Inhibition of Membrane Type-1 Matrix Metalloproteinase by Cancer Drugs Interferes with the Homing of Diabetic T Cells into the Pancreas*

We have discovered that clinically tested inhibitors of matrix metalloproteinases can control the functional activity of T cell membrane type-1 matrix metalloproteinase (MT1-MMP) and the onset of disease in a rodent model of type 1 diabetes in non-obese diabetic mice. We determined that MT1-MMP proteolysis of the T cell surface CD44 adhesion receptor affects the homing of T cells into the pancreas. We also determined that both the induction of the intrinsic T cell MT1-MMP activity and the shedding of cellular CD44 follow the adhesion of insulin-specific, CD8-positive, K<sup>+</sup>-restricted T cells to the matrix. Conversely, inhibition of these events by AG3340 (a potent hydroxamate inhibitor that was widely used in clinical trials in cancer patents) impedes the transmigration of diabetogenic T cells into the pancreas and protects non-obese diabetic mice from diabetes onset. Overall, our studies have divulged a previously unknown function of MT1-MMP and identified a promising novel drug target in type 1 diabetes.

Insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated, autoimmune disease. The pathogenesis of IDDM involves the activation of autoimmune T cells followed by their homing into the pancreatic islets (1). In the islets, T cells destroy insulin-producing β cells (2). The CD44 surface receptor is elevated in activated T cells and, via its interactions with endothelial hyaluronan, contributes to T cell adhesion on the endothelium and the subsequent transmigration events (3). MT1-MMP, a multifunctional membrane-tethered enzyme, functions in cancer cells as one of the main mediators of pericellular proteolytic events and directly cleaves cell receptors (4, 5). CD44, a major hyaluronan receptor, is a well-established target of MT1-MMP in tumor cells (6–9). MT1-MMP cleavage releases the extracellular domain of CD44 from the cell surfaces and inactivates the functionality of the CD44 adhesion receptor. Evidence suggests that MT1-MMP proteolysis of CD44 significantly affects the adhesion and migration of malignant cells (6, 9, 10).

Consistently, we hypothesized that MT1-MMP also targets CD44 in T cells and that these proteolytic events regulate transmigration of the diabetogenic T cells into the pancreatic islets. In agreement with our hypothesis, we have determined here that pharmacological inhibition of T cell MT1-MMP enhances adhesion of the diabetogenic T cells to the pancreatic endothelium. Conversely, the enhanced adhesion of T cells significantly diminishes their subsequent homing into the pancreatic islets. Our results also imply that the inhibition of T cell MT1-MMP is key to the delay of the onset of diabetes in non-obese diabetic (NOD) mice, a well-accepted rodent model of IDDM.

**EXPERIMENTAL PROCEDURES**

*Mice and Cells—NOD mice of NOD/LtJ strain were obtained from the Jackson Laboratory. IS-CD8<sup>+</sup> T cells (insulin-specific, CD8-positive, K<sup>+</sup>-restricted T cells of the TGNFCS clone from the pancreas of NOD mouse) (11) were maintained in Click’s medium supplemented with 5% fetal calf serum, 2 μM mercaptoethanol, 20 mM penicillin-streptomycin, 3 mg/ml L-glutamine, and 5 units/ml recombinant murine interleukin-2 (12). Every 3 weeks IS-CD8<sup>+</sup> cells were mixed with irradiated NOD splenocytes (2000 rads) loaded with the L<sup>18</sup>YLVCGERG<sup>23</sup> insulin B chain peptide (10 μg/ml) (13). The animal treatment protocols have been reviewed and approved by The Burnham Institute review committee.

*Induction of Diabetes in NOD Mice—IS-CD8<sup>+</sup> cells were incubated both with and without AG3340 (50 μM or 21 μg/ml) for 2 h and then injected intravenously into the irradiated (725 rads, 24 h in advance), 5–8-week-old mice (1 × 10<sup>7</sup> cells/animal). Mice were monitored for 21 days. On days 0, 2, 4, 6, 8, and 10 following the injection of the cells, mice received intraperitoneal injection with AG3340 (30 mg/kg or 1 mg/kg) or phosphate-buffered saline alone. The onset of diabetes was identified by assessing urine glucose levels with Diastix strips. Mice with urine glucose levels of ≥2000 mg/dl for 3 consecutive days were considered diabetic. AG3340 (molecular mass = 421 Da) was a kind gift of Dr. Peter Baciu (Allergan, Irvine, CA). According to numerous publications (e.g. Ref. 14), the level of glucose in the urine closely follows that in the blood. The measurement of glucose in urine is a widely accepted method to follow the development of diabetes in NOD mice (15).

*Fluorescent Tracing and Morphometric Analysis—For trafficking studies, IS-CD8<sup>+</sup> T cells were incubated at 1 × 10<sup>7</sup> cells/ml for 30 min at 37 °C in the dark in complete Click’s medium containing 5% fetal calf serum and 0.0075 mg/ml of the fluorescent dye 1,1′-disodicyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). After incubation, the cells were washed three times with phosphate-buffered saline to remove excess DiI. Labeled IS-CD8<sup>+</sup> cells (1 × 10<sup>7</sup>) were injected intravenously in 0.2 ml of phosphate-buffered saline into irradiated (725 rads, 24 h in advance) NOD mice. Mice were sacrificed 24 h after injection of DiI-labeled cells. The spleen and the pancreata were excised and fixed in 0.1 M periodate-lysine-paraformaldehyde phosphate buffer. The organs were next sucrose-saturated,
MT1-MMP Regulates Pancreatic Homing of T Cells

MT1-MMP Sheds Cellular CD44—We determined that MT1-MMP proteolysis of T cell CD44 regulates adhesion and subsequent transmigration and homing of T cells into the pancreas.

FACS analyses with MT1-MMP and CD44 antibodies and fluorescent isothiocyanate-labeled hyaluronan demonstrated the presence of high levels of cell surface-associated MT1-MMP and CD44 in IS-CD8<sup>+</sup> cells in suspension (Fig. 1A). IS-CD8<sup>+</sup> cells recognize an insulin B chain-derived L<sub>1</sub>LVLVGERG<sub>23</sub> peptide in the context of the K<sub>i</sub> major histocompatibility complex class I molecule (13). Injection of IS-CD8<sup>+</sup> cells induces diabetes in sub-lethally irradiated NOD/LtJ mice in 1 week (12). NOD mice are widely used as the best animal model of IDDM (20). Because the Ab815 antibody against MT1-MMP recognizes the hinge domain of the protease, FACS tests do not distinguish the pro-enzyme, the activated enzyme forms, and the inert complexes of MT1-MMP with tissue inhibitors of metalloproteinases including tissue inhibitor of metalloproteinase-2.

The levels of CD44 were significantly reduced in the majority of IS-CD8<sup>+</sup> cells co-incubated with the external, purified, catalytically potent MT1-MMP-CAT (Fig. 1A). This treatment did not affect the levels of other T cell receptors including CD3, CD8, CD29, and CD49 or the viability of T cells (data not shown). To support these observations, we also surface-labeled IS-CD8<sup>+</sup> cells with membrane-impermeable biotin and then co-incubated them with MT1-MMP-CAT. The liberated, soluble CD44 fragments were next captured on streptavidin-agarose beads and detected by Western blotting. These data confirmed that during co-incubation of MT1-MMP-CAT with IS-CD8<sup>+</sup> cells, the limited quantities of MT1-MMP-CAT released by the E1A and MMP-2 cDNAs (19) were either allowed to adhere for 4 h in serum-free unsupplemented Click’s medium to plastic coated with 2% gelatin or kept in solution. Under these experimental conditions, the vast majority of cells became attached to gelatin. In 4 h, media samples (30 μl each) were withdrawn and analyzed by gelatin zymography (17) to identify the proteolytic activity and the activation status of MMP-2. The catalytic domain of MT1-MMP (MT1-MMP-CAT; 3 μg) was co-incubated for 2 h at 37 °C with IS-CD8<sup>+</sup> cells (1 x 10<sup>6</sup>) in 0.2 ml of 50 mM HEPES, 10 mM CaCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>, and protease inhibitor mixture containing phenylmethylsulfonyl fluoride (1 mM) and aprotinin (1 μg/ml each). Biotin-labeled CD44 was captured from the cell lysate and from the medium aliquots on streptavidin-agarose beads. The captured samples were examined by Western blotting with the CD44 (clone IM7.8.1) antibody to determine the released, soluble, CD44 ectodomain in the medium samples and the residual, membrane-anchored, cellular CD44 in the cell lysates.

MT1-MMP-dependent MMP-2 Activation and Gelatin Zymography—IS-CD8<sup>+</sup> cells (1 x 10<sup>6</sup>) were either allowed to adhere for 4 h in serum-free unsupplemented Click’s medium to plastic coated with 2% gelatin or kept in solution. Under these experimental conditions, the vast majority of cells became attached to gelatin. In 4 h, media samples (30 μl each) were withdrawn and analyzed by gelatin zymography (17) to identify the proteolytic activity and the activation status of MMP-2. The catalytic domain of MT1-MMP (MT1-MMP-CAT; 3 μg) was co-incubated for 2 h at 37 °C with IS-CD8<sup>+</sup> cells (1 x 10<sup>6</sup>) in 0.2 ml of 50 mM HEPES, 10 mM CaCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>, and protease inhibitor mixture containing phenylmethylsulfonyl fluoride (1 mM) and aprotinin (1 μg/ml each). Biotin-labeled CD44 was captured from the cell lysate and from the medium aliquots on streptavidin-agarose beads. The captured samples were examined by Western blotting with the CD44 (clone IM7.8.1) antibody to determine the released, soluble, CD44 ectodomain in the medium samples and the residual, membrane-anchored, cellular CD44 in the cell lysates.

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IS-CD8<sup>+</sup> cells co-incubated with MT1-MMP-CAT were also labeled with a fluorescent DiI dye and then injected into irradiated NOD mice. In 24 h, labeled cells were counted in the
pancreatic islets. MT1-MMP proteolysis of CD44 caused an ~4.5-fold decrease in cell homing and almost a 2-fold delay of the onset of diabetes in mice (Fig. 1C). These results suggest that cleavage of T cell CD44 by the external MT1-MMP-CAT decreased the number of the IS-CD8^+ cells that were capable of adhering to hyaluronan of the pancreatic endothelium. Consequently, following co-incubation with MT1-MMP-CAT, the number of transmigrating cells was also low.

**MT1-MMP Is Activated in Adherent IS-CD8^+ Cells**—Endogenous MT1-MMP is latent in non-adherent IS-CD8^+ cells, whereas adhesion of IS-CD8^+ cells induces the activation of MT1-MMP, the cleavage of CD44, and the stimulation of T cell transmigration. Thus, IS-CD8^+ cells were capable of activating MMP-2, the enzyme known to be directly activated by MT1-MMP, only after their adhesion to gelatin (Fig. 2). Non-adherent cells did not activate MMP-2. In agreement, release of the CD44 proteolytic fragments into medium was detected only in adherent IS-CD8^+ cells. CD44 remained intact in non-adherent cells. GM6001 blocked both the activation of MMP-2 and the shedding of CD44 in adherent cells (Fig. 2). These results suggest that MT1-MMP proteolysis of CD44 is induced only following adhesion of the diabetogenic cells to the substratum. It is likely that, following adhesion, activated MT1-MMP cleaves CD44, and this event promotes the activation of T cells through the endothelium and their homing into the pancreatic islets. Conversely, we suggest that inhibition of MT1-MMP enhances the adhesion of T cells and represses their transmigration efficiency.

**Inhibition of MT1-MMP Represses the Diabetogenicity of IS-CD8^+ Cells**—To confirm the role of MT1-MMP in T cell transmigration, homing, and diabetogenesis, we used another potent hydroxamate inhibitor, AG3340 (21). AG3340 inhibits MT1-MMP with a Ki in a sub-nanomolar range. AG3340 was used in cancer Phase I–III clinical trials (22). To evaluate AG3340, we used both IS-CD8^+ cells and the splenocytes isolated from newly diabetic NOD mice (12). The cells were co-incubated with AG3340 or left untreated and then injected in NOD mice, which then received AG3340 (30 mg/kg and 1 mg/kg) or solvent alone (control). AG3340 at a concentration as low as 1 mg/kg delayed the onset of diabetes ~2-fold compared with the control (Fig. 3A). These data are consistent with earlier work showing that inhibiting CD44 with neutralizing antibodies protects NOD mice from IDDM (23).

In agreement, AG3340, by increasing the number of IS-CD8^+ cells that remained firmly adhered to the hyaluronan of the pancreatic endothelium, significantly delayed the process of T cell entry into the pancreatic islets. IS-CD8^+ cells co-incubated with AG3340 and labeled with Dil were injected into NOD mice. In 24 h, labeled IS-CD8^+ cells were counted both at the periphery and inside the islets (Fig. 3, B and C). In the presence

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**Fig. 2.** Proteolytically active MT1-MMP activates MMP-2 and cleaves cellular CD44 in adherent IS-CD8^+ cells. IS-CD8^+ cells were either allowed to adhere to plastic coated with gelatin or kept in solution. Top panel, cells adherent to gelatin-coated plastic (A) and non-adherent cells in suspension (NA) were co-incubated with purified MMP-2 (MMP-2 alone; no cells). In 4 h, media samples were withdrawn and analyzed by gelatin zymography to identify the proteolytic activity and the activation status of MMP-2. To observe the activation of MMP-2 naturally synthesized by T cells, no external MMP-2 was added to the two samples on the left. P, I, and E, the 68-kDa pro-enzyme, the 64-kDa intermediate, and the active 62-kDa mature enzyme of MMP-2. Bottom panel, cells were either surface-biotinylated and allowed to adhere to gelatin or kept in suspension. Cell lysate and medium aliquots were captured with streptavidin-agarose beads. CD44 was analyzed in the captured samples by Western blotting with a CD44 antibody.

**Fig. 3.** A hydroxamate inhibitor, AG3340, inactivates MT1-MMP, blocks CD44 shedding in T cells, and delays the onset of transferred diabetes in NOD mice. A, AG3340 delays the onset of adoptively transferred diabetes in NOD mice. IS-CD8^+ cells and the splenocytes were each injected intravenously into NOD mice (1 × 10^7 and 1.5 × 10^7 cells/mouse, respectively; 6 mice/group). On day 0, 2, 4, 6, 8, and 10 following injection of the cells, mice received intraperitoneal injection with AG3340 (30 and 1 mg/kg). B, AG3340 inhibits the transmigration of IS-CD8^+ cells into the pancreatic islets. IS-CD8^+ cells were co-incubated for 2 h with and without AG3340 (50 µM or 21 µg/ml) and then labeled with Dil. The labeled cells were then injected intravenously into NOD mice. In 24 h, the labeled cells at the entrance of the islet and within the pancreatic islets were each counted in the cryostat sections of the entire pancreas (12). n, total number of islets in each experimental group. C, representative images of the pancreatic islets from NOD mice that received injection with Dil-labeled IS-CD8^+ cells. Images were taken 24 h after injection. Dotted line surrounds the islet. White solid line indicates the islet-relevant area, within which the Dil-labeled cells were counted. Bottom panel, cells were pre-incubated with AG3340; top panel, intact cells. Note that AG3340 blocks the entrance of IS-CD8^+ cells into the islet. D, MT1-MMP proteolysis dynamically regulates the functionality of T cell CD44 in diapedesis. Low levels of MT1-MMP stimulate adhesion of T cells to the hyaluronan-rich endothelium. After T cell adhesion, T cell MT1-MMP is activated. High levels of MT1-MMP activity cause a CD44 deficiency. This event stimulates the transendothelial migration of T cells. Persistent CD44 excess reduces T cell homing and diapedesis.
of AG3340, T cells were detected at the islet entrance, and a smaller number of cells were found inside the islets. In the absence of AG3340, the situation was reversed, and T cells efficiently transmigrated into the islets. Our findings suggest that inhibition of T cell MT1-MMP is key to delaying the onset of diabetes in a well-accepted model of IDDM in rodents. The putative mechanism by which MT1-MMP proteolysis of CD44 regulates diapedesis is explained in Fig. 3D.

Interestingly, injection of the broad spectrum MMP inhibitor KB-R7785 at 100 mg/kg twice daily (i.e. 200 mg/kg/day) for 4 weeks resulted in a significant decrease in plasma glucose levels in a rodent model of type II diabetes in KKAy mice. The proposed mechanism and the target proteins were, however, distinct from those determined in our model of type I diabetes (24). In addition, the dosages of the inhibitors used in our work were ~100-fold lower. Additional studies that suggested the importance of MMP action in diabetes complications including cataract development, proteinuria, and periodontitis were reported by Ryan et al. (25). Overall, these earlier works and our studies imply that the broad spectrum MMP inhibitors may be used to control diabetes by targeting MT1-MMP and probably also disintegrin and metalloproteinase (ADAM)-like proteases (8).

We conclude that pharmacological inhibition of easily accessible, T cell surface-associated MT1-MMP by low dosages of the readily available anticancer hydroxamate class drugs will reduce transmigration of cytotoxic T cells into the pancreatic islets. We believe that our results have the potential for therapeutic translation and need further testing (which is in progress) in a spontaneous animal model of IDDM.

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