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Advances in Neutrophil Testing In Type 2 Diabetes Mellitus

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Abstract

Patients with type 2 diabetes mellitus (T2DM) suffer from impaired glucose metabolism which results in low-grade inflammation and activation of the innate immune system. Neutrophils the key effector cells of the innate immune system and heavily implicated in the pathogenesis of T2DM, are promising cell-based inflammatory biomarkers for immune health profiling, provided that they can be rapidly purified and measured with sufficient precision. In this review, we highlight recent advances in neutrophil isolation and functional assay using microfluidics technologies and the potential of their functional phenotype as a novel biomarker of vascular risk in diabetes.

Keywords: Neutrophils; Diabetes; Point-of-care; Microfluidics; Immunology

Introduction

With the increasing aging population worldwide, metabolic disorders such as diabetes mellitus (DM) and cardiovascular diseases (CVDs) have become the main public health challenges with rising premature morbidity and associated mortality, as well as escalating healthcare costs [1]. DM is characterized by chronic hyperglycemia resulting in increased oxidative stress, inflammation and endothelial dysfunction [2,3]. Patients with CVDs or type 2 diabetes mellitus (T2DM) often exhibit low-grade inflammation, and are assessed based on established cardiovascular risk factors (glycemic control, blood pressure and lipids). Immune health is evaluated by differential leukocyte count and circulating biomarkers (cytokines and C-reactive protein (CRP), which are suboptimal for monitoring stage-dependent pathogenesis, advocating the need to develop new cell-based biomarkers that can quantity specific immune functions in addition to leukocyte enumeration.

Neutrophils, the key effector cells of the innate immune system, play a pivotal role in T2DM and CVDs pathogenesis [4]. Various neutrophil dysfunctions have been reported in T2DM patients including cell stiffening [5,6] impaired chemotaxis [7,8] and phagocytosis which lead to increased susceptibility to bacterial infections [9]. Despite the adverse changes of leukocytes in diabetes, there are currently no specific measurements to assess patient's leukocyte phenotypes or inflammatory status. As distinct neutrophil subsets exhibit functional and phenotypic differences [10] a better understanding of their phenotype and pathophysiological relevance requires novel neutrophil separation tools (independent of surface markers) to improve their predictive capabilities as novel biomarkers [11]. Microfluidics, also known as "lab-on-a-chip" technologies, is a powerful toolbox for rapid sample preparation and detection with its low consumption of sample and reagents, device miniaturization, and single-cell analysis [12]. In this short review, we will highlight recent advances in microfluidics-based neutrophil testing technologies, and the potential of neutrophilfunctional phenotype as biomarkers for diabetes testing.

Discussion

Neutrophil isolation

Neutrophil polymorphonuclear granulocytes (PMN) are the most abundant leukocytes (~50-70%) in humans, with

~2-5×106 neutrophils per mL of whole blood (~109 RBCs). They are short-lived (~5-24hr), prone to activation [13] and should be processed quickly within 2-4 hours of collection. Conventional neutrophil isolation methods include density gradient centrifugation and RBCs lysis, which are laborious (~1-3hr) and require large blood volume (~10mL). Commercial kits based on magnetic bead-based affinity binding (MACS xpress® Neutrophil Isolation Kit (Miltenyi Biotec) and Easy SepTM neutrophil enrichment kit (STEMCELL Technologies) provide high neutrophil yield and purity by negative selection, but is expensive for large volume processing.

Microfluidics technologies for neutrophil isolation have been developed based on affinity binding to functionalized surfaces using common neutrophil markers (CD66b, P-selectin) [14,15]. However, these methods require on-chip cell analysis as it is non-trivial to elute the purified neutrophils off-chip for downstream assays. Our group has previously developed an efficient size-based cell sorting technique known as Dean Flow Fractionation (DFF) based oninertial focusing phenomenon in micro channels [16]. In DFF systems, fluid flowing through a curvilinear (spiral) channel experiences centrifugal acceleration directed radially outward, leading to the formation of two counter-rotating vortices known as Dean vortices [17]. Besides inertial lift forces (FL) particles experience lateral Dean drag force (FD) due to these transverse Dean flows, which results in superior separation resolution as both forces (FL and FD) scale non-linearly with particle size [18-20].

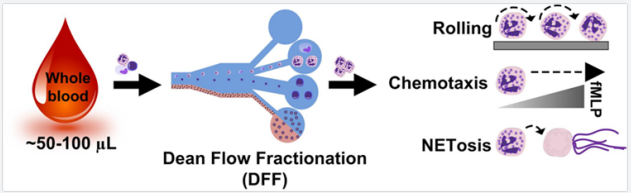

Figure 1: Microfluidic technologies for neutrophil testing in diabetes. Label-free and untouched neutrophils purified using Dean Flow Fractionation (DFF) are continuously eluted off-chip for downstream characterization of different critical functional phenotypes including cell rolling, chemotaxis and formation of neutrophil extracellular traps (NETosis).

We first applied DFF technology to isolate circulating tumor cells (CTCs) [21] and microorganisms from whole blood whereby the size ranges of the target cells are distinctly different from RBCs. By exploiting the subtle size differences between major leukocyte subtypes (neutrophils/monocytes~10-12µm; lymphocytes~7-9µm), we recently developed a novel DFF spiral micro device to purify neutrophils rapidly from whole blood for functional phenotyping in T2DM [22]. The developed technology enables single-step neutrophil isolation (>90% purity) without immune-labeling, saving both time and cost. In addition, the sorted "untouched" neutrophils are continuously eluted off-chip with simultaneous buffer exchange, facilitating user operation and eliminating the need for centrifugation. Moreover, as the method only requires small blood volumes (finger prick ~50-100µL) it can be easily integrated with other cellular assays or detection modules for point-of-care (POC) testing (Figure 1).

Neutrophil rolling

During endothelial inflammation, leukocyte adhesion cascade is a multi-step process involving cell rolling, adhesion and transmigration through blood vessel walls to the site of injury [23]. Neutrophil rolling is widely considered a critical step as it can affect cell adhesion with impaired cell tethering or increased rolling speeds [24,25]. Several microfluidics-based cell rolling assays have been reported previously to study rolling behavior under physiological flow conditions (~1-10dynecm-2), but not in disease-specific context [26-28]. In our study, we combined DFF neutrophil sorting method and microfluidics assay to measure neutrophil rolling speed on E-selectin, a cell adhesion molecule expressed by activated endothelium to initiate leukocyte recruitment. This neutrophil-endothelial interaction is mediated by several sialyl Lewisx presenting ligands expressed on leukocytes including P-selectin glycoprotein ligand 1 (PSGL1), glycosylated CD44 and E-selectin ligand 1 (ESL1) [29].

In our clinical validation, we observed a significant down regulation of neutrophil PSGL-1 expression in T2DM patients. Using automated cell tracking algorithm, we further showed that rolling trajectories of T2DM neutrophils were more discontinuous and irregular as compared to healthy neutrophils. Interestingly, diabetic neutrophils had ~20% higher rolling speeds, which correlated with neutrophil activation, PSGL-1 expression, as well as established cardiovascular risk factors (cholesterol, CRP and HbA1c). Taken together, the data support the hypothesis that neutrophil-endothelial interactions are impaired in T2DM patients which can lead to defective neutrophil recruitment, and thus increased patient susceptibility to infection.

Neutrophil chemotaxis

Chemotaxis, a dynamic process where cells sense and move in response to chemical gradients, is traditionally studied using Boyden chamber (transwell), Dunn chamber and micropipette assay [30]. However, these methods suffer from poor reproducibility and ill-defined chemical gradients, which could be overcome by using microfluidics technologies to generate stable and linear chemo attractant gradient in small length scale (~µm) [15]. Moreover, most microfluidic chemotaxis assays only require ~102-3 neutrophils, and facilitate real-time imaging of cell movement at single cell resolution [31]. First performed clinical testing of patients with burn injury using microfluidics, and observed that neutrophils suffered from impaired directionality or slower migration speed, which were associated with degree of burn injury.

Similarly neutrophils from asthmatic patients also displayed significantly slower migration speed as compared to healthy

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subjects, suggesting its use as a novel diagnostics marker [32]. As impaired neutrophil chemotaxis behavior was reported previously in diabetic patients our group has developed an integrated micro device for neutrophil chemotaxis assay using a drop of blood. The novelty lies in the single-step enrichment of neutrophils using biomimetic cell margination [33] and affinity capture, followed by simultaneous exposure to chemotactic gradient without requiring additional user manipulation [34]. In our preliminary clinical data we also observed signification suppression of chemotaxis behavior in T2DM patient, which can be mitigated by short exposure to metformin in vitro. Besides diagnostics applications, microfluidics chemotaxis assays also enable study of complex chemoattractant gradients with high precision [35], well-controlled spatial and temporal gradients to probe cell migration pattern [36,37], as well as effect of inflammatory mediators in neutrophil-monocyte interactions [38].

Neutrophil extracellular traps (NETs)

First discovered in 2004, formation of neutrophil extracellular traps (NETs) is an innate key defense mechanism against bacterial infections through the release of nuclear and granular contents to contain and kill pathogens [39]. Upon activation or exposure to bacteria, histones undergo citrullination, followed by chromatin decondensation. Nuclear membrane will degrade, leading to DNA release into the cell, and subsequently extrusion out of neutrophils. Secreted NETs (process known as NETosis) then form a sticky scaffold consisting mainly of microbicidal proteases/elastase and cytotoxic molecules (histones). Interestingly, recent work have shown that diabetic neutrophils were more susceptible to NETosis [40], which can mediate delayed wound healing [41].

NETs components (elastase, histones, neutrophil gelatinase-associated lipocalin, and proteinase-3) are also elevated in the blood of patients with diabetic foot ulcers, and were associated with infection or worsening of ulcer [42]. Overall these clinical evidences suggest a major role of NETosis in diabetes pathophysiology and endothelial damage making it a novel biomarker for early detection of diabetes-related vascular or end-organ complications. Compared to chemotaxis development of microfluidics NET osis assay is still at its early infancy with a recent reported assay based on fluorescent imaging of nucleus degradation [43]. Nevertheless given the increasing importance of NETosis and easy quantification using imaging, we expect more development of novel tools to measure NETosis phenotype in POC settings.

Conclusion

Multidimensional neutrophil phenotypic markers will significantly improve their predictive capabilities as inflammatory biomarkers provided that they can be rapidly purified and measured with sufficient precision. Microfluidics technologies are not only useful for efficient neutrophil purification but they can also be readily developed and integrated into POC testing plat forms to look at the sum effects of diabetes, hypertension and hyperlipidemia. This enables proper identification of high risk patients with appropriate follow up, reduces the risks in different aspects of the endothelial activation pathway and in time, the effects of therapeutics can also be studied in diabetes and other dysmetabolic diseases.

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