Ectopic PDX-1 expression in liver ameliorates type 1 diabetes

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Abstract

Type 1 diabetes mellitus (T1DM) results from a specific autoimmune mediated destruction of the pancreatic β-cells. PDX-1 induced developmentally redirected liver cells were suggested to restore the ablated pancreatic function in chemically induced diabetes. However, developmentally redirected liver cells, may have acquired along with the desired β-cell characteristics and functions, also undesired sensitivity to autoimmune attack and therefore may be inefficient in ameliorating T1DM.

This study analyzes whether subjects with β-cell autoimmunity could benefit from Ad-CMV-PDX-1 gene therapy. Using the model of cyclophosphamid-accelerated diabetes in non-obese diabetic (CAD-NOD) mice, we report that recombinant adenovirus mediated PDX-1 gene therapy, ameliorates hyperglycemia in CAD-NOD mice.

Our data demonstrate that 43% of the overtly diabetic CAD-NOD mice treated with Ad-CMV-PDX-1 became normoglycemic and maintained a stable body weight. Ectopic PDX-1 expression induced pancreatic gene expression and insulin production in the mice livers. The amelioration of hyperglycemia, in PDX-1 treated diabetic mice was associated with an immune modulation manifested by Th1 to Th2 shift in the autoimmune T-cell response to antigens associated with NOD diabetes. Thus, liver-to-pancreas transdifferentiation ameliorates T1DM in a process which is associated with a concomitant modulation of the autoimmune attack. Our findings suggest a beneficial therapeutic effect of the PDX-1 gene therapy for treating autoimmune type 1 diabetes mellitus (T1DM).

Keywords: Autoimmune diabetes; Developmental redirection; Gene therapy; Immune modulation

1. Introduction

Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of the insulin-producing β-cells of the pancreatic islets [1]. Pancreas transplantation and islet cell implantation are being explored as replacement therapies for T1DM. Several factors limit their use; vulnerability to reoccurring autoimmune attack, the life-long immunosuppression needed to prevent the allogeneic transplants and the shortage of tissue from cadaver donors [2].

Insulin gene therapy was suggested as a potential approach for treating T1DM in a mode that might overcome these limitations. However, the simple replacement of the insulin gene expression by genetic engineering is not likely to result in continuous normoglycemia, unless the hormone secretion is tightly regulated by glucose within a narrow physiological range [3,4].

A novel approach for generating an autologous insulin producing tissue is the induction of developmental redirection of liver to pancreas. This approach has been demonstrated in mice, xenopus and human tissues [5–17]. In this approach,
the ectopic expression of pancreatic and duodenal homeobox gene 1 (PDX-1), delivered in vivo by a recombinant adenovirus (Ad-CMV-PDX-1), induced a wide, functional and long-lasting developmental redirection process that ameliorated hyperglycemia in STZ-induced diabetic mice [5,6].

Although liver cells are resistant to the selective aggression of the immune system against insulin-secreting β-cells [18], the developmentally redirected liver cells may have acquired undesired sensitivity to pro-inflammatory cytokines and toxins, together with the desired β-cell characteristics and function.

None of the studies that demonstrated the use of developmentally redirected liver cells in treating hyperglycemia analyzed the efficiency of this approach in treating autoimmune T1DM. Here, we demonstrate that systemic Ad-CMV-PDX-1 administration induced a functional liver-to-pancreas developmental redirection process in cyclophosphamide-accelerated non-obese diabetic (CAD-NOD) mice that are under an active autoimmune process.

The non-obese diabetic (NOD) mouse is a common model of T1DM. This mouse develops diabetes as a consequence of a spontaneous autoimmune process [19]. The diabetic process can be accelerated and synchronized by the administration of cyclophosphamide (Cy), a drug that is thought to deplete immune regulatory cells [20]. In contrast to chemical induction of diabetes by STZ, the Cy accelerated destruction ensues from autoimmune attack and is associated with insulinis [20].

Both spontaneous NOD diabetes and cyclophosphamide-accelerated diabetes (CAD) are autoimmune disorders characterized by increased Th1 responses to several auto-antigens, including the 60 kDa heat-shock protein (HSP60) [21], glutamic acid decarboxylase (GAD) [22,23] and insulin [24]. When compared with spontaneous NOD diabetes, CAD stands as a more robust experimental model of T1DM [25–27].

In this work, we suggest that despite the autoimmune process, PDX-1-induced liver-to-pancreas transdifferentiation can effectively be used to treat diabetes in NOD mice, in a process associated by an induced immune-modulation.

2. Materials and methods

2.1. Mice

Male NOD/LtJ, NOD/SCID and BALB/c mice (Harlan Laboratories, Jerusalem, Israel) were bred and housed under pathogen-free conditions in the Animal Breeding Centre of the Sheba Medical Center or Weizmann Institute. Experiments were carried out under the supervision and guidelines of the Institutional Animal Welfare Committee.

2.2. CAD induction

Diabetes onset was accelerated by administration of cyclophosphamide (Cy, Sigma) as previously described [28]. Briefly, 4–5-week-old male NOD mice received an intra-peritoneal (i.p.) injection of 200 mg/kg of Cy. The process was repeated twice more with a 10-day interval between injections. A mouse was considered diabetic when its blood glucose level was higher than 300 mg/dl on two consecutive examinations monitored 2 days apart. The diabetic mice were then injected with the recombinant adenoviruses and blood glucose levels were measured twice weekly using an AccuTrend®GC Glucose Analyzer (Boehringer Mannheim, Mannheim, Germany).

2.3. Glucose tolerance test

Fasting mice (4 h) were injected interperitoneally (i.p.) with 1 g/kg glucose. Blood glucose levels were monitored at the indicated time points in samples drained from the tail vein [29].

2.4. Recombinant adenoviruses

Ad-CMV-PDX-1 was constructed as described [30], containing the cDNA of the rat homologue of PDX-1. Ad-Rip-β-galactosidase (Ad-Rip-β-Gal) was a gift from C.B. Newgard, Duke, NC, USA. Then, 3–5 × 1010 pfu/200–250 μl of the indicated recombinant adenovirus were injected into the tail vein of 8–10-week-old diabetic CAD-NOD mice (20–22 g).

2.5. Peptides and antigens

Peptides were synthesized by a standard Fmoc procedure, purified by reverse-phase HPLC, and their compositions were confirmed by amino acid analysis as previously described [27]. Two peptides derived from HSP60 were used in this study: peptide p12 (EEIAQVATISANGDKDIGNI) [27] and peptide p277 (VLLGGVALLRVPALDSTPANE) [21] corresponding to the 166–185 and to the 437–460 regions, respectively. Peptide p277 was stabilized by substituting its two cysteins at positions 442 and 447 for valines. These substitutions do not affect the immunological properties of p277 [31]. In addition, two peptides derived from GAD were used: peptide p34 (IPPSLRTLEDNEERMRLS) [22] and peptide p35 (SRLSKVAPVIKARMMEYGTT) [22], corresponding to the 509–528 and to the 524–543 regions, respectively. Insulin, glutamic acid decarboxylase (GAD), ovalbumin (OVA) and concanavalin A (Con A) were purchased from Sigma (Rehovot, Israel). Recombinant HSP60 and glutathione-S-transferase (GST) were prepared as described [27].

2.6. RNA isolation and RT-PCR analysis

Total RNA isolation, cDNA synthesis and RT-PCR reactions were performed as previously described [6].

2.7. Pancreas and liver histology

Histological and immunohistochemical staining were performed on pancreata and livers as previously described [5,6]. Briefly, slides were analyzed using the Histomouse™-SP Kit (Zymed Laboratories, South San Francisco, CA, USA), with a monoclonal antibody to human insulin (1:1000; 1:200, respectively, Sigma) or a polyclonal antibody to PDX-1 (1:1000, a gift from C.V. Wright), or monoclonal Ki67 (1:25, Novocastra).
2.8. Determination of insulin content

Liver extracts were prepared as previously described [5,6]. Hepatic insulin and serum insulin levels were determined by RIA (SRI-13K Linco, Missouri, USA). The insulin content was normalized to the wet weight of each organ; average of 120 ± 30 mg for pancreatic tissue and average of 2.5 ± 0.84 g for hepatic tissue.

2.9. T-cell proliferation

NOD mice were sacrificed 20–40 days after viral administration, their spleens were removed and the splenocytes were isolated as previously described [27]. The splenocytes were incubated for 72 h at 37 °C in a humidified atmosphere with 7.5% CO₂. T-cell proliferation was quantified by incorporation of [methyl-³H]thymidine (Amersham, Buckinghamshire, UK; 1 µCi/well) for the last 18 h of incubation. The stimulation index (SI) was calculated as the ratio of the mean cpm of antigen or mitogen to control cells cultured with medium alone [27].

2.10. Cytokine assays

 Supernatants were collected after 72 h of stimulation of the isolated splenocytes with test antigens, Con A or medium alone. IL-10 and IFNγ were quantified in the culture supernatants with an enzyme linked-immunosorbent assay (ELISA; Pharmingen San Diego, USA, [27]). Cytokine levels in supernatants are expressed as pg/ml, the lower limits of detection for the experiments described in this paper were 15 pg/ml for IL-10 and IFNγ.

2.11. Adoptive transfer

Splenocytes were isolated from diabetic NOD mice as described [28]. 2.5 × 10² splenocytes were injected (i.p.) into 5–6-week-old SCID-NOD mice, 1 week after viral administration. Blood glucose levels were measured weekly to detect the onset of diabetes. Insulin content was analyzed as described above.

2.12. Statistical analysis

Statistical analyses were performed using the two-sample Student’ t-test assuming unequal variances.

3. Results

3.1. PDX-1 induces pancreatic lineage in the liver of diabetic CAD-NOD mice: a molecular and cellular analyses

Previous studies have demonstrated that systemic PDX-1 administration induces liver to pancreas developmental redirection in chemically induced diabetic mice [5,6]. To analyze whether liver to pancreas developmental redirection process can occur also, under autoimmune attack, the activation of pancreatic lineage was analyzed at distinct levels. More than 80% of PDX-1 treated mice exhibited the endocrine hormones Insulin, Glucagon and Somatostatin gene expression in their livers (Fig. 1a). The hepatic insulin content of PDX-1 treated mice that became normoglycemic, increased by 55-fold compared to untreated mice (17.75 ± 7 ng/organ vs. 0.325 ± 0.175 ng/organ, respectively Fig. 1b). Immunohistochemistry staining revealed PDX-1 and insulin-positive cells in livers of PDX-1 treated mice (Fig. 1c-1d). The hepatic insulin-producing cells were located close to central veins, as previously described [5,6]. No insulin-positive cells were detected in livers of untreated or Ad-Rip-β-Gal-treated mice (Fig. 1c6).

These data demonstrate that PDX-1 induces pancreatic hormone gene expression and protein production, suggesting that PDX-1 induces the liver-to-pancreas developmental redirection process in overtly diabetic T1DM mice.

3.2. Ad-CMV-PDX-1 treatment ameliorates autoimmune diabetes

To analyze the potential therapeutic effect PDX-1 has in T1DM, overtly diabetic CAD-NOD mice were treated by Ad-CMV-PDX-1, Ad-Rip-β-Gal or remained untreated. To evaluate the state of diabetes, blood glucose levels, serum insulin levels and body weight were monitored. The non-treated and Ad-Rip-β-Gal treated mice remained hyperglycemic (Fig. 2a) and their serum insulin levels were low (0.14 ± 0.07 ng/ml, 0.18 ± 0.1 ng/ml, respectively (Fig. 2b)). The diabetic mice were sacrificed within 2 weeks due to severe diabetes. PDX-1 treatment resulted in reversal of hyperglycemia in 43% (13/30) of mice that became normoglycemic (non-fasting blood glucose ≤200 mg/dl; Fig. 2a). Serum insulin level in normoglycemic PDX-1 treated mice was similar to that of Balb/c control mice (1.16 ± 0.15 ng/ml versus 0.9 ± 0.1 ng/ml, respectively (Fig. 2b)). Moreover, PDX-1 treated mice maintained a stable body weight for the whole duration of the experiment, while non-treated and Ad-Rip-β-Gal treated mice severely lost weight (Fig. 2c).

To assess the functionality of Ad-CMV-PDX-1 therapy, we conducted a glucose-tolerance test in normoglycemic PDX-1 treated CAD-NOD mice, 2–3 weeks after viral administration. The rate of glucose clearance in the PDX-1-treated CAD-NOD mice was similar to that of normoglycemic Balb/c mice (Fig. 2d). In contrast, diabetic mice treated by Ad-Rip-β-Gal failed to show glucose clearance and remained hyperglycemic throughout the test.

These findings suggest that PDX-1 treatment ameliorates autoimmune diabetes.

3.3. Reversal of CAD is associated with down-regulation of specific T-cell proliferation: mechanistic analysis of the therapeutic outcome

T1DM results from an autoimmune attack directed specifically against pancreatic β-cells [1]. While pancreatic islets of all CAD-NOD mice groups showed lymphocyte infiltration
(Fig. 1c4), insulin positive cells in the livers of normoglycemic PDX-1 treated mice (Fig. 1b) did not exhibit any signs of inflammation (Fig. 1c6). The distinct effects on insulin producing cells could be interpreted as a lack of recognition of insulin producing cells in the liver as a target for autoimmunity, or it may suggest a possible cessation of the autoimmune process.

To analyze if PDX-1 treatment affected the immune system in CAD-NOD mice, we analyzed the immunological profile of the mice. We studied the proliferative T-cell responses to insulin, to GAD and its p34 and p35 peptides, as well as to HSP60 and its two immuno-regulatory peptides, p12 and p277, which can treat [32] or prevent [21,22] spontaneous NOD diabetes. Recombinant GST and Con A were used as negative and positive controls, respectively. Diabetic NOD mice were treated with Ad-CMV-PDX-1, and 20–33 days later, splenocytes were prepared from those mice that manifested normoglycemia. As controls, we used splenocytes taken from non-treated diabetic mice or from mice treated with the control virus Ad-Rip-β-gal 10–14 days after administration.

The splenocytes obtained from control mice (non-treated or treated with Ad-Rip-β-Gal) showed significant proliferative responses to insulin, GAD and HSP60 (Fig. 3). In contrast,
the splenocytes from mice that manifested normoglycemia following Ad-CMV-PDX-1 treatment showed a diminished proliferative response to this panel of antigens associated with diabetes. The groups showed no significant proliferative responses to the control antigen GST (Fig. 3). Thus, normoglycemia induced by Ad-CMV-PDX-1 treatment was associated with a decrease in the diabetogenic proliferative T-cell response. This effect seems to be antigen specific, since the

Fig. 2. Ad-CMV-PDX-1 ameliorates diabetes in CAD-NOD mice. Diabetic CAD-NOD mice were treated with Ad-CMV-PDX-1 (n = 13, ■), Ad-Rip-β-gal (n = 9, ▲) or left untreated (n = 7, ●). (a) Blood glucose levels, (b) serum insulin levels and (c) body weight were monitored. In (b) a group of normoglycemic Balb/c mice (P.C. n = 10) was included. (**p < 0.1 compared to untreated mice). (d) Glucose tolerance test in CAD-NOD mice treated by Ad-CMV-PDX-1 (n = 5, ■), Ad-Rip-β-gal (n = 3, ▲) or normoglycemic control Balb/c (n = 8, ●) mice. The data are presented as means ± SE for each group.

Fig. 3. Reversal of CAD is associated with down-regulation of specific T-cell proliferation. Twenty to forty days after recombinant adenovirus administration, spleens were removed and T cell proliferate responses to (a) insulin, HSP60, p277, p12, p34, p35, (b) GAD, (c) GST or Con A were studied. The data are presented as mean SI ± SE for 4–6 individual samples per group (*p < 0.05 compared to untreated group).
groups did not show significant differences in their proliferative responses to Con A.

3.4. Reversal of CAD is associated with a Th1 to Th2 shift of the autoimmune T-cell cytokine response

The T cells that mediate the destruction of the insulin-producing pancreatic β-cells in CAD secrete Th1 cytokines, such as IFNγ [33]. Moreover, immunomodulatory therapies that arrest the diabetogenic autoimmune process usually lead to a Th2 shift in the autoimmune T-cell response, marked by the increased production of IL-10 [27]. To further characterize the autoimmune response in mice treated with Ad-CMV-PDX-1, we studied IFNγ and IL-10 secretion by splenocytes stimulated with insulin, GAD, p34, p35, HSP60, p12 or p277. The splenocytes taken from the different experimental groups did not differ in the amounts of IFNγ or IL-10 released upon activation with Con A, and were not stimulated with the control antigen GST. However, mice that manifested a reversal of hyperglycemia showed a significant decrease in IFNγ secretion.

![Graph](image-url)

Fig. 4. Reversal of CAD is associated with a Th1 to Th2 shift of the autoimmune T-cell cytokine response. Twenty to forty days after treatment by recombinant adenoviruses, spleens were removed and studied for the secretion of IFNγ (a–c) and IL-10 (d–f) upon stimulation with (a,d) insulin, HSP60, p277, p12, p34, p35, (b,e) GAD, (c,f) GST or Con A. The data are presented as means ± SE for 4–6 individual samples per group (*p < 0.05 compared to the untreated group).

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and a significant increase in the secretion of IL-10 triggered by these antigens associated with the progression of diabetes (Fig. 4). These findings suggest that normoglycemia following Ad-CMV-PDX-1 therapy is associated with a Th1 to Th2 shift in the autoimmune response that drives CAD progression.

3.5. Delayed adoptive transfer of diabetes mediated by T-cells from PDX-1 treated normoglycemic mice

We detected the persistence of diabetogenic T-cells in Ad-CMV-PDX-1-treated mice by transferring their splenocytes to NOD/SCID mice. Twenty to thirty days following virus injection, splenocytes were prepared from mice treated with Ad-CMV-PDX-1, or with the control vector Ad-Rip-β-Gal. The spleen cells were injected into NOD/SCID mice, and the development of diabetes was monitored. All the recipient mice developed diabetes. However, the induction of hyperglycemia was delayed in mice treated by splenocytes isolated from normoglycemic Ad-CMV-PDX-1 mice compared to these isolated from Ad-Rip-β-Gal treated hyperglycemic mice (Fig. 5). Sixty-five percent of SCID/NOD mice treated by Ad-Rip-β-Gal-derived cells became diabetic within 8 weeks and 3 weeks later, the whole group became hyperglycemic. At the same time only 10% and 50%, respectively, of mice treated by splenocytes from normoglycemic Ad-CMV-PDX-1 mice became overtly diabetic. Thus, although the stable normoglycemia triggered by Ad-CMV-PDX-1 therapy was associated with immune-modulation of the diabetogenic T-cell response in vitro, Ad-CMV-PDX-1 therapy alone did not completely eliminate potentially diabetogenic autoimmune T cells detectable upon adoptive transfer in vivo.

4. Discussion

This study demonstrates that direct systemic administration of PDX-1 using the first generation recombinant adenovirus ameliorates diabetes in the autoimmune, T1DM mouse model—the CAD-NOD mice. Mice that reverted to normoglycemia (43%) exhibited a normal rate of glucose clearance in a glucose-tolerance test (Fig. 2d). The induction pancreatic lineage in PDX-1 treated liver of these diabetic mice, have been demonstrated at a molecular (Fig. 1a), cellular (Fig. 1b,c) and functional level (Fig. 2). Notably, successful reversal of hyperglycemia in diabetic CAD-NOD mice associated with immune modulation, manifested by a shift from Th1- to Th2-dominated response (Figs. 3,4) and with a delay in the capacity of their splenocytes to adaptively transfer diabetes to SCID-NOD mice (Fig. 5).

Hepatic insulin production was relatively low, thus it may not solely explain the therapeutic outcome of PDX-1 administration. In addition to the effects of the secreted hormone, both ectopic PDX-1 expression and the hepatic insulin production could have increased the rate of glucose clearance by the liver, by promoting glucokinase expression and activity [5,6,16,34] and hence, lowering the blood glucose levels.

The frequency of the functional therapy process in PDX-1 treated CAD-NOD mice was lower than the frequency reported for STZ-induced diabetic mice [5,6]. Several factors could contribute to the lower efficacy and the differential therapeutic effect of PDX-1 treatment between these two models. First, the autoimmune disease, and especially one accelerated by cyclophosphamide, can be more severe than that induced by STZ [5]. Second, we have recently studied the immune response of NOD mice to CAD using antigen arrays and have found that, although shared patterns of antibody reactivity characterize diabetic or healthy NOD mice, each mouse still manifests an individual sub-pattern of autoimmune reactivity [35]. Thus, the immune systems of individual diabetic mice express different states of autoimmunity, and it is conceivable that the immune systems of different mice may be more or less prone to respond to PDX-1 treatment.

Our data indicate that PDX-1 treatment is associated with Th1-to-Th2 shift. Could the adenoviral vector for PDX-1 delivery itself have induced the immune modulation? It was previously suggested that viral [36], bacterial [37], parasitic [38] infections, and even microbial components such as LPS [35,39] or bacterial DNA [18], can down-regulate the diabetogenic response in NOD mice. However, in our study that is unlikely, since mice treated with the control virus Ad-Rip-β-Gal, showed a Th1 response. Thus, the PDX-1 gene and its subsequent effect in liver, and not the virus itself, were necessary to induce immune modulation.

The exact mechanism that underlies the immune modulation associated with PDX-1 treatment is as yet unknown. The capacity of the liver to induce immune tolerance has been shown in several experimental systems [37]. Allogeneic liver transplants can be accepted across MHC barriers [40], antigens administered via the portal vein induce antigen-specific immune tolerance [38] and direct venous drainage from an organ transplant into the portal vein can result in increased graft acceptance [41]. Therefore, following PDX-1 delivery, hepatic presentation of β-cell antigens might lead to the down-regulation of the diabetogenic autoimmune response.

It is noteworthy that the immune modulation associated with a satisfactory response to gene therapy was incomplete: splenocytes from normoglycemic PDX-1-treated mice could...
still transfer diabetes to NOD/SCID mice, however, with a delayed pace. Thus, the control of pathogenic autoimmunity reported in this paper might depend on a continuous interaction of the diabeticogenic cells with transdifferentiated cells in the liver and this interaction could be interrupted upon adoptive transfer into the NOD/SCID recipients. However, this and other alternative explanations need to be tested experimentally.

Several studies documented that insulin-producing extra-pancreatic tissues do not become a target for autoimmune attack [42–44]. It is possible that insulin producing cells in liver do not become a target for autoimmune attack because their developmental redirection is incomplete and the insulin producing cells do not expose antigens, which are recognized by activated splenocytes. Indeed, although the developmentally redirected liver cells express in addition to insulin, many β-cell-specific markers (Fig. 1 and [5,6]), as opposed to normal pancreatic β-cells, developmentally redirected liver cells do not express diabeticogenic antigens such as GAD-65 (ML and SF, unpublished data).

In conclusion, the present study demonstrates that following PDX1 delivery, transdifferentiated liver cells might play a double role in controlling hyperglycemia: The liver cells synthesize and secrete insulin in a physiologically regulated way and, simultaneously, the hepatic presentation of β-cell antigens might lead to the down-regulation of the diabeticogenic autoimmune response.

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