Thalidomide attenuates multiple low-dose streptozotocin-induced diabetes in mice by inhibition of proinflammatory cytokines

K. Amirshahrokhi a,⁎, M. Ghazi-Khansari b

a Department of Pharmacology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran
b Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Thalidomide is an immunomodulatory and anti-inflammatory agent and is used in autoimmune disorders. It has been shown that thalidomide inhibits proinflammatory cytokines production. The purpose of this study was to investigate the effect of thalidomide on the prevention of autoimmune diabetes in mice. Diabetes was induced by multiple low-dose of streptozotocin (MLDS) injection. Mice were treated with thalidomide (300 mg/kg/day orally) for 21 days. Plasma levels of glucose, insulin and nitrate/nitrite as well as pancreatic cytokine levels were measured. Pathological examinations of the pancreas revealed that thalidomide reduced the islet inflammation (insulitis) and destruction of beta cells. Thalidomide treatment prevented hyperglycemia and preserved pancreatic insulin secretion in the diabetic mice. Thalidomide treatment also significantly decreased plasma levels of nitric oxide and pancreatic proinflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12, IL-17 and interferon (IFN)-γ] while increased anti-inflammatory cytokine IL-10. In conclusion, these findings indicate that thalidomide may have a protective effect against the autoimmune destruction of the pancreatic beta-cells during the development of MLDS-induced type 1 diabetes in mice.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Type 1 diabetes is a T-cell mediated autoimmune disease characterized by the selective destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. Chronic pancreatic inflammation (insulitis) and destruction of islet β-cells in type 1 diabetes is mediated by the immune cells, particularly autoreactive CD4 and CD8 T lymphocytes, B-cells, macrophages and dendritic cells [1]. T-cells can directly destroy β-cells through a cytotoxic process, and they can also destroy β-cells through the secretion of proinflammatory cytokines. Moreover, in response to cytokine stimulation, β-cells generate reactive oxygen species (ROSs) and reactive nitrogen species such as nitric oxide (NO), which facilitate their destruction [2]. NO is also synthesized within cytokine-activated macrophages by inducible nitric oxide synthase (iNOS) [3]. T helper 1 cells produce proinflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-12) which activate macrophages and cytotoxic T cells to destroy β-cells, whereas IL-4 and IL-10 cytokines that are produced by activated T helper 2 cells, prevent β-cell destructive insulitis [4].

Experimental insulin-dependent diabetes can be induced by multiple low doses of streptozotocin (STZ) (MLDS) in rodents [5]. MLDS is a commonly used animal model that has many histological and clinical features similar to those of human type 1 diabetes and involves the participation of macrophages and T cells. STZ is a pancreatic β-cell toxin that induces inflammation of the islets by immune cells when it is given in multiple low doses [6]. The MLDS model has been used widely to study the immunological pathways that lead to β-cell death and progressive hyperglycemia.

It has been shown that some drugs such as thalidomide have immunomodulatory and anti-inflammatory activity, which might represent a potential preventive therapy for autoimmune diseases. Thalidomide (α-N-phthalimido glutarimide) is a glutamic acid derivative that was first introduced in 1954 as a sedative drug but was withdrawn from the market due to its teratogenic effects. Thalidomide has various pharmacological properties and it has been used successfully for various inflammatory and autoimmune diseases. Thalidomide was approved by the FDA for the treatment of erythema nodosum leprosum (ENL) and multiple myeloma [7]. It has also been demonstrated that thalidomide or its analogs are effective in the treatment of rheumatoid arthritis, Crohn's disease, prostate cancer, Behcet's disease, chronic host-versus-graft disease, lupus erythematosus and HIV-associated oral ulcers [8–11]. However, teratogenicity, peripheral neuropathy and other adverse effects of thalidomide have led to the design of its new analogs.
New analogs of thalidomide exhibit low toxicity and enhanced potency in blocking cytokine production [12].

In the present study, we hypothesized that thalidomide, due to its anti-inflammatory and immunosuppressive activity, may affect autoimmune diabetes. Consequently, we decided to investigate whether thalidomide treatment could prevent the development of MLDS-induced diabetes in mice.

2. Materials and methods

2.1. Animals and materials

Male Swiss albino mice, weighing 20–25 g, were housed in a room with a 12-h light/dark cycle. The mice had free access to tap water and ad libitum food. All the chemicals were purchased from Sigma.

2.2. Animal treatments

Experimental diabetes was induced by MLDS, as described previously [6]. Briefly, STZ was dissolved in 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally (i.p.), within 10 min of preparation, at a dose of 40 mg/kg/day for 5 consecutive days. Plasma glucose was monitored weekly over the following 21 days. The blood samples were obtained from the tail vein of non-fasted mice and glucose was measured using a glucometer (Accu-Chech Active). Mice were considered diabetic when their non-fasting plasma glucose levels were >250 mg/dl. Mice were euthanized and their pancreatic tissues were removed on day 21 for cytokine and histological examination. Plasma, separated from blood by centrifugation, was stored at −80 °C until nitrate/nitrite and insulin assay.

Our preliminary experiment showed that the threshold dose of thalidomide for a significant inhibitory effect on plasma glucose level was 300 mg/kg/day. Thalidomide was dissolved in 0.5% carboxymethylcellulose (CMC) and administered orally by an intragastric tube. Thalidomide (300 mg/kg) was administered 1 h prior to STZ injection and continued for 21 days. Mice were divided into four groups (7 mice in each group):

- Normal control group: received citrate buffer (i.p.) and CMC orally.
- Thal group: received citrate buffer (i.p.) and thalidomide (300 mg/kg/day, orally).
- MLDS + Thal group: received STZ (i.p.) and thalidomide (300 mg/kg/day, orally).
- MLDS group: received STZ (i.p.) and CMC orally.

2.3. Plasma insulin determination

Before mice were euthanized, blood was collected from each mouse on day 21 in heparinized tubes. Plasma was separated and stored at −80 °C until plasma insulin assay. The insulin level of plasma was determined using a mouse insulin ELISA kit (Mercodia).

2.4. Cytokine assay

Pancreas tissues were rapidly removed from mice, snap frozen in liquid nitrogen and stored at −80 °C. The samples (100 mg) were homogenized in 1 ml Tris–HCl buffer (pH = 7.4) containing protease inhibitors. All homogenized samples were centrifuged (20,000g, 4 °C) in a refrigerated centrifuge for 30 min, and the supernatant was taken and frozen at −80 °C. Supernatant samples were thawed and analyzed for murine cytokine concentrations using mouse-specific ELISA kits (eBioscience). Cytokine levels in the pancreas were expressed as pg cytokine/mg tissue [13].

2.5. Determination of plasma nitrate/nitrite levels

Plasma levels of nitrate/nitrite as a marker of total nitric oxide concentration were determined by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company).
Fig. 3. Effect of thalidomide treatment on the cytokine levels of TNF-α (A), IL-1β (B), IFN-γ (C), IL-6 (D), IL-12 (E), IL-17 (F) and IL-10 (G) in the pancreatic tissue of mice. Thalidomide (300 mg/kg/day, orally) prevented the MLDS-induced increase in TNF-α, IL-1β, IFN-γ, IL-6, IL-12, and IL-17. Thalidomide treatment increased the level of IL-10 (G). Data are means ± SEM. *P < 0.001 vs. normal control group; $P < 0.01 vs. MLDS + Thal group.
2.6. Histological examination

For histological examination of mouse pancreatic islets, the pancreas from each study group was removed on day 21 and fixed in 10% formalin. Specimens were embedded in paraffin and sections were stained with hematoxylin and eosin. The slides were evaluated for presence of islet atrophy and islet inflammation by light microscopy (Olympus).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey’s test were used for multiple comparisons between groups. Data are expressed as means ± SEM. P < 0.05 was considered significant.

3. Results

3.1. Effects of thalidomide on MLDS-induced hyperglycemia and plasma insulin level

In order to study the effect of thalidomide on the course of diabetes, we first tested the effect of it in the MLDS model of diabetes in mice. When mice were injected with multiple low doses of STZ, 1 week after the last injection of STZ, mice presented a progressive hyperglycemia (Fig. 1A) and increased incidence of diabetes (Fig. 1B). Oral administration of thalidomide (300 mg/kg) starting from the first STZ injection for 21 days reduced hyperglycemia and incidence of diabetes. Plasma glucose concentration in control (vehicle-treated) and Thal group remained unchanged and was normal throughout the study.

To evaluate β-cell function in terms of insulin release, we measured the plasma insulin levels on day 21. As shown in Fig. 2, plasma insulin level was significantly decreased in MLDS-treated mice compared with that in the normal control group. Thalidomide prevented the MLDS-induced reduction in plasma insulin, indicating a possible protective effect of thalidomide against β-cell damage. Administration of thalidomide alone (Thal group) had no effect on plasma insulin levels when compared to mice receiving vehicle (control group).

3.2. Effects of thalidomide and MLDS on pancreatic cytokines

Because cytokines play a central regulatory role in the destruction of insulin-producing islet β-cells during development of diabetes, we measured the pancreatic levels of TNF-α, IL-1β, IFN-γ, IL-6, IL-12, IL-17 and IL-10 in all four study groups on day 21 (Fig. 3). MLDS-treatment caused significant increase in the pancreatic levels of proinflammatory cytokines TNF-α, IL-1β, IFN-γ, IL-6, IL-12 and IL-17, while decreased the level of anti-inflammatory cytokine IL-10 as compared with those in the control group. Treatment of mice with thalidomide significantly decreased MLDS-induced production of TNF-α, IL-1β, IFN-γ, IL-6, IL-12 and IL-17, while increased IL-10 as compared with those in MLDS group. Administration of thalidomide alone in normal mice did not influence cytokine levels (except IL-10). There was a significant increase in IL-10 level in Thal group compared to control group (Fig. 3G). These results propose that thalidomide may prevent pancreatic β-cell destruction by influencing the balance between anti- and proinflammatory cytokines.

3.3. Effects of thalidomide and MLDS on plasma nitrate/nitrite levels

Since nitric oxide is an effector molecule that mediates cytokine-induced destruction of pancreatic β-cells, we evaluated the plasma levels of nitrate/nitrite as an indicator of plasma NO levels. As shown in Fig. 4, plasma nitrate/nitrite levels were increased in MLDS-treated mice. However, treatment of mice with thalidomide significantly decreased MLDS-induced production of nitrate/nitrite.

3.4. Histological studies

Histological analysis of pancreatic sections, on day 21, revealed that MLDS-treatment caused islet shrinkage and inflammation and β-cell loss as compared with the normal control group (Fig. 5). Histopathological changes in the thalidomide plus MLDS group were significantly lower than those in the MLDS group, indicating that thalidomide ameliorated MLDS-induced islet inflammation and β-cell destruction. No histological changes were observed in normal control mice. Also, Thalidomide treatment alone had no effect on the pancreatic islets.

4. Discussion

In this study, we found that thalidomide protected the pancreas from immune-mediated β-cell death and ameliorated the development of type 1 diabetes in mice. This protection occurs by blocking nitric oxide production and inhibiting pancreatic proinflammatory cytokines.

Type 1 diabetes as an autoimmune disease is believed to result from perturbed immune regulation. In the MLDS model of diabetes, STZ promotes the invasion of immune cells into islets and trigger inflammation. Several types of immune cells and various factors released from them are involved in the pathogenesis of the disease. The CD8+ T cells and The pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6 and IL-12, are cytotoxic to β-cells by inducing the formation of reactive oxygen species, and NO metabolites inside the β-cells. In contrast, anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10 prevent pancreatic β-cell damage [2,14]. IL-10 has been shown to prevent the onset of diabetes in mice [15]. More recently, The IL-17-Producing T cells have been demonstrated in the development of several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and type 1 diabetes. IL-17 is a proinflammatory cytokine that is detrimental to pancreatic islet cells. It has been shown that the proinflammatory effect of IL-17 depends noticeably on its capability to induce inducible NOS and subsequent release of NO that interferes with β-cell function and cause β-cell destruction [16,17]. Furthermore, IL-17 promotes infiltration of neutrophils and macrophages and stimulates the production of other proinflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12, by activated macrophages [18].
TNF-α initiates the release of cascade of other proinflammatory mediators and has a central regulatory role in β-cell destruction during development of type 1 diabetes. It has been reported that inhibitors of TNF-α prevent MLDS-induced diabetes in mice [19]. It has been proposed that some drugs which prevent the action of TNF-α may exhibit a protective effect against the development of diabetes. Thalidomide as a known inhibitor of TNF-α, displays anti-inflammatory, immunomodulatory, and antiangiogenic activities. It has been shown that thalidomide suppresses or modifies cytokine production and therefore immune responses. Thalidomide exhibits a dose-dependent inhibition of the pro-inflammatory cytokines IL-6 and TNF-α in human peripheral blood mononuclear cells (PBMC) [20]. It also induces production of IL-4 and IL-5 (Th2 cytokine) and concomitantly inhibits IFN-γ (Th1 cytokines) in PBMC cultures [21]. Other studies show that thalidomide and its analogs significantly inhibit lipopolysaccharide-induced TNF-α, IL-1β, IL-12 and IL-6 and increase anti-inflammatory IL-10 production in vivo and in vitro [22–24]. The use of thalidomide in treating various immune-mediated diseases is considerably based on its ability to inhibit the production of TNF-α. Thalidomide inhibits synthesis of TNF-α by inducing TNF-α mRNA degradation, then suppressing the release of this proinflammatory cytokine from macrophages and monocytes [25]. Various studies indicate that the mechanism underlying the therapeutic effect of thalidomide on Crohn's disease [26], experimental pulmonary fibrosis [27] and pancreatitis [28] is related to the suppression of proinflammatory cytokines, especially TNF-α, IL-1β, IL-6 and IL-12.

As mentioned above, the anti-inflammatory and cytokine-modulatory properties of thalidomide have prompted us to study its therapeutic potential in type 1 diabetes. We investigated the effect of thalidomide on the development of MLDS-induced diabetes and the possible mechanisms involved. In our study, thalidomide treatment reduced MLDS-induced hyperglycemia and incidence of diabetes, and preserved pancreatic insulin secretion in the diabetic mice. We showed that chronic thalidomide treatment had the ability to reduce the MLDS-induced proinflammatory mediators, including TNF-α, IL-1β, IFN-γ, IL-6, IL-12 and IL-17 in the pancreas of mice. This is a very important observation, since these cytokines have main role in the destruction of insulin-producing islet β-cells. Indeed, blocking the action of the proinflammatory cytokines by thalidomide decreases insulinitis and suppresses specific pathways that stimulate macrophages and cytotoxic T cells to destroy β-cells. In addition, we demonstrated that the pancreatic level of anti-inflammatory cytokine IL-10 is elevated in response to thalidomide. IL-10 suppresses the induction and progression of autoimmune diabetes.

We supposed that NO could be another target molecule of thalidomide, since thalidomide has been found to exhibit NOS-inhibiting activity [29]. NO is one of the key effector molecules and a nitrogen free radical involved in inflammatory destruction of islet β-cells in diabetes. In our study plasma levels of nitrate/nitrite as a marker of nitric oxide formation in the MLDS group was significantly higher than in other groups. This observation is in agreement with the previous studies showing that nitric oxide production increases in MLDS-treated mice [30,33]. Thalidomide administration significantly decreased the elevation of NO production in the MLDS-treated group. This finding indicates that thalidomide can improve pancreatic islet function by decreasing NO-mediated cytotoxicity against β-cells.

Our findings are consistent with previous studies, showing that the most probable mechanism of thalidomide in limiting β-cell destruction and the progression of diabetes is modulation of immune system. Also, our histological analysis clearly showed the ability of thalidomide to ameliorate β-cell destruction and development of pancreatic islet inflammation by autoreactive lymphocytes. In conclusion, it seems that thalidomide treatment has a protective effect against MLDS-induced diabetes in mice.

Acknowledgments

This study was supported by a grant from Vice Chancellor of Research of Ardabil University of Medical Sciences. We also wish to thank Dr. MR Vahabzadeh for technical assistance in histological analysis.

References


