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Signaling, stress response and apoptosis in pre-diabetes and diabetes: restoring immune balance in mice with alloxan-induced type 1 diabetes mellitus

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ABSTRACT

The aim of this study was to compare immune imbalances in "pre-diabetic" and diabetic mice and to evaluate the efficacy of several agents in improving the immunity of mice with type 1 diabetes. Pre-diabetic and diabetic models generated by a single or double alloxan injection were monitored for plasma glucose and pancreas immunohistochemistry. To study the immunity in pre-diabetic and diabetic Balb/C male mice; the levels of cytokines; synthesis of inducible heat shock proteins HSP72 and HSP90α; activity of the NF-κB, IFR3, SAPK/JNK, and TLR4 pathways; and apoptosis levels in thymuses were measured. Pre-diabetes resulted in a decrease in IL-4, IL-5 and IL-10 in plasma; in diabetic mice, plasma IFN-gamma, IL-6, TNF-alpha, and IL-10 were decreased. The NF-κB alternative pathway activity and TLR4 expression were significantly increased only in pre-diabetic mice, whereas SAPK/JNK activation was observed at both stages of diabetes. Other measured parameters also showed distinct altered patterns in the immunity of pre-diabetic and diabetic mice. Treatment with an inhibitor of NF-κB, thymulin, or a diet with an antioxidant improved or normalized the immune balance in diabetic mice and also notably decreased pancreatic cell damage in pre-diabetic mice.

1. Introduction

Type 1 diabetes (T1D) is characterized by an autoimmune response in which cellular immunity plays a pivotal role in the selective destruction of insulin-producing pancreatic islet beta cells, thus resulting in impaired glucose metabolism. Only 40% of T1D incidents result from a genetic predisposition; more frequently, the disease is caused by various exogenous and endogenous factors. It is well established that cytotoxic T lymphocytes (CTLs) play a dominant role in the pathogenesis of T1D. These CTLs (notably CD8(+) T cells) recognize and kill insulin-secreting pancreatic beta cells, thereby reducing their numbers by approximately 90% [1].

Pre-diabetes, defined as an impaired glucose tolerance or a combination of impaired fasting glucose and impaired glucose tolerance in T2D, may be associated with a higher future risk of stroke [2]. Pre-diabetes is generally diagnosed in epidemiological studies, which are usually referred to as other diseases. People with pre-diabetes should be aware that they are at an increased risk of future diabetes progression. In addition, pre-diabetes is not only a risk factor for diabetes, but it is also frequently associated with other major cardiovascular risk factors [3]. The most accurate estimate should account for the nature and strength of the relationship between pre-diabetes and a risk of future type 1 diabetes progression. Such information may clarify the extent to which pre-diabetes itself is a risk factor, or whether it simply reflects the risk associated with the development of overt diabetes.

The various components of the immune system may be of specific interest as therapeutic targets for disease prevention or treatment. Indeed, numerous reports have demonstrated that T1D can profoundly affect the immune system status in human and animal subjects. However, there is little data on the activities of the NF-κB, IRF3, SAPK/JNK, and TLR4 signaling cascades. In addition, the expression of heat shock proteins has not yet been explored in diabetic mice.

The present study was designed to examine the effects of pre-diabetes and diabetes on the cytokine response in tandem with examining the following: the synthesis of inducible forms of heat shock proteins HSP72 and HSP90α; activities of the NF-κB, IRF-3 and SAPK/JNK signaling pathways; p53 phosphorylation and caspase 3 expression associated with apoptosis in the thymus; and TLR4 expression in splenic lymphocytes. The NF-κB pathway is important for the expression of genes that are involved in the control of the host inflammatory response. The constitutive activation of this pathway is associated with a wide variety of diseases, including inflammation. It has been reported

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that LPS interacts with the LPS-binding protein and CD14, which presents LPS to TLR4, thereby activating inflammatory gene expression via NF-κB signaling [4]. We have recently shown that IKK Inhibitor XII, a selective inhibitor of NF-κB signaling that functions by decreasing NF-κB phosphorylation and thereby reduces translocation of NF-κB into the nucleus, also prevented SAPK/JNK activation in LPS-treated cells [5]. These results were consistent with findings demonstrating that the SAPK/JNK signaling is regulated by the NF-κB pathway [6,7]. In addition, previous reports have demonstrated the roles of Toll-like receptor 4 (TLR4) and interferon-regulated factor 3 (IRF-3) in cellular stress responses involving NF-κB and heat shock proteins [8,9]. Our characterization of these processes will provide new information that should clarify the mechanisms of immune dysregulation during diabetes progression.

Animal models play an important role in the development of the present concepts concerning T1D pathogenesis and therapeutic approaches. Nonobese diabetic (NOD) mice spontaneously develop T1D, and the disease shares many characteristics with human T1D [10]. Because the developed diabetes is actually observed only in aged NOD mice, the main deficiency of the NOD model relates to an overlap of two factors, diabetes and aging.

Several experimental models of diabetes have been developed for the study of T1D, including alloxan- or streptozotocin-induced disease. Indeed, chemically induced diabetic models have varying diabetes durations, but diabetes progresses faster compared with NOD mice [11]. The authors of the above mentioned work induced diabetes in mice using five intraperitoneal streptozotocin injections on five consecutive days until diabetes had developed.

In the present study, we used one or two injections of alloxan to induce so-called "pre-diabetes" or advanced diabetes, respectively. Chronic low-grade inflammation, which is present in type 1 diabetics, results in the release of inflammatory mediators, particularly IL-1beta and TNF-alpha, which promote systemic insulin resistance and beta cell damage. Chronic inflammation in diabetic humans and animals generally results in oxidative stress [12] and the suppression of thymic function. Beginning with fetal life, the thymus plays a central role in the establishment of central immune self-tolerance to neuroendocrine protein families, including insulin-secreting β cells in the pancreatic islets of Langerhans. Indeed, it was established that several neuroendocrine-related genes are transcribed in the thymic epithelial cells of animals and humans [13]. Recently it was shown that peptides produced by thymic epithelial cells, such as thymulin, can modulate an inflammatory response and are prospective anti-inflammatory drugs [5]. Moreover, there is now evidence that primary or acquired failure in thymus-dependent central self-tolerance in β cells may play a primary role in T1D pathogenesis. This novel knowledge is currently being translated into the development of innovative tolerogenic/regulatory approaches that are designed to reprogram specific immune self-tolerance in islet β cells [14].

Using mouse models for T1D, we assessed the capacity of a thymic peptide, antioxidants, and an NF-κB inhibitor to modulate immune regulation and alter autoimmune responses in pre-diabetes and advanced disease.

2. Methods and materials

2.1. Animals, diabetes models, and drug supplements

Six- to 8-week-old male Balb/c mice (22–25 g) were maintained under standard laboratory conditions (20–21 °C, 10–14 h light–dark cycle, 65% humidity) with food and water provided ad libitum. Standard food pellets contained a balanced diet containing proteins, vitamins, and minerals. The procedures used in this study were approved by the ethics committee of the institution and were performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals.

Two phases of diabetes progression, which were indicated as "pre-diabetes" and "advanced diabetes," were developed. Pre-diabetes was induced by a single injection of 250 mg/kg alloxan (Sigma-Aldrich, USA), and the immune status of the animals was examined 11 days after injection, when the blood glucose index was lower than animals with diagnosed diabetes, in the range of 6 to 9 mmol/l (Table 1). The results of our pilot experiments using 50 animals, which were subjected to a single alloxan injection followed by regular glucose testing, demonstrated fluctuations in glucose levels. In these experiments, a fraction of the animals (approximately 15%) became diabetic 1.5–2 months after a single treatment with alloxan. We considered this condition to represent an increased risk of T1D in mice. However, advanced diabetes was modeled by two sequential injections of 250 and 300 mg/kg alloxan with an interval of eleven days. The analysis was performed ten days after the second injection, when the index of blood glucose fluctuated, but was consistently greater than 18 mmol/l (Table 1), at which mice can be considered diabetic with certainty.

The daily antioxidant diet was supplemented with four naturally occurring lipo-soluble antioxidants (8 mg/kg of ubiquinone Q9, 2 mg/kg of β-carotene, 2 mg/kg of α-tocopherol, and 5 mg/kg of vitamin E, all from Sigma, USA) and was initiated after the first alloxan injection and lasted throughout the entire observation period (20 days). Thymulin solution was prepared from serum thymic factor (American Peptides, Sunnyvale, CA, USA) with an equimolar concentration of $ZnCl_2$. 1 h after the alloxan injection, thymulin, at a dose of 0.15 mg/kg of body weight, was injected intraperitoneally every other day throughout the entire observation period (20 days). The IKK Inhibitor XII (Merck, Germany), at a dose of 1.8 mg/kg, was injected intraperitoneally every other day throughout the entire observation period (20 days).

2.2. Blood plasma and cells

Plasma was isolated from blood collected during the decapitation of the animals. Blood samples were kept for 3–5 h at 4 °C and centrifuged at 200 ×*g*, and supernatants were subsequently collected for cytokine assays. Splenic lymphocytes were isolated in DMEM media (Sigma, USA) containing 1% 1 M HEPES solution, 100 μg/ml streptomycin and 10% fetal bovine serum. Erythrocytes were lysed in Tris-buffered ammonium chloride (0.009 M Tris–HCl, 0.135 M NaCl, 0.151 M, pH 7.2). After washing, the samples were stored at a concentration of 1×10^8 cells/ml in RPMI 1640 medium at −20 °C until Western blotting analyses were performed. Thymic lymphocytes were isolated and cultured in the same manner, but without erythrolysis.

2.3. Cytokine measurements

ELISA was used to determine the concentration of cytokines in blood plasma. ELISA Development Kits for mouse TNF-α, IL-4, IL-5, IL-6, IL-10,

Table 1
Effects of thymulin, IKK Inhibitor XII and diet with antioxidants on beta cell mass (mg/pancreas) (A) and on the level of blood glucose (mmol/l) (B) in pre-diabetic and diabetic mice.

	Without additions	Thymulin	Inhibitor	Diet
A				
Control	1.1 ± 0.3	0.99 ± 0.3	1.0 ± 0.3	1.1 ± 0.3
Prediabetic	1.0 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	1.0 ± 0.3
Diabetic	*0.5 ± 0.05	**0.85 ± 0.2	**0.88 ± 0.2	**0.8 ± 0.2

Each value is the mean ± SD calculated for 15 sections/pancreas for each of three mice.

B				
Control	3.8 ± 1.0	4.0 ± 1.5	3.9 ± 0.9	3.7 ± 1.6
Prediabetic	*7.9 ± 2.2	7.6 ± 2.3	8.0 ± 2.8	7.2 ± 3.3
Diabetic	*18.5 ± 2.3	**13.6 ± 3.2	**14.3 ± 2.2	**12.9 ± 2.5

Each value is the mean ± SD calculated for 10 mice.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the alloxan group, $p < 0.05$.