Specific Inhibition of Autoimmune T Cell Transmigration Contributes to β Cell Functionality and Insulin Synthesis in Non-obese Diabetic (NOD) Mice*

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The pathogenesis of IDDM2 involves the activation of autoimmune T killer cells. Activated autoimmune T cells transmigrate from the bloodstream through the pancreatic endothelium and into the islets of Langerhans, where they destroy insulin-producing β cells. We suggested and then proved in our current work that the inhibition of T cell transmigration and homing would effectively control islet destruction and stimulate the functional recovery of the insulin-producing β cells and the regeneration of the pancreatic islets.

Mice of the NOD inbred strain develop a spontaneous disease closely resembling human IDDM and have been widely and successfully used as a model of IDDM (1–3). CD8+ T lymphocytes are involved in diabetogenesis in NOD mice; mice lacking CD8+ T cells do not develop diabetes (4, 5). The cell surface CD44 levels are elevated in activated T cells (6). Via its interactions with endothelial hyaluronan, CD44 functions as a prominent adhesion receptor in autoimmune T cell adhesion on the endothelium and the subsequent transmigration events (7–11). Membrane type-1 matrix metalloproteinase (MT1-MMP) (12, 13) is the most important cell surface-associated proteinase that contributes to the shedding CD44 in the adher- en-ant autoimmune CD8+ T cells (11, 14). As demonstrated in our earlier cell-based tests (8, 9, 14), MT1-MMP cleavage releases the extracellular domain of CD44 from T cell surfaces and inactivates the CD44 cell receptor function. Similarly, the cleavage of CD44 by MT1-MMP plays a significant role in the regulation of tumor cell migration (15, 16). By means of this regulatory proteolysis, which our earlier data suggest (8, 9, 14), MT1-MMP appears to control the severity of the diabetic disease and mediates the transition of T cell adhesion on endothelium to transendothelial migration that results in T cell homing into the pancreatic islets. The inhibition of MT1-MMP proteolysis of CD44 leads to an extended instead of a temporal adhesion of cytotoxic autoimmune T cells on the vascular endothelium. In a similar fashion, tissue inhibitor of metalloproteinases-2 (TIMP-2; a potent inhibitor of MT1-MMP) but not tissue inhibitor of metalloproteinases-1 (TIMP-1; a poor inhibitor of MT1-MMP) decreased T-cell transmigration and preserved insulin production in a type 1 diabetes organ culture model (17). The long term immobilization on the endothelium impedes the transmigration and homing efficiency of the diabetogenic CD8+ T cells. These combined events delayed the onset of the transferred diabetes in NOD mice (8). Consistent with our data, a recent publication suggests that MT1-MMP plays an important role in regulating monocyte transendothelial migration (18).

Therapies of IDDM will require the repair of immunological tolerance breakdown, restoration of insulin-producing β cell mass, or both (19). Because in IDDM the de novo developing β cell populations, which are believed to originate from stem cell precursors (20–25), are continually destroyed in the islets by the transmigrating autoimmune T cells, we hypothesized that diminishing the rate of T cell transmigration and homing would lead to both the restoration of the β cell mass (25) and a clinically relevant increase in insulin production in acutely diabetic NOD mice. Here, using acutely diabetic NOD mice and performing subsequent extensive morphometric analyses and the measurement of both the C-peptide blood levels and the pro-
insulin mRNA in the islets, we experimentally confirm that this hypothesis is correct.

MATERIALS AND METHODS

**Animals**—Mice of NOD/LtJ (NOD), C57Bl/6, and NOD.CB17-Prkdc scid (NOD-scid) strains were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in the pathogen-free animal facility of the Burnham Institute for Medical Research. All animal treatment protocols have been reviewed and approved by the Burnham Institute Animal Care Committee. NOD female mice developed diabetes in ~5 months following birth. The onset of spontaneous diabetes was identified by assessing urine glucose levels with Diastix strips (Bayer, Tarrytown, NY) and verified by blood glucose measurement using an Ascensia Elite One touch blood glucose monitor (Bayer). Mice with blood glucose levels >450 mg/dl for three consecutive measurements were considered diabetic.

**Adoptive Transfer**—Adoptive transfer of diabetes was performed by intravenous injection of freshly isolated splenocytes from diabetic NOD animals into irradiated (725 Roentgens, 24 h in advance) syngeneic NOD recipients (1.5 × 10^7 cells/animal) (26). Both males and females 6–10 weeks of age were used. Because neither sex nor age affected the incidence and the time of diabetes onset, the results were pooled. The recipients of adoptive transfer were monitored daily by blood glucose measurements for 30 days for diabetes development. Animals with blood glucose levels >450 mg/dl for three consecutive measurements were considered diabetic.

**Multidose STZ Treatment**—To induce diabetes, streptozotocin (STZ) (40 mg/kg in 0.1 M citrate buffer, pH 4.5) was injected intraperitoneally daily for 5 consecutive days into 8-week-old C57BL/6 female mice (27). Blood glucose levels were monitored every second day. Animals were considered diabetic when their blood glucose levels were >450 mg/dl in three consecutive measurements. Mice in the control group received an equal volume of sodium citrate buffer alone.

**AG3340 Treatment**—Porcine insulin (Sigma; ≥27 USP units/mg; 15–20 units/kg; one injection every 2–3 days) was injected subcutaneously into female NOD mice that had already developed acute spontaneous diabetes and also into C57BL/6 animals with STZ-induced diabetes. Insulin injections lasted for 20 days to ensure total destruction of any residual degranulated β cells (28). The spontaneously diabetic NOD- and STZ-treated C57BL/6 control animals (four and five mice/group, respectively) then continued to receive insulin alone. Experimental groups of spontaneously diabetic NOD- and STZ-treated C57BL/6 (six mice/group each) received insulin subcutaneously jointly with AG3340 intraperitoneally (5 mg/kg; one injection every 2–3 days) for 30 days, and then insulin injections were stopped in all groups, whereas experimental groups continued to receive AG3340 intraperitoneally (5 mg/kg; one injection in every 2–3 days) for the following 30 days. Every other day, animals were subjected to measurements of blood glucose. Animals with blood glucose levels >450 mg/dl for three consecutive measurements were considered diabetic and were then sacrificed.

**Intraperitoneal Glucose Tolerance Test**—Mice were fasted for 16 h. Glucose (2.0 g/kg, 20% solution in 0.9% NaCl) was administered intraperitoneally. Blood glucose was analyzed at 0, 10, 20, and 60 min after injection using a blood glucose monitor (Bayer).

**Immunohistochemistry, Morphometry, and Pancreatic Regenerative Capability Analyses**—Pancreata were weighed, fixed by immersion in 10% formaldehyde, and paraffin-embedded. Paraffin-embedded sections were stained with guinea pig anti-insulin polyclonal serum (Linco Research, St. Charles, MO) and rabbit polyclonal antibody to glucagon (DacoCytozation, Carpinteria, CA) followed by species-specific secondary horseradish peroxidase-conjugated antibodies. The Vector VIP kit (Vector Laboratories, Burlingame, CA) was used to visualize horseradish peroxidase-stained tissue. Sections were counterstained with hematoxylin, mounted, and analyzed.

Morphometry and a pancreatic regenerative capability analyses were performed using serial 5-μm-thick longitudinal paraffin sections of pancrea separated by a 50-μm interval and immunostained for insulin. The microscopic images of pancreatic sections were taken using the Olympus BX51 microscope (Olympus America, Center Valley, PA) connected through a video camera to a computer. Images were analyzed using the Metamorph (Molecular Devices, Sunnyvale, CA) and Image Pro Plus software (Media Cybernetics, Bethesda, MD). The β cell representation was measured using our assumption that all insulin-positive areas were represented by β cells. Morphometric analysis was performed using a grid system. At least 300 fields/mouse were examined. Relative β cell, non-β cell, and total exocrine tissue areas were calculated. The β cell mass was then calculated by multiplying the relative β cell representation by the corrected pancreatic weight.

For the analysis of the pancreatic regenerative capability, we used 5-μm-thick longitudinal paraffin sections of pancrea separated by a 50-μm interval. The sections were immunostained for insulin. The size of the individual functional islets, defined as the insulin-positive area of the sections, was measured using an Olympus BX51 microscope with a ×200 magnification connected through a video camera to a computer. The data were analyzed by Metamorph software and expressed in pixels (29). According to our observations, 85–90% of the functional islets in adult NOD-scid mice exceeded 80 μm (2000 pixels) in size. Infiltration-free, insulin-positive, and ≥2000 pixel in size islets were considered to be either regenerating or newly formed. These miniature islets were counted in the sections of the entire pancreas and recorded. 4–6 mice/group were analyzed by an observer under double-blinding conditions. The data were expressed as mean value ± S.E.

**Measurement of Serum Insulin and C-peptide Levels**—Mice were fasted for 16 h. Glucose (2.0 g/kg body weight, 20% solution in 0.9% NaCl) or an equal volume of 0.9% NaCl was administered intraperitoneally. The blood was withdrawn from the orbital sinus 30 min after injection. The serum insulin and the C-peptide levels were measured in triplicate using the insulin enzyme-linked immunosorbent assay kit (Crystal Chemistry, Downers Grove, IL) and the C-peptide radioimmunoassay kit (Linco), respectively. The data were expressed as mean ± S.E.

**Q-RT-PCR**—Total RNA was extracted from fresh pancreatic tissue using the RNeasy Maxi kit (Qiagen, Valencia, CA). Reverse transcription was performed using 3 μg of RNA. Quan-
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Inhibition of T Cell MT1-MMP in Spontaneously Diabetic NOD Mice—To generate proof-of-principle data that the inhibition of T cell MT1-MMP and the subsequent reduction of autoimmune T cell transmigration and homing would lead to regeneration of insulin-producing β cells, NOD female mice were first allowed to develop spontaneous IDDM. To compare the effects of an AG3340 treatment on spontaneous and STZ-induced diabetes (27), we also used multiple injections of STZ (40 mg/kg/day; intraperitoneally for 5 days) to cause diabetes in 8-week-old C57BL/6J.six mice. Diseased mice then received insulin alone (15 units/kg subcutaneously) for 20 days to ensure the complete destruction of residual degranulated β cells (28). For the next 30 days, the control mice continued to receive insulin, whereas the experimental groups received insulin jointly with AG3340 (5 mg/kg).

Because of the considerations described below, we specifically selected this hydroxamate inhibitor for our studies. AG3340/Prinomastat (3(S)-2,2-dimethyl-4[(4-pyridin-4-yloxy)-benzensulfonyl]-thiomorpholine-3-carboxylic acid hydroxamate) was used earlier in phase III trials in cancer patients and is a highly potent hydroxamate inhibitor of MMPs and especially of MT1-MMP (Kᵢ = 40 pM) (30, 31).

The Kᵢ values of AG3340 against MMP-2, MMP-3, and MMP-13 are higher, and hence, AG3340 is less efficient (~100, 300, and 200 pM, respectively). Other individual MMPs are significantly less sensitive to AG3340 inhibition (e.g., the Kᵢ values for MMP-1 and MMP-7 are 10 and 55 nM, respectively) (30, 32, 33). Our previous extensive studies involving AG3340, a recently designed thirane MMP-2/MMP-9 inhibitor SB-3CT (4-phenoxyphenylsulfonyl) butane-1,2-dithiol) (34, 35), and epigallocatechin-3-gallate (EGCG), a major natural catechin of green tea and a nonspecific inhibitor of MMPs (36–38), dem-
In addition, AG3340 caused a recovery of insulin-producing limited peri-islet insulitis in spontaneously diabetic NOD mice. An increase in the number of intact islets and the islets with glucagon. Consistent with our earlier data (9), AG3340 caused excised, sectioned, and stained with antibodies to insulin and erating or the newly formed islets, the murine pancreata were stopped at day 50. The experimental mice continued to receive AG3340 injections. Both in the control and in the experimental mice, insulin injections were treatment of acutely diabetic mice with insulin. Day 20 indicates the beginning of the treatment of the normal level of glucose observed in disease-free mice (200 g/dl) and mild hyperglycemia conditions induced diabetes. The proinsulin mRNA level was close to zero in mice with STZ-induced diabetes. In turn, the pancreatic insulin mRNA content in AG3340-treated diabetic NOD mice was accompanied by a 9-fold increase in β cell mass (0.386 ± 0.037 mg in insulin + AG3340 mice versus 0.048 ± 0.02 mg in the insulin alone NOD control; p < 0.05) (Fig. 3, B and C). In diabetic NOD mice that received the inhibitor, β cell mass was equal to 40% of the mass of healthy NOD−/− mice.

To further support our data, an observer under double-blind conditions counted the newly formed, infiltration-free, insulin-positive islets having the size of islet-like structures were predominantly observed in those spontaneously diabetic NOD animals that received the inhibitor. Similar structures were infrequent either in diabetic NOD mice that received insulin alone or in mice with STZ-induced diabetes regardless of the inhibitor treatment. These islet-like structures were free from mononuclear infiltration and produced insulin and glucagon, thus providing evidence of the functional recovery of the hormone-secreting cells. In STZ-treated mice, the insulin staining was insignificant, suggesting a complete or near complete destruction of β cells (Fig. 3A).

Because insulin synthesis is a surrogate measure of β cell mass, we directly determined the β cell mass in mice (28, 42). β cell mass was calculated from pancreatic sections made throughout the whole organ and stained for insulin. We used a morphometric analysis with a grid system to analyze at least 300 fields/animal. The significant increase in the pancreatic insulin mRNA content in AG3340-treated diabetic NOD mice was accompanied by a 9-fold increase in β cell mass (0.386 ± 0.037 mg in insulin + AG3340 mice versus 0.048 ± 0.02 mg in the insulin alone NOD control; p < 0.05) (Fig. 3, B and C). In diabetic NOD mice that received the inhibitor, β cell mass was equal to 40% of the mass of healthy NOD−/− mice.

Regeneration of β Cells in Acutely Diabetic NOD Mice—To examine the functionality of the surviving and either the regenerating or the newly formed islets, the murine pancreata were excised, sectioned, and stained with antibodies to insulin and glucagon. Consistent with our earlier data (9), AG3340 caused an increase in the number of intact islets and the islets with limited peri-islet insulitis in spontaneously diabetic NOD mice. In addition, AG3340 caused a recovery of insulin-producing β cells in the pancreatic parenchyma. Intriguingly, the small-sized, presumably rescued, regenerating, or newly formed, was 30–40% of that detected in healthy NOD−/− mice. The proinsulin mRNA level was close to zero in mice with STZ-induced diabetes.

FIGURE 1. AG3340 reduces blood glucose levels in diabetic NOD mice. A, blood glucose in spontaneously diabetic NOD mice. B, blood glucose in C56BL/six mice with STZ-induced diabetes. At the indicated times, the levels of glucose were measured with a blood glucose monitor. Day 2 indicates the beginning of the treatment of the experimental mice with AG3340. Both in the control and in the experimental mice, insulin injections were stopped at day 50. The experimental mice continued to receive AG3340 injections. Lines in A and B show the normal level of glucose observed in disease-free mice (200 g/dl) and mild hyperglycemia conditions (≥450 g/dl). N, number of mice/group. C, glucose tolerance test. Three control NOD−/− mice (open symbols) and three acutely diabetic experimental NOD mice (filled symbols) were treated for 50 days with insulin. The experimental mice began receiving AG3340 injections for 30 days starting at day 20. At day 54, mice received glucose (2.0 g/kg intraperitoneally). Blood glucose was then measured at the indicated times after injection.
In general agreement with our data, TIMP-1 has been shown to inhibit cytokine-induced apoptosis in multiple cell lines (43) and, in addition, TIMP-1 overexpression in mouse cells enhanced the replication of cells in TIMP-1 transgenic mice and contributed to the regeneration of cell mass in the STZ model of IDDM (44).

The Assessment of Immunosuppression—To determine whether AG3340 specifically represses the T cell transmigration processes as opposed to causing immunosuppression, we used an adoptive transfer of diabetes model. For this purpose, NOD mice with spontaneously developed diabetes were treated with insulin and AG3340 or insulin alone. In 25 days, the total splenocyte population was isolated from the spleens of experimental and control animals. The isolated splenocytes (1.5 × 10^7) were injected intravenously into young irradiated (725 Roentgens, 24 h in advance) syngeneic NOD recipients (six mice/group) (26). The onset of spontaneous diabetes was monitored daily by assessing urine glucose levels and verified by blood glucose measurements. Mice with blood glucose levels >450 mg/dl for three consecutive days were considered diabetic. There was no difference in the onset of diabetes among the groups, suggesting that no specific immunosuppressive effects were induced by AG3340 treatment (Fig. 4).

In conclusion, we are now confident that inhibition of T cell transmigration by the MT1-MMP antagonists including AG3340 provides a level of protection against diabetes by controlling islet-destructive autoimmunity and stimulating the
functional recovery of the insulin-producing β cells. Our data provide a rationale for conducting similar tests of the inhibitors of MT1-MMP in IDDM in humans.

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