction events lead to the activation of NFκB and transcription of proinflammatory chemokines such as monocyte chemoattractant protein–1 and cytokines such as IL-1β, IL-6, and tumor necrosis factor [6-11]. In addition to showing that TLR2 and TLR4 surface expression is increased on monocytes isolated from T1DM-MV compared with T1DM, we demonstrate increased NFκB DNA binding activity as well as increased IL-1β release from monocytes of T1DM-MV compared with T1DM. There was also a significant correlation between the increased TLR2 and TLR4 expression, NFκB activity, and IL-1β release, indicating a direct relationship between TLR2 and TLR4 activities and increased inflammation in T1DM-MV. Furthermore, it is important to note that macronutrient intake increases whereas insulin suppresses TLR2 and TLR4 expression [13,14]; and the latter may contribute in part to increased TLR2 and TLR4 in T1DM, an insulin-deficient state.

In conclusion, this is the first demonstration of increased TLR2 and TLR4 expression and activity in T1DM-MV monocytes. Future studies will examine molecular mechanisms for increased TLR2 and TLR4 expression, determine their contribution to microvascular complications of T1DM, and examine their modulation.

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