Recovery of endogenous beta cell function in non-human primates following chemical diabetes induction and islet transplantation

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ABSTRACT

Objectives: To describe the ability of non-human primate endocrine pancreata to re-establish endogenous insulin production after chemical beta cell destruction.

Research Design and Methods: Eleven monkeys (Macaca fascicularis) were rendered diabetic with streptozotocin. Eight diabetic monkeys received intraportal porcine islet transplantation.

Results: Two monkeys transplanted after 75 days of insulin dependent diabetes, showed recovery of endogenous C-peptide production a few weeks after transplantation, concomitant with graft failure. Histological analysis of the pancreas of these monkeys showed insulin-positive cells, single or in small aggregates scattered in the pancreas and adjacent to ducts. Interestingly, numerous CK19<sup>+</sup> cells co-stained with proinsulin and PDX-1 antibodies. Furthermore, the peculiar double phenotype glucagon<sup>+</sup>/GLUT2<sup>+</sup> was observed. In these monkeys as well as in all others the original islets showed no insulin staining.

Conclusions: Our data provide evidence that in non-human primates the pancreas can re-establish endogenous insulin production after chemical beta cell destruction. This seems to be a non-generalizable event with only two out of eleven monkeys recovering beta cell function. In these two monkeys, younger age and islet graft behaviour might have played a role in triggering endogenous beta cell recovery.
Until a few years ago, lesions of the endocrine pancreas, as occur in type 1 diabetes, were thought to be permanent and irreversible since diabetic patients require hormone replacement therapy for life (1). Despite the clinical evolution of the disease, it is still unknown whether the islet beta cells possess, at least in part, the ability to heal from an injury (2).

Animals. Fifteen male Cynomolgus monkeys (Macaca Fascicularis) (Three Spring Scientific, Perkasie, PA), 2-4 years old and of 2.6-4.7 Kg (median 3.6 Kg), were included in the study.

Four wild type retired breeder pigs (Wally Whippo, Enon Valley, PA) and three GT-DKO pigs (alpha 1,3-Ga lactosyltransferase KO pigs. Revivicor, Blacksburg, VA) of similar weight were used as sources of pancreata for islet isolation. One wild type pig was used for two transplants. All animal care procedures were in accordance with the institutional Principles of Laboratory Animal Care.

Induction of diabetes. Diabetes was induced in 11 monkeys with 125-150 mg/kg i.v. of Zanosar Streptozotocin (STZ) (Sicor Pharmaceuticals, Irvine, CA, USA) in a single dose (12).

In humans, the ability of the post-natal pancreas to expand the beta cell mass after injury is still debated (9,10). Spontaneous recovery of beta cell function has been reported in only few patients previously diagnosed with type 1 diabetes (11).

Islet preparation and transplantation. Following pancreatectomy in the anesthetized donor pig, islet isolation was carried out according to a modification of the method described for human islets, optimized for pigs (15). Intraportal injection of islets (an average of 60,000 Islet Equivalents/Kg body weight with a range of 40,000 to 100,000 Islet Equivalents/Kg) resuspended in plain CMRL-1066 with addition of heparin (70-140 U/kg; Baxter, Deerfield, IL) or dextran sulfate (MW ~5000d; 4 mg/kg; Fluka, Buchs, Switzerland) was carried out under general anesthesia of recipients.

In two recipients where pig islet grafts functioned for a few weeks and eventually failed, we observed increasing endogenous C-peptide production paralleled by metabolic improvement. We reviewed all metabolic data, did extensive histological analysis and here report evidence of recovery of endogenous insulin production in these two monkeys.

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heparin or dextran sulfate, Prostacyclin (GlaxoSmithKline, Research Triangle Park, NC) and Aspirin (14).

**Diabetes and graft/endogenous beta cell function monitoring.** Blood glucose (mmol/L) was measured in whole blood with Free Style, Lifescan. Primate C-peptide (nmol/L) levels were measured by radioimmunoassay (Linco Research, St. Charles, MO, USA). Confirmatory post-STZ C-peptide levels were measured using a chemoluminescent-based technology (Bayer Centaur, Tarrytown, NY). Porcine C-peptide (nmol/L) was detected by radioimmunoassay (Linco Research). Intra Venous Glucose Tolerance Tests (IVGTT) were carried out before and after transplantation, as described (14).

**Fixation and immunostaining of specimens.** Monkeys were euthanized at the time of rejection or when the vascular lines stopped working. Specimens of the pancreatic tissue were obtained, fixed and analysed as described in Supplemental Methods. Morphometric analysis was conducted as per Ref. 16.

**RESULTS**

**Recovery of endogenous C-peptide.** All monkeys treated with STZ became hyperglycemic (blood glucose >15 mmol/L) within 48 hours, requiring exogenous insulin. Fasting C-peptide was under detection levels (<0.16 nmol/L) in all treated animals using a chemoluminescent method. Using commercially available ultra-sensitive RIA kits, the monkeys showed an overall reduction in C-peptide of at least 75% compared to the pre-STZ values (Figure 1A). Lack of C-peptide increase 5 minutes after glucose infusion during IVGTT after STZ treatment characterized all treated monkeys. Diabetic monkeys that did not undergo transplantation (N= 3) and islet recipients with early graft loss (N=6), characterized by undetectable or low porcine C-peptide levels for less than 2 weeks) showed no increase of the autologous C-peptide over the post-STZ basal levels (Figure 1B). Exogenous insulin requirements following graft failure or in non-transplanted monkeys remained unchanged over time (data not shown). The pancreatic islets of STZ treated monkeys showed complete absence of insulin immunostaining (Supplemental figure 1). Monkeys M4804 and M5204, with C-peptide levels below detection using both detection methods for more than two months after STZ treatment, showed substantial recovery of basal endogenous C-peptide after a period of three weeks with islet graft function (Figure 1B). Interestingly, the curves representing endogenous versus porcine C-peptide followed opposite trends (Figure 1C,E). Glycemia did not worsen after graft failure, exogenous insulin requirement was lower than before transplantation, possibly because of the autologous insulin production (Figure 1D,F) and body weight increased at the pace of healthy monkeys. Several weeks after transplantation, endogenous C-peptide levels were not only detectable but showed a low but measurable response to glucose stimulation (Supplemental figure 2). Following islet graft failure in all other recipients, glycemia and insulin requirements returned to pre-transplantation levels or worsened (data not shown) while endogenous C-peptide did not increase. In monkeys with long-term graft function (3 and 12 months), recovery of endogenous insulin production was not observed (our unpublished data).

**Histological findings.** The pancreata of all the monkeys were analysed for the presence of proinsulin+ cells. The two monkeys that re-established endogenous C-peptide production showed several proinsulin+ cells grouped in small aggregates, or single cells scattered throughout the pancreas but distant from aggregates of glucagon+ cells (Figure 2). We quantified the number of proinsulin+ cell clusters with area equal or smaller than 30 µm² (i.e., cell clusters not included in the
islets of Langerhans existing before STZ treatment nor in the ducts). Proinsulin+ cell clusters covered 0.23 and 0.18% of the whole section area in M5204 and M4804, respectively. These values were higher than in STZ-diabetic controls, (0.05±0.0004%; N=4 different monkey pancreata) but lower than non-diabetic monkeys (0.30±0.01%; N=3 monkeys pancreata). Proinsulin+ cells co-stained with PDX-1 (Figure 3N and Supplemental figure 3). Interestingly, anti-CK19 antibody, used to identify the ductal/epithelial cells, co-stained with proinsulin+ cells in these monkeys (Figure 3A-H). The pancreata of these monkeys showed stronger CK19 expression in comparison to non-diabetic and STZ-diabetic, transplanted and non-transplanted controls (Supplemental figure 4).

Additionally, the monkeys with recovered beta cell function presented double PDX-1+/CK19+ staining (Figure 3K-M). PDX-1+ cells were also found in STZ-diabetic control monkeys but they did not co-stain with CK19 (Figure 3J) nor with proinsulin (data not shown). The atypical GLUT2/glucagon phenotype was found in islets devoid of insulin-producing cells in M4804 and M5204 (Figure 4A, and lower panel) whereas it was not observed in STZ-diabetic (Figure 4C) nor in non-diabetic control monkeys (Figure 4B). Anti-Ki67 antibody was used as a nuclear marker of active cell proliferation. Anti-Ki67 stained CK19+ cells and fibroblasts, but not proinsulin+ cells (data not shown).

**DISCUSSION**

In rodents regenerative properties of beta cells have been unveiled (6,7). In humans, there is no clear similar indication. The autoimmune process that causes type 1 diabetes in the first place might also be responsible for halting potential attempts at restoring insulin production (2). Nonetheless it is unclear, even in the absence of the immune attack, if the pancreatic beta cell function can recover efficiently (17). Anecdotic reports describing return to a normoglycemic status in patients diagnosed and treated for type 1 diabetes seem to prove that islet function can be re-established in humans, concurrent with the disappearance of autoimmunity (11). In non-human primates used for pre-clinical investigation of type 1 diabetes treatments, hyperglycemia can be induced by administration of STZ. Once C-peptide is significantly reduced and exogenous insulin administration begins, in contrast to that observed in rodents, spontaneous normoglycemia is believed to be unrecoverable (18).

However, in our hands two non-human primates rendered diabetic with STZ and with virtually no endogenous residual beta cell function for two months regained endogenous insulin production concomitant with pig islet graft failure. At the time they were sacrificed, endogenous C-peptide levels were more than 10 times higher than after STZ treatment with associated lower insulin requirements, despite C-peptide being below the normal range of a non-diabetic cynomolgous monkey (14).

The post-transplantation clinical course of these two monkeys was characterized by glycemias persistently higher than in the normal physiologic range, despite graft insulin production, but well below the range recorded in non-transplanted diabetic monkeys. It is a common notion that chronically elevated blood glucose levels have a negative impact on beta cell function but it is also known that glucose infusion and mild hyperglycemia may stimulate growth of the beta cell mass. It is unclear whether a threshold for beneficial/toxic effect indeed exists in monkeys and if this effect may have played a role in triggering recovery of insulin production in our monkeys. We observed that in the six monkeys with short graft function and consequent severe hyperglycemia and those that returned to stable normoglycemia after transplantation (our unpublished data,
and 19), no recovery of the endogenous function occurred.

Histological examination of the pancreas of these two monkeys showed scattered proinsulin\(^+\) cells, mostly organized as single cells or in small clusters, not associated with glucagon\(^-\) cells, but often to ducts, similar to the ones just recently described (8,20). The frequency of small proinsulin\(^+\) cell aggregates was higher in the monkeys with recovered beta cell function compared to diabetic controls but lower than in non-diabetic monkeys. While on one hand we may hypothesize that new cells formed, data do not allow to rule out that proinsulin\(^+\) cells result from a degranulation-regranulation process as described by Sherry in the autoimmune NOD mouse model (21). However, if regranulation were the mechanism of recovery of the beta cells, proinsulin\(^+\) cells associated with the damaged islets (thus near large glucagon\(^+\) cell aggregates) should have been seen.

Interesting features in the pancreas of these monkeys were the presence of proinsulin\(^+\) cells expressing CK19 within and outside the ducts as well as CK19\(^-/\)/PDX-1\(^+\)/proinsulin\(^+\) cells, suggesting that beta cells may have formed ex novo from duct progenitors, recapitulating pancreatic organogenesis and neogenesis. In agreement with this observation seem to be the molecular analysis of factors physiologically involved in organogenesis, although the modest numbers of monkey tissue for this study limits the soundness of the conclusion (Supplemental figure 5).

An additional characteristic of the pancreas of these monkeys was the presence of glucagon\(^+\)/GLUT2\(^-\) cells. A lack of evidence for glucagon\(^+\)/proinsulin\(^+\) cells suggests that glucagon\(^+\)/GLUT2\(^-\) cells were unlikely committed to becoming beta cells, recalling embryonic development phases when GLUT2 is expressed temporarily in pancreatic non-beta cells, likely acting as a signal for further development (22).

The histological findings indicate that damage secondary to STZ may be itself a trigger for pancreatic regenerative responses, however it seems unable to sustain sufficient beta cell recovery. In order to explain the phenomenon observed in these two monkeys, additional stimuli and peculiar conditions must be contemplated. One factor of interest was age: these monkeys were the youngest in the study (38 and 26 months compared to 49±6 months all others).

The failure of the graft, i.e., islet beta cell dysfunction and death, may also have fostered regenerating signals, as described in other forms of pancreatic injury (23). This would be in line with reports indicating that patients experiencing islet allograft loss can still exhibit detectable C-peptide levels and a better management of the diabetic status (24,25), even if their immunosuppressive regime included sirolimus and tacrolimus, both known to limit beta cell regeneration (7).

Recovery of the beta cell function can occur in non-human primates: the mechanisms that lay behind it remains, however, to be demonstrated.

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Abbreviations
STZ: streptozotocin; CK19: cytokeratin 19; DKO: Double knock out; GT: gal-transferase
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Figure 1. A: Endogenous C-peptide levels before and after STZ treatment. Closed triangles indicate the values of C-peptide in the two monkeys that recovered autologous insulin production. B: Relative increase of endogenous C-peptide over basal post-STZ values. Closed lines indicate STZ-diabetic monkeys that were not transplanted (N=3) as well as STZ-diabetic recipients that experienced early graft loss (N=6). Dotted lines represent the monkeys with recovered beta cell function (N=2). C, D, E, and F: Metabolic profile of the monkeys with recovered endogenous C-peptide (M4804 and M5204). C and E: Graft (porcine) and endogenous (primate) C-peptide levels. (D and F): Blood glucose and exogenous insulin administration.
**Figure 2:** Proinsulin+ cells in the monkeys with recovered endogenous beta cell function. (A-C) Proinsulin+ cells are organized in small aggregates or scattered as single cells in the pancreas, not associate with glucagon+ cells. White arrows: proinsulin+ cells. Yellow arrows: glucagon+ cells, presumably islets with damaged beta cells. (D-F): Higher magnification. Pictures are representative of both monkeys with recovered endogenous beta cell function.
Figure 3. A-H: Presence of double phenotype CK19/proinsulin in monkeys with recovered endogenous beta cell function. A,B,C, and D: Monkeys with recovered endogenous beta cell function show co-expression of CK19 with proinsulin (yellow). E,F,G, and H: Detail of a pancreatic duct. I-N: Double PDX-1+/ CK19+ in the pancreas of monkeys with recovered function (K with detail in L, and M). CK19 and PDX-1 do not co-stain in non-diabetic healthy monkeys (I); PDX-1+ cells are found scattered throughout the pancreas of STZ-diabetic monkeys, but do not co-localize with CK19 (J); M and N: consecutive pancreatic sections in M5204 (monkey with recovered beta cell function) showing CK19+/PDX-1+ (M) and CK19+/proinsulin+ (N) cells, respectively. Arrows show PDX-1+ cells in J, double positive PDX-1+/CK19+ in K, L and M and PDX-1+/proinsulin+ in N.
Figure 4: Double GLUT2+/glucagon+ cells in the pancreas of monkeys with recovered endogenous beta cell function. Co-expression of glucose transporter GLUT2 with glucagon (A) and not with insulin (D) within islets damaged by STZ in M 4804 (monkey with recovered beta cell function). B and E: non-diabetic monkey islet. C and F: STZ-diabetic monkey islet. Double GLUT2+/glucagon+ and GLUT2+/proinsulin+ cells stain in yellow. Lower panel shows GLUT2/glucagon+ cells in Monkey M5204 (higher magnification).