Evaluation of Colchicine Effect on Cytokine Production from Peripheral Blood Mononuclear Cells of Type I Diabetes

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ABSTRACT

Problem: Type 1 diabetes mellitus (DM) results from autoimmune destruction of insulin-producing β cells and is characterized by the presence of insulitis and β-cell autoantibodies. Cytokines are known to play an important role in autoimmunity and have been suggested to be involved in the pathogenesis of insulin-dependent diabetes (IDDM). The aim of the present study was to evaluate the levels of major cytokines such as IL-2, IL-6, and IL-10 in Type I DM and in apparently healthy volunteers (control) and to study the effect of colchicine on the capacity of peripheral blood monocytes isolated from Type I diabetes patients and healthy volunteers with or without immune stimulation to produce IL-2, IL-6, and IL-10. Experimental approach: Fifty type I diabetes patients were randomly recruited from the Diabetes/Endocrine Clinics along with 15 healthy subjects. Levels of cytokines were measured from cultured blood monocytes using standard ELISA kits. Major findings: results revealed that basal levels of IL-2, IL-6, and IL-10 were significantly higher in diabetes group compared to healthy group ($P < 0.05$). LPS or PHA-induced production of these cytokines was significantly higher in diabetes group ($P < 0.05$). Moreover, pretreatment of stimulated monocytes of DM patients with colchicine inhibited cytokine levels to be comparable to that of cell cultured from healthy individuals. Conclusions: These results provide a new perspective on the protective role of colchicine against inflammatory long-term complications associated with type I DM.

Keywords: IDDM, IL-2; IL-6; IL-10, Pro-Inflammatory Cytokines, Anti- Inflammatory Cytokines.

1. INTRODUCTION

Type I diabetes mellitus is an autoimmune disease characterized by gradual destruction of β-cells of the pancreas.[1] The development of diabetes mellitus type I is mainly related to genetic factors,[2-3] and usually results in a long and short term complication.[4-7] Unawareness of the disease is common in about 25% patients.[8-9] Diabetic patients may enter a long asymptomatic period with a hyperglycemia that consequently results in short term complications such as ployuria, polydipsia, polyphagia, glycosuria, ketonuria, blurred vision, and weight loss.[8] In addition, chronic hyperglycemia may lead to long term complication, including neuropathy, hypertension, kidney damage, poor blood circulation, neurological and vascular lesions, that might cause increase in the incidence of infectious diseases, foot ulcers, amputation, development of macrovascular complications-related to cardiovascular morbidity and mortality.[5, 10-12] These complications are well linked to several mediators released from different cell types, including immune cells and adipocytes.[13-14] These mediators such as

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proinflammatory cytokines have been identified as being involved in inflammatory response among type I diabetes patients, and believed to play a pivotal role in the development of complications of the disease.[14-16]

Previous studies have shown that diabetic patients experience accumulation of inflammatory cells in about two folds more than non diabetic patients.[17-19] This has been shown to be responsible for the destruction of pancreatic β-cells, induction of metabolic defects such as transient alteration in lipids, peripheral insulin resistance, decreasing glucose uptake in adipocytes, and interference with proper wound healing. Cytokines have short term endurance and function as part of the host response to infection and acute inflammatory response with leukocytes such as macrophages.[20] In a one model, production of cytokines was triggered by elevated levels of glycated proteins with chronic hyperglycemia that result in inflammatory responses.[12] The later was well linked to increased risk of cardiovascular diseases including hypertension, stroke, recurrent bacterial infections, and neurological defects such as peripheral neuropathy.

The cytoskeleton targeting drug, colchicine, is known to have an anti-inflammatory and anti-mitotic effect, commonly used in the treatment of gouts, pericarditis, and as anticancer agent.[21-22] It is able to inhibit secretion of endogenous mediators such as cytokines and chemokines that are known to be involved in the complications of diabetes.[23] The current study evaluated the levels of circulating cytokines such as IL-2, IL-6, and IL-10, in type I diabetes patients. In addition, the potential of the cytoskeleton targeting drug, colchicine, to inhibit cytokines levels was studied among diabetes patients as well as healthy volunteers.

Results

As shown in Table 1, both the DM and control groups had similar age ranges (7 to 35 years, and 6-35 years, respectively). Additionally, biochemical analyses revealed that BMI, RBCs count, FBG, HbA1c, and TG levels of the control group fall within the normal range. In contrast, type I DM patients showed significantly higher BMI, RBCs count, FBG, HbA1c, and TG compared to the apparently healthy (control) group ($P < 0.05$, Table 1).

Table 1. Demographic and biochemical characteristics of the study's population

Parameter	Control group	DM group	P-Value
Gender n (%)			0.511
Male	6 (50.0%)	18 (36.0%)	
Female	6 (50.0%)	32 (64.0%)	
Age* (Years)	24.08 ± 6.454	23.40 ± 7.972	0.783
BMI*	20.02 ± 1.421	23.73 ± 4.863	0.01
Smoking n (%)	5 (33)	10 (20)	
RBCs* (x10^{12}cell/L)	5.10 ± 0.542	12.02 ± 5.215	0.010
FBG* (mmol/L)	4.37 ± 0.527	10.75 ± 4.868	0.001
HbA1c*	5.45 ± 0.901	9.63 ± 2.343	0.001
TG* (mmol/L)	0.918 ± 0.565	3.13 ± 0.540	0.001

* Values are mean ± SD and statistical analysis was carried out using un-paired t-test.

In the present study, patients have developed type I DM since different periods of time, where 15(30%) of the patients had DM type I since 2-5 years, 21 (42%) had the disease since 5-10 years and 9(18%) had the disease since >10 years. The therapeutic modalities used to control the hyperglycemia in most type I DM patients were insulin

and diet procedure, and most common concurrent disease or complication among those patients were hypertension 21 (42.0%), retinopathy 15 (30.0%), foot ulceration 10 (20%), foot infection 4 (8%) and UTI 4 (8%).

Previous studies have shown that different cytokines might be implicated in the development of microvascular and macrovascular complications in type I DM patients. Results in Table 3 revealed that the levels of the proinflammatory cytokines including IL-2, IL-6, and IL-10 were significantly higher in patients compared to the control group. Moreover, the response in terms of cytokine production was found to be significantly higher in patients compared to the control individuals (Table 2).

Table 2. Cytokine levels (ng/ml) among healthy and diabetes groups (mean ± SD)

Cytokines	**IL-2**	**IL-6**	**IL-10**
Control	31.12±2.89	77.32±18.87	9.11±1.55
Control-Induced	71.70±11.95	155.88±30.01	17.01±3.60
DM	67.50±23.67	105.8±14.18	23.69±7.04
DM-Induced	103.10±29.09	320.69±30.30	44.63±18.26
DM-Induced/colchicine	28.50±12.33	81.44±11.88	23.11±11.33
P-Value*	0.0001	0.0001	0.0001
Control Vs DM	P < 0.001	P < 0.001	P < 0.001
Control Vs Control-Induced	P < 0.001	P < 0.001	P > 0.05
Control Vs DM-Induced	P < 0.001	P < 0.001	P < 0.001
Control Vs DM-Induced/colchicine	P > 0.05	P > 0.05	P < 0.001
DM Vs Control-Induced	P > 0.05	P < 0.001	P > 0.05
DM Vs DM-Induced	P < 0.001	P < 0.001	P < 0.001
DM Vs DM-Induced/colchicine	P < 0.001	P < 0.001	P > 0.05
Control-Induced Vs DM-Induced	P < 0.001	P < 0.001	P < 0.001
Control-Induced Vs DM-Induced/colchicine	P < 0.001	P < 0.001	P > 0.05
DM-Induced Vs DM-Induced/colchicine	P < 0.001	P < 0.001	P < 0.001

* Data analysis was carried out using one-way ANOVA followed by Tukey's post-hoc

To evaluate the colchicine potential to inhibit cytokine production, blood samples were taken from the control individual. Cultured Cells were treated with different colchicine concentrations (0, 12.5, 25, and 50 ng/mL) as described in the Methods. Colchicine inhibited the production of different cytokines in a dose-dependent manner where the optimal inhibitory concentration of colchicines was 50 ng/mL (data not shown). Table 2 shows the levels of cytokines (IL-2, IL-6, and IL-10) in cultured cells from DM patients after pretreatment of cultures with colchicines (50 ng/ml) as compared to other treatment group. Colchicine inhibited production of cytokines (IL-2 and IL-6, but not IL-10) to levels comparable to that in non-stimulated cells from control group.

Discussion:

Type 1 diabetes mellitus (DM) results from autoimmune destruction of insulin-producing β cells and is characterized by the presence of β-cell autoantibodies. Moreover, pro-inflammatory cytokines are known to play an important role in autoimmunity and have been suggested to be involved in the pathogenesis of the disease.[24-26] In the present study, we evaluated the incidence of long-term complications of type 1 DM and the levels of pro-inflammatory cytokines such as IL-2,

IL-6, and IL-10. Results indicated the presence of a number of type I DM complication including hypertension, retinopathy, urinary tract infections, and lymphadenopathy in diabetes group but not in the controls. Additionally, results in the present study showed that significant differences exist between type I DM patients and control subjects with detectable supernatant levels of IL-2, IL-6, and IL-10 of cultured monocytes, indicating that the presence of these cytokines in peripheral blood might be a distinctive feature of DM. In other autoimmune diseases, raised levels of IL-l, IL-2 and IFN-γ have been reported,[27-29] suggesting that production of these cytokines at the site of target organ may be reflected by measurable levels in circulation.

The inflammatory cytokines IL-1, tumor necrosis factor, interferon γ, IL-6, IL-10, IL-15, IL-17, and monocyte chemoattractant protein-1 have been implicated in the development of microvascular and macrovascular complications in both type I and II DM.[5-6, 13, 30] While several studies have demonstrated strong correlations between inflammatory cytokines and endothelial damage, it remains to be demonstrated whether a cause-and-effect relationship exists between inflammatory cytokines and the pathogenesis of diabetes complications. Whereas some investigators hypothesize that cytokine alterations play a despicable role in disease pathogenesis, others contend that cytokine changes are a protective physiologic response to hyperglycemia-induced stress.[31-33] In addition, the pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, have cytotoxic, or cytocidal actions to pancreatic islets by inducing nitric oxide (NO) production.[34] Our results tend to suggest that increased levels of cytokines might contribute to the development of DM-associated complications.

Based on the above anti-inflammatory and antifibrotic effects of colchicine, we studied the effect of colchicine on the capacity of peripheral blood monocytes isolated from apparently healthy or from Type I diabetes patients and stimulated with or without LPS or PHA to produce IL-2, IL-6, and IL-10. Results revealed a potent inhibitory effect of colchicines against cytokines secretion, particularly in cells isolated from diabetes patients. These findings might strengthen the rational use of colchicines in several diseases associated with inflammatory responses such as familial Mediterranean fever,[37] primary biliary cirrhosis,[38] and Behcet's syndrome.[39]

The beneficial effects of colchicine have also been demonstrated in several experimental kidney disease models. McClurkin et al. showed that colchicine treatment protected renal function and reduced fibrosis in a rabbit model of severe crescentic glomerulonephritis.[40] They observed significantly lower serum creatinine concentrations and less renal interstitial fibrosis in colchicine-treated animals compared with the vehicle-treated group.[40]

CONCLUSION:

The finding that IL-2, IL-6, and IL-10 from patients with type I DM at diagnosis is suggestive of an increased production rate which may take place at pancreatic level and be reflected by measurable levels in the circulation. If this observation is confirmed in a larger number of cases, levels of these cytokines could become a very useful marker to monitor the disease long-term complications progression. In addition, these findings may provide a new perspective on the protective role of colchicine against inflammatory long-term complications associated with type I DM.

EXPERIMENTAL SECTION

Study design and subjects:

This is a comparative cross sectional study. The study was approved by the Institutional review boards (IRB) of both Jordan University of Science and Technology and from the Ministry of Health in Jordan for study application at Princess Basma Teaching Hospital. Sample recruitment started in February 2009, and ended in August 2010. The study included 50 type I diabetes patients –according to endocrinologist diagnosis- who were recruited from the Diabetes/Endocrine Clinics of the Princess Basma Teaching Hospital. As controls, 15 age matched apparently healthy subjects were recruited from

same clinic's waiting area as they were mostly patient's relatives or friends. At the clinic visit, the study procedure and goals were explained to patients both verbally and through the designed consent form. Patients, who were approved for participation, signed the consent form and were interviewed by the researcher using a previously validated questionnaire. Patient's medical profiles were reviewed for further collection and confirmation of patient's demographics, anthropometric, biochemical, and clinical history.

Blood samples collection and handling:

Overnight-fasting blood samples were withdrawn from participants by a specialized laboratory technician. Each sample was distributed in an evacuated EDTA tube (5 mL blood) as well as an anticoagulant-free plain tube (10 mL blood). Blood samples distributed in plain tubes were centrifuged at 4000 xg for 4 minutes. 500µL of each serum sample were used for analysis of FBS, $HbA1_C$ and triglycerides).

Anthropometric measurements

Using patient's reported height and weight; BMI was calculated using the following equation: BMI = weight (kg) / (height (m))^2. Patients were stratified as previously described [41-42].

Cell culture:

Twelve milliliters (12ml) whole blood samples were drawn from patients aseptically and placed in sterile heparinized tubes for cytokine level determination. The cell culture was set as isolated PBMC's from whole blood diluted (1/1 v/v) by gradient density centrifugation over Ficoll-Histopaque. PBMC's at concentration of $2*10^6$ cells/ml were suspended in RPMI supplemented with 10% fetal calf serum (FCS) and antibiotics. Cultures were incubated for 24 hour at 37ºC in a humid environment with approximately 5% CO_2. Then, the excess media were collected and centrifuged to remove non-adherent cells. The adherent cells that remained in the wells were treated as the following groups: group 1: negative control with 10% RPMI only; group 2: cells were induced for cytokine production with lipopolysaccharides (LPS 10µg/ml); group 3: cells were pretreated with colchicine at different concentrations (0, 12.5, 25, and 50ng/ml) before adding LPS. After 24 hours, supernatants were aspirated for the quantification of IL-2, and IL-6. Detached cells at concentration of $2.5*10^5$ were cultivated treated as the following: Group 4: negative control with culture media; group 5: cells were treated with phytohemaglutinin (PHA) at 20µg/ml; and group 6: cells were pretreated with colchicine for 2 hours before adding PHA. After 72 hours of incubation at 37ºc in a humid environment with 5%CO_2, supernatants were collected for IL-10 measurement. The supernatant were stored at -70ºC for ELISA work.

Measurements of IL-2, IL-6, and IL-10:

The collected supernatants (see above) were used for measurements of IL-2, IL-6, and IL-10 concentrations using enzyme linked immune-sorbent assay (ELISA) kits (DuoSet ELISA Development System). Each sample ran in duplicate, with 9 levels of 2 fold serial dilution standard and two blanks. All procedures were performed according to the manufacturer's instruction. Plates were scanned at 450 nm using a plate reader (Stat Fax 2100 plate reader, Awareness Technology, USA)

Statistical analysis

Data was analyzed using SPSS package version 17 (SPSS Inc, Chicago, USA) for windows. Data was expressed in various tables; discrete variables are expressed as counts and frequencies, and were compared using Chi-square test. Continuous variables are expressed as mean±SD, and were analyzed using un-paired Student t-test or one-way ANOVA followed by Tukey's post-hoc as appropriate. $P < 0.05$ was considered significant.

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تقييم تأثير الكولشيسين على إنتاج السيتوكينات من الخلايا في الدم المحيطي وحيدات النوى من مرضى السكري النوع الأول

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ملخص

يعد داء السكري النوع الأول ناتجا عن تدمير الخلايا المنتجة للانسولين، ويتميز بوجود التهاب في الخلايا المنتجة للانسولين، بالإضافة إلى وجود مستويات عالية من الأجسام المضادة لهذه الخلايا. لقد كان الهدف من هذه الدراسة تقييم مستويات بعض السيتوكينات المهمة مثل IL-2، IL-6، وIL-10–في مرضى السكري من النوع الأول وعلى متطوعين أصحاء، وذلك لدراسة تأثير الكولشيسين على قدرة حيدات الدم المحيطي المعزولة من النوع الأول ومرضى السكري ومتطوعين أصحاء مع أو من دون التحفيز المناعي لإنتاج IL-2، IL-6، وIL-10. تم قياس مستويات السيتوكينات باستخدام مجموعات ELISA القياسية. وكشفت النتائج أن مستويات هذة السيتوكينات كانت أعلى، وبشكل ملحوظ في مرضى السكري مقارنة مع مجموعة الأصحاء قبل وبعد تحفيز الخلايا المناعية بواسطة PHA، وعلاوة على ذلك، فان المعالجة بالكولشيسين حالت دون زيادة مستويات السيتوكينات في كلتا المجموعتين . وتخلص نتائج هذه الدراسة إلى ان الكولشيسين قد يحد من حصول بعض التعقيدات المزمنة في مرضى السكري النوع الاول والناتجة عن ارتفاع مستويات هذة السيتوكينات.

الكلمات الدالة: IDDM، IL-2، IL-6، وIL-10 السيتوكينات، والسيتوكينات المضادة للالتهابات.

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