Preventive and Therapeutic Potential of p38α-Selective Mitogen-Activated Protein Kinase Inhibitor in Nonobese Diabetic Mice with Type 1 Diabetes

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ABSTRACT

Mitogen-activated protein kinases (MAPKs) and heat shock proteins (HSPs) are ubiquitous proteins that function within T cells in both normal and stress-related pathophysiological states, including type 1 diabetes. The nonobese diabetic (NOD) mouse spontaneously develops T cell-mediated autoimmune pancreatic beta cell destruction that is similar to type 1 diabetes in humans. Because p38 MAPKs have been shown to modulate T cell function, we studied the effects of a p38α MAPK-selective inhibitor, indole-5-carboxamide (SD-169), on the development and progression of type 1 diabetes in the NOD mouse. In preventive treatment studies, SD-169 significantly reduced p38 and HSP60 expression in T cells of the pancreatic beta islets. Following treatment, the incidence of diabetes as determined by blood glucose levels was significantly lower, and immunohistochemistry of pancreatic beta islet tissue demonstrated significant reduction in CD5+ T cell infiltration in the SD-169 treatment group as compared with untreated NOD mice. In therapeutic studies using mildly and moderately hyperglycemic NOD mice, SD-169 treatment lowered blood glucose and improved glucose homeostasis. Furthermore, following cessation of SD-169 treatment, NOD mice showed significant arrest of diabetes. In conclusion, we report that this p38α-selective inhibitor prevents the development and progression of diabetes in NOD mice by inhibiting T cell infiltration and activation, thereby preserving beta cell mass via inhibition of the p38 MAPK signaling pathway. These results have bearing on current prophylactic and therapeutic protocols using p38α-selective inhibitors in the prediabetic period for children at high risk of type 1 diabetes, in the honeymoon period, and for adults with latent autoimmune diabetes.

Type 1 diabetes is a multifactorial disease in which autoimmunity plays a prime role. The disease becomes clinically manifest when the majority of endocrine beta cells have been destroyed in a T cell-mediated process (Yoshida and Kikuta, 2000). Methods that predict the development of type 1 diabetes may allow the evaluation of pharmacological treatment strategies that halt or even prevent beta cell destruction (Debussche et al., 1993; Keymeulen and Somer, 1993; Mahon et al., 1993; Ryu et al., 2001; Shapiro et al., 2002). Following the initiation of insulin therapy, 60 to 80% of patients with type 1 diabetes will experience a transient remission known as the honeymoon period (Keymeulen and Somer, 1993), and therapeutic strategies have been described to prolong this period by slowing the ongoing self-destruction of the insulin-producing beta cells (Hosker and Turner, 1982; Heinze and Thon, 1985; Crump, 1987; Palmer and McCulloch, 1990; Herold et al., 2002; Shapiro et al., 2002). Between 5 and 30% of adults presenting with mild hyperglycemia suggestive of type 2 diabetes may actually have a slow-progressive form of type 1 diabetes designated latent autoimmune diabetes of adults (Casteels et al., 1998). Immunosuppressive agents prevent the onset of type 1 diabetes in NOD mice and humans (Mori et al., 1986; Mahon et al., 1993; Casteels et al., 1998; Tabatabaie et al., 2000; Shapiro et al., 2002). Most of these agents have been studied in a prophylactic context, before the appearance of overt diabetes or insulitis (Mori et al., 1986; Mahon et al., 1993; Casteels et al.,

ABBREVIATIONS: NOD, nonobese diabetic; MAPK, mitogen-activated protein kinase; IL, interleukin; HSP, heat shock protein; SD-169, indole-5-carboxamide (an ATP competitive inhibitor of p38 kinase); ELISA, enzyme-linked immunosorbent assay; IFN, interferon; LC, liquid chromatography; MS/MS, tandem mass spectrometry; p-p38, phosphorylated p38; PBS, phosphate-buffered saline; FR167653, pyridinyl imidazole compound, specific inhibitor of p38 pathway.
and CD4 function contributing to the destruction of pancreatic beta cells in NOD mice (Ando et al., 2004). The evidence suggests that CD25+ T cells are also downregulated after treatment with the p38α-selective MAPK inhibitor. In addition, we show that p38 MAPK inhibition improves glucose homeostasis in a therapeutic protocol involving mildly and moderately diabetic NOD mice.

**Materials and Methods**

**Animals**

Female NOD mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our animal facility. Mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Scios, Inc. All mice were kept under conventional conditions at a constant temperature (22–25°C) and fed a commercial powdered Purina chow diet and tap water ad libitum (Purina, St. Louis, MO).

**Materials**

Powdered diet (Lab Diet-5015) was from Dean’s Animal Feeds (San Carlos, CA). SD-169 was added to diet using a Cuisinart Mini-prep Plus (Cuisinart, East Windsor, NJ). Blood glucose levels were measured with glucose test strips and LifeScan One Touch Glucose Meter (Milpitas, CA) using a sample taken from the tail. Serum insulin was quantitated using a mouse insulin ELISA kit (ALPCO Diagnostics, Windham, NH). ELISA kits for IFNγ and IL-10 were obtained from BD Biosciences (San Diego, CA). The primary antibody for HSP60 was purchased from Lab Vision (Fremont, CA). Phospho p38 was from Santa Cruz Biotechnology (Santa Cruz, CA). Absorbance and secondary antibodies for insulin were obtained from PharMingen (San Diego, CA). Phospho p38 was from PharMingen (San Diego, CA). Absorbance and secondary antibodies for insulin were obtained from BD Biosciences (San Diego, CA).

**Chemical Description of SD-169**

SD-169 is indole-5-carboxamide, an ATP competitive inhibitor of p38 kinase. It is a small-molecule, orally active inhibitor of p38α MAPK.

**SD-169 Potency and Specificity**

IC₅₀ of SD-169 against the human p38MAPKs (α, β, γ, and δ isoforms) was previously measured in vitro using purified preparations of enzymes and quantifying the incorporation of radiolabeled ATP into myelin basic protein (MDS Pharma, Tampa, FL). SD-169 demonstrated 38-fold potency against p38α MAP kinase (IC₅₀ = 3.2 nM) than p38β MAP kinase (IC₅₀ = 122 nM). When tested in vitro at a concentration of 50 μM, SD-169 demonstrated no inhibitory activity against a panel of other kinases including p38γ MAP kinase, extracellular signal-regulated kinase 2, c-Jun N-terminal kinase-1, and mitogen-activated protein kinase-activated protein kinase-2.

**Methods**

**Dose Selection Studies**

SD-169 powder was admixed on a weekly basis in fresh powdered mouse chow at low (200 mg/kg) and high (600 mg/kg) doses. Ten NOD mice were given free access to SD-169 mixed with chow from ages 8 to 18 weeks and at an average of 3.5 g/day. Blood samples were collected by tail vein at week 13 and again at 18 weeks of age. SD-169 was measured by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Approximately 0.1 ml of plasma was prepared from each rat, frozen on dry ice, and then stored at −80°C until analysis. SD-169 and the internal stan-
dard, SD-282, were extracted from plasma using a protein precipitation method. The extract was analyzed by LC/MS/MS on a Symmetry C18 column (Waters, Milford, MA).

**Preventive Studies.** Four independent experiments were conducted to evaluate the effects of SD-169 in NOD mice. The first and second experiments examined the effects of SD-169 on the incidence of diabetes, pancreatic T cell infiltration, blood glucose and insulin, CD4+ and CD8+ cell ratios, and phosphorylated p38 (p-p38) expression in the pancreas. Animals were randomized into three groups: group 1 mice (n = 20) received standard chow/vehicle, group 2 mice (n = 20) received chow with low-dose SD-169 (200 mg/kg, which yields 0.6 μM exposure levels, see Results), and group 3 mice (n = 20) received high-dose SD-169 (600 mg/kg which yields 1.8 μM exposure levels, see Results). The design of the third and fourth experiments was the same as the first two experiments except that SD-169 was administered only at high dose, and additional endpoints were studied, including IFNγ and IL-10 levels in the peripheral blood; CD45RA+ and CD45RB+ cell profiles in the spleens; and immunohistochemistry of insulin and HSP60 expression in pancreas. The number of mice used in the standard chow/vehicle group and SD-169 at high-dose groups were nine to 10 each. In all the experiments, treatment was initiated in animals at 8 weeks of age and continued until the 18th week of age. Food intake, body weight, and blood glucose levels were measured once per week. Mice with fed blood glucose levels higher than 150 mg/dl on 3 consecutive days were considered diabetic and were eligible for the preventive studies outlined above. This cutoff was determined from previous experiments showing that such mice when left untreated invariably progressed to hyperglycemia and were considered diabetic and were eligible for the preventive studies outlined above. The inhibitory activity of IFNγ and IL-10 in the plasma was determined by ELISA kits according to manufacturers’ protocols. The frequency of CD45RA+ and CD45RB+ T cells was determined by staining splenocytes with anti-CD3, anti-CD4, and anti-CD45RA or anti-CD3, anti-CD4, and anti-CD45RB. All acquisitions were performed using a fluorescence-activated cell sorter and analyzed with Cell Quest software (BD Biosciences). SD-169 concentrations in the plasma were measured by LC/MS/MS. At the end of the study, pancreata were collected from all mice for histology and immunohistochemistry.

**Histology.** Pancreata were fixed in 10% buffered formalin solution, embedded in paraffin, and stained with H&E.

**Immunohistochemistry.** Pancreata were embedded longitudinally in Tissue-Tek OCT Compound (Miles Scientific, Naperville, IL) and quickly frozen in a methylbutane tank and stored at −70°C. Tissues were sectioned (4 μm) on a cryostat from the middle part of the pancreas. Consecutive sections from the middle area of the pancreas were sectioned and placed onto positively charged slides. After sections were dried, they were fixed in acetone at −20°C. Dry slides were then stored at −70°C. Immunohistochemical staining for T cells (CD3+, CD4+, CD8+, CD5+), p-p38, and HSP60, were performed as follows. Slides were removed from the freezer and allowed to come to room temperature before being rinsed two to three times in PBS. All sections were immersed in 3% hydrogen peroxide for 20 min at room temperature to block endogenous peroxidases completely. To prevent nonspecific reaction with primary antibody, sections were pretreated with 5% normal goat serum. Sections stained for HSP60 were also treated with rodent block (Lab Vision, catalog no. TA-125-RB) for 1 h. After three washes with PBS, the sections were incubated with primary antibodies at room temperature for 1 h. The primary antibodies used in this study were rat anti-mouse CD5+ (T cells) monoclonal antibody diluted at 1:25 (BD Pharmingen catalog no. 550289), rat anti-mouse CD4+ (T helper cell) monoclonal antibody diluted at 1:25 (BD Pharmingen catalog no. 550278), rat anti-mouse CD8+ (T suppressor/cytotoxic cells) monoclonal antibody diluted at 1:25 (BD Pharmingen catalog no. 550282), rabbit p-p38 polyclonal antibody diluted at 1:50 (Santa Cruz catalog no. SC-9715-R), mouse HSP60 (ready to use, Las Vision, catalog no. MS-120-R7), and goat CD3 at 1:50 (Santa Cruz Biotechnology catalog no. sc-1127) respectively. After three washes with PBS, the sections were incubated with secondary antibodies at room temperature for 30 min. The secondary antibody used for CD5+, CD4+, and CD8+ was biotinylated goat-anti-rat (Chemicon International, Inc. catalog no. AP183B) diluted at 1:2000, and normal rat IgG was used as a negative control. The secondary antibody used for p-p38 was goat-anti-rabbit biotinylated IgG (Chemicon International, Inc. catalog no. AP187B) diluted at 1:2000, and the negative control in this case was normal rabbit IgG. The secondary antibody used for HSP60 was biotinylated goat-anti-mouse IgG1 (Chemicon International, Inc. catalog no. AP181B) diluted at 1:2000, and the secondary antibody used for CD3+ was biotinylated donkey anti-goat-IgG diluted 1:2000 (Chemicon International, Inc. catalog no. AP180B) diluted at 1:2000. The negative control for HSP60 and CD3+ was normal mouse IgG. After treatment with secondary antibodies, all sections were then treated with ABC reagents (Vector Laboratories Inc. catalog no. 6100) and finally stained with diaminobenzidine (Research Genetics Inc., now Invitrogen, Carlsbad, CA). Mouse anti-insulin monoclonal antibody, Clone Z006 (Zymed catalog no. 08-0066 Ready to use) was employed as the primary antibody for these studies. Donkey anti-mouse biotinylated IgG (Chemicon International, Inc. catalog no. AP192B) was used as the secondary antibody at a dilution of 1:2000. NeoMarkers normal mouse IgG1 (Lab Vision catalog no. NC-748-P), and 1:400 was used as negative control. After secondary antibody treatment, all sections were treated with ABC reagents (Vector Laboratories Inc.) and finally stained with diaminobenzidine (Research Genetics Inc.). Following several rinses, the sections were counterstained with hematoxylin and subsequently overslipped with permanent mounting medium.

**Histology Evaluation.** The extent of lymphocytic infiltration (intensity of insulitis) in islet cells was evaluated by histologists blinded to the treatment regimen of the samples. The middle part of the pancreas was evaluated, allowing examination of cross sections of at least 20 islets. Histopathological changes for diabetes were evaluated by the development of insulitis in this NOD model. Insulitis was evaluated by the total number of inflamed islets and the percentage of area in an islet that was infiltrated with T lymphocytes. Samples were scored as: 0, indicating no lymphocyte infiltration or absence of insulitis; 1, indicating 1 to 5% of area of islet infiltrated by lymphocytes; 2, indicating 5 to 25% of area of islet infiltrated by lymphocytes; 3, indicating 25 to 50% of the area of islet infiltrated by lymphocytes; and 4, indicating 50 to 75% of the area of islet infiltrated by lymphocytes. The total number of inflamed islets was also counted in each animal sample. Ten islets were evaluated for p-p38 and HSP60 content. p-p38 immunohistochemistry analysis was evaluated by the number of the positively stained cells in an islet and scored as: 0, indicating no positively stained cells in an islet; 1, indicating intense staining of one to five cells per islet; 2, indicating intense staining of five to 10 cells per islet; 3, indicating intense staining of 10 to 15 cells per islet; and 4, indicating intense staining of more than 15 cells per islet. HSP60 immunohistochemistry analysis was evaluated by the percentage of positively stained T lymphocytes in the inflamed islets.

**Therapeutic Studies**

**Studies on Mildly and Moderately Hyperglycemic Mice.** Four independent experiments were conducted to evaluate the effect of SD-169 on the blood glucose levels in mildly hyperglycemic mice. In the first experiment, NOD mice 13 to 14 weeks old with blood glucose levels around 150 mg/dl for 3 consecutive days were randomized to three treatment groups: group 1 (n = 7) received powdered chow (vehicle), group 2 (n = 7) received chow with low-dose SD-169 (200 mg/kg, which yields 0.6 μM exposure levels, see Results), and group 3 (n = 7) received chow with high-dose SD-169 (600 mg/kg, which yields 0.6 μM exposure levels, see Results). Treatment was maintained for 17 days. Body weights and blood glucose were measured every 3 days, and serum insulin was taken at the time of sacrifice. In the second experiment, additional data on fasting blood glucose,
glucose levels and oral glucose tolerance were collected. Mildly hyperglycemic NOD mice 13 to 14 weeks old (blood glucose levels ~150 mg/dl consecutively for 3 days) were randomized into two treatment groups: group 1 mice (n = 6) received powdered chow/vehicle, and group 2 mice (n = 6) received chow with high-dose SD-169. Treatment was for 17 days. Blood glucose levels were measured on day 17 following an overnight fast. Glucose tolerance was evaluated on day 17 following an overnight fast. Blood glucose was measured immediately prior, 30, 60, and 120 min following an oral glucose challenge (2 g/kg/body weight). In the third experiment, additional data on the effect of withdrawal of SD-169 treatment on blood glucose levels was collected. Moderately hyperglycemic NOD mice around 14 weeks old (blood glucose levels ~250 mg/dl consecutively for 3 days) were randomized into two treatment groups: group 1 mice (n = 8) received powdered chow (vehicle), and group 2 mice (n = 8) received chow with high-dose SD-169. The study lasted 47 days; SD-169 treatment was withdrawn on day 28 and blood glucose levels were monitored for an additional 19 days. In the fourth study, severely hyperglycemic NOD mice around 16 weeks old, having a blood glucose of approximately 450 mg/dl, were randomized into two treatment groups: group 1 mice (n = 6) received standard powdered chow (vehicle), and group 2 mice (n = 6) received chow containing high-dose SD-169. Treatment was maintained for 17 days. Body weight and blood glucose were measured every 3 days.

**Statistical Analysis.** Differences between SD-169 (low and high doses) and vehicle-treated groups in body weight, blood glucose, and plasma insulin were analyzed for statistical significance with one-way analysis of variance followed by post hoc Bonferroni correction. A two-tailed unpaired test was used to determine statistical significance in studies involving two treatment groups (high-dose SD-169 and vehicle). The nonparametric two-tailed test (Mann-Whitney) or nonparametric analysis of variance (Kruskal-Wallis) was used to compare vehicle and SD-169 treatment on p38 MAP kinase, HSP60, Th1, and Th2 cell populations and insulitis. All analyses were performed using InStat (GraphPad version 3.0; GraphPad Software Inc., San Diego, CA).

### Results

#### Dose Levels of SD-169

Preliminary studies demonstrated that 200 and 600 mg/kg doses of SD-169 in powdered chow result in 0.6 (low dose) and 1.8 (high dose) μM circulating concentrations, respectively, at initial and end of the studies. Food intake was not affected by the presence of drug in the diet.

**Preventive Studies.** Prediabetic NOD mice treated with SD-169 for a period of 10 weeks had higher body weights, lower blood glucose levels, and higher insulin levels compared with the vehicle-treated group (Fig. 1, A–C). There was a statistically significant and dose-dependent delay in the onset of diabetes as defined by blood glucose levels greater than 150 mg/dl (Fig. 1D). By week 18, 60% of the vehicle group had developed diabetes, whereas in the low- and high-dose SD-169-treated groups, only 26 and 15% of the mice developed diabetes, respectively.

The pancreata of NOD mice from the vehicle group showed destruction of the islets of Langerhans with massive lymphocytic infiltration at week 18 (Fig. 2A). In contrast, the pancreata of mice treated with SD-169 at both low and high doses showed only minor lymphocytic infiltration (Fig. 2, B and C). Quantitative histological assessment showed that SD-169 treatment significantly halted lymphocytic infiltration into the pancreas (Fig. 2D). In mice treated with and without SD-169, 90% of the infiltrating lymphocytes were shown by immunohistochemistry to be CD5+ T cells (Fig. 3A). Of the infiltrating T cells, 80% were CD4+ (Fig. 3B) and 20% were CD8+ (Fig. 3C), and SD-169 treatment did not alter this distribution (Figs. 3, D–F). The heavier immunohistochemical signal in the peripheral regions of the islets suggests progression from a typical peri-insulitis to destructive insulitis. Destructive insulitis is more prominent in the

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**Fig. 1.** Preventive effects of SD-169 on the development of diabetes in NOD mice. Prediabetic NOD mice treated with low (200 mg/kg) or high (600 mg/kg) doses of SD-169 for 10 weeks had higher body weights (*, p < 0.05 versus vehicle group) (A), lower blood glucose levels (*, p < 0.05; **, p < 0.01 versus vehicle group) (B), and higher insulin levels (, p < 0.05 versus vehicle group) (C) compared with the vehicle-treated group. There was a statistically significant (*, p < 0.01 versus vehicle group) and dose-dependent delay in the onset of diabetes as defined by blood glucose levels greater than 120 mg/dl (D). By 18 weeks, 60% of the mice fed standard chow (vehicle) had developed diabetes, whereas in the low- and high-dose SD-169-treated groups, only 30 and 10% of mice developed diabetes. Values are reported as the mean ± S.D. (n = 20). Open circles, vehicle group; open triangles, SD-169 at low-dose groups; hatched circles, SD-169 at high-dose groups.
vehicle-treated group, whereas peri-insulitis is apparent in the SD-169-treated groups.

Neither IFNγ (representative Th1 cytokine) nor IL-10 (representative Th2 cytokine) was detected in the peripheral blood of vehicle or treated NOD mice at 13 or 18 weeks (limit of detection 3.1 pg/ml, data not shown). SD-169 at the high dose significantly lowered both CD45RA⁺ and CD45RB⁺ cells in the spleens at the early stage of the disease (13 weeks).
weeks) but had little effect at the late stage (18 weeks). For mice treated during early stage disease, percent positive CD45RA<sup>+</sup> cells were 10 ± 1 in vehicle group and 6.7 ± 1 in SD-169 treatment group (p < 0.03), whereas intensity of CD45RB<sup>+</sup> staining was 1685 ± 70 in vehicle group and 1550 ± 40 in SD-169 group (p < 0.05) (data not shown).

HSP60 was located in the pancreatic beta cells as well as in the infiltrating T cells, in the lymphocytes inside the small blood vessel close to the pancreatic beta cells, and in the lymphocytes around the lymphatic vessel. All of these HSP60-positive T cells were confirmed by IHC stain for CD3, a surface marker for T lymphocytes. Enhanced HSP60 expression was observed in the cytoplasm of the T cells infiltrating into the pancreatic beta cell mass of the vehicle-treated group in 13- and 18-week-old NOD mice (Fig. 4A). SD-169 at high dose significantly reduced HSP60 expression in the T cells as well as in the pancreatic beta cells (Fig. 5, B and C) (data shown at 18 weeks). P-p38 MAPK expression was observed in both the cytoplasm and the nucleus of the T cells infiltrating into the pancreatic beta cell mass in 13- and 18-week-old NOD mice of the vehicle-treated group (Fig. 5A). In contrast, SD-169 significantly reduced p38 MAPK expression in the T cells (Fig. 5, B and C) (data shown at 18 weeks).

**Therapeutic Studies.** The effects of SD-169 treatment of mildly hyperglycemic NOD mice on body weight, blood glucose, and serum insulin levels are shown in Fig. 6. SD-169 treatment resulted in a dose-dependent favorable improvement in body weight (Fig. 6A). Although blood glucose levels rose significantly in the vehicle group during the 17-day treatment period, mice receiving SD-169 maintained low glucose values in a dose-dependent manner (Fig. 6B). In the high-dose SD-169 group, blood glucose levels remained constant during the entire treatment period. Although plasma insulin levels were higher in the SD-169 groups (1.09 ± 0.4 ng/ml in low-dose groups and 1.01 ± 0.2 ng/ml in high-dose groups) than in the vehicle group (0.50 ± 0.15 ng/ml), this difference did not reach statistical significance; statistical significance was demonstrated in the pancreatic insulin-positive cell numbers as determined by immunohistochemistry (Fig. 6C). At the end of the study, insulin index scores in the vehicle and SD-169 at high dose were 0.63 ± 0.80 and 30 ± 16, respectively (p < 0.001) (Fig. 6C3). In a second study of mildly hyperglycemic mice treated with either vehicle or high-dose SD-169, similar beneficial effects on body weight and blood glucose were seen. After 17 days of treatment, fasting glucose was lower in the SD-169 group as compared with the vehicle group (196 ± 18 versus 241 ± 27 mg/dl, respectively; p < 0.05). In addition, there was a statistically significant improvement in glucose tolerance at 30 min for the SD-169 versus the vehicle treatment groups (314 ± 21 and 411 ± 25 mg/dl, respectively; p < 0.001). In a third study of moderately hyperglycemic mice (blood glucose ~250 mg/dl) treated with either vehicle or high-dose SD-169, significant arrest of hyperglycemia was seen by day 28 and was associated with body weight gain (Fig. 6D). Cessation of SD-169 treatment from day 28 onwards was not associated with any further increase of blood glucose levels when mice were monitored for an additional 19 days (Fig. 6D). This is a durable effect, as evidenced by steady glucose levels and body weights for up to 47 days of evaluation. In the fourth study, we examined in severely hyperglycemic (blood glucose around 450 mg/dl) NOD mice that SD-169 had no effect on body weight, blood glucose, and serum insulin levels even when given at the high dose (data not shown).

**Discussion**

In this study, we have shown that a p38α-selective inhibitor, SD-169, can prevent the development of diabetes and alleviates mild and moderately hyperglycemic states in NOD mice. SD-169 treatment of NOD mice is associated with reduced T cell infiltration of pancreatic islets and improved β cell islet function as judged by reduced blood glucose in fed and fasted mice, improved glucose tolerance, elevated pan-

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**Fig. 4.** HSP60 expression in the T cells that have infiltrated pancreatic beta cells of vehicle (A)- and SD-169 (B)-treated NOD mice. After 10 weeks of treatment, enhanced HSP60 kinase expression was observed (dark staining) both in cytoplasm and nucleus of the T cells that had infiltrated the beta cell mass of the vehicle-treated group. In contrast, SD-169 significantly reduced HSP60 expression in the T cells. Summarized results on HSP60 expression are shown as grades (C). Treatment with SD-169 significantly decreased the HSP60 expression in the T cells (+, p < 0.01 versus vehicle group). Open bars, vehicle; hatched bars, SD-169 at high-dose group. Values are reported as the mean ± S.D. (n = 10).
creatic insulin levels, and reduced expression of p38 MAPK and HSP60. Notably, transient treatment of moderately hyperglycemia with SD-169 has a prolonged effect for at least 19 days after withdrawal of treatment. There are several possible explanations for this enduring effect. It is possible that this state is temporary, as is the honeymoon period seen in children during early insulin treatment. It may reflect a carryover inhibition of not only p38 MAPK activity but also carryover inhibition of its expression. Alternatively, this enduring effect could reflect a transient role for p38 MAPK in the development of insulitis and progression to diabetes, such that inhibition during a key period (i.e., when there are some residual beta cells preserved in the pancreas) is sufficient to reverse mild hyperglycemia and halt progression of the disease. These current data do not allow us to draw a conclusion as to the mechanism of the enduring effect but certainly provoke further interest in examining blood glucose levels and serum insulin levels beyond 40 days in SD-169-treated NOD mice. As would be expected, SD-169 has no effect on body weight, blood glucose, or serum insulin levels when mice are severely hyperglycemic, at which time the pancreas is devoid of islets.

p38 MAPK and HSP60 are believed to play important roles in T cell function, including the control of cytokine expression (Brudzynski, 1993; Schafer et al., 1999; Gregori et al., 2002; Rincon and Pedraza-Alva, 2003). In addition, p38 MAPK and/or HSP60 appear to be involved in antigen presentation and mediating the activation of T cells, thus increasing cellular immunity (Benagiano et al., 2005). The majority of infiltrating T cells express activated p38 MAPK and HSP60, both of which are reduced by SD-169 treatment. It appears, therefore, that blocking p38 activity with SD-169 attenuates HSP60 expression in the pancreatic T cells of NOD mice.

The molecular mechanisms by which p38α MAPK inhibition reduces T cell infiltration remain unclear. Consistent with previous reports, we were unable to detect Th1 or Th2 cytokines (IFNγ or IL-10) in the serum or pancreas of vehicle-treated NOD mice (Cohen, 1997), and this was also true for NOD mice treated with SD-169. We also examined various subsets of T cell that have been implicated in the disease. Previous evidence from animal experiments suggests that both CD4+ and CD8+ T cells are required for the islet destruction in NOD mice (Casteels et al., 1998). It has been reported that p38α MAPK is the major p38 MAPK isoform expressed by CD4+ T cells (Prakken et al., 2003). Our p38α-selective inhibitor, SD-169, dramatically reduced the number of T cells in the islets of NOD mice at 13 and 18 weeks, but the ratio of CD4+ to CD8+ was unchanged. This probably reflects previous evidence that p38 MAPK regulates CD4+ and CD8+ cells in the prediabetic stage only. Intriguingly, SD-169 positively regulated different populations of T cells (CD45RA+ and CD45RB+) only at the early stage (13 weeks) of the diabetes but not late stage (18 weeks). Although inconclusive by themselves, these data provide a framework for further mechanistic studies and highlight the need to examine the critical role of p38α activity in the earlier stages of autoimmune destruction of beta cells in NOD mice.

Unlike other agents that also prevent development of diabetes in NOD mice, SD-169 might prove useful as an early intervention to delay the progression of diabetes. For example, several immunosuppressive agents halt T cell infiltration into the beta cells of the islets and prevent the develop-
ment of diabetes in NOD mice (Mori et al., 1986; Mahon et al., 1993; Casteels et al., 1998; Tabatabaie et al., 2000; Shapiro et al., 2002), but have no glucose-lowering effects in moderately or even mildly hyperglycemic NOD mice. Prophylactic treatment with the p38 MAPK inhibitor FR167653 has been shown to reduce pancreatic insulitis and reduce beta cell destruction in NOD mice (Ando et al., 2004). Although both FR167653 and SD-169 are specific p38 MAPK inhibitors, SD-169 is p38α-selective, whereas FR167653 is not. Ando et al. (2004) studied p38 pharmacology in a prophylactic mode but not in a therapeutic mode. In the present therapeutic modality study, SD-169 significantly lowered blood glucose levels not only in mildly (blood glucose levels around 150 mg/dl) but also moderately (blood glucose levels around 250 mg/dl) hyperglycemic NOD mice. This indicates that SD-169 treatment may improve glucose homeostasis by preserving some beta cell mass in a moderately damaged pancreas, an effect shown to coincide with improved tolerance (i.e., return of insulin release) in response to a glucose load.

The honeymoon period in children with type 1 diabetes is characterized by preserved beta cell function due to some residual beta cells. Therefore, interruption of beta cell loss in these individuals by a p38α MAPK inhibitor may be a viable therapy. It is also presumed that early, aggressive control of blood glucose levels with p38α MAPK inhibitor should remediate the relative beta cell exhaustion and allow for short-term glucose homeostasis without exogenous insulin. Since serum autoantibodies can be detected while islet cells are being destroyed during the prediabetic period, intervention with a p38α MAPK inhibitor might be expected to help during the onset of the disease in children at high risk. Similarly, therapeutic intervention with a p38α MAPK inhibitor may be useful for adult with latent autoimmune diabetes, which can be diagnosed up to 3 years prior to full disease onset. If the autoimmune process can be detected and halted, one may be able to preserve the remaining beta cell function, thus preventing type 1 diabetes.

In summary, we report that an α-selective p38 MAPK inhibitor not only prevents the development of diabetes and alleviates mild hyperglycemia in NOD mice by inhibiting T cell infiltration and preserving beta cell mass via p38 MAPK signaling but shows a prolonged effect that continues after withdrawal of treatment. These results have bearing on current prophylactic and therapeutic protocols using p38 inhibitors in the prediabetic period of children at high risk of type 1 diabetes or in the honeymoon period, as well as in adults with latent autoimmune diabetes.

Fig. 6. Therapeutic effects of SD-169 on the blood glucose levels in mildly hyperglycemic NOD mice. Mildly hyperglycemic NOD mice treated with SD-169 for 17 days showed less weight loss than did untreated NOD mice (*, p < 0.05 versus vehicle group) compared with the vehicle-treated group (A). In vehicle-treated NOD mice, significant hyperglycemia developed by day 17 compared with its baseline value, whereas SD-169 dose dependently prevented the development of hyperglycemia, and the mice are only mildly hyperglycemic by day 17 (*, p < 0.01 versus baseline value) (B). Immunohistochemical staining for insulin-positive cells in the islets of vehicle (C1) and SD-169 (C2)-treated NOD mice. Treatment with high-dose SD-169 significantly increased pancreatic insulin-positive cells (*, p < 0.001 versus vehicle group; C3). Moderately hyperglycemic mice were treated with either vehicle or high-dose SD-169 for a period of 47 days. In vehicle-treated NOD mice, significant hyperglycemia developed by day 28 compared with its baseline value (*, p < 0.001 versus baseline value). Significant arrest of hyperglycemia at the level of treatment initiation was seen with SD-169 at high dose by day 28 (*, p < 0.01 versus vehicle group). Cessation of SD-169 treatment on day 28 was not associated with any further increase of blood glucose levels when mice were monitored for another 19 days, i.e., day 47 (**, p < 0.01 versus vehicle group) (D). Open circles, vehicle group; open lined bar/triangle, SD-169 at low-dose groups; hatched bar/circles, SD-169 at high-dose groups. Values are reported as the mean ± S.D. (n = 7 to 8).


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