Oral therapy for type 1 diabetes mellitus using a novel immunomodulator, FTY720 (fingolimod), in combination with sitagliptin, a dipeptidyl peptidase-4 inhibitor, examined in non-obese diabetic mice

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ABSTRACT

Aims/Introduction: The therapeutic effectiveness against type 1 diabetes mellitus of a novel immunomodulator, FTY720 (fingolimod), in combination with sitagliptin, a dipeptidyl peptidase-4 inhibitor, was examined in the non-obese diabetic (NOD) mouse model.

Materials and Methods: Female NOD mice that had developed type 1 diabetes mellitus spontaneously were divided into four groups according to which therapy they received: (i) FTY720 (0.1 mg/kg, orally, six times a week) plus sitagliptin (1 mg/kg, orally, six times a week); (ii) FTY720 (0.1 mg/kg, orally, six times a week); (iii) sitagliptin (1 mg/kg, orally, six times a week); and (iv) the vehicle (water) alone. Therapeutic efficacy was evaluated in terms of survival rate, ratio of insulin-positive β-cells/total islet area, extent of islet inflammation (insulitis score) and blood-glucose level.

Results: The therapeutic administration of FTY720 plus sitagliptin significantly improved survival (83% at 70 days after onset, P < 0.05) compared with sitagliptin alone (17%) or vehicle alone (0%). The fasting-blood glucose level, the ratio of insulin-positive β-cells/total islet area and the insulitis score in the surviving mice, which had been treated with FTY720 plus sitagliptin, were improved to the normal levels as in age-matched NOD mice with normoglycemia.

Conclusions: Combination therapy with FTY720 and sitagliptin is a promising candidate for type 1 diabetes mellitus treatment, and might allow the treatment of type 1 diabetes mellitus with only oral agents. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2012.00218.x, 2012)

KEY WORDS: FTY720, Non-obese diabetic mouse, Type 1 diabetes mellitus

INTRODUCTION

The novel immunomodulator, FTY720 (fingolimod), is a synthetic structural analog of myriocin (ISP-I), a metabolite of Isaria conelarita1,2, and was developed by Tetsuro Fujita (F), of our group, in collaboration with Taito Co. (T; Mitsui Sugar, Tokyo, Japan) and Yoshitomi Pharmaceutical Industries, Ltd (Y; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) in Japan. FTY720 has been reported to be effective not only in preclinical transplantation models, but also in several models of immunological disease, including rheumatoid arthritis3, myasthenia gravis4, multiple sclerosis5, type 1 diabetes mellitus6 and atopic dermatitis7. The mechanism of action of FTY720 differs from that of established immunosuppressants, such as tacrolimus hydrate and cyclosporine. FTY720 blocks S1P signaling by inducing internalization and intracellular partial degradation of the receptors8–10. As a result, it suppresses immune response by sequestering circulating mature lymphocytes from blood and peripheral tissues to the secondary lymphoid tissues and thymus11,12.

Sitagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, represents a new therapeutic approach for the treatment of type 2 diabetes mellitus. DPP-4 degrades incretin hormones, such as glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP), and so sitagliptin causes an increase in the levels of the intact (active) form of these hormones. Active GLP-1 and GIP stimulate glucose-dependent insulin biosynthesis and release, and GLP-1 also suppresses
glucagon release, delays gastric emptying and increases satiety. DPP-4 inhibitors improved glycemic control, insulin secretion and β-cell function in rodents.

The non-obese diabetic (NOD) mouse is an excellent animal model of spontaneous type 1 diabetes mellitus, showing many of the characteristics of human type 1 diabetes mellitus; that is, autoreactive T cells attack islet β-cells, resulting in depletion of insulin secretion in these mice.

In general, type 1 diabetes mellitus develops in childhood and is treated with intensive insulin therapy, consisting of four or more insulin injections daily. Insulin self-injection imposes a huge burden on patients, and it is difficult to achieve effective blood-glucose control, especially during the period of development of secondary sexual characteristics. Alleviation of this burden is an important goal, and we have reported the therapeutic effectiveness of FTY720 in combination with once-daily injection of insulin glargine in the present study, we examined the efficacy of a purely oral combination therapy consisting of FTY720 and sitagliptin. Our results show that this oral therapy is as beneficial as the combination of FTY720 with insulin glargine.

**MATERIALS AND METHODS**

**Animals and Ethics**

Female NOD mice bred under specific pathogen-free conditions were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were given γ-ray-irradiated food (CRF-1; Oriental Bio Co., Kyoto, Japan) and distilled water for injection (Ohtsuka Pharmaceutical Co. Ltd, Tokyo, Japan). The pancreas was excised from mice under sodium pentobarbital anesthesia after the observation period. Tissues were fixed with 10% buffered formalin solution (Wako Pure Chemical Industries Ltd, Osaka, Japan) and embedded in optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan), then 10-μm sections were cut from each sample at 200-μm intervals. Five pancreatic sections from each animal were stained for insulin using monoclonal rat anti-insulin antibody (R&D Systems Inc., Minneapolis, MN, USA) and peroxidase-conjugated monoclonal mouse anti-rat IgG2a antibody (Invitrogen, Carlsbad, CA, USA), and visualization was carried out with diaminobenzidine. The ratio of insulin-positive β-cells/total islet area was evaluated by using Image J software (downloaded from the NIH website, http://rsbweb.nih.gov/ij/index.html, accessed 9 February 2011).

**Extent of Insulitis**

The extent of insulitis was evaluated in sections stained with hematoxylin–eosin using Mayer’s Hematoxylin Solution (Wako Pure Chemical Industries Ltd) according to the standard method, and scored according to the following criteria:

- grade 0, no mononuclear cell infiltration;
- grade 1, mononuclear cell infiltration around the islet, but no intra-islet infiltration;
- grade 2, mononuclear cell infiltration in and around the islet, but intra-islet infiltration in less than one-third of the islet area;
- grade 3, intra-islet mononuclear cell infiltration in one-third to half of the islet area;
- grade 4, extensive intra-islet infiltration occupying more than half of the islet area.

**Immunostaining for CD4⁺ and CD8⁺ T cells**

The pancreas was excised from surviving mice under sodium pentobarbital anesthesia after the observation period. Tissues were fixed with 4% buffered paraformaldehyde solution (Wako Pure Chemical Industries Ltd) and embedded in optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan), then 10-μm frozen sections were cut from each sample at 200-μm intervals. Five pancreatic sections from each animal were stained for CD4 using monoclonal rat anti-mouse CD4/ L3T4 antibody (Beckman Coulter Inc., Fullerton, CA, USA) and N-Histofine Simple Stain Mouse MAX PO (Rat; Nichirei Biosciences Inc., Tokyo, Japan), and visualization was carried out with diaminobenzidine. The adjacent section was stained for CD8 using monoclonal rat anti-mouse CD8a/Lyt-2 antibody (Beckman Coulter Inc.) and peroxidase-conjugated monoclonal mouse anti-rat IgG2a antibody (Invitrogen), and visualization as
described earlier. The number of CD4+ and CD8+ T cells/islet area (mm2) was evaluated by using Image J software.

**Statistical Analysis**

Unless noted otherwise, data are presented as the mean ± SD. The significance of differences in the blood-glucose levels, the ratio of insulin-positive β-cells/total islet area and insulitis score was evaluated by using the Mann–Whitney U-test, and the significance of differences in survival rate was evaluated by using the log–rank test. P < 0.05 was considered statistically significant.

**RESULTS**

**Therapeutic Effect of FTY720 in Combination with Sitagliptin on Established Type 1 Diabetes Mellitus in NOD Mice**

We previously reported that therapeutic administration of FTY720 (0.1 mg/kg, orally, twice a week) prolonged the survival (45% at 70 days after onset) of NOD mice with overt type 1 diabetes mellitus, and FTY720 in combination with once-daily injection of insulin glargine further improved the survival to 85%6. In the present study, the efficacy of purely oral agents (FTY720 in combination with sitagliptin, which is widely used to increase insulin secretion) was examined in the NOD mouse model.

NOD mice with hyperglycemia; that is, overt type 1 diabetes mellitus, were divided into the following four groups: (i) combination therapy group (n = 6); (ii) FTY720 group (n = 6); (iii) sitagliptin group (n = 6); and (iv) placebo group (n = 6). The four groups were matched for age (28 ± 8, 27 ± 5, 24 ± 7 and 27 ± 49 weeks-of-age, respectively) and blood-glucose level at the onset of treatment (454 ± 91, 421 ± 71, 492 ± 67 and 406 ± 102 mg/dL, respectively). Survival curves in the four groups are shown in Figure 1. In the placebo group, all mice died within 58 days after the onset. In the sitagliptin group and the FTY720 group, one mouse (17%) and three mice (50%), respectively, survived during the observation period (up to 70 days after the onset), whereas five mice (83%) in the combination therapy group survived during the observation period. The difference in the survival rates between the combination therapy group and the sitagliptin group (as well as the placebo group) was significant (P < 0.05, log–rank test). In addition, the survival rate in the combination therapy group was similar to that found in the FTY720 plus insulin glargine group (85%) in a previous study6.

To confirm the therapeutic efficacy of the combination therapy biochemically, immunohistochemically and histochemically, we examined blood-glucose level, the ratio of insulin-positive β-cells/total islet area (insulin-positive cell ratio) and the extent of islet inflammation (insulitis score). The insulin-positive cell ratio and the insulitis score were evaluated at the end of the observation period in surviving mice or at the time of death. First, the fasting-blood glucose (FBG) levels in surviving mice after 28, 42 and 56 days of treatment and in age-matched NOD mice with normoglycemia were measured (Figure 2). The FBG level in the surviving mice in the combination therapy group was lowered from 454 ± 91 mg/dL (before treatment) to 182 ± 132 mg/dL (28 days of treatment), 161 ± 109 mg/dL (42 days of treatment) and 77 ± 35 mg/dL (56 days of treatment; Figure 2). The level after 56 days of treatment was similar to that in age-matched NOD mice with normoglycemia (n = 5, 91 ± 21 mg/dL; Figure 2) and was significantly lower than that in the surviving mice in the FTY720 group (n = 3, 160 ± 35 mg/dL). The FBG level in the dead mice in all groups was higher than those of the surviving mice in the combination therapy and the FTY720 groups after 56 days of treatment, except for a few of the dead mice, in which the FBG level was lowered at the end-stage because of poor performance status, especially abnormal eating behavior. Second, the insulin-positive cell ratio in the combination therapy group (n = 6, 0.56 ± 0.40) was similar to that in age-matched NOD mice with normoglycemia (n = 5, 0.69 ± 0.18; Figure 3). In contrast, the ratios in the FTY720 group (n = 6, 0.23 ± 0.29), the sitagliptin group (n = 6, 0.05 ± 0.13) and the placebo group (n = 4, 0) were significantly (P < 0.05) lower than that in age-matched NOD mice with normoglycemia. In addition, the ratio in the combination therapy group was significantly (P < 0.05) higher than that in the sitagliptin group and the placebo group, and tended to be higher than that in the FTY720 group. Insulin-positive β-cells were still present in the surviving mice in the FTY720 group and the sitagliptin group, whereas no insulin-positive β-cells were detected in dead mice of any group (Figure 3). Third, the insulitis scores in the combination therapy group (n = 6, 2.0 ± 0.9) and the FTY720 group (n = 6, 2.3 ± 0.4) were similar to that in the...
Figure 2 | Effect of therapeutic administration of FTY720 in combination with sitagliptin on fasting-blood glucose (FBG) levels in non-obese diabetic (NOD) mice with overt type 1 diabetes mellitus. Female NOD mice with hyperglycemia; that is, overt type 1 diabetes mellitus, were treated with: (i) FTY720 plus sitagliptin (n = 6, combination therapy group; FTY720, 0.1 mg/kg, orally, six times a week plus sitagliptin, 1 mg/kg, orally, six times a week); (ii) FTY720 (n = 6, FTY720 group; 0.1 mg/kg, orally, six times a week); (iii) sitagliptin (n = 6, sitagliptin group; 1 mg/kg, orally, six times a week); and (iv) vehicle alone (n = 6, placebo group). FBG levels after 28, 42 and 56 days of treatment in the four groups and age-matched NOD mice with normoglycemia (n = 5) were measured by using Glucocard (diameter 4 mm; Arkray Inc., Kyoto, Japan) after an overnight fast. Each symbol indicates an individual mouse, and mean values of the surviving mice are indicated by the horizontal bars. The opened and closed symbols denote surviving mice and dead mice, respectively. The significance of differences in the blood glucose levels was examined by using the Mann–Whitney U-test; NS, not significant.

Figure 3 | Effect of therapeutic administration of FTY720 in combination with sitagliptin on the ratio of insulin-positive β-cells/total islet area in non-obese diabetic (NOD) mice with overt type 1 diabetes mellitus. Female NOD mice with hyperglycemia; that is, overt type 1 diabetes mellitus, were treated with: (i) FTY720 plus sitagliptin (combination therapy group; FTY720, 0.1 mg/kg, orally, six times a week plus sitagliptin, 1 mg/kg, orally, six times a week); (ii) FTY720 (FTY720 group; 0.1 mg/kg, orally, six times a week); (iii) sitagliptin (sitagliptin group; 1 mg/kg, orally, six times a week); and (iv) vehicle (placebo group). Pancreas sections from surviving and dead mice (combination therapy group; surviving mice n = 5, dead mice n = 1), FTY720 group (surviving mice n = 3, dead mice n = 3), sitagliptin group (surviving mice n = 1, dead mice n = 5) and placebo group (surviving mice n = 0, dead mice n = 4), and age-matched NOD mice with normoglycemia (n = 5) were stained with monoclonal rat anti-insulin antibody and the insulin-positive cell ratio was evaluated. Representative pictures illustrating different insulin-positive cell ratio values are also shown. Each symbol indicates an individual mouse, and mean values are indicated by the horizontal bars. The opened and closed symbols denote surviving mice and dead mice, respectively. The significance of differences was examined by using the Mann–Whitney U-test. ND, not detectable; NS, not significant.
age-matched NOD mice with normoglycemia (n = 5, 2.0 ± 0.7; Figure 4). In contrast, the scores in the sitagliptin group (n = 6, 3.5 ± 0.6) and the placebo group (n = 4, 3.4 ± 0.6) were significantly (P < 0.05) higher than that in the age-matched NOD mice with normoglycemia. In addition, the scores in the combination therapy group were significantly (P < 0.05) lower than that in the sitagliptin group and the placebo group, and tended to be lower than that in the FTY720 group (Figure 4). To examine the difference in population of infiltrated lymphocytes, the frozen pancreas sections from the surviving mice were stained with anti-CD4 (Figure 5) and anti-CD8 antibodies. In terms of CD8+ T cells, no relationship was observed between the therapeutic effect of the combination therapy and the number of the infiltrated CD8+ T cells (data not shown). Whereas, the number of CD4+ T cells in the combination therapy group (n = 5, 757 ± 565/mm²) was significantly lower than that in age-matched NOD mice with normoglycemia (n = 5, 2090 ± 1930), and tended to be lower than those in the FTY720 group (n = 3, 3760 ± 3840) and sitagliptin group (n = 1, 815).

**DISCUSSION**

The novel immunomodulator, FTY720 (fingolimod), has a number of attractive properties. The mechanism of its immunosuppressive effect is different from those of established immunosuppressants, as described in the Introduction. At therapeutic doses, FTY720 does not affect T cell and B cell responses in vitro or in vivo. As FTY720 treatment allows preservation of many aspects of immune function, including the total number of lymphocytes, the capacity for lymphocyte activation in lymph nodes and tissues, the capacity for generating antibodies, and innate immune responses, there is only a limited increase in susceptibility to infectious diseases, including herpes virus infection, urinary tract infection and so on. Furthermore, immune memory function is not impaired. FTY720 was recently approved by the Food and Drug Administration (USA) for treatment of multiple sclerosis (http://www.gilenya.com, accessed 30 September 2010).

Our previous study showed that FTY720 partially protected β-cells from autoimmune destruction and maintained the insulin-secretory function. However, as the therapeutic effectiveness of FTY720 alone in animals with overt type 1 diabetes mellitus was limited, it was necessary to develop a more effective regimen to treat type 1 diabetes mellitus. The reductions in circulating T cells by FTY720 are predominantly seen in naïve T cells and central memory T cells (TCM) subsets. This might relate to the fact that naïve T cells and TCM express high levels of...
debated. In contrast, effector memory T cells (TEM) lack expression of CCR7, and thus do not recirculate regularly through lymph nodes; that is, the majority of T lymphocytes in the peripheral blood and tissues are TEM23. This might be the reason why the therapeutic efficacy of FTY720 alone was limited. Itoh and Maki24 reported that surgical removal of 90% of pancreatic tissue before onset of insulin induced a long-term diabetes-free condition in NOD mice, but pancreatectomy after development of moderate insulin had no effect on the course of type 1 diabetes mellitus. Therefore, the essential requirements of a more effective regimen are: (i) the regimen should regulate the autoreactive T cells, that is both TCM and TEM; and (ii) maintain and/or increase the number of functional β-cells to more than 10% of healthy mice. These considerations led us to examine combination therapy of FTY720 with sitagliptin.

Sitagliptin, a new hypoglycemic agent for type 2 diabetes mellitus, is an orally administrable, highly selective DPP-4 inhibitor. In sitagliptin-treated rodents, approximately 80% inhibition of plasma DPP-4 activity and two- to threefold elevation in active GLP-1 levels were observed in association with a reduction in glucose excursion after an oral glucose tolerance test. In addition, chronic treatment with sitagliptin decreased FBG and was associated with a low incidence of hypoglycemia.25 GLP-1 has been shown to promote the proliferation and differentiation of β-cells in vivo26 and in vitro27, and treatment with sitagliptin increased the number of insulin-positive β-cell in islets in a rodent model28. In addition, Kim et al.29 reported that sitagliptin suppressed the migration of CD4+ T cells. Therefore, we chose sitagliptin as the combination partner of FTY720.

In the present study, we found that therapeutic administration of FTY720 in combination with sitagliptin significantly improved survival, and 83% of NOD mice with overt type 1 diabetes mellitus survived to the end of the observation period. The beneficial effects in the treatment of type 1 diabetes mellitus were similar to those found for FTY720 in combination with insulin glargine.30 The blood-glucose level was ameliorated at 56 days after the onset of treatment in the combination therapy group (Figure 2). These results were confirmed immunohistochemically and histochemically in terms of the ratio of insulin-positive β-cells/total islet area, and the extent of islet inflammation, respectively. No insulin-positive β-cells were detected in dead mice (Figure 3), and the insulitis score in dead mice was higher than that in surviving mice in the combination group (Figure 4). In addition, the ratio of insulin-positive β-cells and the insulitis score in the combination therapy group (0.56 ± 0.40 and 2.0 ± 0.9, respectively) were similar to those in age-matched NOD mice with normoglycemia (0.69 ± 0.18 and 2.0 ± 0.7, respectively), and were also consistent with those found30 in NOD mice with overt type 1 diabetes mellitus treated with FTY720 and insulin glargine (0.60 ± 0.24 and 1.5 ± 0.1, respectively). In the FTY720 group, the survival rate after the observation period was 50%. Thus, although β-cells are protected from autoimmune destruction by FTY720, the increased insulin-secretory activity might lead to depletion of releasable insulin (β-cell exhaustion). Accordingly, sitagliptin might promote the proliferation and differentiation of the remaining insulin-secretory β-cells in the combination therapy group. In addition, the infiltrated CD4+ T cells in the combination therapy group were decreased or tended to decrease as compared with those in the FTY720 group, the sitagliptin group and age-matched NOD mice with normoglycemia. The ameliorating effect of the combination therapy might depend in part on this observation. In terms of populations of infiltrated lymphocytes, Phillips et al.30 reported that both CD4+ and CD8+ were required to develop type 1 diabetes mellitus in NOD mice; however, no association of the number of infiltrated CD4+ and CD8+ T cells to islet with stage of the disease was clarified31. Therefore, more detailed study is required to understand the difference in the population of infiltrated lymphocytes. At present, the principles underlying combination therapy are considered as follows: (i) FTY720 is expected to protect β-cells from autoimmune destruction by sequestering naïve T cells and TCM to the secondary lymphoid tissues and thymus; and (ii) sitagliptin is expected to compensate for the decreased insulin secretion by increasing the number of β-cells,
promoting glucose-stimulated insulin secretion and suppressing the migration of CD4+ T cells, especially TEM. The next step will be to develop criteria for identifying those individuals for whom combination therapy would be appropriate.

In conclusion, the results of the present study suggest that the combination of FTY720 and sitagliptin is a promising candidate for therapy of type 1 diabetes mellitus at an early phase, when some β-cell function still remains. This approach to the treatment of type 1 diabetes mellitus with only oral agents is expected to alleviate the critical problems of intensive insulin therapy, that is, the burden on patients and the difficulty of achieving good control of blood glucose.

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