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Original Article

Toll-like receptor 2 expression on monocytes and microvascular complications in type 2 diabetic patients

Rasha Emad Badr [a,*], Mona Ibrahim Salama [a], Asmaa Kamal Abd-Elmaogood [b], Abd El-Raouf Mohamed Eldeib [c]

[a] Lecturer of Clinical and Chemical Pathology Department, Faculty of Medicine, Portsaid University, Portsaid, Egypt
[b] Lecturer of Clinical and Chemical Pathology Department, Faculty of Medicine, Suez Canal University, Egypt
[c] Professor of Internal Medicine Department, Faculty of Medicine, Suez Canal University, Egypt

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ABSTRACT

Diabetes mellitus (DM) is a chronic debilitating illness, and atherosclerotic changes are inevitable and usually neglected during the follow-up of diabetic patients. Toll-like receptor 2 (TLR2) is under trial in many studies to hold responsibility for atherosclerosis process progression as they suggest a malfunction of these receptors expressed on monocytes in diabetic patients. This study aimed to assess the association between the TLR2 and type 2 diabetes mellitus (T2DM) in Egyptian diabetic patients and to investigate its relationship with some diabetic complications.

Methods: This study included a 60 diabetic patients group 1 (diabetic complicated), group 2 (diabetic non-complicated) and 30 age-matched normal healthy blood donors.

Results: Toll-like receptors (TLRs) expression was significantly associated with T2DM. In this study, the mean fluorescent intensity (MFI) of TLR2 was 596.9 ± 84.78 in group 1, 326.23 ± 62.98 in group 2 while in group 3 it was 208.47 ± 156.73. There was a significant correlation between MFI of TLR2 and random blood sugar (RBS) and glycated haemoglobin (HbA1c) ($p < 0.05$).

Conclusion: TLR2 was overexpressed in diabetic patients with microvascular complications compared to diabetic non-complicated patients and normal healthy controls.

1. Introduction

Diabetes mellitus (DM) is a major public health problem worldwide. Type 2 diabetes mellitus (T2DM) is becoming a major chronic disease health burden in Africa. Prevalence ranged from 2.6% in rural Sudan to 20% in urban Egypt [1]. According to the international diabetes federation, there were 8,222,600 cases of diabetes in Egypt in 2017 [2].

Toll-like receptors (TLRs) are an evolutionarily conserved family of pattern recognition receptors expressed on innate immune cells. They play a pivotal role in the inflammatory responses in DM. Moreover, they mediate the pathophysiological process of atherosclerosis [3–5]. Emerging data *in vitro* and *in vivo* suggest that systemic inflammation plays a role in the pathogenesis of DM complications via innate immune receptors [6].

TLR expression has also been detected on cardiac, epithelial, endothelial and vascular smooth muscle cells. Moreover, mesenchymal and parenchymal cells of different organs and tissue such as kidney, heart, lung, liver, skin, brain and intestine express TLR, but their functional role and relevance are not yet fully understood. Modulating these TLRs could be beneficial in forestalling diabetic complications giving the pivotal role of inflammation in both microvascular and macrovascular complication [7]. Currently, there is great interest in the development of TLR small molecule modulators for interrogating TLR signalling and treating diseases caused by TLR signalling malfunctions [8]. We aimed in this work to assess the Toll-like receptor 2 (TLR2) expression on monocytes in type 2 diabetic patients with microvascular complications compared to type 2 diabetic patients without microvascular complications and in reference to healthy normal age-matched controls.

1.1. Subjects

This study is a case-control study that included 60 diabetic patients, recruited from the Diabetes outpatient clinic in the internal

* Corresponding author.
E-mail address: dr.rashaemad@hotmail.com (R.E. Badr).

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medicine department of Cairo-Suez Canal University hospital. The studied subjects were distributed into two groups according to the inclusion and exclusion criteria for each group,

Group 1: (n = 30) Type 2 Diabetic patients showing signs of microvascular complications, e.g. retinopathy and/or nephropathy.
Group 2: (n = 30) Type 2 diabetic patients with no signs of microvascular complications.
Group 3: (n = 30) consisted of healthy age-matched controls selected from blood donors attending the blood bank hospital.

Exclusion criteria for all the groups were as follows; Age <18, Type 1 diabetic patients, the presence of hypercholesterolemia and patients with glycated haemoglobin (HbA1C) more than 10% over the last year.

After applying the inclusion and exclusion criteria, a sample frame was designed, and a listing of the patients was done. Their selection came through systematic random sampling.

2. Materials and methods

All study participants were subjected to full history taking, general, neurological and fundus examinations, to assess their current disease status and the presence of any of its complications. Fundus and neurological examinations were done to assess different degrees of retinopathy and diabetic neuropathy.

After obtaining a written informed consent from all participants and approval of the University ethical committee; 10 ml of the peripheral blood (PB) from all the study participants were obtained aseptically. Also, the random urine sample was obtained from all the study participants. Blood samples were divided as the following: 4 ml in plain tube 1 for biochemical studies, 2 ml in sodium fluoride tube for the assessment of random blood sugar (RBS), 2 ml in dipotassium ethylenediaminetetraacetic acid (EDTA) tube for HbA1c and finally 2 ml in EDTA tube for TLR2 expression examination.

The following investigations were done for all diabetic patients:

1 Biochemical studies: RBS, HbA1c, lipid profile and urine microalbumin concentration using (Hitachi 912, Boehringer Mannheim Diagnostic, USA).
2 Immunophenotyping study: Assessment of TLR2 expression on monocytes by the (FACSCalibur™, BD Biosciences, USA).

2.1. Immunophenotyping

For each sample, two tubes were labelled, the first was unstained tube and the second contained a panel of conjugated monoclonal antibodies (MoAb) to monocyte marker (FITC MoAb against CD 14 Reagent, Becton Dickinson BD, USA) for the sorting of monocyte and PE labelled monoclonal antibodies against TLR 2 (Becton Dickinson BD, USA). One hundred microlitres of the PB were delivered in each tube, and 5 μl of each MoAb were added to the respective tubes, then the tubes were vortexed and incubated in the dark in cold environment for 25 min. After that, 3 ml of prediluted (1:10) lysing solution pH adjusted at 7.2 was added to each tube, then the tubes were vortexed and incubated for 10 min in the dark at room temperature.

For three times, tubes were centrifuged at 3000 round per minute (rpm) for 5 min, and the supernatant was discarded, then the cells were washed with 2 ml of phosphate buffered saline (PBS) (with pH adjusted at 7.3 ± 0.2). Cells were suspended in 500 μl PBS to be processed by the Flow Cytometry machine.

2.2. Data interpretation

Flow cytometric analysis was done using Cell Quest software (Tree Star, USA). A minimum of 40,000 events was acquired. Gating was done on the lymphocyte population based on the forward scatter (FSC) and side scatter (SCC) properties followed by the gating of CD 14 + events. Then, the expression of TLR2 was assessed and presented as a percentage, absolute count and mean fluorescent intensity (MFI).

3. Statistical methods

Data were analysed using software package for statistical analysis, SPSS version 16 under Windows XP operating system for IBM compatible. Qualitative data were described in the form of numbers and percentages while quantitative data were described in the form of mean ± Standard deviation (SD) and Range. The comparison between the two groups regarding quantitative data was performed using independent student t-test or Mann Whitney test according to the normality of the data.

3.1. Pairwise correlations

Statistics between two variables was done using Spearman rank order correlation test and multicomparison using Bonferroni Post hoc test.

4. Results

Participants of the study were all around their sixth decade with a female predominance. The age and sex of the study population were compared; there was no significant statistical difference ($p > 0.05$) among them which fulfils the goal of proper cross matching within the groups. The selected biochemical tests performed were compared among the study groups; there was a statistically significant difference ($p < 0.01$) between them regarding RBS, lipid profile and HbA1c percentage (Table 1). In the complicated group, microalbuminuria (7, 23.33%) signified diabetics with nephropathy in pace with history and examination. While retinopathy represented 43.33% (13/30) of them which was confirmed via fundus examination. Only 10 patients showed both the complications together.

Flow cytometric analysis of the monocytes and their expression of TLR revealed a statistically significant difference in the monocyte percentage among the three groups, being the highest in the complicated patients. Also, the expression and the MFI of TLR 2 were highest among the same group (Table 2).

Table 3 showed that the MFI was highest among those with retinopathy yet the difference between the MFI between patients having only retinopathy or only nephropathy or both were statistically significant.

Pairwise correlation of the study characteristics revealed that $CD14^+$ TLR + monocytes increased significantly with age and with worsening of the diabetic parameters like RBS and HbA1c, as shown in Table 4.

5. Discussion

T2DM is a growing health problem in developed and developing countries [8] and is characterised by impaired insulin secretion, peripheral insulin resistance and increased hepatic glucose output leading to hyperglycaemia [9,10]. Most individuals with T2DM suffer serious complications of chronic hyperglycaemia including nephropathy, neuropathy, retinopathy and accelerated development of cardiovascular disease [11].

Table 1
Baseline laboratory findings of the participants.

	Complicated Diabetic (n = 30)	Non Complicated Diabetic (n = 30)	Control (n = 30)	p-value
RBS mg/dl	221.87 ± 61 212(139–410)	194.13 ± 59.2185(135–412)	90.77 ± 9.76 90(72–110)	0.0001*†‡
HbA1C (%)	7.69 ± 0.75 7.65(6.6–9)	7.33 ± 0.8 7(6.4–9)	4.76 ± 0.46 4.8(4–5.6)	0.0001*†‡
LDL mg/dl	96.23 ± 18.97 99.8(57–130)	88.81 ± 17.7 89(60–123)	77.52 ± 18.27 75.4(50–120)	0.0007*†
Cholesterol mg/dl	215.37 ± 15.5219(185–239)	210.9 ± 11.53 213.5(184–227)	151.97 ± 16.54 149(129–195)	0.0001*†‡
Triglycerides mg/dl	175.4 ± 9.13 175(153–198)	159.73 ± 13.29 163(130–180)	128.23 ± 11.19 125(110–148)	0.0001*†‡$

*p-value is significant (p < 0.05).
†Bonferroni post hoc test revealed that the comparison of the complicated group to control group is significant.
‡Bonferroni post hoc test revealed that the comparison of the non-complicated group to control group is significant.
$Bonferroni post hoc test revealed that the comparison of the complicated group to non-complicated group is significant.

Table 2
Comparison of TLR2 MFI, the percentage of $CD14^+$ TLR+ and percentage of monocytes among the study groups.

	Complicated Diabetic	Non-Complicated Diabetic	Control	p-value
Monocytes (%)	9.54 ± 2.17 10.05(4.8–13.1)	9.1 ± 1.76 9.4(5.6–12.4)	6.94 ± 12.55 7.05(2.3–10)	0.0001*
$CD14^+$ TLR2+ (%)	96.17 ± 3.04 97(88–100)	89 ± 5.29 89.5(78–98)	67.99 ± 12.55 70.5(44.5–90)	0.0001*
TLR2 MFI	596.9 ± 84.78 584.5(350–780)	326.23 ± 62.98 313.5(220–500)	208.47 ± 156.73 184(74–826)	0.0001*

*p-value is significant (p < 0.05).

Table 3
Comparison of TLR2 MFI according to the types of complications among the complicated diabetic patients.

TLR2 MFI	Nephropathy	Retinopathy	Nephropathy & Retinopathy	p-value
Mean ± SD	620.4 ± 70.6	630.5 ± 80.2	536.7 ± 72	0.02*
Median(Min-Max)	632(512–712)	600(498–780)	553.5(350–600)	

*p-value is significant (p < 0.05).

Table 4
Pairwise correlations of the study characteristics.

		Age	RBS	HbA1c	LDL	HDL	TG	CHOL	MFI
RBS	R	0.68							
	P	0.00*							
HbA1c	R	0.73	0.81						
	P	0.00*	0.00*						
LDL	R	0.35	0.32	0.35					
	P	0.00*	0.00*	0.00*					
HDL	R	−0.17	−0.15	−0.17	−0.43				
	P	0.11	0.16	0.12	0.00*				
TG	R	0.10	0.20	0.18	−0.07	−0.08			
	P	0.37	0.06	0.09	0.52	0.45			
CHOL	R	0.34	0.28	0.34	0.92	−0.21	0.07		
	P	0.00*	0.01*	0.00*	0.00*	0.05	0.49		
MFI	R	0.65	0.54	0.56	0.09	0.11	0.05	0.14	
	P	0.00*	0.00*	0.00*	0.42	0.29	0.67	0.18	
CD14 + TLR+	R	0.63	0.63	0.73	0.32	−0.08	0.06	0.32	0.58
		p0.00*	0.00*	0.00*	0.00*	0.45	0.57	0.00*	0.00*
Monocytes	R	0.42	0.43	0.50	0.13	−0.11	0.11	0.13	0.28
	p	0.00*	0.00*	0.00*	0.21	0.32	0.31	0.21	0.01*

R: Pearson coefficient.
p: p-value, *p-value is significant (p < 0.05).

In this study, it was found that TLRs were overexpressed in type 2 diabetic patients with microvascular complications than in non-complicated type 2 diabetic patients. Both Diabetics groups showed higher expression of TLR2 than in the normal healthy controls.

These results were also consistent with the findings of Dasu et al., in 2010 [12], who determined the levels of TLR2 and TLR4 expression by flow cytometric analysis in control and type 2 diabetic subjects. He found that monocyte surface expression of TLR2 and TLR4 was significantly elevated in type 2 diabetes compared with the control in both complicated and non-complicated diabetic subjects (p‹0.005). Also, he investigated the TLR2 and TLR4 messenger ribonucleic acid (mRNA) levels by real-time RT-PCR and found a corresponding increased TLR2 and TLR4 mRNA expression in type 2 diabetes compared with the control subjects. He further confirmed the increased monocyte TLR2 and TLR4 protein content using Western blot assay.

The results of our study also agreed with a study of Creely and his team in 2007 [13], who showed increased TLR2 expression in the adipose tissue of type 2 diabetic patients with strong correlations to endotoxin levels. These observations taken together suggest a potential role for TLR2 and TLR4 in the pathology of diabetes with limited mechanistic details.

Our findings were also consistent with the findings of Devaraj and his team in 2011 [14], who found that the TLR2 and TLR4 surface expression was increased on monocytes isolated from Type 1 diabetes (T1DM) patients with microvascular complications (T1DM-MV) compared to those without T1DM and healthy controls. Also, he demonstrated a significant increase of nuclear factor κB binding activity, as well as increased IL-1β release in resting and activated monocytes of T1DM-MV compared to T1DM patients and controls.

Also, in 2008, Devaraj et al. [15] showed increased TLR2 and TLR4 expression, intracellular signalling and TLR-mediated inflammation in monocytes with significant correlation to HbA1c levels in type 2 diabetic patients.

These findings were also consistent with the findings of Rajamani et al., in 2014 [16], who had the first report on TLR demonstration on microvascular endothelium that was increased with hyperglycaemia. TLR-2/4 mediated myeloid differentiation factor 88 (MyD88) pathway was upregulated as shown by increased MyD88 protein levels and nuclear p65. Additionally, the TLR-4-mediated Non-MyD88 pathway was also activated as indicated by the increased protein levels of TIR-domain-containing adapter-inducing interferon-β (TRIF) and interferon regulatory factor 3 (IRF3). These findings are in line with previous studies implicating hyperglycaemia in renal microvascular complications.

Kaur et al., in 2012 [17] reported increased TLR-4 expression and activity under hyperglycaemia in renal mesangial cells

incriminating TLR-4 in contributing to diabetic nephropathy. Lin et al. showed increased TLR-4 expression under hyperglycaemic conditions in human proximal tubular epithelial cells pointing towards a role for TLR-4 in tubulointerstitial inflammation in diabetic nephropathy [18].

In conclusion, DM is a major global health issue. Egypt has a high prevalence rate of DM which is about 20%. The current study demonstrated TLR2 overexpression in diabetic patients with microvascular complications than in diabetic non-complicated patients and normal healthy controls. TLR2 expression on monocytes was increased in type 2 diabetic patients and was related to microvascular complications.

Conflicts of interest

We, the authors of "**Toll-like receptor 2 expression on monocytes and microvascular complications in Type 2 diabetic patients**", declare that there is no financial or personal relationship which could result in a conflict of interest with regard to the publication of the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2019.01.038.

References

[1] Bos M, Agyemang C. Prevalence and complications of diabetes mellitus in Northern Africa, a systematic review. BMC Public Health 2013;13:387.
[2] https://www.idf.org/our-network/regions-members/middle-east-and-north-africa/members/34-egypt.html.
[3] Li H, Sun B. Toll-like receptor 4 in atherosclerosis. J Cell Mol Med 2007;11:88–95.
[4] Uematsu S, Akira S. Toll-like receptors and innate immunity. J Mol Med 2006;84:712–25.
[5] Mullick AE, Tobias PS, Curtiss LK. Toll-like receptors and atherosclerosis: key contributors in disease and health? Immunol Res 2006;34:193–209.
[6] Ramirez RS, Dasu RK. Toll-like receptors and diabetes complications: recent advances. Curr Diabetes Rev 2012;8(6):480–8.
[7] Jialal I, Kaur H. The role of toll-like receptors in diabetes-induced inflammation: implications for vascular complications. Curr Diabetes Rep 2012;12(2):172–9.
[8] van Dieren S, Beulens J, vander Schouw Y, Grobbee D, Neal B. The global burden of diabetes and its complications: an emerging pandemic. Eur J Cardiovasc Prev Rehabil 2010;17(1):3–8.
[9] American Diabetes Association. Standards of medical care in diabetes-2012. Diabetes Care 2014;35(Suppl 1):S11–63.
[10] American Diabetes Association. Standards of medical care in diabetes- 2014. Diabetes Care 2014;37(suppl 1):14–80.
[11] Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Thorsteinsdottir U. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet 2006;38(3):320–3.
[12] Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care 2010;33(4):861–8.
[13] Creely SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab 2011;292:740–7.
[14] Devaraj S, Jialal I, Yun JM, Bremer A. Demonstration of increased toll-like receptor 2 and toll-like receptor 4 expression in monocytes of type 1 diabetes mellitus patients with microvascular complications. Metabolism 2011;60(2):256–9.
[15] Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a proinflammatory state. J ClinEndocrinolMetab 2008;93(2):578–83.
[16] Rajamani U, Jialal I. Hyperglycemia induces Toll-like receptor-2 and -4 expression and activity in human microvascular retinal endothelial cells: implications for diabetic retinopathy. J Diabetes Res 2014:15.
[17] Kaur H, Chien A, Jialal I. Hyperglycemia induces toll like receptor 4 expression and activity in mouse mesangial cells: relevance to diabetic nephropathy. Am J Physiol Renal Physiol 2012;303(8):1145–50.
[18] Lin M, Yiu WH, Wu HJ, Chan LY, Leung JC, Au WS, et al. Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. J Am Soc Nephrol 2012;23(1):86–102.