The Effect of Pro-Inflammatory Conditions on Neutrophil Rolling Adhesion

CURRENT STATUS: POSTED

Keith Taverner  
The University of British Columbia

Yousif Murad  
The University of British Columbia

Adam Yasunaga  
The University of British Columbia

Christine Furrer  
The University of British Columbia

Jonathan Little  
The University of British Columbia

Isaac TS Li  isaac.li@ubc.ca  
University of British Columbia Okanagan  
Corresponding Author  
ORCID: 0000-0002-8450-5326

DOI:  
10.21203/rs.2.12835/v1

SUBJECT AREAS  
Epidemiology

KEYWORDS  
HL-60, Neutrophils, Type 2 diabetes mellitus, cell rolling adhesion, P-selectin, PSGL-1
Abstract

Objective: Type 2 diabetes mellitus (T2D) is the result of a dysregulation of insulin concentrations and signaling, leading to an increase in both glucose concentration and proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α. Previous work showed that T2D patients exhibited immune dysfunction associated with increased adhesion molecule expression on endothelial cell surfaces, accompanied by decreased neutrophil rolling velocity on the endothelial cell surface. Changes in cell rolling adhesion have direct vascular and immune complications such as atherosclerosis and decreased healing time seen in T2D patients. While previous studies focused primarily on how endothelial cells affect neutrophil rolling under T2D conditions, little is known on changes to neutrophils that affect their rolling. In this study, we aim to show how the rolling behaviour of neutrophils are affected by T2D conditions on a controlled substrate.

Results: We found that neutrophils cultured in T2D-serum mimicking media showed an increase in cell rolling velocity compared to neutrophils under normal conditions. Specifically, glucose alone is responsible for higher rolling velocity. While cytokines further increase the rolling velocity, they also reduce the cell size. It is likely that both glucose and cytokines reduce the PSGL-1 expression level on neutrophils.

Introduction

Neutrophils are the most numerous immune cells, and some of the first responders to infection. Under physiological conditions, neutrophils flow freely in the blood stream. However, during the inflammatory response, neutrophils are captured out of the blood stream and roll on the endothelial wall of postcapillary venules.[1] This is mediated by the rapid formation and dissociation of interactions between P-selectin glycoprotein ligand 1 (PSGL-1) on neutrophil membranes and P-selectin expressed on endothelial surfaces under
inflammatory conditions.[1, 2] This tightly regulated process is essential for immune responses mounted by neutrophils. Therefore, any changes in rolling adhesion can have profound effects on the immune system.

Diabetes is a condition resulting from insufficient insulin production and/or impaired insulin response with associated hyperglycemia (elevated blood glucose concentration). T2D patients represent 90.9% of the 23.1 million adults with diabetes according to the National Health Interview Survey.[3] T2D is associated with high basal cytokine levels and a wide range of innate immune responses.[4] Hyperglycemia has been linked to a proinflammatory state leading to increased production of interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α).[5, 6] Hyperglycemia can also cause insulin resistance from chronic exposure to glucose and reactive oxygen species formation.[5, 6] Patients with T2D can experience several complications such as cardiovascular disease, atherosclerosis, blindness, and kidney failure.[7, 8] Atherosclerosis is associated with chronic inflammation possibly due to the increased recruitment of immune cells including neutrophils.[9] Previous studies have shown patients with T2D to exhibit immune dysfunction related to neutrophil chemotaxis, rolling, and adhesion.[7, 10, 11] Specifically, research has shown PMNs (polymorphonuclear neutrophils) taken from T2D patients demonstrated a decrease in cell rolling velocity compared to a control population.[7, 11] These studies examined cell rolling on an activated human umbilical vein endothelial cells (HUVEC) surface where variable concentrations of P-selectin and other adhesion molecules can be present.[7, 9, 11] This surface receptor heterogeneity may have significant effects on cell rolling behaviour.[9, 12] Additionally, these studies used a singular wall shear stress value of 0.07 Pa which may not be sufficient to demonstrate the full range of effects imparted by diabetic growth conditions.[7, 11] To better understand to role of the PSGL-1 in T2D, we investigated neutrophil rolling on a surface with a controlled P-selectin density.
Additionally, we chose a range of shear stress levels to mimic conditions found in both arterial (1–7 Pa) and venous (0.1–0.6 Pa) blood flow.[13] PSGL-1 is both a key ligand for rolling adhesion and a receptor to enable intracellular signaling, inducing cytokine secretion, activation of SRC family kinase, β2-integrin, and potential activation of Phosphoinositide 3-kinase (PI3K) signaling in either neutrophils or the endothelial cells they are rolling on.[14]

In this paper, rolling adhesion is used as an assay to directly probe how PSGL-1 expression is affected by T2D conditions using controlled substrate P-selectin concentration. Our aim is to investigate how neutrophil rolling is affected by T2D-like pro-inflammatory conditions, such as hyperglycemia, and supraphysiological TNF-α, IL-6, and insulin concentrations, related solely to the interaction between PSGL-1 and P-selectin.

Methods

Flow chamber construction

The flow chamber construction has been reported previously[15]. Briefly, the flow chamber consisted of a coverslip for the bottom (rolling surface) and a glass slide for the top. The cover glass was passivated by polyethylene glycol (PEG) and functionalized by PEG-biotin (Laysan Bio) at 20:1 ratio. Once the flow chamber was assembled, a protein scaffold made up of 1 mg/mL streptavidin (Thermofisher), 100 μg/mL protein G (Thermofisher) and 10.6 μg/mL of P-selectin-Fc (R&D System) was functionalized through PEG-biotin onto the surface.

Cell growth and rolling experiment

HL-60 cells were cultured in 25 cm² culture flasks (VWR) containing glucose-free RPMI-1640 media (Thermofisher), 10% FBS (VWR), and 1% pen/strep (Gibco). Cell cultures were maintained in 5% CO₂ at 37°C. HL-60 cells were differentiated into neutrophils using 1.5%
dimethyl sulfoxide (DMSO). To make up the final concentrations of pro-inflammatory conditions, 40% glucose (VWR), 1000 pM insulin (Humulin), 10 ng/mL TNF-α (PeproTech), and 10 ng/mL IL-6 (PeproTech) were used. Studies have demonstrated the pro-inflammatory effects of these concentrations of peptides [16–18]. Glucose concentration was measured using a commercial glucose monitor (Contour).[19] Two mL of cell culture were centrifuged for 3.5 minutes at 300 rcf. Approximately 1.7 mL of liquid was removed, and cells were re-suspended in rolling buffer and added to the flow chamber. [15] Darkfield microscopy at 10x magnification was used to capture cell rolling movies at 30 fps. A syringe pump (Harvard Apparatus) was used to control the flow rate. Flow rates between 0.5 and 15 mL/hour were used in our experiments. Data acquisition length was set to allow cells to cross the entire field of view, generating longest possible tracks. After each run, all remaining cells were washed out. Videos were analyzed using custom MATLAB code for cell tracking and quantification.

Results And Discussion

Effect of Glucose on Cell Rolling

Hyperglycemia is mimicked in our experiments by culturing neutrophils (differentiated of HL-60 cells) in glucose rich media. To measure the effect of hyperglycemia on neutrophil rolling adhesion and neutrophil phenotype in general, we tested neutrophils cultured at three different glucose concentrations: 8, 19 and 25 mM. Neutrophil rolling experiments were carried out at a range of physiologically relevant shear stresses (0.17, 0.41, 0.83 and 1.24 Pa) to determine the physiological relevance of any effects observed.

As a control, we observed a linear relationship between mean cell rolling velocity and the applied shear stress as has been reported in the literature.[20] This result holds true at all glucose concentrations. However, under hyperglycemia conditions, higher glucose concentration results in increased cell rolling velocity (Figure 1a). The slope of rolling
velocity as a function of glucose concentration also increases, demonstrating that the change in neutrophil rolling velocity is most evident under high shear stress. Therefore, hyperglycemic conditions lead to an increase in neutrophil rolling velocity which may have immunological consequences \textit{in vivo} such as increasing the difficulty of extravasation into tissues.

There are two possible mechanisms that could lead to such increase in rolling velocity under hyperglycemic exposure: 1. a decrease in PSGL-1 expression on neutrophil surface leading to weaker adhesive interactions with the P-selectin coated surface and/or 2. an increase in cell size where shear flow exerts a greater force to move the cells. Under different glucose concentration at different shear stresses, we did not observe significant changes in cell size (Figure 1b). We used the projection area of a cell as a proxy for cell size. The lack of significant changes of cell size across different glucose concentrations indicates that glucose alone is not changing the phenotypic size of the cell. Furthermore, cells maintain the same size over a large range of shear stresses, indicating that the cells remain rigid and shear stresses used in the experiment are not deforming the cells.

Next, we examine whether the effect of glucose on cell rolling velocity require chronic glucose exposure or is acute glucose shock sufficient to induce such effect. We mimicked the effects of acute hyperglycemia exposure on neutrophils by exposing cells cultured under various glucose concentrations at the time of rolling experiments only (on the minute timescale). Our result indicates that neither the cell rolling velocity (Figure 1c) or cell size (Figure 1d) are affected by acute glucose exposure over a wide range (6.4 mM to 30.8 mM) glucose concentrations. This result shows the importance of chronic glucose exposure (culturing media for days) in affecting neutrophil phenotypes.

Lastly, we examine whether chronic exposure to non-metabolic sugar such as mannitol induces similar effects as glucose. We replaced glucose with mannitol under the exact cell
culturing and rolling conditions. The cell rolling velocity increased only slightly (<10%) at high mannitol concentration (Figure 1e) compared to an increase of up to 400% in the case of glucose (Figure 1a). Furthermore, different concentrations of mannitol induced no change in the cell size across the whole range of shear stress (Figure 1f). This result shows that chronic exposure specifically to glucose has a significant effect on cell rolling behaviour. Exposure to mannitol also serves as a control for osmotic pressure on the cell caused by the increase of sugar concentration, which in our case, did not change the size of the cell and hence, insignificant effect on the cell rolling velocity. From these evidences, it is more likely that such large effect of glucose on cell rolling velocity is the result of changes to cell surface protein (i.e. PSGL-1) expression than physical size changes. Such change may be the result of increased growth rate under high glucose concentration. Indeed, the rate of cell growth increases with glucose in the culturing media, which is particularly true when considering that the insulin resistant glucose transporter GLUT1 is expressed in neutrophils. [21]

**Effect of Pro-inflammatory Cytokines on Cell rolling**

Cells cultured in media containing the diabetic cocktail (hyperglycemic media plus TNF-α, IL-6 and insulin, see Table 1) were compared to those cultured under only hyperglycemic conditions in rolling experiments. We observed a small increase in cell rolling velocity over a range of shear stresses for neutrophils cultured under these conditions (Figure 2a). However, the effect of the diabetic cocktail compounds on cell size is quite significant (Figure 2b). Here, we observe a significant decrease in cell projection area (up to 25%) upon chronic exposure to the diabetic cocktail compared to hyperglycemic exposure alone. When plotting the cell size against mean rolling velocity (Figure 2c), two clusters corresponding to hyperglycemic and diabetic cocktail conditions clearly emerge. The decrease of cell size is generally accompanied by a decrease in rolling velocity, as the
shear force acting on a smaller cell is lower. However, the fact that the smaller cells under diabetic cocktail condition roll faster than larger cells under just hyperglycemic condition came as a surprise. Because of our surface passivation and functionalization, only P-selectin are presented on the surface, enabling only adhesive interactions between P-selectin and PSGL-1. We are directly probing the rolling adhesion behaviour due to the P-selectin, PSGL-1 interaction. Hence a further decrease of PSGL-1 expression under the diabetic cocktail condition comparing to hyperglycemic condition is the most likely cause for the observed behaviour. This is also supported by the need for chronic exposure to hyperglycemia and the lack of effect upon acute exposure which does not allow for the time to affect surface protein expression.

Limitations

There are several limitations in this work:

The rolling velocity of neutrophils are dictated by both the phenotype of the neutrophil and the endothelial cells. Our study focuses exclusively on how neutrophils are affected given the substrate they roll on remain unchanged. Hence, the rolling velocity changes should be considered only within the context of the current experiment. It would be difficult to draw direct extrapolations towards an in vivo system in terms of the neutrophil rolling velocity. Although differentiated HL-60 cells are terminally differentiated and do not proliferate, they still metabolize glucose in the media. We notice the glucose concentration drop up to 20% over the 48 hours. The values reported are average glucose concentration. Neutrophils were obtained by differentiating HL-60 cells. The differentiation process does not produce 100% neutrophil, as up to 20% cells remain undifferentiated. This may cause bias in the observation, but we do not have means to separate the differentiated cells from undifferentiated cells. Lastly, we found that it is challenging to precisely control the surface density of P-selectin in our flow chamber. We observed up to 15% differences in rolling velocities on different functionalized coverslip surfaces while keeping other experimental conditions constant. Additionally, the high shear flow also removes P-selectin functionalized on the surface over time (~ 30–60 mins). This makes it challenging to assign statistical significance if the true rolling velocity is less than 15% different. In order to be able to quantitatively compare rolling velocity with the same surface density, we created multiple channels on the same functionalized surface and recorded data only in the first 30 mins. However, replicates across different functionalized surface is simply averaged for the lack of a better normalization method.

Declarations

Ethics approval and consent to participate
Consent for publication
Not applicable

Availability of data and materials
The datasets used in the current study are available from the corresponding author on request.

Competing interests
The authors declare that they have no competing interests

Funding
Experiment design, data collection and analysis are supported by funding from the Natural Sciences and Engineering Research Council (NSERC). Instrument used in the study was supported by the Canada Foundation for Innovation (CFI).

Authors’ contributions
I. T. S. L., J. L., C. F. and K. T. designed the research; K. T., Y. M., and I. T. S. L. wrote the manuscript; all authors have read and edited the manuscript; K. T. and Y. M. analyzed and interpreted the data; A. Y., Y. M., and I. T. S. L. wrote the program for data analysis; and K. T. preformed the experiments. All authors have read and approved the manuscript. No conflict of interest was declared by the authors.

Acknowledgements
Not applicable

References
1. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nature Reviews Immunology. 2007;7:678-89.
2. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. J Exp Med. 2006;203:2569–75. doi:10.1084/jem.20060925.

3. Bullard KM, Cowie CC, Lessem SE, Saydah SH, Menke A, Geiss LS, et al. Morbidity and Mortality Weekly Report Prevalence of Diagnosed Diabetes in Adults by Diabetes Type—United States, 2016. 2016;67:2016–8.

4. Marc Y. Donath and Steven E. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol. 2011;11:98.

5. Dasu MRMR, Devaraj S, Zhao L, Hwang DHDH, Jialal I. High glucose induces toll-like receptor expression in human monocytes. Diabetes. 2008;57:3090.

6. Siklova M, Simonsen L, Polak J, Stich V, Bülow J. Effect of short-term hyperglycemia on adipose tissue fluxes of selected cytokines in vivo during multiple phases of diet-induced weight loss in obese women. J Clin Endocrinol Metab. 2015;100:1949–56.

7. Rovira-Llopis S, Bañuls C, Apostolova N, Morillas C, Hernandez-Mijares A, Rocha M, et al. Is Glycemic Control Modulating Endoplasmic Reticulum Stress in Leukocytes of Type 2 Diabetic Patients? Antioxid Redox Signal. 2014;21:1759–65. doi:10.1089/ars.2014.6030.

8. Burrows NR, Li YF, Gregg EW, Geiss LS. Declining rates of hospitalization for selected cardiovascular disease conditions among adults aged 35 years with diagnosed diabetes, u.S., 1998–2014. Diabetes Care. 2018;41:293–302.

9. Omi H, Okayama N, Shimizu M, Okouchi M, Ito S, Fukutomi T, et al. Participation of high glucose concentrations in neutrophil adhesion and surface expression of adhesion molecules on cultured human endothelial cells: effect of antidiabetic medicines. J Diabetes Complications. 2001;16:201-8.

10. Alba-Loureiro TC, Munhoz CD, Martins JO, Cerchiaro GA, Scavone C, Curi R, et al.
Neutrophil function and metabolism in individuals with diabetes mellitus. Brazilian J Med Biol Res. 2007;40:1037-44.

11. Diaz-Morales N, Rovira-Llopis S, Bañuls C, Lopez-Domenech S, Escibano-Lopez I, Veses S, et al. Does Metformin Protect Diabetic Patients from Oxidative Stress and Leukocyte-Endothelium Interactions? Antioxid Redox Signal. 2017;27:1439-45. doi:10.1089/ars.2017.7122.

12. Li ITS, Ha T, Chemla YR. Mapping cell surface adhesion by rotation tracking and adhesion footprinting. Sci Rep. 2017;7:44502.

13. Paszkowiak JJ, Dardik A. Arterial wall shear stress: Observations from the bench to the bedside. Vasc Endovascular Surg. 2003;37:47-57. doi:10.1177/153857440303700107.

14. Tinoco R, Otero DC, Takahashi AA, Bradley LM. PSGL-1: A New Player in the Immune Checkpoint Landscape. Trends Immunol. 2017;38:323-35. doi:10.1016/j.it.2017.02.002.

15. Li ITS, Ha T, Chemla YR. Mapping cell surface adhesion by rotation tracking and adhesion footprinting. Sci Rep. 2017;7:44502.

16. Lademarco MF, Barks JL, Dean DC. Regulation of Vascular Cell Adhesion Molecule-1 Expression by IL-4 and TNF-a in Cultured Endothelial Cells Key words: cell adhesion molecules - half-life * messenger RNA * inflammation * selective leuko- cyte recruitment. Clin Invest. 1995;95:264-71. doi:10.1172/JCI117650.

17. Afford SC, Pongracz J, Stockley RA, Crocker J, Burnett D. The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J Biol Chem. 1992;267:21612-6.

18. Ma H, Zhang S, Xu Y, Zhang R, Zhang X. Analysis of differentially expressed microRNA of TNF-α-stimulated mesenchymal stem cells and exosomes from their
culture supernatant. Arch Med Sci. 2018;14:1102–11.

19. Bailey TS, Wallace JF, Pardo S, Warchal-Windham ME, Harrison B, Morin R, et al. Accuracy and User Performance Evaluation of a New, Wireless-enabled Blood Glucose Monitoring System That Links to a Smart Mobile Device. J Diabetes Sci Technol. 2017;11:736–43.

20. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. Cell. 1991;65:859–73.

21. Daoud AK, Tayyar MA, Fouda IM, Harfeil NA. Effects of diabetes mellitus vs. in vitro hyperglycemia on select immune cell functions Diabetes mellitus suppresses select immune cell functions Daoud et al. J Immunotoxicol. 2009;6:36–41.

Table

**Table 1.** Cell growth conditions.

| Test Group           | Glucose Concentration (mM) | IL-6 Concentration (ng/mL) | TNF-α Concentration (ng/mL) | Insulin Concentration (nM) |
|----------------------|---------------------------|---------------------------|-----------------------------|---------------------------|
| Control              | 5                         | N/A                       | N/A                         | N/A                       |
| Hyperglycemic        | 25                        | N/A                       | N/A                         | N/A                       |
| Diabetic Cocktail    | 25                        | 10                        | 10                          | 1                          |

Figures
Effects of hyperglycemia on neutrophil rolling adhesion. (a) Neutrophil rolling velocities increase as a function of shear stress under different glucose concentrations (chronic exposure, Dark to light red represent decreasing glucose concentration). Error bars represent the first and third quartiles. (b) Cell projection area is not changed as a function of media glucose concentration. (c, d) Acute changes in glucose concentration have no detectable effect on neutrophil rolling velocity (c) or size (d). All data correspond to rolling at 0.29 Pa. (Sample size between 400-1700 cells per set.) (e, f) Neutrophils cultured under the same glucose concentration but differing mannitol concentrations in media exhibit similar rolling velocity and size. (Dark and light green represent high and low mannitol concentration. Sample size between 400-1100 cells per set.)
Effects of pro-inflammatory conditions on neutrophil rolling adhesion. (a) Neutrophil rolling velocity as a function of shear stress under hyperglycemic (red) and diabetic cocktail (blue) conditions. (b) Neutrophil cell projection area as a function of shear stress under hyperglycemic (red) and diabetic cocktail (blue) conditions. (c) Mean cell area vs. mean rolling velocity at different shear stresses (0.17 Pa – 1.24 Pa) shows two clusters, indicating cells chronically exposed to diabetic cocktail are smaller and rolls faster.