Poly (ADP-Ribose) Polymerase Inhibition Prevents Spontaneous and Recurrent Autoimmune Diabetes in NOD Mice by Inducing Apoptosis of Islet-Infiltrating Leukocytes

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Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that consumes NAD in response to DNA strand breaks. The PARP inhibitor nicotinamide prevents NAD consumption and protects islet β-cells from chemically induced necrosis but not cytokine-induced apoptosis. Therefore, it is unclear how nicotinamide protects NOD mice from autoimmune diabetes in which apoptosis is the mode of β-cell death. To investigate the mechanism of diabetes prevention by PARP inhibition, we studied the effects of a novel, potent PARP inhibitor, PJ34, a phenanthridinone derivative, on diabetes development in NOD mice and on diabetes recurrence in diabetic NOD mice transplanted with syngeneic islets. PJ34 administration from age 5 or 15 weeks significantly decreased insulitis, β-cell destruction and diabetes incidence, and protection from diabetes continued for 12 weeks after PJ34 therapy was stopped. Similarly, syngeneic islet graft survival was prolonged and outlasted therapy in PJ34-treated mice. Immunohistochemical studies revealed significantly fewer leukocytes in islet grafts of PJ34-treated mice, together with increased apoptosis of these cells and decreased expression of the T helper 1–type cytokine interferon (IFN)-γ. These results suggest that PARP inhibition protects against autoimmune β-cell destruction in NOD mice by inducing apoptosis of islet-infiltrating leukocytes and decreasing IFN-γ expression in the islets. Diabetes 52: 1683–1688, 2003

Type 1 diabetes is characterized by a leukocytic infiltration of the pancreatic islets of Langerhans and autoimmune destruction of islet β-cells. Nonobese diabetic (NOD) mice develop spontaneous disease similar to human type 1 diabetes. A variety of effector mechanisms that could lead to β-cell death in NOD mice have been identified, including CD4+ and CD8+ T-cells, proinflammatory cytokines, and free radicals. The main mode of β-cell death by one or more of these cytotoxic effector mechanisms is apparently by apoptosis (1–3).

Apoptosis is an active form of cell death in which the cell undergoing apoptosis contributes to its own demise (4). Cell death can also involve DNA strand breaks, activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), consumption of NAD, and a decrease in cellular ATP levels (5,6). When ATP levels decrease below a critical threshold following proapoptotic insults, necrotic death ensues; if ATP levels are at least partially preserved, the apoptotic program can be completed (7). PARP inhibitors prevent NAD consumption and decreases in cellular ATP content, thereby preventing necrosis and either rescuing the cells or allowing apoptosis to proceed (5,6). For example, studies with isolated islets in vitro have shown that the PARP inhibitor nicotinamide protects islet β-cells from chemically induced necrosis but not cytokine-induced apoptosis (8). Also, nicotinamide protects NOD mice from autoimmune diabetes (9); however, the mechanism of this protection is unclear because apoptosis is apparently the main mode of β-cell death in NOD mice (1–3), and PARP inhibitors do not prevent cytokine-induced apoptosis of β-cells (8).

In the present study, we investigated the mechanism of diabetes prevention by a potent novel PARP inhibitor. We found that PARP inhibition prevented diabetes by reducing β-cell apoptosis; however, this was the indirect result of inducing apoptosis and deletion of islet-infiltrating leukocytes.

RESEARCH DESIGN AND METHODS

Animals. Female NOD mice, 4 weeks of age, were purchased from Taconic (Germantown, NY). The mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care.

Diabetes prevention studies. Female NOD mice were treated with a PARP inhibitor, PJ34, or PBS vehicle. PJ34, the hydrochloride salt of N-(5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide, was synthesized as described (10–12). PJ34 is based on a modified phenanthridinone structure and is ~10,000 times more potent than the prototypical PARP inhibitors nicotinamide and 3-aminobenzamide (10–12). The mice were treated with 15 mg/kg PJ34 or PBS vehicle, injected intraperitoneally twice a day. This treatment regimen has previously been shown to inhibit PARP activation in various tissues in rodent models in vivo (10–12). Treatments were begun at age 5 weeks (early insulitis) and 15 weeks (advanced insulitis, just before diabetes.
The mice were monitored daily for diabetes onset by urine testing using Keto-Diastix (Bayer, Etobicoke, ON, Canada). Diabetes was diagnosed by the presence of glucosuria (>6 mmol/l) and ketonuria (>1.5 mmol/l) as well as a tail vein blood glucose ≥12 mmol/l on two consecutive days, measured by glucometer (Glucometer Elite, Bayer). In the first study, PJ34 and vehicle treatments were begun at age 5 weeks, and diabetes incidence was monitored to age 30 weeks. In the second study, PJ34 and vehicle treatments were begun at age 5 weeks; at age 15 weeks, the mice were killed by sodium pentobarbital overdose and pancreata were collected for histological examination and insulin content assay. In the third study, PJ34 and vehicle treatments were begun at age 15 weeks and stopped at age 30 weeks; diabetes incidence was monitored until age 47 weeks.

**Islet transplantation studies.** Female NOD mice were allowed to develop diabetes, without any treatments, and then were treated with daily subcutaneous injections of 1.0 units of a 1:1 mix of regular and NPH pork insulin for 4–6 weeks before receiving transplants of syngeneic islets. Islets were isolated from 4-week-old female NOD mice by collagenase digestion of the pancreas and Ficoll density gradient centrifugation and then hand-picked (13). A total of 500 islets (pooled from three to four donor NOD mice) were transplanted under the left renal capsule in each diabetic NOD mouse, according to a previously described procedure (14). The mice were treated, from the time of transplantation, with 15 mg/kg PJ34 or vehicle, injected intraperitoneally twice a day. Transplantation was considered successful if the nonfasting blood glucose returned to normal (<7.0 mmol/l) within the first 24 h after transplantation. Urine was monitored daily and if glucosuria and ketonuria appeared, then tail vein blood glucose was measured. Graft rejection was diagnosed by return of hyperglycemia (blood glucose ≥12 mmol/l) accompanied by glucosuria and ketonuria on 2 consecutive days. In the first study, the mice were monitored for diabetes recurrence (islet graft destruction). PJ34 and vehicle treatments were stopped at 35 days after islet transplantations, while the mice continued to be monitored for diabetes recurrence. In the second study, diabetic NOD mice received syngeneic islet transplants and were treated with PJ34 or vehicle, as in the first study, and then the islet grafts were removed at 10–14 days after transplantation and examined by immunohistochemistry for cell composition and by RT-PCR for cytokine mRNA expression.

**Histological studies.** Pancreata were divided longitudinally and one-half was fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm, mounted on slides, and stained with hematoxylin and eosin. Coded slides were read by light microscopy. Islet inflammation (insulitis) was graded 0 to 3, according to the extent of islet infiltration by leukocytes: 0 = none, 1 = only peri-islet leukocytes, 2 = ≤50% islet area infiltrated by leukocytes, 3 = >50% islet area infiltrated. A mean insulitis score was calculated for each pancreas, by dividing the sum of the insulitis score for individual islets by the number of islets examined (≥30 per pancreas).

**Pancreas insulin assay.** The other half of each pancreas was weighed, minced with fine scissors, and incubated in acidified ethanol (75% ethanol, 1.5% 12 mol/l HCl, and 23.5% H2O) for 18 h at 4°C to extract insulin from cells. The ethanol extracts were diluted in insulin assay buffer, and insulin was measured using a radioimmunomassay with rat insulin as standard (Linco Research, St. Charles, MO).

**Islet graft cell preparations.** Islet grafts were removed and transferred into Ependorf tubes containing 500 μl Ca2+/Mg2+-free PBS with 0.2 mg/ml EDTA (cell dissociation buffer; Gibco-BRL, Burlington, ON, Canada). While on ice, the islet grafts were cut into small pieces with fine scissors and dissociated into single cells by incubation in the cell dissociation buffer at 35°C for 20 min, followed by syringing through progressively narrower gauge needles (sizes 16–22). Dissociated cells were washed twice in PBS, pH 7.4, and total cell counts were determined using a Neubauer hemacytometer. Cells were identified from the same cDNA preparation.

**Immunohistochemical studies.** Leukocytes and islet β-cells isolated from the grafts were identified by immunohistochemical methods as previously described (15). Briefly, cells were thawed and treated to block nonspecific binding with peroxidoxblock at 25°C for 10 min, followed by Blocker block kit (Zymed Labs, San Francisco, CA) at 25°C for 2 h. Primary antibodies were a rat monoclonal antibody (mAb, Leu-7, Pharmingen) to mouse total leukocytes (CD45+ cells) or rat IgG2 control antibody (Cedarlane, Hornby, ON, Canada), and a guinea pig anti-insulin antibody (Linco) or guinea pig control antibody. Secondary antibodies were biotinylated goat anti-rat mouse-absorbed IgG (for CD45+ cells) and biotinylated goat anti-guinea pig Ig (for insulin+ cells). Next, cells were incubated with streptavidin–alkaline phosphatase conjugate, and then the phosphatase Fast-blue chromogen. Leukocytes (CD45+ cells) were stained blue on the surface and, on other slides, β-cells (insulin+) were stained blue in the cytoplasm.

**RT-PCR analyses.** RNA was extracted from islet grafts by a modification of the guanidinium thiocyanate method (18), and interferon (IFN)-γ, interleukin (IL)-2, and IL-4 mRNA transcripts were measured in a semiquantitative RT-PCR assay, as described (19). The values obtained for each cytokine PCR product were normalized as a percentage of cyclophilin PCR product amplified from the same cDNA preparation.

**Statistical analyses.** Diabetes incidence data were compared for significant differences by Fisher’s exact test. Islet graft survival times (median days after transplantation) were compared for significant differences by the Mann-Whitney rank sum test. All other data are expressed as means ± SE, and differences between means were analyzed by Student’s unpaired t test; P < 0.05 was considered significant.
with PJ34, from age 5 weeks, delayed diabetes onset to age 16 weeks, and diabetes incidence at age 30 weeks was 33% (6 of 18 mice) ($P = 0.0059$ vs. vehicle).

In a separate study, NOD mice were similarly treated with PJ34 and vehicle, from age 5 weeks, and at age 15 weeks pancreata were collected from normoglycemic mice in both groups. PJ34 significantly decreased leukocytic infiltration of islets (insulitis) and significantly increased pancreatic insulin content (Fig. 2).

Next, NOD mice were treated with PJ34 starting at age 15 weeks, just before diabetes onset. Diabetes incidence was significantly reduced at age 30 weeks in PJ34-treated mice (42%, 13 of 31) compared with vehicle-treated mice (80%, 20 of 25) ($P = 0.0061$) (Fig. 3). Furthermore, diabetes incidence remained at 42% for 12 weeks after PJ34 therapy was stopped at age 30 weeks, then the incidence increased to the level observed in vehicle-treated mice by age 46 weeks.

**Islet transplantation study.** We investigated the ability of PJ34 to block an established autoimmune response by transplanting syngeneic islets into diabetic NOD mice. We found that diabetes recurred in all six mice treated with vehicle by day 18 after islet transplantation (median islet graft survival, 12 days), whereas diabetes recurrence was delayed for as long as 85 days after islet transplantation in PJ34-treated mice (median islet graft survival, 68 days; $P = 0.0007$ vs. vehicle) (Fig. 4). Also, 6 of 10 PJ34-treated mice (60%) that were normoglycemic when PJ34 treatment was stopped at 35 days after islet transplantation remained normoglycemic for another 25 to 50 days before diabetes recurred.

**Phenotype, PARP activity, and apoptosis of cells in islet grafts.** Immunohistochemical analysis of the cell composition of islet grafts revealed that PJ34 therapy significantly decreased the number of leucocytes (CD45$^+$) in islet grafts, and $\beta$-cells (insulin$^+$) were significantly increased (Fig. 5). The expected effect of PJ34 to inhibit PARP enzyme activity was detected by the biotinylated NAD cytochemical method, and this showed that PJ34 significantly decreased PARP activity in both leucocytes and $\beta$-cells in islet grafts. In contrast, apoptotic leucocytes (TUNEL$^+$ CD45$^+$ cells) were significantly increased whereas apoptotic $\beta$-cells (TUNEL$^+$ insulin$^+$) were significantly decreased in islet grafts from PJ34-treated mice (Fig. 5). Also, PJ34 therapy induced apoptosis of leucocytes (TUNEL$^+$ CD45$^+$ cells) in the spleen in these experiments: 40 ± 2% of CD45$^+$ splenic cells were TUNEL$^+$ in PJ34-treated mice compared with 19 ± 2% of CD45$^+$ cells that were TUNEL$^+$ in spleens of vehicle-treated mice (means ± SE; $n = 6$; $P < 0.01$), and total CD45$^+$ splenic cells were somewhat decreased in PJ34-treated mice (170 ± 8 × 10$^6$ cells) compared with vehicle-treated mice (184 ± 6 × 10$^6$ cells; NS).

**Cytokines expressed in islet grafts.** RT-PCR analysis of cytokine mRNA levels in islet grafts revealed that IFN-$\gamma$ mRNA expression was significantly decreased in PJ34-treated mice, whereas IL-4 and IL-10 mRNA levels were not significantly changed (Fig. 6). Also, the ratio of Th1/Th2-type cytokines (IFN-$\gamma$/IL-4 + IL-10) expressed in islet grafts of PJ34-treated mice (0.6 ± 0.2, $n = 12$) was...
significantly decreased compared with that in vehicle-treated mice (1.2 ± 0.3, n = 9; P < 0.05).

**DISCUSSION**

In this study, we found that a novel PARP inhibitor, PJ34, significantly delayed and decreased the incidence of autoimmune diabetes development in diabetes-prone NOD mice, as well as disease recurrence in diabetic NOD mice transplanted with syngeneic islets. Interestingly, diabetes prevention outlasted administration of the PARP inhibitor in both experimental models. This suggested that PARP inhibition may have targeted immune effector mechanisms responsible for autoimmune β-cell destruction. This interpretation is supported by the following findings. First, PJ34 significantly decreased leukocytic infiltration of pancreatic islets in diabetes-prone NOD mice, as well as the number of leukocytes that infiltrated syngeneic islets transplanted into diabetic NOD mice. Second, PJ34 inhibited PARP activity in leukocytes and significantly increased the numbers of these cells undergoing apoptosis in islet grafts, which could account for their reduced numbers in islet grafts of PJ34-treated mice. Third, expression of IFN-γ, a Th1-type cytokine, was significantly decreased in islet grafts of PJ34-treated mice, whereas expression of IL-4 and IL-10, Th2-type cytokines, was not changed. Taken together, these findings suggest that PARP inhibition may protect against autoimmune β-cell destruction in NOD mice as a secondary consequence of inducing apoptosis of islet-infiltrating leukocytes and decreasing IFN-γ expression in the islets. Because PJ34-induced leukocyte apoptosis was not restricted to islet grafts, but also occurred in spleens, it is likely that apoptosis of islet-infiltrating leukocytes was a direct result of PARP inhibition in leukocytes and not secondary to reduced β-cell death.

Previous studies have shown that the PARP inhibitor nicotinamide protects NOD mice from developing diabetes (9,20). In the latter study, nicotinamide was reported to prevent cytophosphamid-induced diabetes in NOD mice by reducing β-cell apoptosis (20). It was not known, however, how nicotinamide prevented β-cell apoptosis because studies with isolated islets in vitro have shown that nicotinamide protects islet β-cells from chemically induced necrosis but not cytokine-induced apoptosis (8). Our study reveals that despite the inhibition of PARP in islet β-cells by PJ34, β-cell apoptosis was prevented rather than increased as would be expected and as was observed in leukocytes in which PARP was inhibited. This suggests that PARP inhibition in vivo prevents β-cell apoptosis indirectly by inducing apoptosis and deletion of islet-infiltrating leukocytes, including IFN-γ-producing cells. The mechanism of leukocyte-induced apoptosis of β-cells does not appear to be cytokine-mediated, because nicotinamide prevents β-cell apoptosis in NOD mice in vivo (20) but does not prevent cytokine-induced apoptosis of β-cells in vitro (8). On the other hand, nicotinamide protects islet β-cells from chemically induced necrosis (8). Therefore, PARP inhibition by PJ34 may have protected β-cells from necrosis, as well as from apoptosis; however, it is not known to what extent, if any, necrosis contributes to β-cell death in autoimmune diabetes.

PARP activation and consequent depletion of cellular NAD⁺ and ATP contents are important pathogenetic fac-

**FIG. 5.** A PARP inhibitor, PJ34, induces apoptosis of leukocytes and prevents apoptosis of β-cells in syngeneic islet grafts in NOD mice. Islet grafts were collected from normoglycemic NOD mice treated with twice-a-day i.p. injections of 15 mg/kg PJ34 (n = 8) or vehicle (n = 7) at 10–14 days after islet transplantation. Leukocytes (CD45⁺ cells) were significantly decreased and β-cells (insulin⁺) were significantly increased in islet grafts from PJ34-treated mice. Both leukocytes and β-cells exhibiting PARP activity were significantly decreased by PJ34 treatment. In contrast, apoptotic leukocytes (TUNEL⁺ CD45⁺ cells) were significantly increased, whereas apoptotic β-cells (TUNEL⁺ insulin⁺) were significantly decreased, in islet grafts from PJ34-treated mice. Values are means ± SE. *P < 0.05, **P < 0.01 vs. vehicle.

**FIG. 6.** Cytokine mRNA expressions in syngeneic islet grafts collected from normoglycemic NOD mice treated with twice-a-day i.p. injections of 15 mg/kg PJ34 (n = 12) or vehicle (n = 9) at 10–14 days after islet transplantation. IFN-γ mRNA is significantly lower in islet grafts of PJ34-treated mice than vehicle-treated mice. Data are means ± SE. *P < 0.05.
tors leading to cell necrosis in various forms of reperfusion injury, shock, inflammation, neurodegeneration, and vascular diseases, and animals treated with PARP inhibitors or those with genetic absence of the PARP enzyme are resistant to these disorders (6,21,22). Similarly, PARP-deficient mice are resistant to toxic diabetes elicited by a single high dose (23–25) and to multiple low doses (26) of streptozotocin. In contrast, a recent study reported that NOD mice with a disrupted PARP-1 gene were not protected from either spontaneous or cyclophosphamide-accelerated diabetes (27). Although this finding is evidence against a role for PARP activation in the pathogenesis of autoimmune diabetes, the PARP-1-deficient NOD mice still expressed PARP-2, and this enzyme may have replaced the deficient PARP-1 activity in PARP-1-knockout NOD mice (27).

Regarding clinical application of PARP inhibitors to prevent type 1 diabetes, preliminary studies suggested beneficial effects (28–30); however, multicenter placebo-controlled clinical trials recently concluded that nicotinamide did not significantly decrease the progression to diabetes in individuals at risk for the disease (31 and E.A. Gale, unpublished observations). Although these findings suggest that PARP activation does not play a role in the pathogenesis of autoimmune diabetes, it is important to note that the nicotinamide has a low potency as a PARP inhibitor. Also, at the doses administered in humans (1.5–3.0 g/day), nicotinamide induces insulin resistance; therefore, a therapeutic effect on preserving β-cell function and glucose homeostasis may be missed (32).

The PARP inhibitor that we administered to NOD mice in the present study, PJ34, is a novel potent PARP inhibitor based on a modified phenanthridinone structure and is ~10,000 times more potent than the prototypical PARP inhibitors nicotinamide and 3-aminobenzamide (10–12). PJ34 was previously evaluated in a cell-free PARP assay, utilizing NAD+ and purified PARP enzyme. PJ34 dose-dependently inhibited PARP activity, with a concentration at half-maximal stimulation (EC50) of 20 nmol/l. The EC50 of the prototypical PARP inhibitor 3-aminobenzamide was 20 μmol/l (10). Also, the dose of nicotinamide (500 mg/kg per day) that prevents diabetes development in NOD mice (9,20) is much greater than the dose of PJ34 (30 mg/kg per day) that prevented diabetes development and recurrence in the present study. Therefore, PARP inhibitors of the phenanthridinone series such as PJ34 may be superior to nicotinamide as candidates for type 1 diabetes prevention.

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