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The Role of Intermediate Monocytes (CD14+CD16+) in Development and Diagnosis of Recent Type 1 Diabetes in Children

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Abstract

Background: Monocytes play an important role in antigen presentation and cytokine production to achieve a proper immune response and are therefore largely implicated in the development and progression of autoimmune diseases such as Type 1 diabetes mellitus.

Objectives: To determine the significance of expanded intermediate monocytes as a predictive factor for the poor residual islet β−cell function in children with recent-onset Type 1 diabetes mellitus.

Subjects and methods: This study was a case- control study carried out at Pediatric Endocrinology Outpatient Clinic and Clinical Pathology Department, Zagazig University Hospital. It included 20 patients with recent onset T1DM and 20 age and sex matched healthy children as a control group. All studied groups were subjected to full history taking, thorough clinical examination and laboratory investigations in the form of CBC, fasting blood glucose, C-peptide and serum HbA1c levels. Cell-surface monocyte phenotypic analysis was performed after staining with human anti-CD14 and anti-CD16 by flow cytometry.

Results: There were highly significant increase in the ratio of intermediate monocytes and significant increase in the ratio of classical, non-classical monocytes in T1DM group compared to control group.

Conclusion: The intermediate monocyte population was expanded in pediatric patients with T1DM. As these cells were shown to have pro-inflammatory activity, they are likely to be implicated in the impaired function of β-cells, with deleterious consequences for the development of T1DM.

Keywords: Children, Type 1 Diabetes Mellitus, Intermediate Monocytes, β-cells.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is considered an autoimmune disease in which the function of pancreatic β-cells, which secrete insulin, is impaired and this is due to auto reactive immune cell-mediated destruction (insulitis). T1DM is also characterized by dysregulation of blood glucose level due to impairment of the pancreatic β-cell function leading to increase hemoglobin A1c level (HbA1c) in peripheral blood [(1)].

There are many studies suggest that the development of T1DM is associated strongly with different immune cell subsets, which include monocytes. This increase in the monocyte population especially the intermediate subgroup has been shown to trigger the destruction of pancreatic β-cell [(2)].

Monocytes are classified into three subgroups based on the differential expression level of CD14 (a lipopolysaccharide (LPS) receptor) and CD16 (FcγRIII). These subgroups are classical (CD14++ CD16-), which make about 85%, intermediate (CD14+CD16+) that is about 5% and non-classical (CD14dim CD16++) that is about 10% [(3)]. Many studies proved that there is expansion in Intermediate monocytes subgroup in many inflammatory and autoimmune conditions, for example chronic kidney disease, active rheumatoid arthritis, heart and coronary artery disease, and type 2 diabetes [(4)]. In addition, monocytes expand in patients with human immunodeficiency virus as they promote expansion in memory T cell [(5)]. The marked expansion in intermediate monocytes in patients with T1DM was demonstrated to produce more tumor necrosis factor-alpha (TNF-α). This factor is an effective inflammatory factor [(6)]. Therefore, this factor TNF-α has been correlated with the severity of T1DM [(7)]. There is evidence that the intermediate monocyte has an antigen-presenting function with a dendritic cell-like feature [(7)]. Upon antigen stimulation, studies proved that intermediate monocytes became the main producers of inflammatory factors, like interleukin (IL)-1α, IL-6, and TNF-α [(8)]. Recent studies showed that the expansion of intermediate monocytes could trigger the destruction process of β-cell [(9, 10)].

The aim of the study was to determine the significance of expanded intermediate monocytes as a predictive factor for the poor residual islet β−cell function in children with recent-onset Type 1 diabetes mellitus.

SUBJECTS AND METHODS

Study design: A case-control study that was performed in Pediatric Endocrinology Outpatient Clinic, Zagazig University Hospital and Clinical

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Pathology Department, Zagazig University in Sharkia Governorate during period from March 2019 to July 2019.

Sample size: Assuming that mean ± SD of CD14+CD16+ monocyte in T1DM and in control group was 2.38 ± 2.2 versus 6.87 ± 6.7 respectively. So, sample size was calculated by Open Epi program to be 40 cases (20 in each group) with confidence level 95% and power of test 80%.

Subjects: This study was conducted on 40 children divided into 2 groups, Group I (T1DM group) included 20 children having recent onset T1DM and Group II (control group) included 20 age and sex matched healthy children.

Inclusion criteria: Children with recent onset T1DM, duration of illness 1-3 months, age between 5 years -15 years and both sexes were included.

Exclusion criteria: Age < 5 years and > 15 years, duration of illness > 3 months, patients with microvascular complications or with coexisting autoimmune, chronic, and acute inflammatory diseases.

All the studied groups were subjected to the following:

1- Complete history taking with special emphasis on history of present illness (onset of diabetes, duration of symptoms, severity of the disease), past history (preceding viral illness as mumps or varicella, medication taken, any complication as DKA) and family history of DM.

2- Clinical examination with special emphasis on general examination including general appearance with specific attention to abnormalities of growth and with stress on signs of dehydration, dry mouth, acidotic breathing, vomiting and abdominal pain. In addition, vital signs were assessed as heart rate, respiratory rate and anthropometric measures, which included weight, height, head circumference and mid upper arm circumference.

3- Investigations included routine laboratory investigations as complete blood count, blood sugar level and ketones in blood, blood gases for assessment of acid-base status, serum electrolyte (Na, K and Ca), calculation of serum osmolarity, urine analysis for glucosuria, ketonuria, serum HbA1c level and C-peptide level. In addition, the investigations included special laboratory investigations as immunophenotyping of peripheral blood specimen on monocytes population and cell-surface monocyte phenotypic analysis that was performed after staining with human anti-CD14 and anti-CD16 by flow cytometry, which was done as follows:

1- Specimen preparation: Intended use of monoclonal antibodies that are designed to quantitatively determine the percentage of cells bearing these monoclonal antibodies within a population and quantitatively determine their density on cell surface by flow cytometry.

2- Sample collection: Serum and peripheral blood mononuclear cells (PBMCs) were isolated from ethylene-diamine-tetra-acetic acid-treated blood samples collected from all participants. The PBMCs were separated by standard Ficoll–Hypaque density centrifugation at 1000 rpm for 20 min.

3- Blood measurements: Surface phenotypic marker expression of the absolute counts of total lymphocytes, monocytes, and neutrophils in the peripheral blood of all subjects were measured by flow cytometry.

4- Principle of the test: Cells are incubated with labeled monoclonal antibody, which binds to cells expressing the antibody of interest, unbound antibody is then washed from the cells, and cells expressing monoclone are fluorescently stained with the intensity of staining directly proportional to the density of expression of the monoclone.

5- Sample staining (surface marker analysis).

6- Flowcytometric analysis: Data were acquired on a FACS caliber flowcytometer (BD immune cytometry systems, San Jose, CA). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers and flowcytometric two parameters dot plots and quadrant statistics were generated by cell quest software (Becton Dickinson immune-cytometer systems). Analysis was performed after manual gating around a monocyte population on a forward scatter versus side scatter dot-plot. A second gate was subsequently put on the CD 14 positive –monocytes population. Results were expressed as percentages of monocytes subsets (CD14++/CD16-, CD14+/CD16+, CD14dim/CD16++).

Ethical and patients' approval: Written informed consent was obtained from all participants and **the study was approved by the Research Ethical Committee, Faculty of Medicine, Zagazig University (Institutional Research Board IRB). The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.**

Statistical Analysis

Data entry, processing and statistical analysis were carried out using MedCalc ver. 18.2.1 (MedCalc, Ostend, Belgium). Tests of significance (Kruskal-Wallis, Wilcoxon's, Chi square, logistic regression analysis, and Spearman's correlation) were used. Data were presented and suitable

analysis was done according to the type of data (parametric and non-parametric) obtained for each variable. $P \leq 0.05$ (5%) was considered to be statistically significant.

RESULTS

Table (1) showed non-significant difference between the studied groups regarding age, sex and anthropometric measurements (Wt. and Ht).

Table (2) showed highly significant increase in FBS and HBA1c and highly significant decrease in C-peptide in patient group compared to control group.

Table (3) showed significant increase in absolute count of monocytes and non-significant increase of neutrophils and lymphocytes counts in patient group compared to control group.

Table (4) showed highly significant increase in the ratio of classical and intermediate monocytes in T1DM group. While, the ratio of non-classical monocytes was non-significantly increased in T1DM group compared to control group.

Table (5) showed that in type 1 diabetes mellitus patients, the CD14+/CD16+monocytes (Intermediate subtype) revealed the highest sensitivity and specificity (75% and 100% respectively) among the other monocytes subtypes as the classical were 30% and 90% respectively and the non-classical were 5% and 95% respectively.

Table (6) showed that HbA1c showed high sensitivity compared to FBS and C-peptide in diagnosis of T1DM (100% versus 90% and 35% respectively) while specificity was the same within the three parameters 75%.

Table (7) showed highly significant positive correlation between intermediate monocytes ratio and FBS and HbA1c. However, there was significant negative correlation between intermediate monocytes ratio and C-peptide.

Table (1): Demographic and anthropometric data of the studied groups

Demographic data	**Group I T1DM n: 20**		**Control n: 20**		**Test of Significance**	**P- value**
Gender	**No**	**%**	**No**	**%**		
Male	9	45%	10	50%	χ^2=0.100	>0.05
Female	11	55%	10	50%		
Age mean ± SD **(**years)	9.3 ± 2.9 (5-15)		9.8 ± 3 (42-68)		t=0.531	>0.05
Weight (Kg)	38.4 ± 10.9 (22-60)		33 ± 1.5 (19-55)		t=1.676	>0.05
Height (cm)	130.9 ± 19.3 (110-170)		131.7 ± 13 (110-155)		t=0.153	>0.05

t= Student t test; $\chi^{2:}$ chi-square test; P > 0.05: not significant; * Significant

Table (2): laboratory characteristic of T1DM in the studied groups

Clinical parameter	**Group I TIDM n: 20**	**Control group N=20**	**t-test**	**P-value**
FBS (mg/dl)	309.2 ± 6.6	82.9 ± 7.1	10.402	0.0001*
HBA_{1C} (%)	11.3 ± 2.06	5.14 ± 0.2	13.310	0.0001*
C. peptide (ng/ml)	0.54 ± 0.1	3.6 ± 0.8	16.595	0.0001*

*p<0.05=: Significance FBS: fasting blood sugar HBA1C: glycated hemoglobin

Table (3): Differential WBCs of CBC of the studied groups

	Group I TIDM n: 20	**Control group n: 20**	**Student 't' Test**	**P-value**
Monocytes (absolute count)	3.5 ± 0.5	2.04 ± 0.3	2.503	0.01*
Neutrophils (absolute count)	1.6 ± 0.3	1.2 ± 0.4	0.707	0.483
Lymphocytes (absolute count)	1.9 ± 0.3	1.5 ± 0.2	0.686	0.496

p>0.05= not significant; *p<0.05=: Significance.

Table (4): Monocyte subsets characterization in the studied groups

	Group I TIDM n: 20	Control group n: 20	Test of significance	P-value
CD14++/CD16- (classical)	89.4 ± 3.6	74.9±12.3	t= 5.059	0.0001*
CD14+/CD16+ (intermediate)	18.5 ± 12.2	4.5±1.9	t= 5.070	0.0001*
$CD14^{Dim}$/CD16++ (non-classical)	6.6 ± 1.5	5.8±1.6	t=1.631	0.111

t: student t test; *p<0.05=: Significance

Table (5): The Sensitivity, Specificity of Monocyte Subsets in T1DM Patients

	Sensitivity	Specificity	PPV	NPV	Positive Likelihood Ratio	Negative Likelihood Ratio	Accuracy
CD14++/CD16- Classical	30%	90%	75%	56.3%	3	0.78	62.5%
CD14+/CD16+ Intermediate	75%	100%	100%	80%	-	0.25	87.5%
$CD14^{Dim}$/CD16++ Non classical	5%	95%	50%	50%	1	1	50%

PPV: Positive predictive value; NPV; Negative predictive value

Table (6): Sensitivity and specificity of laboratory data characteristic of T1DM

	Sensitivity	Specificity	PPV	NPV	Positive Likelihood Ratio	Negative Likelihood Ratio	Accuracy
FBS	90%	75%	78.6%	88.2%	3.6	0.13	82.5%
HbA1c	100%	75%	80%	100%	4	0	73.2%
C. peptide	35%	75%	58.3%	53.6%	1.40	0.87	30%

p>0.05= not significant; *p<0.05=: Significance

Table (7): Correlations between Laboratory parameters and circulating Monocytes subsets cells in patients with type 1 diabetes

	CD14++/CD16- Classical		CD14+/CD16+ Intermediate		$CD14^{Dim}$/CD16++ Non classical	
	r	P	r	P	r	P
FBS	0.361	0.117	0.452	004*	-0.145	0.541
Hb A_{1c}	0.146	0.537	0.760	0.0001*	0.108	0.649
C. peptide	0.183	0.439	-0.477	0.03*	-0.103	0.665

p>0.05= not significant; *p<0.05=: Significance

DISCUSSION

Type 1 diabetes mellitus (DM1) is caused by autoimmune selective destruction of pancreatic ß-cells. As a multifactorial disease, it is caused by a complex combination of genetic and environmental factors triggering autoimmunity [(11)].

All complications of diabetes are related to the associated microangiopathy. The onset of microangiopathy is associated with infiltration by inflammatory cells, as well as elevated levels of C-RP and proinflammatory cytokines. Infiltration by neutrophils, monocytes and firm adhesion of these leukocytes to vascular endothelial cells are one of the earliest events present for many years before overt retinopathy or nephropathy [(7)]. Peripheral blood monocytes are heterogeneous population and until recently were only divided into two subsets based on CD16 expression – CD16 and CD16+. However, minor CD16+ population can be further subdivided into CD14brightCD16+ and CD14dimCD16+ cells. Therefore, there are three distinct subsets of monocytes: the classical CD14brightCD16, the intermediate CD14brightCD16+, and the non-classical CD14dimCD16+ [(3)].

The main objectives of this study were to study the relation between change in intermediate monocyte level and development of recent onset T1D and to determine if level of intermediate monocyte is a predictive factor for poor residual islet cells function that is determined by level of HA1c, insulin and c-peptide. This study was conducted on 40 participants divided into two groups, 20 patients with T1DM, 9 were males (45%) and 11 were females (55%) with mean age of 9.3 ± 2.9 years and a control group including 20 healthy children, 10 were males (50%) and 10 were females (50%) with mean age 9.8 ± 3 years. This study showed that there was non-significant difference between the studied groups regarding age, sex and anthropometric measurements (Weight and Hight).

Monocytes are comprised of heterogeneous subgroups that can be classified as classical (CD14++CD16−) about 85%, intermediate (CD14+CD16+) about 5% and non-classical (CD14dimCD16++) about 10% based on the differential expression levels of CD14 (a lipopolysaccharide (LPS) receptor) and CD16 (FcγRⅢ) **(3)**.

The current study showed that there was significant increase in absolute count of monocytes and non-significant increase regarding neutrophils and lymphocytes counts in patient group compared to control group. The results of the current study are in line with study of **Ryba-Stanisławowska and colleagues (12)** as they found that comparison of the CD16+ monocyte counts between TIDM patients and healthy subjects showed considerably higher counts of CD14brightCD16+ and CD14dimCD16+ monocytes in the patients as compared to healthy individuals. This is consistent with the study of **Ramirez and colleagues (13),** which showed that CD16+ subsets of monocytes are expanded in some autoimmune diseases and may be involved in the induction of inflammatory immune response.

In this study, there was highly significant increase in the ratio of classical and intermediate monocytes in T1DM group. While the ratio of non-classical monocytes was non-significantly increased in T1DM group compared to control group. The results of the present study are in agreement with study of **Ren and colleagues (14)** as they reported that intermediate monocytes were increased compared to the healthy cohort.

The present study showed that in type 1 diabetes mellitus patients the CD14+/CD16+monocytes (Intermediate subtype) revealed the highest sensitivity and specificity (75% and 100% respectively) among the other monocytes subtypes as the classical were 30% & 90% respectively and the non-classical were 5% & 95% respectively. In TIDM patients the CD14+/CD16+ monocytes were active and expanded in patients with FBS (with high glucose level), high HB A1c and with low c-peptide levels with sensitivity of 90%, 100% & 35% respectively and specificity of 75% within the three parameters. HbA1c showed high sensitivity compared to FBS and C-peptide in diagnosis of T1DM (100% versus 90%. 35%), while specificity was the same within the three parameters 75%. The results of the present study are in agreement with study of **Ren and colleagues (14).** They reported that all of T1DM patients recruited in this study showed a dramatic increase in HbA1c (11.8% ± 2.5, reference value 4.0–6.0%), and a significant decrease in both serum insulin concentration (3.2 ± 2.3 IU/ml, reference value 6.0–27 IU/ml) and serum C-peptide level (0.9 ± 0.7 ng/ml, reference value 1.1–5.0 ng/ml)**.**

The data of the present study showed that there was highly significant positive correlation between intermediate monocytes ratio and FBS and HbA1c. However, there was significant negative correlation between intermediate monocytes ratio and C-peptide. These data agree with the study of **Ren and colleagues (14)** who revealed that T1DM patients showed higher HbA1c levels had greater numbers of peripheral blood intermediate monocytes. Moreover, an inverse correlation was observed between the absolute number of intermediate monocytes and the concentration of insulin and C-peptide, respectively, in T1DM children**.** Furthermore, **Ryba-Stanisławowska and colleagues (12)** observed that increased level of HbA1c was correlated with the frequency of intermediate and non-classical subsets of CD16+ monocytes. The stronger correlation was seen between HbA1c level and percentage of non-classical CD14dimCD16+ cells and this is not consistent with our results**.** The levels of insulin and C-peptide are reliable clinical indicators for the competent function of β−cell. **Dereke and colleagues (15)** demonstrated that the absolute number of intermediate monocytes was negatively correlated with the concentrations of C-peptide and insulin. Collectively, these results suggest that expanded intermediate monocytes might play a detrimental role for β−cell function in children with T1DM.

CONCLUSION

The intermediate monocyte population was found to be expanded in pediatric patients with T1DM. As these cells were shown to have pro-inflammatory activity, they are likely to be implicated in the impaired function of β−cells, with

deleterious consequences for the development of T1DM.

RECOMMENDATIONS

Further studies on large geographical scale and on larger sample size to emphasize our conclusion. Further characterization of the function of intermediate monocytes could pave the road for improving understanding of the relationships between these pro-inflammatory cells and the destruction of β−cells in children with T1DM and give important data to understand pathogenesis of type 1 diabetes and its complications.

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