Impairment of Mitochondrial–Nuclear Cross Talk in Neutrophils of Patients with Type 2 Diabetes Mellitus

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Abstract Increased leukocyte apoptosis is intrinsically linked to disease patho-physiology, susceptibility to and severity of infections in type 2 diabetes mellitus (T2DM) patients. A consistent defect in neutrophil function is considered central to this increased risk for infections. Although redox imbalance is considered a potential mediator of these associated complications, detailed molecular evidence in clinical samples remains largely undetected. The study consisted of three groups ($n = 50$ each) of Asian Indians: early diagnosed diabetic patients, cases with late-onset diabetic complications and age and gender-matched healthy controls. We evaluated mitochondrial oxidative stress, levels of nuclear DNA damage and apoptosis in peripheral blood neutrophils isolated from T2DM patients. We observed that in both early and late diabetic subjects, the HbA1c levels in neutrophils were altered considerably with respect to healthy controls. Increased oxidative stress observed in both early and late diabetics imply the disentanglement of fine equilibrium of mitochondria-nuclear cross talk which eventually effected the augmentation of downstream nuclear γ H2AX activation and caspase-3 expression. It would be overly naïve to refute the fact that mitochondrial deregulation in neutrophils perturbs immunological balance in type 2 diabetic conditions. By

virtue of our data, we posit that maneuvering mitochondrial function might offer a prospective and viable method to modulate neutrophil function in T2DM. Nevertheless, similar investigations from other ethnic groups in conjunction with experimental evidences would be a preeminent need. Obviously, our study might aid to comprehend this complex interplay between mitochondrial dysfunction and neutrophil homeostasis in T2DM.

Keywords Mitochondria dysfunction · Type 2 diabetes mellitus · Free radical injury · Translational research

Introduction

Over the past three decades, the number of people diagnosed globally with diabetes mellitus has more than doubled. In 2010, an estimated 285 million people worldwide had diabetes mellitus and figure is projected to rise to 439 million by 2030, which represents 7.7 % of the total adult population of the world aged 20–79 years [1]. Of this global burden, 90 % is shared by type 2 diabetic mellitus (T2DM). The causes of T2DM epidemic are embedded in a very complex framework of genetic and epigenetic interactions [2]. Increased leukocyte apoptosis in T2DM is intrinsically linked with disease patho-physiology and enhances susceptibility to and severity of infections [3]. A consistent defect in neutrophil function is considered central to this increased risk for infections [4, 5]. As neutrophil function requires energy, a compromised metabolic state in diabetic conditions reduces their chemotactic, phagocytic and microbicidal activities [6].

Neutrophil apoptosis regulates neutrophil functional longevity and is considered as an integral component of the physiologic process. Considerable research has demonstrated

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that free radical injury at the level of mitochondria severely affects neutrophil functional longevity [7]. Recently, we and others have delineated the role and clinical significance of mitochondrial dysfunction in peripheral blood lymphocytes of diabetic subjects [8, 9]. In continuation to our previous investigation in the T2DM cohort, we speculate that perturbed interplay of mitochondria-nuclear cross talk may as well create an imbalance in oxidative function of neutrophils in T2DM subjects contributing to increased cellular demise. To specifically address this molecular missing link, we directly examined the levels of mitochondrial oxidative stress, nuclear DNA damage and mitochondrial mediated apoptosis in peripheral blood neutrophils of T2DM patients (both early diagnosed patients and cases with late-onset complications) along with an equal number of age and gender-matched healthy controls.

Materials and Methods

Subjects Selection

Our study included 150 Asian Indians categorized into three groups ($n = 50$ each). Group 1 consisted of healthy controls (age and gender-matched); group 2, early diagnosed T2DM patients (with 1–2 years of disease history); and group 3, T2DM patients with late complications (≥ 5 years disease history) [9]. Informed consents were obtained from all subjects who participated in the investigations as per Institutional Review Board norms. Prior to recruitment of subjects, general health criteria viz. age, gender, tobacco use, alcohol consumption, and dietary patterns were examined. Healthy controls recruited in the study had no clinical history of metabolic syndrome. The diabetic participants were required to have relatively stable glycaemic control, manifested by at least two successive glycated hemoglobin (HbA1c) previous assay results with no more than 1 % variation. Patients with history of medication for hypertension and dyslipidemia were

excluded from the study. T2DM subjects and healthy controls recruited in the study were not on any type of antioxidant supplementations. All diabetic subjects were treated with insulin and oral hypoglycemic agents (sulfonylurea and/ metformin) as described in Table 1.

Sample Collection and Reagents

All the investigations were performed in both fasting and glycated hemoglobin state and were conducted between 8:30 to 9:30 a.m. (IST). Subjects with T2DM were instructed to hold their morning oral diabetes medications prior to the blood sample collection. From each participant (T2DM and healthy controls), 20 mL of venous blood samples were collected and of which, 5 mL was used for pathological investigations. With the help of fully automated analyzer (Hitachi 912, Boehringer Mannheim, Germany), serum clinical chemistry was investigated. Estimated HbA1c values were calculated in accordance with the conversion formula established by Rohlfing et al. [10]. Remaining 15 mL was used for isolation of peripheral blood mononuclear cells through Lymphoprep[®] density gradient centrifugation, as described previously [11].

Isolation of Neutrophils

Neutrophils were isolated from peripheral blood of both healthy and diabetic volunteers by dextran sedimentation and density gradient centrifugation protocol with appropriate modifications [11]. Fifteen milliliter of venous blood was diluted with 9 % NaCl in 1:1 ratio, over layered on Lymphoprep[®], and centrifuged at $250 \times g$ for 45 min at 4°C . Dextran 3 % was added to the pellet followed by incubation for 1 h and centrifugation at 2,400 rpm for 10 min. The supernatant was discarded and to the pellet, the red cell lysing solution was added and centrifuged. Finally, the cells were washed with $1 \times$ PBS. Neutrophils thus separated were

Table 1 Clinical and laboratory characteristics of the studied groups; healthy controls (group 1), early diagnosed (group 2); and cases with late T2DM complications (group 3)

	Control	Early T2DM	Late T2DM
Age (years)	44 $\pm$ 8	42 $\pm$ 9	47 $\pm$ 5
BMI (kg/m^2)	26.2 $\pm$ 4.1	28.2 $\pm$ 4.8	31.2 $\pm$ 6.1
Waist circumference (cm)	96 $\pm$ 12	110 $\pm$ 14	108 $\pm$ 16
Treatment (single) (insulin, metformin, sulphonylurea)	–	22	24
Treatment (combined) (insulin + metformin; metformin + sulphonylurea)	–	28	26
Hb1Ac (%)	5.36 $\pm$ 0.53	6.52 $\pm$ 0.68*	8.29 $\pm$ 0.82*
LDL cholesterol (mg/dL)	112 $\pm$ 24	98 $\pm$ 18	96 $\pm$ 13
HDL cholesterol (mg/dL)	56 $\pm$ 8	53 $\pm$ 12	54 $\pm$ 10
Total serum cholesterol (mg/dL)	184 $\pm$ 36	176 $\pm$ 41	178 $\pm$ 38
Serum triglycerides (mg/dL)	114 $\pm$ 42	134 $\pm$ 48	142 $\pm$ 52

* $P \leq 0.001$; values represent mean $\pm$ SE ($n = 50$)

examined for viability using trypan-blue dye exclusion test and counted by Neubaer's haemocytometer.

Analysis of Intracellular Oxygen Radical Species

Reactive oxygen species (ROS) was measured by washing cells with PBS followed by incubation with 50 μ L of working solution of fluorochrome marker CM-H2DCFDA [5-(and 6)-chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate acetyl ester] (final working concentration adjusted to 2.5 μ g/50 μ L) for 2 h. The cells were harvested, washed in PBS and cell-associated fluorescence was qualitatively assessed by applied spectral bio-imaging system with nucleus counter-stained by Hoechst [12]. While quantitative measurement was performed by flow cytometry [13].

Measurement of Anti-oxidant Defense Enzymes

Levels of the antioxidant defense system enzyme GR in the cells were measured through a spectrophotometric ELISA, in which the oxidation of NADPH to NADP⁺ is monitored by the decrease in absorbance at 340 nm, which is directly proportional to the GR activity in the sample [14]. SOD in the cells was also evaluated through ELISA, as reported previously [15]. The percentage inhibition of the formation of nitro blue tetrazolium (NBT)-diformazan by SOD was converted to the relative activity of the sample, expressed as mU/mL at the absorbance of 450 nm.

Evaluation of Nuclear DNA Damage

A marker for DNA damage, γ H2AX was evaluated both qualitatively and quantitatively. For qualitative analysis, cells were fixed in 10 % formaldehyde for 1 h and permeabilized with 0.1 % Triton X-100 for 30 min, blocked with 3 % BSA for 3 h, and then incubated with primary antibody against γ H2AX (dilution 1:500; Calbiochem, Nottingham, UK) for 3 h and Texas-Red conjugated secondary antibody (dilution 1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h, while nucleus was counterstained with Hoechst (Sigma-Aldrich Co., St. Louis, MO, USA) and immediately mounted with anti-fade solution, cover-slipped, and stored in the dark at 4 °C [16, 17]. Images were acquired by a spectral bio-imaging system and analyzed with version 4.0 software (Applied Spectral Imaging, Edingen, Neckarhausen, Germany). For quantitation of percent positive cells for γ H2AX, indirect immunofluorescence method was performed through flow cytometry as described previously [18].

Assessment of Mitochondrial Mediated Apoptosis

The expression of caspase-3, a marker of mitochondrial mediated apoptosis, was measured qualitatively through immunocytochemistry using monoclonal anti-caspase-3 antibody (Abcam, Cambridge, UK). Nuclei were counter-stained using Hoechst [19]. For quantitative flow cytometric analysis of caspase-3, 1×10^6 cells were processed using PE Active Caspase-3 Apoptosis kit from BD™ Biosciences, San Diego, CA, USA. The cells were washed and analyzed by flow cytometry in FL2 channel. A total of 10,000 events were recorded [20].

Statistical Analysis

The data were expressed as mean $\pm$ standard error (SE). Differences in measured parameters among group 1 (control), group 2 (early), and group 3 (late) T2DM subjects were assessed by the Student's *t* test and analysis of variance. *P* value <0.05 was considered statistically significant. All analyses were performed with SPSS software package version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Oxidative Stress

Generation of intracellular ROS (H₂O₂) in terms of DCFH oxidation was measured as an initiator for oxidative stress. In comparison to controls, qualitative immunofluorescence analysis demonstrated higher levels of H₂O₂ in neutrophils of late T2DM subjects as marked by accumulated DCF (green) fluorescence (Fig. 1a). Mean percent positive cells with DCF fluorescence as measured through flow cytometry were 40.86 ± 4.98 , 14.70 ± 2.74 and 4.31 ± 0.12 in late, early diagnosed T2DM patients and healthy subjects respectively (Fig. 1b).

Anti-oxidant Status

As a defense against these reactive free radicals, the body produces antioxidant enzymes and SOD and GR are the main antioxidant enzymes long known for their protective function against oxidative cell damage. Figure 2 shows a consistent diminution in levels of GR and SOD activity in peripheral neutrophils of T2DM patients. The observed mean GR activity in controls was 398.06 ± 54.24 mU/mL, whereas in T2DM patients with early and late complications, GR activity was 327.16 ± 28.13 and 198.10 ± 22.36 mU/mL, respectively. The mean SOD activity in controls was $296.52 \pm$

Fig. 1 a Representative immunofluorescence microphotographs showing higher DCF fluorescence in neutrophils of late T2DM subjects as compared to healthy controls ($\times 200$). **b** Bar diagram showing mean percent DCF fluorescent positive cells in controls, early and late T2DM subjects as quantitated through flow cytometry. Values expressed as mean $\pm$ SE ($n = 50$ in each group). ** $P < 0.001$

Fig. 2 Histogram showing levels of anti-oxidant enzymes GR and SOD in controls, early and late T2DM subjects. Values expressed as mean $\pm$ SE ($n = 50$ in each group). * $P < 0.05$; ** $P < 0.001$; ns not significant

29.41 mU/mL, whereas it was 216.14 ± 20.52 and 143.78 ± 18.08 mU/mL in early and late T2DM subjects respectively.

DNA Damage Response

Upon DNA damage, the histone H2AX is phosphorylated and acts as a one of the major downstream signal for recruitment of DNA damage proteins to the damaged site. Immunocytochemical analysis of neutrophils from late T2DM patients showed higher numbers of γ H2AX foci as compared to controls (Fig. 3a). Flow cytometric evaluation of γ H2AX positive cells (%) in late, early T2DM subjects and healthy controls were 37.52 ± 3.11 , 8.33 ± 2.22 , and 2.82 ± 1.07 respectively (Fig. 3b).

Activation of Caspase-3

Caspase-3 mediated proteolysis is a critical element of the apoptotic process mediated through mitochondria. Active caspase-3 comprises a heterodimer of 17- and 112-kDa subunits, which in turn are derived from a 32-kDa proenzyme, a marker for cells undergoing apoptosis. Active caspase-3 proteolytically cleaves and activates other caspases and relevant targets in the cytoplasm. Immunocytochemical analysis illustrated hyper-activated caspase-3 in late T2DM cohort in comparison to healthy controls (Fig. 4a). Quantitative evaluation of percent active caspase-3 positive cells in

Fig. 3 a Fluorescence microphotographs illustrating higher number of γ H2AX foci in neutrophils of late T2DM subjects in comparison to healthy controls ($\times 200$). **b** Graph depicting percent positive γ H2AX cells measured through flow cytometry in controls, early and late T2DM subjects. Values expressed as mean $\pm$ SE ($n = 50$ in each group). * $P < 0.05$; ** $P < 0.001$

Fig. 4 **a** Qualitative fluorescent microscopic analysis demonstrating representative images of caspase-3 expression in neutrophils of control and late T2DM subjects ($\times 200$). **b** Flow cytometric quantification of active-caspase-3 positive cells in control, early and late T2DM subjects. Values expressed as mean $\pm$ SE ($n = 50$ in each group). * $P < 0.05$; ** $P < 0.001$

late T2DM subjects were found to be elevated with values 26.64 ± 4.57 in comparison to other two groups (early T2DM 4.66 ± 1.04 and control group 1.78 ± 0.57 respectively) (Fig. 4b).

Discussion

Neutrophils are a critical component of the innate immune system with several effector and immune regulatory functions. They are the most abundant cell type among circulating white blood cells and play a central role in host defense against the invading pathogens [21]. Approximately 10^{11} cells are produced daily and the average half-life of non-activated neutrophils in circulation is about 4–10 h. Upon activation, they undergo extravasation and migrate into tissues, where they survive for 1–2 days. Neutrophils are constantly produced in larger number in bone marrow, and to maintain cellular homeostasis an equal number die within a definite period through apoptosis [22, 23]. However, neutrophils in diabetic condition, present decreased functional longevity which might attribute to their defective immune regulation, and increased susceptibility and severity of infections [24]. In the present

study, we demarcated the liaison between impaired mitochondrial–nuclear cross talk in T2DM neutrophils and subsequent homeostasis. We observed that in both early and late diabetic subjects, the HbA1c levels in neutrophils were altered considerably. Simultaneously, increased oxidative stress observed in both early and late diabetics imply the disentanglement of the fine equilibrium of mitochondria–nuclear cross talk which eventually effecting downstream γ H2AX as a DNA damage response. Evaluation of caspase-3 in late and early diabetics showed a hyper-activated response of the marker indicating a mitochondrial mediated cellular phenomenon and further signifying an impaired neutrophil homeostasis.

The metabolic cues or the damage that occurs within the mitochondria can culminate in wide-ranging changes in nuclear gene expression via a retrograde signaling from mitochondria to nucleus [25, 26]. Recent investigations have pointed to a role of mitochondrial oxidative stress in modulating neutrophil functional ability in T2DM [27, 28]. On similar lines, in our study the free radical overproduction observed in the form of H_2O_2 generation along with diminished antioxidant enzymes SOD and GR activities in neutrophils of both early and late diabetic groups was attributed to an adaptive reaction of oxidative stress in mitochondria (Figs. 1, 2). Impaired oxidative metabolism within the mitochondria might activate signaling cascade of events damaging nuclear DNA that further exacerbates the neutrophil function. Incidentally, our findings in early and late diabetic neutrophils evidenced such scenario of nuclear DNA damage response in terms of elevated γ H2AX foci formation (Fig. 3). Hyper-activated DNA damage response beyond the scope of repair can drive the cell into an irreversible process of cellular demise or apoptosis. Apoptosis is the regular fate of neutrophils and apoptotic neutrophils are disposed of by macrophage-mediated phagocytosis. The translocation of pro-apoptotic proteins such as caspases to mitochondrial outer membrane has been demonstrated to precede nuclear DNA fragmentation and apoptosis in neutrophils [29]. Furthermore, type 2 diabetic neutrophils possess highly deregulated mitochondrial morphology with putative involvement in apoptosis [30]. In the present study, activation of caspase-3 measured cytometrically recorded significant higher apoptotic index in both early and late diabetic groups compared with healthy controls (Fig. 4).

Mitochondrial–nuclear crosstalk is critical for maintaining cellular homeostasis. Mitochondrial genome encodes for two rRNA, 22 tRNA and 13 proteins. These proteins, including those involved in the replication, transcription and translation of mtDNA, are encoded by nuclear DNA. Although physical location of mitochondrial and nuclear DNA is apart, there is constant communication between the two genomes to carry out essential

mitochondrial functions [31]. Nucleus-encoded factors regulate integrity, replication and expression of the mitochondrial DNA including mitochondrial DNA encoded respiratory chain components. Deregulation in mitochondrial machinery signals a negative feedback on nuclear transcriptional activity. It has been suggested that this retrograde signaling influences the bidirectional communication between the nucleus and mitochondria affecting metabolism [32, 33]. It has been reviewed that mitochondrial–nuclear crosstalk in neutrophils operates at least in two distinct ways. Firstly, they contribute essential subunit polypeptides to important mitochondrial proteins; second, they collaborate in the synthesis and assembly of these proteins. While the first kind of interaction is critical for the regulation of oxidative energy production, second type requires the bidirectional flow of information between the nucleus and the mitochondrion. In neutrophils, the mitochondria lack life-supporting activity, yet regulate neutrophil apoptosis by releasing a number of proapoptotic proteins into the cytosol [34, 35].

Our results decipher the first mechanistic insights of the mitochondrial deregulation in neutrophils that might aid in explicating the clinical importance of immunologic aberrations observed in type 2 diabetic conditions. By virtue of our data, we posit that contriving mitochondrial function offers a prospective and viable method to modulate neutrophil function in type 2 diabetics. However, further investigations involving interventions that alter the mitochondrial–nuclear cross talk in neutrophils of type 2 diabetic patients would be a preeminent need. Nevertheless, studies in other ethnic cohorts of larger sample size in conjunction with experimental evidence might not only enhance our knowledge of this multifaceted association between mitochondria dysfunction and neutrophil homeostasis in T2DM, but would also underlines the need to develop novel drug entities that can directly target mitochondria and modulate its function.

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Disclosures None declared.

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