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Fasting Glucose Level Modulates Cell Surface Expression of CD11b and CD66b in Granulocytes and Monocytes of Patients with Type II Diabetes

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Abstract

Cardiovascular complications are the leading cause of mortality in type II diabetes (T2DM), in which onset and progression of atherosclerosis is linked to chronic inflammation. Activation status of innate immune cells (granulocytes Gc, monocytes Mc), as reflected by increased CD11b, CD66b and other surface markers, increases their endothelial and cytokines/chemokines release. While this inflammatory activation appears inversely related to poor glycemic control, the effect of acute spontaneous hyperglycemia on innate immune cell activation remains unclear. Expression of key markers (CD11b, CD14, CD16, CD62L, and CD66b) was therefore determined by flow cytometry on whole blood of healthy subjects and patients with T2DM with spontaneous, fasting eu- or hyper-glycemia both at baseline and after 30, 90, and 240 min. of room temperature incubation. Hyperglycemic patients with T2DM had significantly higher Gc and Mc CD11b and Gc CD66b surface mean fluorescence intensity (MFI) as compared to euglycemic patients with T2DM whose values were similar to healthy controls. CD16 expression in CD14+CD16+ Mc was elevated in all patients with T2DM, regardless of glycemic levels. Our data suggest that while the presence of diabetes per se may have a pro-inflammatory effect, hyperglycemia seems further acutely exacerbate innate cell inflammatory status, and their consequent endothelial adhesion and vascular damage potential.

Keywords

Glucose; Granulocytes; Monocytes; Type II Diabetes

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INTRODUCTION

T2DM is increasingly considered a chronic inflammatory condition in which frequency and intensity of inflammatory exacerbations likely to determine the rate of onset and progression of related long-term cardiovascular complications¹. Activation of innate immune cells appears to play a key role in these processes². Activated granulocytes (Gc) and monocytes (Mc) enhance diapedesis and migration kinetics across the endothelium; this favors the initiation of microvascular damage as observed for instance in diabetic retinopathy³, as well as the progression of atherosclerosis in larger vascular districts leading to coronary artery disease, myocardial infarction, and other ischemic events⁴. The activation of Gc and Mc is characterized by the expression of a number of cell surface markers (clusters of differentiation, CD) among which integrin, CD11b, selectin CD62L and the Gc-specific marker, CD66b, function as cellular anchors to the endothelium. The expression of lipopolysaccharide (LPS) co-receptor, CD14, and FC receptor CD16 are also widely accepted markers of monocyte activation^{5, 6}.

Increased activation of Gc and Mc has been broadly, albeit inconsistently, associated with obesity, hyperlipidemia, and diabetes. Gc CD11b was shown to be elevated in rat models of obesity⁷ and type 1 diabetes (T1D)⁸, but reduced in rats with T2DM⁹. In humans, while some studies reported no difference^{10, 11} or even reduced¹² surface marker expression in T2DM as compared to healthy controls, others have shown in T2DM increased Gc counts¹³ and increased Gc expression of CD11b, CD66b and other surface markers both in patients with¹⁴ and without¹⁵ microangiopathy which was partly reversible with bariatric surgery and diet¹⁶. Even in subjects who do not have T2DM, the number of CD11b expressing polymorphonuclear neutrophils can be increased acutely by a hyperinsulinemic clamp¹⁷, chronically the CD11b expression of polymorphonuclear leukocytes by the presence of hyperlipidemia¹⁸; and isolated Gc are activated *in vivo* by byproducts of hyperglycemia (methylgluoxal, AGEs)¹⁹.

A similar pattern also exists for Mc. *In vitro*, Mc cell lines have been reported to increase the expression of CD14 and other markers when exposed to glucose²⁰. AGEs²¹ and FFAs²² increase expression of pro-inflammatory cytokines during hyperglycemia²³, one study, however, also reported decreased CD11b expression in Mc of rats with T2DM⁹. *In vivo*, increased expression of inflammatory surface markers has been reported in obese^{24,25}, and patients with T1D^{25,26} and T2DM^{27,28} which improved with anti-diabetic treatment²⁹ and weight loss²⁴. The patients' quality of glycemic control appears to also play a role, as poorly controlled patients with T2DM displayed higher intracellular expression of pro-inflammatory cytokines³⁰ and increased endothelial adhesion and differentiation changes associated with macrophages³¹.

Overall, therefore, the presence and magnitude of inflammatory activation in T2DM displays a considerable variability rendering very difficult to define the actual level of cardiovascular risk to which a patient with T2DM is exposed at a specific time in the course of the disease. More importantly, it is difficult to ascertain from existing reports whether the increase of inflammatory processes is due to the presence of diabetes per se and therefore irreversible, or due to the effect of hyperglycemia or other exacerbating stimuli such as hyperlipidemia and therefore amenable for correction. While both components may be simultaneously at play, it is likely that inflammatory patterns may be constantly shifting. Our earlier work shows, in fact, that even in healthy subjects, high concentrations of glucose have a proinflammatory effect on Gc and Mc³², while in children with T1D, systemic levels of pro-inflammatory cytokines were proportional to the magnitude of hyperglycemia occurred both earlier the same day³³ or during the previous three days³⁴.

Obtaining a firmer understanding about the mechanisms modulating inflammation in T2DM, and innate immune cell activation in particular, may critically improve the design, implementation, and monitoring of therapeutic strategies against diabetic cardiovascular complications. This may be especially relevant in patients in whom good glycemic control is an unrealistic treatment goal, which unfortunately counts for a very large share of the diabetic population. To date, however, information as conceptually simple as assessing if the presence or absence of real-life hyperglycemia can in short-term affect inflammatory activation of innate immune cells in T2DM, is not available. We therefore designed the present study based on the hypothesis that by affecting surface marker expression, fasting blood glucose levels have a short-term modulatory effect on the activation of Gc and Mc in patients with T2DM.

METHODS

Subjects

Nine patients with T2DM and eleven healthy controls were enrolled in the study; subjects' characteristics are described in Table 1. Prior the study, UCI Institutional Review Board (IRB) approval was received and the subjects signed and given informed consent.

Subjects in the T2DM group had been diagnosed with the condition at least 2 years prior to study enrollment, were non-smoking, in general good health, reported no recent trauma or infection, had no evidence or history of other acute or chronic pathology, and took no medications other than oral hypoglycemic agents. Except for diabetes history and related medications, identical enrollment criteria were used for the control group.

Based on whether fasting blood glucose levels on study day was below or above 8 mM, Patients with T2DM were later divided into a Low Glucose (n=4) or High Glucose (n=5) group. The two subgroups displayed a similar medication profile, most of them taking metformin and the two of each group a second oral hypoglycemic agent.

Study Procedures

The subjects were instructed to fast after midnight the night before testing but allowed to drink water ad libitum. On study day, participants reported at the UC Irvine Institute of Clinical and Translational Science at 7:30 am, where an 8 ml blood sample was drawn by a research nurse into tubes containing potassium EDTA. Subjects were then fed and dismissed.

Whole blood samples were placed into a 37°C 5% CO₂ incubator for 240 minutes. At timepoints, 0, 30, 90, and 240 min 100 µL blood samples were taken from the incubator for staining of cell surface markers and flow cytometry. Each 100 µL sample was slightly mixed and incubated in the dark for 15 minutes with fluorescent-conjugated monoclonal antibodies were specific to CD11b-phycoerythrin (PE) (Becton Dickinson/Pharmingen San Diego, CA), CD14-fluorescein isothiocyanate (FITC), (AlexaFluor 647) (Biolegend San Diego, CA), CD16-PE (Biolegend San Diego, CA), CD62L-FITC (Becton Dickinson/Pharmingen San Diego, CA), and CD66b-FITC (Becton Dickinson/Pharmingen San Diego, CA). Red blood cells were then lysed with 2 ml 1X FACS lysing solution (Becton Dickinson, San Jose, CA) and the samples were incubated for 15 minutes in dark. The samples were centrifuged at 600 g for 10 minutes and washed in 2 ml 1 X wash buffer containing 1% fetal calf serum and 0.02% sodium azide in 1 X phosphate buffered saline (PBS) without calcium and magnesium. Each sample was centrifuged at 600 g for 10 minutes, the supernatant was removed and the pellet was resuspended in 500 microliter 1% formaldehyde in PBS.

Flow Cytometry

An Accuri C6 (BD Accuri Cytometers Ann Arbor, MI) flow cytometer was used for data acquisition and analysis. Each event was recorded in a forward-side scatter plot in which lymphocytes, monocytes, and granulocytes were located according to their low, medium, or high forward scattering and the data was reported in mean fluorescence intensity (MFI)

Statistics

Values reported as group means and standard error of the mean (SEM). Within-group differences over time were investigated utilizing One-Way Analysis of Variance (ANOVA) with Dunnett's Multiple Comparisons while across-group differences with Two-Way ANOVA followed by Bonferroni Correction tests. Statistical significance was defined as $p < 0.05$. Graphpad Prism 4 software (GraphPad Software Inc. San Diego, CA) was used for all statistical analyses.

RESULTS

At baseline the High Glucose group displayed ~60% greater granulocyte expression CD11b ($p < 0.01$) and about 30% greater expression of CD66b ($p < 0.05$) as compared to healthy controls (Figures 1a, 1b), the levels of expression in the Low Glucose group was similar to healthy controls although it did not reach statistical significance when compared to High Glucose. Granulocyte CD62L was similar across groups (Figure 1c). When blood samples were incubated for 240 min at 37°C, i.e. subtracting them from the continuous exposure to novel stimuli in the blood stream, levels of surface markers expression decreased similarly in all groups, while differences across groups were maintained. Statistical difference between controls and the High Glucose group was not only present overall, but also at each individual timepoint. (Figure 1d).

Similar to granulocytes at baseline, monocyte expression of CD11b was ~60% greater in High Glucose subjects (Figure 2a) as compared to controls ($p < 0.01$) who displayed values similar to the Low Glucose group. Again this difference was maintained for at least 240 min when whole blood was incubated at 37°C (Figure 2d). Expression of CD16 on CD14+CD16+ monocytes markedly elevated in groups with T2DM as compared to controls but the elevation did not reach statistical significance in either group (Figure 2b). If the two groups were combined however, the mean levels of CD16 expression was significantly greater than in controls ($p < 0.05$) The CD62L expression levels similar to granulocytes were also approximately the same in all three groups (Figure 2c).

DISCUSSION

The main finding of this study is in a group of patients with T2DM, levels of *in vivo* surface marker expression in Gc and Mc appeared to parallel levels of spontaneous morning glycemia. In subjects with high fasting blood sugar, baseline expression of CD11b in both cell types and CD66b in Gc was significantly higher than in healthy controls. For these markers, in euglycemic subjects with T2DM mean values, while indistinguishable from healthy controls, did not reach statistical significance vs. the hyperglycemic group, likely due to a combination of small sample size and greater variability. Expression of CD16 in CD14+CD16+ Mc, while not affected by the level of hyperglycemia, was higher in all patients with T2DM as compared to control. All differences observed at baseline persisted after whole blood was incubated at room temperature for 4 hours.

The main surface markers, on which this study was focused, independent of the diabetic or metabolic state of subjects, have been clearly linked to the development of cardiovascular disease. Increased expression of Gc and Mc surface markers such as CD11b and CD66b has

been associated with increased ability of activated leukocytes to cause endothelial damage, leukocyte aggregation, and capillary obstruction, leading to atherosclerosis and other cardiovascular complications³⁵. Further, high counts of neutrophils expressing CD66b have been reported from human carotid atherosclerotic plaques³⁶. Our results show a marked, significant increase of both Gc CD11b and CD66b expression in patients with T2DM with high fasting blood sugar. CD16 expression of CD14+CD16+ monocytes has also become an established marker of cardiovascular risk. CD14+CD16+ monocyte levels have been correlated with increasing BMI status and obesity^{24,37}, with presence and outcome of coronary artery disease^{38,39} with intima-media thickness⁴⁰, with the likelihood of cardiovascular events in patients with kidney disease⁴¹, and with systemic concentrations of atherogenic lipoprotein levels⁴². In line with this prior evidence, in patients with T2DM as a whole, CD16 expression of CD14+CD16+ monocytes was significantly elevated as compared to controls. Unlike other surface markers, however, no difference was observed between High- and Low-glucose patients, possibly indicating that this marker may be a better indicator of inflammatory status due to the presence of diabetes per se, rather than reflecting short-term inflammatory changes. CD62L is abundantly expressed on the surface of neutrophils in which facilitates attachment to the endothelium^{43,44}, and on monocytes from which it is shed during activation⁴⁵. Little data is available on CD62L in patients with T2DM with one study indicating no difference vs. control¹⁵; and another reporting lower levels in patients with T2DM with microangiopathy¹⁴. In our experimental conditions, similar levels of CD62L expressions were observed across all groups.

To our knowledge, our study is the first to document the effect on leukocyte surface markers, of acute differences of spontaneous, *in vivo* glycemic levels in patients with T2DM. The concept that exposure to hyperglycemia may acutely activate several leukocyte cell lines however, supported by multiple lines of evidence. A strong activating effect of and/or byproduct of hyperglycemic metabolism (AGE's, methylglyoxal) has in fact been reported *in vitro* both on freshly isolated leukocytes¹⁹ and on established cell lines for both Gc's dHL-60,⁴⁶ and Mc's THP-1^{21,23} U937²⁰. In these studies, pro-inflammatory activation was reflected by increased intracellular expression of inflammatory cytokines²³, increased surface expression of CD11b^{19,21} enhanced cell aggregation, impaired phagocytosis, and induction of reactive oxygen species (ROS)⁴⁶. Importantly, hyperglycemia appears to further facilitate cell adhesion by increasing surface expression of adhesion molecules (VCAM-1) on vascular endothelial cells (ECV304)⁴⁷, thereby amplifying the endothelial damaging potential of activated leukocytes. Circulating leukocytes do not adhere to healthy endothelial cells; unless they are stimulated to express adhesion molecules⁴⁸. Determining whether this endothelial activation was also exacerbated in our study with higher glycemia, would have been unquestionably interesting; we will have to remand this experimental question, however, for future studies.

The above evidence should logically be paralleled, *in vivo*, by increased inflammatory activation in the presence of chronic or acute hyperglycemia. Surprisingly little, however, has been published on this issue: poorly controlled patients with T2DM, when compared to a well-controlled group displayed elevated expression of Mc IL-6³⁰ and MCP-1³¹ mRNA and increased Mc surface expression of CD36 and CD68³¹. In terms of the effect of short-term hyperglycemia, the only currently available data are on patients with T1D, in which the severity of morning hyperglycemia³³ or the mean levels of hyperglycemia during the previous 3 days³⁴. While these findings are certainly compatible with our current results, our data are the first to report simultaneous activation of multiple surface markers on both Gc and Mc in T2DM, and the effects of different spontaneous glycemic status.

A number of issues should be taken into account when evaluating our results. A possible limitation is the small number of study participants, which complicates interpretation of

statistical results, limiting the meaning of evaluation normal distribution of data, and rendering impossible to apply multivariate regression analyses. In light of this, results should be seen as part of pilot study to be confirmed in future, larger protocols. Additionally, data interpretation could be possibly confounded by the fact that differences existed across groups in a number of characteristics, such as age, BMI and glycemic control. There was, for instance, an evident age difference between healthy controls and subjects with T2DM. We believe however that this should have had a negligible, if any, effect on our results, as age does not appear to be a contributing factor to CD11b expression of neutrophils^{49, 50}. Even within our T2DM group, whose age range spanned over 20 years, no trend was observed of greater inflammatory expression in older subjects. BMI was also significantly lower in healthy subjects than in either T2DM subgroup, and this variable is indeed associated with a proinflammatory status⁵¹. Considering a greater BMI an intrinsic component of the large majority of cases of T2DM, however, we decided not to normalize it in our control group. Had the level of increased BMI been the deciding factor in GC and MC surface marker expression, its effect should have been similar in both T2DM group; this may therefore be the case in the expression of CD16 in CD14+CD16+ Mc. For the other markers, however, a clear difference was observed across T2DM groups independent of BMI status. Finally, differences in HbA1C also existed between the Low- and High-Glucose T2DM groups, averaging just over 6 % in the former, and just shy of 9% in the latter. This obviously allows the interpretation that the chronic, rather than acute level of hyperglycemia may have determined the reported level of pro-inflammatory leukocyte activation. Our experimental design, however, sought the effect of *spontaneous* morning hyperglycemia, which by definition is more likely to occur in patients with poor glycemic control; overall, in fact, in our patients with T2DM a very strong correlation existed ($R^2 > 0.95$) between fasting glycemia on study day and HbA1c. Additionally, our group has previously demonstrated that the effect of prior hyperglycemia on subsequent inflammatory status follows a hierarchical pattern, with most recent hyperglycemia (i.e. immediately preceding the study) exerting a much stronger effect than average glycemia over previous days or months³⁴. We therefore believe that while the difference in HbA1c may have played a role, the greater part of the reported differences in leukocyte surface markers activation was indeed due to the acute effect of glycemic status immediately before study samples were drawn.

Our findings, although clearly preliminary in nature and based on a small sample size, may have some conceptual relevance that could be translated, if confirmed by larger scale trials, to every-day clinical management of T2DM. While in fact the consistent prevention of hyperglycemia must remain the preeminent target of every therapeutic approach in this population, it is the sad reality of most clinical practices that optimal (or often just acceptable) glycemic control is an unrealistic objective for a large share of these patients. It is conceivable, therefore, that in at least some refractory cases, the progression of inflammation-related cardiovascular complication may be curbed by careful, tailored use of anti-inflammatory medications. Non-steroid anti-inflammatory drugs (NSAID's), for instance, by inhibiting cyclo-oxygenase (COX), not only reduce inflammation but also positively affect glucose-induced insulin release and glucose tolerance⁵². While "classic" NSAID's significant side-effects and lack of enzyme specificity may limit their practical applicability, novel anti-inflammatory molecules are being aggressively developed, able to target single or small numbers of components of the pro-inflammatory cascade. In this context, it becomes critical to definitely identify which cells and which compounds are involved, and to what degree, in each aspect of diabetes-related pro-inflammatory activation. Gaining this knowledge will require a series of complex, more ambitious studies; our results on Gc and Mc can however be seen as a very small but possibly meaningful contribution in this direction.

In conclusion, our study showed that a simultaneous activation in both Gc and Mc, as reflected by expression of pro-inflammatory surface markers, is present in patients with T2DM. While some markers, such as CD16 in CD14+CD16+ Mc appear to be consistently activated in T2DM, independent of current glycemic levels, expression of others such as CD11b and CD66b, appears acutely enhanced by hyperglycemia. These data may provide the conceptual rational for the development of specific molecular targets for tailored anti-inflammatory interventions, which could prevent or delay the development of diabetic cardiovascular complications in cases in which optimal glycemic control is not achievable.

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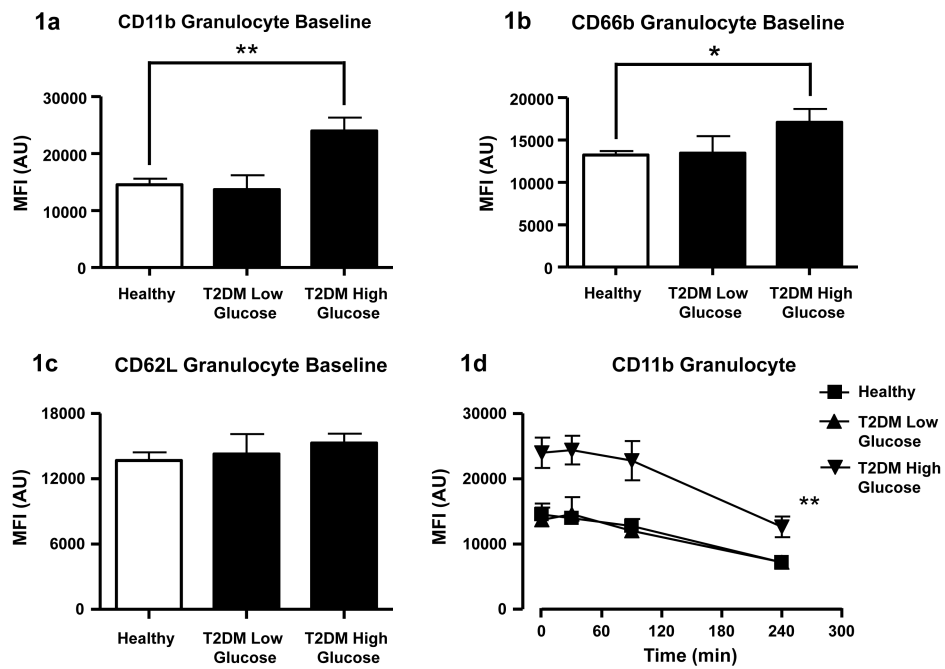


Figure 1. Baseline granulocyte expression of CD11b (**p=0.0016) (a), CD66b (*p=0.045) (b), CD62L (p=0.5389) (c), and CD11b during 240 min incubation (**p=0.0015) (d) in healthy subjects (n=11) and patients with T2DM with low (n=4) and high fasting blood sugar (n=5).

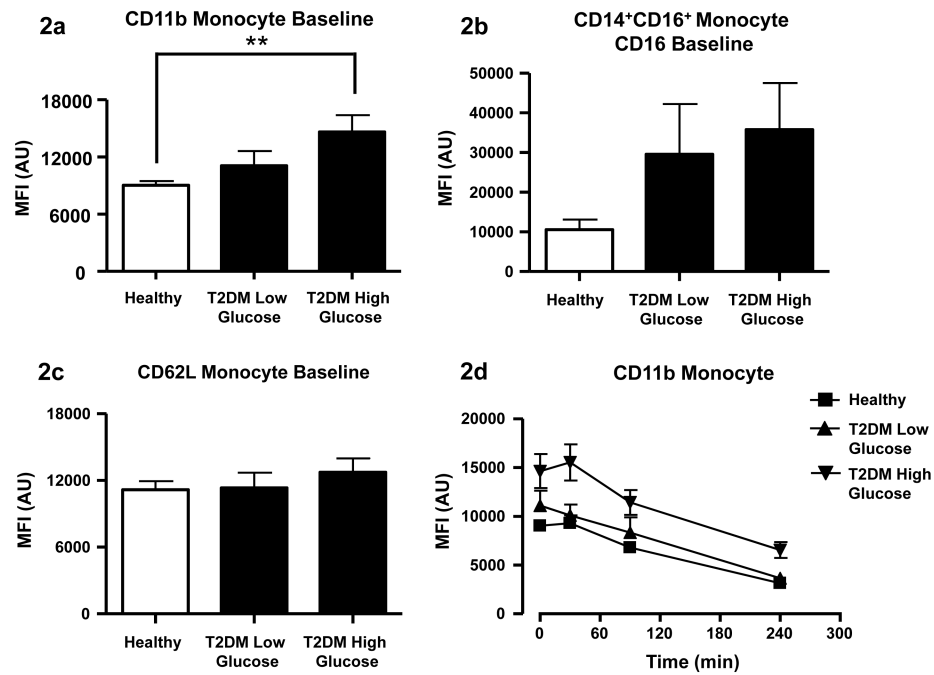


Figure 2. Baseline monocyte expression of CD11b (** $p=0.0031$) (a), CD16 in CD14⁺CD16⁺ cells ($p=0.1875$) (b), CD62L ($p=0.5417$) (c), and CD11b during 240 min incubation ($p=0.3145$) (d) in healthy subjects ($n=11$, $n=5$ for double-stained samples) and patients and T2DM with low ($n=4$) and high fasting blood sugar ($n=5$).

TABLE 1

Subject Demographics

Subject Groups (males)	Age (years)	Height (cm)	Weight (kg)	BMI	Fasting Blood Glucose (mM)
Healthy (11)	30.4±3.7	179.6±1.8	78.6±3.3	24.4±0.9	4.8±0.3
Glucose Low (4)	42.7±4.2	175.7±1.4	113.3±13 *	36.6±3.7 ***	5.9±0.4
Glucose (High) (5)	49.2±4.5 *	173.6±3.4	111.2±9.5 ***	37.1±3.5 ***	15.1±2.3 ***

* p<0.05,

*** p<0.01 Glucose vs Healthy. Data are mean±SE