



All-trans retinoic acid prevents the development of type 1 diabetes by affecting the levels of interferon gamma and interleukin 4 in streptozotocin-induced murine diabetes model

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ABSTRACT. The aim of this study was to explore the molecular mechanism by which all-trans retinoic acid (ATRA) prevents type 1 diabetes mellitus (T1DM). Fifty ICR mice were randomly assigned to three groups: prevention group [N = 20; mice received 10 mg/kg ATRA daily for 5 days and then 60 mg/kg streptozotocin (STZ) for 5 days]; diabetic group (N = 20, mice received 95% sterile peanut oil and 5% dimethyl sulfoxide for 5 days and then 60 mg/kg STZ for 5 days); and control group (N = 10, mice received 95% sterile peanut oil and 5% dimethyl sulfoxide for 5 days and then citrate buffer for 5 days). Blood glucose was measured using blood glucose test strips and serum insulin was measured by radioimmunoassay. Islets cell morphology was assessed by microscopy and ELISA was used

to measure the serum levels of interferon gamma (IFN- γ) and interleukin 4 (IL-4). In the prevention group, blood sugar levels were found to be reduced and serum insulin levels increased compared with the levels in the diabetic group ($P < 0.05$), indicating that ATRA prevented the STZ-induced damage to islet cells. Meanwhile, ATRA was shown to decrease the levels of IFN- γ and increase the levels of IL-4 as well as the IFN- γ /IL-4 ratio in STZ-treated animals ($P < 0.05$). These findings suggest that ATRA prevents the recurrence of autoimmune insulinitis. This study demonstrated that ATRA effectively prevents the progression of T1DM in a murine model of the disease by reducing IFN- γ levels and increasing IL-4 levels.

Key words: All-trans retinoic acid; Type 1 diabetes mellitus; Interferon gamma; Interleukin 4

INTRODUCTION

Type 1 diabetes mellitus (T1DM) results from the T cell-mediated destruction of β -cells in the pancreas-resident islets of Langerhans. Autoimmune diseases are characterized by a significant reduction in insulin secretion; and imbalance and destruction of immune tolerance cytokines cause autoimmune disorders due to this lack of insulin (Berman et al., 1996; Rabinovitch and Suarez-Pinzon, 1998).

Vitamin A is an essential micronutrient and all-trans retinoic acid (ATRA) plays an important role in maintaining the active form of vitamin A in the context of immune regulation (Napoli, 1996). It has been shown that ATRA has protective effects in animal (Zunino et al., 2007) and *in vitro* cell culture (Kang et al., 2004) models of T1DM; however, the molecular mechanisms of the protective effects of ATRA on pancreatic islet remains unknown.

Here, a mouse model of T1DM was established by intraperitoneal injection of streptozotocin (STZ). The protective effects of ATRA on pancreatic β -cells of T1DM mice was investigated by assessing changes in the levels of interferon gamma (IFN- γ) and interleukin 4 (IL-4) as well as in the IFN- γ /IL-4 ratio. All the results will provide the basic information for the prevention of human T1DM.

MATERIAL AND METHODS

Reagents

STZ was purchased from Sigma (St. Louis, MO, USA) and was suspended in 0.05 M citrate buffer, while 10 mM solution of ATRA (Sigma) was prepared in 100% ethanol for storage.

Animals

Fifty male diabetic ICR (Institute of Cancer Research) mice weighing ~16-18 g were purchased from the Basic Medical Experimental Animal Center, Jilin University.

Grouping

Fifty ICR mice were fed 1 week after adaptation and were randomly assigned to three

groups: the prevention group (N = 20), in which mice received subcutaneous injection of ATRA (10 mg/kg daily) for 5 days followed by 5 days intraperitoneal injection of STZ (60 mg/kg) for 5 days; diabetic group (N = 20), in which mice received subcutaneous injections of 95% sterile peanut oil and 5% dimethyl sulfoxide for 5 days followed by intraperitoneal injection of STZ (60mg/kg); and the control group (N = 10), in which mice received subcutaneous injections of 95% sterile peanut oil and 5% DMSO for 5 days followed by intraperitoneal injection of citrate buffer for five days. Mice with blood glucose levels of >16.7 mM after 5 days of STZ injection were considered a suitable model for T1DM.

Blood and tissue analyses

Blood samples (1 mL) were collected from the tails of the experimental animals weekly and serum was collected from the blood by centrifugation. Blood glucose concentration was measured using blood glucose test strips (Roche, Indianapolis, IN, USA) and serum insulin levels were measured using an insulin radioimmunoassay kit (Weifang Three-dimensional Bio-engineering Co. Ltd., Weifang, China). Pancreatic pathology was assessed using hematoxylin-eosin staining and light microscopy of islet cells. INF- γ and IL-4 levels were measured using a human interferon- γ ELISA kit and an interleukin-4 ELISA Kit (Dakota for the Biotechnology Co., Ltd., Shenzhen, China), respectively.

Statistical analysis

All data were analyzed by SPSS11.0 and are reported as means \pm SD. Paired *t*-tests were used to compare data in the same group before and after treatment and ANOVA was used to compare data between groups. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

ATRA improves the physiology of T1DM in mice

All mice in the control group grew normally and exhibited normal activity, weight, food and water intake, and urine output. In the diabetic group, most parameters (growth rate, daily activity, food and water intake, weight gain, and urine output) decreased compared with those in a control group. One mouse in the diabetic group died due to severe T1DM after 5 days of STZ administration. One mouse in the prevention group also died; however, most other mice in this group exhibited normal for growth rate, daily activity, food and water intake, weight gain, and urine output before and after intraperitoneal injection with STZ, indicating that ATRA improves the physiology of T1DM in mice by preventing the destruction caused by STZ.

ATRA reduces the levels of blood glucose in T1DM mice models

Before STZ treatment, there were no significant differences in the blood glucose levels between the mice in the three different groups (ATRA, diabetic, and control; $P > 0.05$, Table 1). After 1 week of STZ treatment, blood glucose levels significantly increased in the diabetic group compared with the ATRA and control groups ($P < 0.05$, Table 1). Blood glucose concentrations peaked in the

diabetic group after 4 weeks of STZ administration ($P < 0.05$, Table 1). Similarly, the concentrations of blood glucose also peaked in prevention group after 4 weeks of STZ administration; however, the peak levels of blood glucose were significantly lower in the prevention group than in the diabetic group at the fourth week ($P < 0.05$, Table 1). In the control group, blood glucose levels did not differ significantly between the first week and the sixth week ($P > 0.05$, Table 1).

Table 1. Effects of ATRA on the blood glucose levels.

| Groups | Cases | Before STZ treatment (mM) | After STZ treatment (mM) | | | | |
|------------|-------|---------------------------|----------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | | | 1 week | 2 weeks | 3 weeks | 4 weeks | 6 weeks |
| Prevention | 19 | 6.02 ± 0.59 | 7.39 ± 3.38 ^c | 9.45 ± 4.85 ^c | 10.90 ± 5.02 ^c | 12.79 ± 4.24 ^c | 12.31 ± 4.36 ^c |
| T1DM | 19 | 6.17 ± 0.63 | 9.81 ± 4.28 ^{a,b} | 12.60 ± 4.62 ^b | 16.07 ± 4.44 ^b | 20.31 ± 2.72 ^b | 20.77 ± 4.15 ^b |
| Control | 10 | 5.99 ± 0.76 | 5.86 ± 0.68 | 6.23 ± 0.61 | 6.05 ± 0.58 | 6.09 ± 0.71 | 6.51 ± 0.67 |

^a $P < 0.05$ versus prevention group; ^b $P < 0.05$ versus control group; ^c $P < 0.05$ versus diabetic group.

ATRA reduces the incidence of T1DM

Before STZ treatment, there were no diabetic cases among the ATRA, the diabetic, and the control group animals (blood glucose < 7 mM in all groups). After 1 week of STZ treatment, there was one diabetes case in the prevention group and two in the diabetic group. The incidence of diabetes increased with time in both the ATRA and diabetic groups (Table 2) and in the diabetic group, the incidence of diabetes was the highest at 4 weeks of STZ injection. In the control group, there were no diabetes cases from the first week to the sixth week (Table 2).

Table 2. Effects of ATRA on the incidence of diabetes.

| Groups | Diabetes incidence (%) | | | | |
|------------|-------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | 1 week | 2 weeks | 3 weeks | 4 weeks | 6 weeks |
| Prevention | 5.3 (1/19) ^a | 10.5 (2/19) ^a | 26.3 (5/19) ^a | 36.8 (7/19) ^a | 36.8 (7/19) ^a |
| T1DM | 10.5 (2/19) | 21.1 (4/19) | 42.1 (8/19) | 78.9 (15/19) | 84.2 (16/19) |
| Control | 0 | 0 | 0 | 0 | 0 |

^a $P < 0.05$ versus the diabetic group.

ATRA improves serum insulin levels

Before STZ treatment commenced, there was no difference in the serum levels of insulin between the ATRA, diabetic, and control groups ($P > 0.05$). After 6 weeks of STZ treatment, there was a marked decrease in the serum insulin in the prevention group compared with the control group ($P < 0.05$, Table 3); however, the serum insulin in the diabetic group was lower than the levels in both the ATRA and control groups ($P < 0.05$, Table 3). In the control group, serum insulin levels did not differ between the different weeks ($P > 0.05$).

Table 3. Effects of ATRA on serum insulin levels.

| Groups | Serum insulin (IU/mL) | | | | |
|------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | 1 week | 2 weeks | 3 weeks | 4 weeks | 6 weeks |
| Prevention | 28.51 ± 3.24 ^{a,b} | 30.21 ± 2.99 ^{a,b} | 31.58 ± 3.25 ^{a,b} | 31.94 ± 3.25 ^{a,b} | 32.64 ± 3.89 ^{a,b} |
| T1DM | 26.77 ± 2.12 ^a | 25.74 ± 2.62 ^a | 24.51 ± 2.84 ^a | 24.89 ± 2.57 ^a | 23.81 ± 2.95 ^a |
| Control | 41.98 ± 6.12 | 42.14 ± 6.88 | 42.77 ± 7.14 | 41.58 ± 6.69 | 42.92 ± 7.7 |

^a $P < 0.05$ versus the control group; ^b $P < 0.05$ versus the diabetic group.

ATRA prevents the development of pancreatic pathology

The islets of mice in the control group of this study were found to have favorable morphological characteristics: most cells were oval-shaped with clear boundaries. A larger number of islet cells were arranged neatly and nuclei were round or oval in shape (Figure 1A). The number of islet cells in the mice in the prevention group was slightly lower than that in control group mice; however, in the prevention group animal islet cells exhibited normal morphology. Most islet cells in the prevention group mice exhibited extensive cytoplasmic swelling and degeneration (Figure 1B). In the diabetic group, islet number was significantly lower than that in the control group and many shrinking cells with indistinct boundaries were observed. Nuclei were furthermore shown to be irregular in shape and degraded. A few infiltrating lymphocytes and mononuclear cells were observed in the diabetic group islets (Figure 1C).

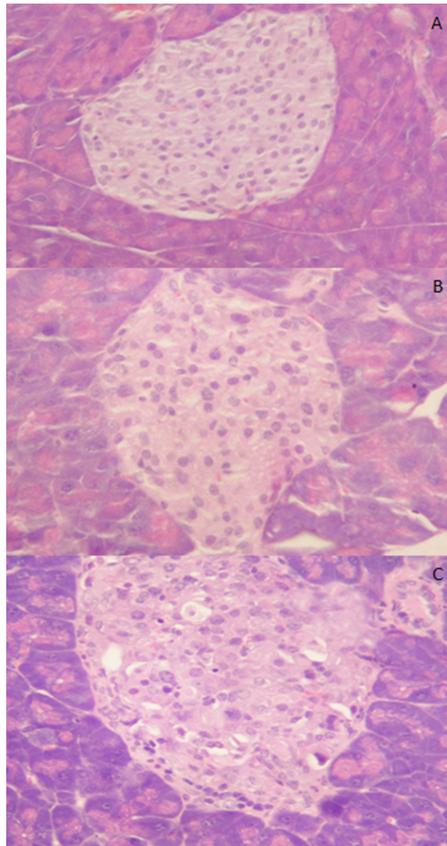


Figure 1. Effects of ATRA on the development of pancreatic pathology. **A.** Islet cells in the control group were oval with clear boundaries. Many islet cells were arranged neatly and nuclei were round or oval. **B.** Islet numbers were slightly lower in the prevention group compared with the control group; however, the islet cells exhibited normal morphology in prevention group mice. Most cells, however, exhibited extensive cytoplasmic swelling or degeneration in control group mice. **C.** Islet numbers were significantly lower in the diabetic group than in the control group. Most cells were shrunken and had undefined boundaries, while nuclei were in irregular shape or degraded. Some infiltrating lymphocytes and mononuclear cells were present.

Effects of ATRA on the serum levels of IFN- γ and IL-4

The serum levels of IFN- γ and IL-4 in the diabetic group animals were shown to be significantly higher and lower, respectively, than those in the prevention and control group animals; while the IFN- γ /IL-4 ratio was found to be markedly higher in the diabetic group than in the prevention and control groups ($P < 0.05$, Table 4). In the prevention group, the serum IFN- γ and IL-4 levels were significantly lower and higher, respectively, than in the diabetic group ($P < 0.05$, Table 4). Compared with the levels measured for the prevention group, the serum levels of IFN- γ were significantly lower in the control group ($P < 0.05$, Table 4), while there was little difference between the levels of IL-4 in the control group compared with prevention group ($P > 0.05$, Table 4). These findings suggest that ATRA reduces the levels of IFN- γ and increases the levels of IL-4 in the serum.

Table 4. Serum levels of IFN- γ and IL-4 after six weeks of STZ treatment.

| Groups | Cases | IFN- γ (pg/mL) | IL-4 (pg/mL) | IFN- γ /IL-4 |
|------------|-------|-------------------------------|-------------------------------|------------------------------|
| Prevention | 10 | 34.09 \pm 3.03 ^b | 39.89 \pm 5.88 ^b | 0.87 \pm 0.15 ^b |
| T1DM | 12 | 53.20 \pm 9.07 ^a | 22.31 \pm 6.00 ^a | 2.54 \pm 0.80 ^a |
| Control | 8 | 29.04 \pm 3.34 | 40.84 \pm 10.54 | 0.74 \pm 0.18 |

^a $P < 0.05$ versus the control group; ^b $P < 0.05$ versus the diabetic group.

DISCUSSION

Patients with T1DM have been shown to have low levels of vitamin A compared with healthy people, suggesting that vitamin A plays an important role in normal metabolism and thus that vitamin A deficiency affects immune regulation, leading to T1DM (Basu and Basualdo, 1997; Baena et al., 2002; Sia, 2005). Vitamin A was previously shown to reduce the incidence of T1DM in nucleotide-binding oligomerization domain (NOD) mice (Zunino et al., 2007) and ATRA is known to protect an insulin-producing cell line (RINm5F) from glucotoxicity by reducing glucose-induced damage (Kang et al., 2004; Jin et al., 2010). In this study, ATRA was shown to preserve islet cell numbers and inhibit STZ-induced increases in cell size. The STZ-induced impairment of insulin secretion was found to be prevented by ATRA in this study, demonstrating a protective effect of ATRA on STZ-treated pancreatic β -cells. Treatment with ATRA was furthermore shown to reverse the concentration decreases and weight loss caused by STZ. Together, these findings suggest that ATRA decreases the incidence of STZ-induced T1DM in a mouse model.

All-trans retinoic acid has been found to regulate immune processes via cytokine regulation. Vitamin A deficiency disrupts the balance of Th1/Th2, which leads to an increase in the levels of Th1 and a decrease in the levels of Th2 (Nikawa et al., 1999; Farhangi et al., 2013). Vitamin A activates Th2 responses and reduces Th1 responses (Lovett-Racke and Racke, 2002); and ATRA has been shown to reduce the expression of Th1 and increase the expression of Th2 in human lymphocytes (Dawson et al., 2006). Increased ATRA levels induce IL-4 gene expression in myelin basic protein-specific T cells, which consequently express a Th1-like phenotype (Lovett-Racke and Racke, 2002), while ATRA deficiency has been found to result in an increase in the number of Th1 cells (Nozaki et al., 2006). Retinoic acid has been shown to delay the differentiation and maturation of dendritic cells, reduce the activity of lymphocyte reaction, reduce Th1 cytokine production, and increase Th2 cytokines, thereby causing a shift of the immune response to Th2 (Bai et al., 2010;

Accordingly, the findings of this study demonstrate that ATRA adjusts the IFN- γ /IL-4 imbalance caused by the STZ-induced decrease in IFN- γ levels, increase in IL-4 levels, and the resulting reduced IFN- γ /IL-4 ratio. In this way, ATRA significantly reduces STZ-induced islet inflammation and prevents damage to pancreatic islet β -cells. The findings reported here are consistent with previous reports (Nieman et al., 2004; Sodhi and Singh, 2013). The effects of ATRA on Th2-type cytokines such as IL-4 are currently being investigated, and ATRA has furthermore been found to reduce the levels of Th1-type cytokines such as IFN- γ and IL-1 β , thereby reducing β -cell cytotoxicity and protecting islet cells from damage caused by, for example, STZ-induced nonspecific insulinitis. Previous research demonstrated that ATRA protects the RIN-m5F β -cell line by decreasing the levels of IL-1 β , TNF- α , and IFN- γ , allowing islet cell numbers to recover to normal levels. This effect may involve the ATRA-induced decrease in NO release; however, further investigation is required to confirm this hypothesis.

In this study, a mouse model of T1DM was established by repeated injection of mice with STZ. Using this model, it was shown that ATRA reduces the incidence of T1DM by protecting islet β -cells, upregulating the levels of the pancreatic β -cell factor IL-4, reducing the cell toxicity-associated levels of IFN- γ in β -cells, and maintaining the Th1/Th2 balance. Vitamin A deficiency is associated with the pathogenesis of T1DM, and the multiple functions of ATRA therefore need further study in the context of T1DM. The findings reported here; however, provide supporting evidence that ATRA may have potential as a drug for the prevention of T1DM in the future.

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