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Effect of redox status of peripheral blood on immune signature of circulating regulatory and cytotoxic T cells in streptozotocin induced rodent model of type I diabetes

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ABSTRACT

Diabetes mellitus is an autoimmune chronic inflammatory disease manifested by hyperglycemia and associated with imbalance in redox status and inflammatory response. Oxidative stress has been reported to affect functions of T cell repertoire- regulatory T cells (T_{regs}) and cytotoxic lymphocytes (CTLs). T_{regs} are involved in prevention against autoreactive T cells and controlling inflammation while CTLs are major mediators of tissue injury. Hence the present study is novel as it contemplates to understand oxidative stress in diabetes *vis-à-vis* T cells. Comparative analysis was carried out between two groups, i.e., healthy Sprague Dawley (SD) and Streptozotocin (STZ) induced SD rat model of type1 diabetes (T1D). Various hematological, biochemical and oxidative stress parameters were assessed in plasma samples in the study. Peripheral blood mononuclear cells (PBMCs), T_{regs} and CTLs were evaluated for intracellular oxidative stress using 2′,7′–dichlorofluorescin diacetate (DCFDA), mitochondrial ROS using Mitosox-red, mitochondrial membrane potential using JC-1 in PBMCs. T_{reg} populations expressing IL-4, IL-6 and IL-10 and CTLs expressing αβ-T cell receptor (αβ -TCR), interferon- γ (IFN-γ), perforin and granzyme were also considered. We found decreased activity of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and reduced glutathione(GSH) and increased lipid peroxidation (LPO) in plasma indicated altered redox state in diabetic animals. Elevated intracellular reactive oxygen species (ROS) and mitochondrial superoxide was observed in T1D group confirming oxidative stress in cell specific manner. Cell population with hyperpolarized mitochondrial membrane potential was found to be elevated in T1D group. We found a decrease in T_{reg} population in T1D group in comparison to healthy group. T_{reg} population expressing IL-4, IL-6 were increased and those expressing IL-10 were found to be reduced in diabetic group. The CTL numbers were dropping whereas αβ-TCR, IFN-γ, perforin and granzyme expressing CTLs were on the rise in diabetic group. Our finding suggested an increased oxidative stress in T_{regs} and CTLs which might be responsible for progressive inflammatory environment built up due to persistent hyperglycemia. This was fortified by the statistical analyses where strong correlation between LPO and CTLs expressing TCR, IFN-γ, perforin and granzyme was noted. Lipid peroxidation was also found to be correlated to intracellular ROS in T_{regs} and CTLs along with other important revelations. The present study gives important insights into the significance of oxidative stress on immune system and its mediators in diabetes.

1. Introduction

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease which is characterized by aggressive β-cell damage leading to impaired or no insulin secretion and abnormally high blood glucose. Due to diminished insulin levels, glucose uptake and utilization are affected, leading to hyperglycemia, glucosuria, ketoacidosis and other complications. According to the estimation of World Health Organization, 347 million people worldwide have diabetes (American Diabetes Association, 2006) and is therefore a major concern. Prolonged

Abbreviations: Tregs, regulatory T cells; T1D, type1 diabetes; PBMCs, peripheral blood mononuclear cells; DCFDA, 2′,7′–dichlorofluorescin diacetate; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GSH, reduced glutathione; LPO, lipid peroxidation; CTLs, cytotoxic lymphocytes; STZ, streptozotocin; ROS, reactive oxygen species; MDA, malondialdehyde; SLE, systemic lupus erythematosus; MHP, mitochondrial inner-membrane hyperpolarization

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hyperglycemia has been reported to be associated with increased production of reactive oxygen species (ROS) through glucose autoxidation (Hunt et al.,1990; Wolff et al.,1991). Looking at the opposite scenario, oxidative stress has also been linked to diabetic state in animals and humans (Kakkar et al.,1995; Ihara et al.,1999; Opara, 2002). Imbalance of redox status might have an important role in pathogenesis of autoimmune diseases by enhancing inflammation, inducing apoptotic cell death, breakdown of immunological tolerance, etc. (Kumagai et al.,2003).

Lipid peroxidation, the non-enzymatic phenomenon of oxidative damage, is involved in various diseases manifestations through generation of a category of molecular patterns known as damage-associated molecular patterns or danger signals. These danger signals, such as oxidized phosphatidylcholine, cardiolipin, and phosphatidylserine; which majorly constitute modified proteins are known to evoke innate immune response (Miller et al., 2011; Chou et al., 2009; Weismann and Binder, 2012). Oxidatively modified molecules are also reported to induce adaptive immunity and play role in autoimmune responses and have been implicated in pathogenesis of various immune and inflammatory diseases (Karin et al., 2006).

Diabetic complications are thought to be mediated by four metabolic pathways (polyol, hexosamine, Protein kinase C activation and Advanced glycation end-product pathways) that are upregulated or activated by sustained hyperglycemia. This can be explained by a theory given by Brownlee that during hyperglycemia increased metabolite flux through metabolic pathways leads to increased oxidative phosphorylation-linked ROS production (Brownlee, 2001). This theory indicates that mitochondria have an important role to play in tissue injuries associated with diabetes. Hence analysis of mitochondrial ROS and mitochondrial membrane potential become mandatory.

On the other hand, autoimmune diseases, are outcome of the lost battle by T_{regs} to auto-reactive T cells, leading to destruction of self-tissue by immune attack. The inflammatory response and hyperglycemia in diabetes leads to activation of endothelial cells and accumulation of macrophages, helper, and cytotoxic T cells. Cytotoxic T cells are involved in inflammatory response mediated manifestations of tissue and cell damage.

In order to understand the immunological and physiological cross-link in diabetes PBMCs, T_{regs} and cytotoxic T cells (CTLs) were chosen, so as to get a better cell specific insight in disease etiology using Streptozotocin (STZ) induced model of T1D in SD rat was used.

In the current study, hematological, enzymatic and non-enzymatic oxidative stress parameters in plasma from T1D and healthy animal groups were analyzed to understand disease status. Histopathological analyses of tissues such as spleen and pancreas from both the groups was carried out. ROS measurements (both intracellular and mitochondrial) in PBMCs, T_{regs} and CTLs were carried out to get a glimpse of cellular and mitochondrial redox states which revealed some interesting readouts.

Immune signatures of T_{regs} by way of expression of intracellular cytokines (IL-4, IL-6 and IL-10) and CTLs (expressing cytotoxic proteins-perforin, granzyme & IFN-γ) were analyzed. The effect of redox status on intracellular ROS and mitochondrial ROS, mitochondrial membrane potential, etc. brought to light the importance of oxidative stress in T1D. Further, correlations between different oxidative and immunological parameters were drawn to unravel their interdependence which can give a better insight into disease progression and its manifestations.

2. Materials and method

2.1. Materials

The chemicals employed in the study were of analytical grade: Streptozotocin (Sigma Aldrich). The fluorochrome tagged monoclonal antibodies were from BD biosciences (US) and Biolegend (USA) and DCFH-DA (Sigma-Aldrich, USA), Mitosox RedTM (Invitrogen, USA), BD MitoscreenTM (JC1) assay kit were used.

2.2. Experimental animals

Male SD rats were acclimatized to laboratory conditions and had ad libitum access to food as well as water (25 ± 3 °C temperature, 50–55 ± 5% humidity and a 12 h light–dark cycle) for at least 7 days prior to commencement of experiments. They were maintained on a regular commercial diet (14% fat, 61% carbohydrate, and 25% protein from total energy). All the experimental procedures were approved by the Institutional Animal Ethical Committee, Panjab University, Chandigarh (IAEC-468 dated 20/09/2014). The study had two animal groups (rats weighing 200–250 g): Healthy group consisting of healthy rats (n = 5–7) and Diabetic group consisting of rats with STZ induced diabetes (n = 5–7).

2.3. Diabetes model in rat

For creating diabetes model in rats, animals were fasted overnight and provided free access to water. A single intraperitoneal dose of STZ 55 mg/kg body weight, dissolved in citrate buffer (pH 4.5) was administered (Gajdosík et al., 1999; Szkudelski, 2001). The rats were supplied with 10% glucose solution after 6 h of streptozotocin administration for the next 24 h to prevent hypoglycemic shock which occurs due to massive insulin release resulting from destruction of pancreatic β-cells. After 24 h of streptozotocin injection, hyperglycemia appeared in rats along with visible signs of polyuria. Rats with blood glucose level of 300 mg/dl and higher were included in further experiments. After induction of diabetes, rats were examined for hematological, biochemical, oxidative stress and immunological parameters. Body weight and food intake of rats were monitored weekly.

2.4. Hematological and biochemical parameters

Peripheral blood was drawn from orbital vein of animals and collected in separate vacutainers for different tests i.e. for hematological parameters in heparin and for renal and liver function tests (serum) in plain vacutainers. Hemoglobin, acetylated hemoglobin (HbA1c), total leukocyte count, differential count (Neutrophil %, Lymphocyte %, Monocyte %, Eosinophils %, Basophils %), total RBC (million/cu mm), Mean corpuscular Hemoglobin (picograms), Mean corpuscular Hemoglobin concentration (g/dl), Platelet count were analyzed using Hematology analyzer. Renal and liver function tests were carried out with samples. Mean blood glucose levels were monitored with blood samples withdrawn from the tail vein of the rats.

2.5. Histopathological analysis

Animals from both diabetic and healthy groups were anesthetized and sacrificed. Pancreas and spleen tissue samples were removed and washed with phosphate buffered saline (PBS) of pH 7.4. Tissues were fixed in 37% formalin and embedded in paraffin wax. Thin sections were prepared and stained with hematoxylin and eosin dyes.

2.6. Oxidative stress parameters

The enzymatic and non-enzymatic parameters of oxidative stress were analyzed in plasma samples of both groups, to understand the redox status in diabetic animal model.

I Enzymatic antioxidant parameters of oxidative stress:

A **Catalase (CAT) Assay (EC 1.11.1.6):** Catalase activity was estimated by the method of Luck (1971) where the rate of decomposition of H_2O_2 (substrate) was taken as measurement of catalase activity. Activity of catalase is expressed as units /mg protein