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Therapeutic approaches based on beta-cell mass preservation and/or regeneration

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1. ABSTRACT

Beta-cell deficiency is a pathophysiologic component of diabetes mellitus and a primary cause of islet dysfunction. Islet dysfunction is a prerequisite for the development of diabetes mellitus since individuals with insulin resistance (e.g. obesity, pregnancy) do not develop hyperglycemia unless beta-cell compensation fails. Therefore, understanding of the biology and mechanisms involved in normal beta-cell adaptation may provide novel therapeutic targets for preservation and/or regeneration of beta-cell mass in diabetes mellitus. Normal adaptation of beta-cell mass occurs by beta-cell replication and/or neogenesis from precursor cells inside the pancreas. However, the relative importance of both processes for successful adaptation is unknown. In type-2 diabetes, the primary defect is increased beta-cell apoptosis. Since replicating beta-cells are more vulnerable to apoptosis, the proapoptotic diabetic milieu limits the regenerative capacity of the islet and directly causes accelerated islet loss. Therapeutic approaches need to address both processes of islet turnover (regeneration and cell loss) in order to be successful. It may be anticipated that such an intervention is also effective early in the course of diabetes or in prediabetic conditions.

2. INTRODUCTION

Beta-cell function is tightly regulated to meet short- (food intake, physical activity) and long-term (insulin resistance) demands for insulin secretion and to maintain glucose homeostasis. Acute functional adaptation is primarily achieved by regulation of insulin secretion on the cellular level (insulin vesicle dynamics, translational and transcriptional activity) (1-3), while chronically increased insulin requirements induce expansion of beta-cell mass (4-6) in order to prevent the development of diabetes mellitus. Since there is no direct longitudinal evidence about the time course and the relative importance of various mechanisms contributing to beta-cell expansion in humans the quantitative potential for plasticity of islet tissue in humans is unknown. Indirect evidence from autopsy studies in diabetic and non-diabetic individuals indicates that tissue renewal and consequently adaptation to insulin resistant states probably requires longer (months to years) (4, 7) than in rodents (days to weeks) (8, 9). In diabetic states, the mechanisms of islet regeneration are functionally intact (4, 10) and may be further enhanced by appropriate interventions. Therefore, detailed insights into the regulatory pathways of islet regeneration may provide the attractive perspective to develop therapeutic strategies

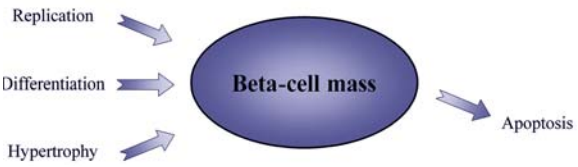

Figure 1. Beta-cell turnover. Replenishment of the beta-cell pool (beta-cell mass) occurs by beta-cell replication, differentiation from stem or progenitor cells or hypertrophy. Cell loss occurs primarily by apoptosis.

in order to induce adequate adaptation of beta-cell mass for stabilization or even improvement of glucose metabolism.

3. NORMAL ADAPTATION OF BETA-CELL MASS

Like other tissues, the islets of Langerhans undergo a permanent turnover of cells with cell renewal from replication of already existing beta-cells, differentiation of stem or progenitor cells and hypertrophy. Cell loss, on the other hand, primarily occurs by apoptosis (Figure 1). It is more than 80 years ago that it was generally hypothesized that the endocrine pancreas may be a self-renewing tissue (11), yet it required studies of islet morphology in animal models to provide evidence that the endocrine pancreas retains the capacity to adapt to changing demands of insulin secretion. Studies with non-diabetic rats directly showed that e.g. glucose infusions over a period of 4 days induce beta-cell replication, hypertrophy and a ~50% expansion of beta-cell mass (12). Other reports confirmed that, in rodents, the endocrine pancreas has a tremendous capacity for regeneration, e.g. after only 6 days of hyperglycemia (*i.v.* glucose) there was a doubling of beta-cell mass in male Sprague Dawley rats (13), 4 days of glucose infusion (*i.v.*) induced a 4-fold increase of beta-cell replication in C57Bl/6 mice (14). In the first of these three studies, the main mechanism of expansion was islet neogenesis from stem or progenitor cells inside islets or the epithelium of exocrine pancreatic ducts. For quantification of islet neogenesis, the percentage of insulin positive cells in the ductal epithelium is determined because it is believed that these cells may bud off from the ducts and then independently continue to grow into mature islets (15). Conceptually, humans are also capable of beta-cell regeneration. Evidence for adaptation of beta-cell mass to changing metabolic demands in humans has been reported for pregnancy (16) and with some more detail for obesity (4). In obese versus lean non-diabetic humans (BMI 36.4. ± 1.2. versus 22.5. ± 0.5. kg/m^2), there is a compensatory increase of beta-cell mass of ~50%. Less is known about the underlying mechanisms of expansion. In that cross-sectional study, there were no differences of beta-cell replication between obese and lean subjects. In contrast, islet neogenesis (percentage of insulin positive cells of the exocrine duct epithelium) was increased in obesity. However, due to the difficulties associated with obtaining pancreatic tissue (difficult access, local complications after biopsy) there are no mechanistic studies examining islet morphology before and after a certain stimulus or pancreatic injury in humans. Therefore, to date there is no direct evidence for active islet regeneration e.g. that insulin positive duct cells proceed to develop into mature islets or that mature islets adapt their size by regulation of beta-cell replication and/or apoptosis. A recent report showed that real-time imaging of the developing pancreas, cell movement and tissue morphogenesis is feasible (17) and may provide important insights into islet turnover if applied to models of endocrine pancreas regeneration. In that study, it was shown that during pancreatic development, scattered beta-cells migrate to cluster into islet like structures. However, for most studies with pancreatic tissue, one important limitation is the cross-sectional nature of the data because measurement of cell replication or beta-cell differentiation technically requires tissue specimens or fixed tissue and thus usually precludes longitudinal analyses of an individual organism. Hence, it is an ongoing debate whether beta-cell regeneration, which is clearly present in humans, occurs primarily by neogenesis, replication or both processes (18) and whether the available techniques are adequate to detect changes of these parameters. Recently, the elegant technique of lineage-tracing (genetic marking) in rodents provided additional insights. In one study, a tamoxifen inducible beta-cell specific label (human alkaline phosphatase) was introduced into mice (19). If the mice are injected over a short period with tamoxifen, differentiated beta-cells will start to stably express the label even after discontinuation of tamoxifen treatment and pass it on to their progeny. Beta-cell turnover was then examined after this pulse period during aging or after pancreatectomy (chase period). In this experimental setting, labeling of differentiated beta-cells during the pulse period occurs with an efficiency of ~30%. These cells subsequently continue to stably express the label during the chase period and one would expect that a) entirely stem cell derived new islets would contain no label, b) within pre-existing islets maintenance by stem cells would result in a gradual time-dependent decrease in the fraction of labeled beta-cells or c) maintenance of islet tissue by beta-cell replication alone would result in a constant fraction of labeled beta-cells inside islets. The experiments showed that the fraction of labeled beta-cells remained constant and the authors therefore concluded that beta-cells are primarily formed by replication rather then stem cell differentiation. The same group also studied another regeneration model with inducible beta-cell specific expression of diphtheria toxin, which leads to apoptosis of 70-80% of beta-cells and diabetes mellitus (20).

Lineage-tracing was again performed with the tamoxifen inducible beta-cell specific label (human alkaline phosphatase). After discontinuation of diphtheria toxin expression there was a full recovery from diabetes mellitus with beta-cell regeneration primarily from replication. Further evidence for the notion that beta-cell replication may be the primary mechanism for maintaining beta-cell mass comes from studies manipulating the cell cycle machinery. A cyclin D2 knockout does inhibit postnatal beta-cell replication and results in a ~75% beta-cell deficit in 14d-old mice (21). Neogenesis counted as the percentage of insulin positive duct cells was not altered in comparison to wild type mice. One would anticipate that beta-cell differentiation from progenitor cells could at least partially

compensate for this deficit if it were an active mechanism of beta-cell formation under these conditions. However, all these results have to be interpreted with caution, because there is also evidence to support the hypothesis that pancreatic duct cells do, in fact, serve as progenitors for new islets (islet neogenesis). First, there are numerous reports in rodents and humans demonstrating the presence of insulin positive cells in the pancreatic ductal epithelium and the induction of these cells under conditions that are believed to activate beta-cell regeneration (4, 8, 22-26). Second, lineage-tracing experiments with genetically marked exocrine duct cells instead of beta-cells using the carbonic anhydrase promoter II showed that at birth and at 4 weeks of age ~15% of beta-cells express this ductal marker (27). Therefore this study suggests that early in life ductal cells serve as progenitors for beta-cells. But, the data from all studies using lineage-tracing was obtained in rodents and it is unclear whether from the outcome extrapolation to islet turnover in humans is reasonable. Also, in most studies no measurement of islet neogenesis using the conventional parameter of insulin positive duct cells was performed to examine whether prior reports on the induction of islet neogenesis and beta-cell replication after e.g. pancreatectomy could be reproduced in the different experimental settings. Moreover, there might also be experimental limitations with these techniques regarding the efficiency and specificity of the labels. Recently, a model was proposed that provides an approach to integrate the controversial data reported for islet turnover in different studies. It hypothesizes that beta-cell replication and differentiation from progenitor or stem cells might be differentially regulated dependent on the intensity of the stimulus for regeneration (18). If the stimulus is low or moderate new beta-cells are primarily formed by replication, while a stronger stimulus (e.g. >50-60% pancreatectomy) also activates differentiation of progenitor or stem cells. This model emphasizes the shortage of experimental data in this field of research and that the experimental techniques may have a strong influence on results.

Another shortcoming of the available data is that it remains unknown how quickly humans can adapt their beta-cell mass in response to insulin resistance. Based on our estimate of islet turnover in human autopsy pancreata, it would require several years (7) until a clinically relevant number of new beta-cells are formed. Also, the overall frequency with one replicating beta-cell (replication marker Ki-67) in ~20 islets was generally low compared to rodents, in which one replicating beta-cell in ~2 islets can be found (8). Indirect evidence from transplantation surgery with living pancreatic organ donors (28) further suggests that the overall regenerative capacity in humans may be rather low. If the distal half of the pancreas is resected for donation to a relative with type-1 diabetes there is a ~50% risk for the development of diabetes mellitus for the donors within the next 9 to 18 years. One conclusion from this data is that in humans there may be no adequate beta-cell compensation after pancreatectomy even over longer periods. On the other hand, the donors were first-degree relatives of a subject with type-1 diabetes, so there is an up to 15% chance that the manifestation of diabetes mellitus was favored by a predisposition for autoimmune diabetes mellitus, although this was carefully excluded prior to organ donation. A recent study analyzed islet turnover in humans after ~50% pancreatectomy when the same individuals subsequently underwent pancreatic surgery again (29). This experimental design, although it is retrospective and included patients with malignant pancreatic disease, provides the unique opportunity to analyze human pancreatic tissue from the same individual at two time points. Interestingly, partial pancreatectomy in humans did neither induce beta-cell proliferation (Ki-67), nor the percentage of insulin positive duct cells (neogenesis). Taken together these studies indicate that the regenerative capacity in humans may be much lower than in rodents, because under conditions that induce deterioration of glucose metabolism there is little evidence for the induction of islet regeneration. There is also evidence for a negative correlation between neogenesis and beta-cell replication with aging (4, 25), which would additionally impair beta-cell regeneration in humans. This association was reproduced in isolated human islets and might be caused by an age-dependent decline of PDX-1 expression in human pancreatic tissue (30).

In order to understand the regulation of islet turnover and develop therapeutic targets, it is also important to understand the origin and the identity of progenitor cells and/or stem cells, whether they reside inside islets, in the pancreatic duct epithelium and/or the bone marrow. Pancreatic islet and ductal cells have successfully been transformed into beta-cells *in vitro* (31-34) and may therefore serve as a source for new beta-cells. For bone marrow cells, the situation is less clear. In rodents, the available data is controversial (35, 36), while there are almost no studies in humans. Recently, it was reported that in recipients of a hematopoietic stem cell transplant there is no evidence of beta-cells in the endocrine pancreas which differentiated from cells of the transplant, whereas in the exocrine pancreas some cells clearly derived from the graft (37). Thus it is unlikely that hematopoietic stem cells are a major source for new beta-cells. Nevertheless, bone-marrow derived cells in islets may be indirectly involved in islet regeneration by the release of locally acting factors which initiate endogenous regeneration (38). Mature pancreatic acinar cells have also been proposed to serve as progenitors for pancreatic islets (39, 40), although it was recently reported that in mice with an inducible genetic marker of acinar cells (lineage-tracing) and induction of islet regeneration by partial pancreatectomy or duct ligation, there is no evidence for labeled (=transdifferentiated) cells in the endocrine cell pool (41). At present, it has to be concluded that progenitors for pancreatic islets do reside in pancreatic islets themselves and in the ductal epithelium.

4. *IN VIVO* REGULATORS OF ISLET REGENERATION

Adaptation of the endocrine pancreas to long-term insulin resistance in humans is characterized by increased insulin secretion and the expansion of beta-cell mass to prevent the development of diabetes mellitus. Little

is known about the factors providing feedback to the endocrine pancreas, about the requirements of whole body metabolism for adaptation. In principle, extrapancreatic factors could directly or indirectly (*via* regulation of intrapancreatic signaling molecules) regulate beta-cell turnover. There is some evidence to suggest that glucose may be an ideal candidate: insulin secretion is tightly coupled to glycemia and beta-cells possess an exquisite sensitivity for metabolic changes. Based on data obtained in rodents, it has been hypothesized for some time now that glucose is a differential regulator of beta-cell plasticity (42-44). Undoubtedly, this has been shown for hyperglycemia, which induces beta-cell toxicity, oxidative stress and programmed cell death (45, 46). Much less data is available for the effects of normoglycemia or mild elevation of glucose concentrations, partly because of the dynamic nature of glucose concentrations throughout the day and the subtle changes that occur in non-diabetic individuals. Some explanation with a broader perspective may be inferred from a model that integrates the variables beta-cell mass, plasma insulin concentrations and plasma glucose concentrations into a regulatory feedback loop in order to clarify the relationship between them (43). This model suggests that during prolonged hypoglycemia, beta-cell mass is decreasing due to beta-cell death exceeding beta-cell replication. At normoglycemia or mild hyperglycemia, beta-cell replication exceeds death and leads to the expansion of beta-cell mass and an increase of insulin concentrations, whereas hyperglycemia induces net cell loss due to increasing beta-cell death. An important issue for this complex regulation of beta-cell turnover is the question how beta-cells could sense small glucose excursions during normoglycemia and translate these signals into tissue plasticity. Recently, it has been reported that glucokinase and insulin receptor substrate-2 (IRS-2) may play a central role in this context (47). Glucokinase has long been recognized as a key component of the glucose sensing apparatus of the beta-cell for glucose stimulated insulin secretion (48). In mice haploinsufficient (glucokinase +/-) of beta-cell specific glucokinase, the adaptation to insulin resistance induced by high fat feeding is impaired (47). In wild type mice, prolonged insulin resistance over a period of 20-weeks induced beta-cell replication and a >2-fold increase of beta-cell mass, while in glucokinase deficient mice the induction of beta-cell replication and expansion of beta-cell mass was absent. IRS-2 expression, which is known to play an important role for beta-cell growth and survival, followed the same pattern with increased expression in wild type mice fed with a high fat diet and reduced expression in glucokinase deficient mice fed with the same high fat diet. Beta-cell specific overexpression of IRS-2 in glucokinase +/- mice reversed the defects of beta-cell replication and allowed expansion of beta-cell mass. These data imply that both glucokinase and IRS-2 are important factors for beta-cell expansion in response to dietary fat induced insulin resistance. It is also evidence that glucose might indeed be an important direct signal not only for the regulation of beta-cell function but also for the regulation of beta-cell turnover *via* glucokinase and IRS-2. Interestingly, high fat feeding also induced the expression of the IGF-1 receptor in wild type mice but not in glucokinase +/- mice, although the functional relevance of this observation remained unclear. It emphasizes however that glucose metabolism is just one component of the complex regulation of beta-cell expansion. In the context of the afore mentioned study, it has been concluded that glucose may be a dominant factor which is directly regulating beta-cell adaptation to insulin resistance. An indirect regulatory mechanism, e.g. *via* stimulation of insulin release and activation of insulin signaling, seems unlikely, since in mice double heterozygous for null alleles in the insulin receptor and insulin receptor substrate-1 genes, a ~50% reduction in expression of these two proteins does not impair beta-cell expansion in the response to insulin resistance (49).

Adaptation of beta-cell mass to insulin resistance in humans probably is a slow process that requires months to years. During pregnancy, expansion of beta-cell mass for the maintenance of glucose homeostasis needs to occur more quickly within weeks to a few months. Therefore, understanding of the mechanisms regulating beta-cell adaptation during pregnancy might identify valuable therapeutic targets which might be effective within short periods of time and therefore particularly attractive for diabetes therapy. Data from animal experiments document the rapid changes occurring in the endocrine pancreas, e.g. in rats expansion of beta-cell mass during pregnancy is realized by a 3-fold increase of beta-cell proliferation probably mediated by placental lactogens and prolactin, while after delivery, involution of the endocrine pancreas occurs within several days by reduced beta-cell replication and increased beta-cell apoptosis (50). However, the molecular mechanisms underlying these changes have not been completely understood. Recently, a more detailed analysis of the mechanisms underlying beta-cell adaptation to pregnancy has been performed with special attention to menin (51). Menin is the protein product of the Men1 gene and known to be an endocrine tumor suppressor and transcriptional regulator (52). In multiple endocrine neoplasia, type-1 mutations of Men1 cause synchronous tumors of the endocrine pancreas, pituitary and parathyroid glands. In mice and humans, it has been shown that reduced expression of Men1 promotes neuroendocrine cell proliferation (53) and hence, it was hypothesized that beta-cell adaptation to pregnancy might be regulated by changes of Men1 expression. In C57Bl/6 mice, it was shown that during pregnancy reduced Men1 expression (mRNA and protein) corresponded to increased beta-cell proliferation in maternal islets (51). The protein levels of the menin responsive factors p27 and p18, which encode for cyclin-dependent kinase inhibitors, were concomitantly reduced, thus providing a potential mechanism *via* cell-cycle regulation for the induction of beta-cell proliferation. Consistent with these findings transgenic expression of menin in maternal beta-cells inhibited beta-cell proliferation and expansion of beta-cell mass during pregnancy resulting in defective glucose metabolism with hyperglycemia and impaired glucose tolerance. Interestingly, lactogen signaling leads to decreased Men1 expression, e.g. prolactin treatment with osmotic pumps over 6 days reduced Men1, p27 and p18 mRNA by ~50% and induced beta-cell proliferation (2-3-fold). Although the complete signaling cascade for beta-cell turnover is likely

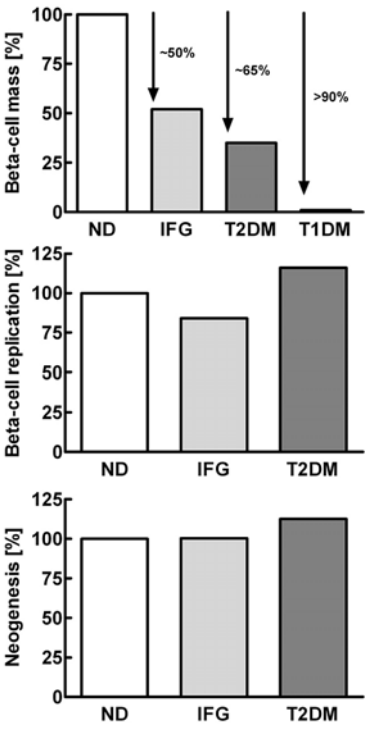

Figure 2. Beta-cell mass (A) is reduced in subjects with impaired fasting glucose (IFG), type-2 diabetes and type-1 diabetes in comparison to non-diabetic individuals. Beta-cell replication (B) and neogenesis (C) are not defective in prediabetic (IFG) and type-2 diabetic subjects. ND: Nondiabetic; IFG: Impaired Fasting Glucose; T2DM: Type-2 Diabetes Mellitus; T1DM: Type-1 Diabetes Mellitus. Modified from (4, 10).

more complex than described here, these studies provide potentially important insights about the hormonal-molecular factors central to beta-cell growth during pregnancy.

Other hormones that are considered to be potentially relevant for beta-cell turnover under physiological conditions are incretin hormones, e.g. glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (54, 55). GLP-1 and GIP are the predominant hormones mediating the incretin effect, which describes the phenomenon that oral glucose elicits a greater insulin response compared to an intravenous isoglycemic infusion (56). Besides acute effects on insulin and glucagon secretion and extrapancreatic effects (deceleration of gastric emptying, promotion of satiety), GLP-1 also promotes beta-cell growth by inducing beta-cell differentiation and replication, as well as inhibiting beta-cell apoptosis (57-60). Experimentally, these effects are also well established for GIP (61), but it remains unknown whether physiological levels of GLP-1 or GIP receptor signaling are essential for the regulation of beta-cell mass. A good model to address this question are GLP-1 or GIP receptor knockout (GLP-1R -/-, GIPR -/-) mice. GLP-1R -/- mice exhibit only mild abnormalities of glucose homeostasis and are characterized by normal beta-cell mass and beta-cell size but defective islet architecture with fewer large islets and smaller islets than in wild type mice (62). Interestingly, the typical distribution of beta-cells in the core of the islets and alpha-cells in the mantle is disturbed and more (2-3-fold) alpha-cells are located centrally. This defect of islet morphology in GLP-1R -/- mice indicates that GLP-1R signaling is one important component of normal islet development. Additional studies analyzed beta-cell regeneration in GLP-1R -/- mice after induction of beta-cell death by streptozotocin injection (60) or reduction of beta-cell mass by partial pancreatectomy (63). Disruption of GLP-1 receptor signaling leads to an increased vulnerability of beta-cells to streptozotocin with increased rates of beta-cell apoptosis and impairs beta-cell regeneration. Whereas wild type mice are capable of complete restoration of beta-cell mass within five weeks after a ~70% pancreatectomy, regeneration in GLP-1R -/- mice is severely impaired with a prolonged deficit of beta-cell mass after five weeks of ~60%. In the postreceptor signaling pathways, expression of the transcription factor PDX1 (pancreatic duodenal homeobox-1) is essential for integration of GLP-1R dependent signals for islet turnover in beta-cells (64). These studies provide evidence that endogenous GLP-1 signaling is necessary for successful adaptation of islet turnover to pancreatic injuries with beta-cell deficit. Qualitatively similar results have been reported from experiments with GIPR -/- mice (61). Although there are no major defects of GLP-1 or GIP secretion in type 2 diabetes, the lack of GIP responsiveness in subjects with type 2 diabetes (65) indicates that this defect may also apply to the induction of beta-cell growth in the face of insulin resistance specifically in subjects with a predisposition for the development of diabetes mellitus. However, at present this remains unknown, since there is no good experimental model of beta-cell regeneration in primary human tissue to address this question in subject groups with different diabetes risks or metabolic status. Recent discussions on the importance of GLP-1 signaling for mechanisms of beta-cell turnover in humans with polymorphisms in the gene coding for transcription factor 7-like-2 (TCF7L2) (66) underscore the need for experimental models of beta-cell regeneration in human tissue.

5. FAILED ADAPTATION OF BETA-CELL MASS IN DIABETES MELLITUS

In diabetes mellitus, the self-renewing capacity of the endocrine pancreas fails and leads to a progressive beta-cell deficit (4, 5, 67). Although in type-2 diabetes mellitus important mechanisms of regeneration (replication and neogenesis) are intact, beta-cell deficiency cannot be overcome (Figure 2). The activities of regenerative mechanisms in the islet in type-1 diabetes are not well characterized, however indirect evidence suggests that even after long-standing type-1 diabetes there still is regeneration occurring (10). The reason for failed adaptation and beta-cell deficiency in diabetes mellitus is increased beta-cell apoptosis (4, 10, 68). Furthermore, replicating beta-cells are more vulnerable to apoptosis than non-replicating cells in a proapoptotic environment, leading to preferential demise of the regenerating cells (69, 70).

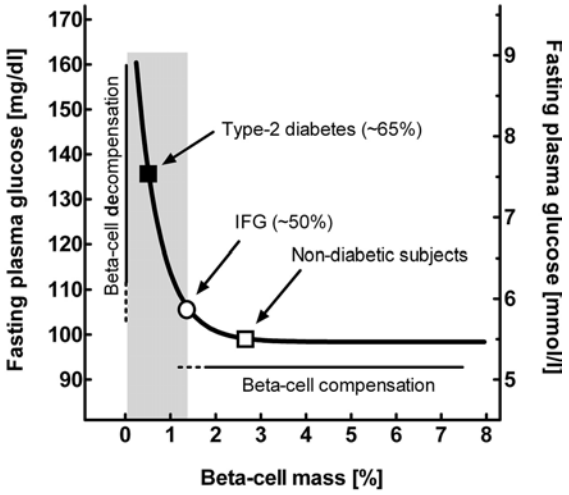

Figure 3. Relationship between beta-cell mass and fasting glycemia in weight-matched, obese humans without antidiabetic therapy. Depicted are mean values. If a critical beta-cell deficit of ~50% is exceeded there is decompensation of glucose metabolism. The shaded area indicates the region with tight coupling between beta-cell mass and fasting glycemia, in which therapeutic interventions to block beta-cell loss and/or expand beta-cell mass would be most effective with respect to glycemia. Modified from (86).

The mechanism leading to the induction of beta-cell apoptosis particularly in the prediabetic individual before decompensation of glucose metabolism remains unknown. Potential factors are adipocytokines (e.g. TNF-α, IL-6), which directly or indirectly influence beta-cell function and survival (71-73). Also, they have been implicated to inhibit the signaling cascade of the insulin receptor leading to insulin resistance (74). Other proinflammatory cytokines (e.g. interleukin-1β) have also been suspected to induce beta-cell destruction in type-1 and type-2 diabetes (75, 76). There is some evidence that islet production of interleukin-1β may be regulated by glucose (77). This scenario would implicate that interleukin-1β is a proapoptotic factor which becomes relevant with the manifestation of hyperglycemia. Another potential cause for increased beta-cell apoptosis is the cytotoxic action of the beta-cell hormone human islet amyloid polypeptide (IAPP), which aggregates into mature amyloid fibrils (69, 78). Oligomeric molecules of this aggregation process interact with and destabilize membranes, induce endoplasmic reticulum stress and apoptosis (69, 78-81). Recent publications describing genetic risk loci for type-2 diabetes provide evidence that a genetic defect of the beta-cell is a prerequisite for the development of diabetes mellitus, since the majority of loci described are functionally associated with beta-cell metabolism, survival and regeneration (82, 83). Therefore, it has to be hypothesized that beta-cell deficiency develops in genetically predisposed individuals due to failed beta-cell regeneration and increased beta-cell apoptosis in the face of insulin resistance, which is most commonly induced by obesity.

In diabetes mellitus with overt hyperglycemia beta-cell apoptosis and loss of beta-cell function is accelerated by chronic hyperglycemia (glucose toxicity), elevated free fatty acids (lipotoxicity) and cytokines. Gluco-lipotoxicity involves decreased expression of the insulin gene, the regulation of transcription factors and leads to chronic oxidative stress, e.g. interaction of advanced glycation end products (AGE) with its receptor RAGE and activation of NFkappa-B. Beta-cells are particularly vulnerable to oxidative stress since their intrinsic antioxidative capacity is low (84). Hyperglycemia also increases the expression of the FAS-receptor and signaling of the extrinsic pathway for induction of apoptosis (45).

Human and animal studies show that there is a causal relationship between beta-cell deficiency and the development of hyperglycemia (Figure 3) (85, 86). In experiments with Sprague-Dawley rats (pancreatectomy or injection of alloxan or streptozotocin), the limit before decompensation of glucose metabolism occurs is a beta-cell deficit of ~80% (87), while in larger animal models (primates, pigs) decompensation occurs earlier when the beta-cell deficit exceeds ~50% (85, 88). In humans, the relationship between beta-cell mass and fasting plasma glucose concentrations is similarly described by a curvilinear function with a steep increase of glycemia when beta-cell deficiency exceeds a critical limit of ~50% compared to non-diabetic individuals (Figure 3). In this zone of decompensation, beta-cell mass is tightly coupled to diabetic glucose concentrations and thus opens the perspective to effectively lower glycemia in diabetes mellitus by even small increases of beta-cell mass. Since the primary reason for beta-cell deficiency is increased apoptosis, therapeutic approaches for preservation or expansion of beta-cell mass have to include mechanisms to induce regeneration and concomitantly inhibit beta-cell apoptosis.

6. THERAPY

Current therapeutic principles for the management of diabetes mellitus possess some differential effects on the activity of progressive beta-cell failure, but do not provide sufficient protection from the chronic progressive course of the disease in order to prevent its severe complications (89, 90). Due to the causal relationship between beta-cell deficiency, islet dysfunction, insulin deficiency and loss of glucose control beta-cell regeneration is one goal for future diabetes therapy. The increasing understanding of the molecular mechanisms of beta-cell turnover provides potential targets for the development of new therapeutic techniques. However, the experimental evidence largely derives from animal studies and it is still uncertain whether extrapolation to humans is possible. Conceptually, beta-cell expansion may be achieved by the induction of endogenous regeneration and/or exogenous cell replacement therapy. *In vitro* production of fully differentiated and mature beta-cells for transplantation into patients with diabetes mellitus has not been accomplished yet and may be associated with immunological problems, if the expanded tissue is not

derived from the recipient, and accelerated cell senescence. This and other adverse effects (e.g. activation of coagulation, local inflammation, transient hypoxia) induce a rapid decline of islet function after transplantation limiting its application. The present report will focus on the approaches to induce endogenous beta-cell regeneration. This has the advantage that physiologically occurring expansion of beta-cell mass for adaptation to new states of insulin resistance like in obesity or pregnancy serves as a model to identify molecular mechanisms and potential therapeutic targets.

6.1. INDUCTION OF BETA-CELL REGENERATION

Expansion of beta-cell mass in obesity provides the basis to compensate for insulin resistance by increasing insulin secretion. As pointed out previously (see 3.1.), it is unlikely that insulin itself is the signal for the endocrine pancreas to expand. Exogenous subcutaneous insulin therapy in diabetes mellitus reproduces the glucose lowering effects of endogenous insulin and is able to acutely improve beta-cell function (91, 92) but most likely does not induce beta-cell regeneration in the clinical setting. Nevertheless, components of the insulin and IGF-I receptor signaling pathways (e.g. IRS-2) are potential targets to therapeutically exploit the effect of insulin and IGF-I receptor signaling for beta-cell growth and differentiation. A proof of principle was reported in experiments with IRS-2 knockout mice (IRS-2 -/-) (93). IRS-2 deficiency induced the development of type-2 diabetes due to insulin resistance and a beta-cell deficit of 83%. This defect is specific for IRS-2, since IRS-1 -/- mice compensate for insulin resistance by expanding beta-cell mass by 85%. Beta-cell specific overexpression of IRS-2 enhances beta-cell growth, survival and function and was able to prevent the development of diabetes mellitus in IRS-2 -/- mice and other rodent models of diabetes mellitus (94) by increasing beta-cell mass. Upregulation of IRS-2 was associated with enhanced expression of the transcription factor PDX1, which is important for glucose sensing of the beta-cell, insulin secretion and suppression of apoptosis (95-97). Expression of the gene for the beta-cell glucose transporter GLUT-2 was also upregulated (3-fold), providing a molecular link for enhanced glucose sensitivity of beta-cells (94). Similarly, transactivation of IRS-2 and other metabolic genes by TFE3, a basic helix-loop-helix protein, reduced glucose levels in diabetic and non-diabetic mice (98). These studies exemplify that therapeutic approaches based on molecular biological techniques to influence the expression of cell signalling molecules, transcription factors and/or cell cycle proteins have a great potential to develop specific and very effective therapeutic targets for the expansion of beta-cell mass. However, therapeutic principles based on molecular biology also bear the burden that they interfere with fundamental processes of cell metabolism and require extensive preclinical testing to identify the potential risks they are associated with. Given the fact that these risks for adverse events during diabetes therapy are sometimes not even completely understood for currently available therapies, which were supposed to be safe, and may be discovered after market approval, it has to be expected that therapeutic approaches against diabetes mellitus using molecular biological techniques will not be available for clinical application within the next 10 years.

The regulation of beta-cell adaptation during pregnancy occurs, at least partially, by hormones (placental lactogen and prolactin; see 3.1.). A stimulatory effect of lactogens on beta-cell proliferation and function has been shown *in vitro* with primary and clonal beta-cells, as well as *in vivo* with continuous infusion (51) and in mice with expression of placental lactogen in the beta-cell (99). Beta-cell selective expression of placental lactogen leads to lowering of blood glucose levels compared to control mice by elevation of plasma insulin concentrations. Beta-cell proliferation and beta-cell mass were increased (2-fold) in these animals. Interestingly, glucose-responsiveness of isolated perifused islets was normal, suggesting that the observed effects on glucose metabolism in vivo result predominantly from increased beta-cell mass and less importantly from increased function of individual beta-cells. In this experimental approach with transgenic expression of murine placental lactogen selectively in beta-cells, there is no elevation of circulating concentrations of placental lactogen despite its property of being a secretory protein. Studies on the functional role of placental lactogens or prolactin in humans are not available. Especially the effects of elevated prolactin concentrations on the endocrine pancreas could be analyzed in patients with prolactinomas before and after antisecretory therapy.

Analogous to the construction of beta-cells expressing placental lactogens tissue engineering techniques might also be used to introduce other molecules into beta- and non-beta-cells. Other hormones or growth factors that might also be valuable for this purpose are e.g. hepatocyte growth factor (HGF), epidermal growth factor (EGF), gastrin and incretin hormones. Transgenic overexpression of HGF in mice induces beta-cell proliferation and activates signaling through protein kinase C in beta-cells (100). Delivery of HGF into diabetic mice (streptozotocin) by rapid injection of DNA from a recombinant human HGF expressing plasmid into the tail vein induced beta-cell proliferation, decreased beta-cell apoptosis and improved glucose levels (101). EGF and gastrin have also been studied without direct genetic manipulation but systemic delivery. Gastrin infusion (*i.v.*) over a period of three days in rats (duct-ligation model of pancreas regeneration) seems to selectively stimulate new islet formation, because there was a 2-fold increase of beta-cell mass with an increased number of individual, extra-islet beta-cells and small beta-cell clusters, while there was no induction of beta-cell proliferation (102). The expectations that gastrin may be an effective therapy to induce beta-cell regeneration were further encouraged by reports that combination therapy with gastrin and EGF induces expansion of beta-cells in isolated human islets (103) and restores normoglycemia in diabetic NOD mice by expansion of beta-cell mass (3-fold) (104). However, the clinical relevance for a systemic exogenous delivery of these molecules is controversial, since even in humans with a gastrin producing tumor islet hyperplasia can only be found in direct proximity of the tumor and not throughout the whole organ (105).

Agonistic signalling at the receptor for the incretin hormone GLP-1 also exerts trophic effects on the endocrine pancreas and induces beta-cell differentiation from progenitor cells and beta-cell proliferation. In islet-like cell clusters from human fetal pancreata, the incretin mimetic exendin-4 induces differentiation into beta-cells and replication *in vitro* (58). This is functionally relevant and has a sustained effect since after transplantation of human islet-like cell clusters under the kidney capsule into athymic nude mice treatment with exendin-4 (*i.p.* over 10 days) but not with saline improves graft function (glucose induced C-peptide release) even six weeks after discontinuation of exendin-4. The mechanism mediating proliferation and differentiation in these experiments is again the transcription factor PDX1. There is some evidence to suggest that the induction of beta-cell proliferation by agonism at the GLP-1 receptor involves transactivation of the EGF receptor in a PI 3-kinase and PKC dependant manner (59). Other important factors of the GLP-1 receptor signalling pathway are IRS-2, cAMP and the cell cycle regulator Cyclin D1 (106). Recently, it has been reported that elevation of endogenous incretin hormone concentrations by inhibition of their inactivating enzyme dipeptidyl peptidase-4 (DPP-4) restores beta-cell mass in streptozotocin-induced high-fat diet diabetic mice (107).

6.2. INHIBITION OF BETA-CELL APOPTOSIS

A physiological mechanism for cytoprotection probably is the auto- and paracrine effect of insulin. In a beta-cell line (108) and in isolated islets (109), it was demonstrated that exogenous but also directly secreted insulin protects from apoptosis and induces cell proliferation. These effects require PI3-kinase signaling and PDX-1. The concentrations of exogenous insulin necessary were clearly in the supraphysiological range (nM to μM). These concentrations might be achieved under physiological conditions if the insulin concentrations in close proximity to actively secreting beta-cells reach the concentrations inside secretory vesicles. However, it seems unlikely that exogenous insulin therapy is capable of reproducing the high local concentrations inside the islets in non-diabetic individuals if insulin is absorbed from a subcutaneous depot. Clinical studies have not been able to show that insulin therapy in type-2 diabetes mellitus does protect from progressive loss of beta-cell function (89, 90). Nevertheless, morphometric analysis of human autopsy pancreata shows that prolonged insulin therapy might be associated with a lower beta-cell deficit compared to di*et al*one without differences of fasting glycemia (4). The mechanism remains unclear because there is not sufficient data about islet turnover from these pancreata.

Incretin hormones not only induce new beta-cell formation but also protect beta-cells from programmed cell death. This has been confirmed for both molecules already approved for the treatment of type-2 diabetes mellitus (exenatide, sitagliptin) and other molecules in development (*e.g.* vildagliptin, liraglutide) (110). The antiapoptotic effects of GLP-1 based therapies have been demonstrated *in vitro* for cytokine induced beta-cell apoptosis (IL 1β, TNFα, interferon γ) in primary cultured rat beta-cells (60). In the beta-cell line MIN6, GLP-1 reduces H_2O_2 induced beta-cell death by cAMP and PI3-kinase dependent mechanisms (111). The same signaling pathways are important for the inhibition of free fatty acid induced beta-cell apoptosis by GLP-1 in RINm5f cells (112). The antiapoptotic effect of GLP-1 receptor agonism is not beta-cell specific, because primary neurons are also protected by GLP-1 and exendin-4 from glutamine induced damage (113). *In vivo* studies have been primarily performed with mice and rat models. GLP-1, incretin mimetics and DPP-4 inhibitors improve function of the endocrine pancreas and improve islet morphology. As mentioned above (see 6.1.), GLP-1 receptor signaling increases beta-cell mass by the induction of beta-cell regeneration (replication and differentiation) and concomitant inhibition of beta-cell apoptosis (60, 64, 114). The antiapoptotic effect of GLP-1 can also be reproduced in isolated human islet tissue. In static incubation experiments with freshly isolated human islets, GLP-1 reduces spontaneous beta-cell apoptosis, improves glucose-stimulated insulin secretion and protects from gluco-lipotoxicity (115, 116). The transcription factor PDX1 is essential for the protective effects of GLP-1, since in mice with a beta-cell specific inactivation of PDX1, Exendin-4 is ineffective (64). On the molecular level, the antiapoptotic effect is also coupled to the reduction of proapoptotic factors like cleaved caspase-3 and the increase of antiapoptotic factors like Bcl-xL, Bcl-2 or IAP-2. In addition to these direct effects, there may also be indirect effects mediating beta-cell protection from the reduction of glucose and FFA levels. However, these indirect effects would also be present during the treatment with other antidiabetic strategies. Due to the low beta-cell turnover in humans, it probably requires several years until it will be clarified, whether incretin mimetics slow or reverse disease progression compared to established therapeutics. Tachyphylaxis does not seem to be a problem during prolonged GLP-1 treatment (117). GLP-1 concentrations used for *in vitro* experiments are higher (nM range) than pharmacological GLP-1 concentrations occurring during antidiabetic therapy (pM range). Since plasma concentrations of GLP-1 *in vivo* after DPP-4 inhibition are in the low pM range and beta-cell protection is still occurring, it seems unlikely that GLP-1 concentrations during diabetes therapy with incretin mimetics are below the threshold for induction of beta-cell regeneration. Clinical studies are underway to examine whether GLP-1 based therapies might also be effective for the treatment or prevention of type 1 diabetes mellitus.

Recently, antagonism at the interleukin-1 receptor with a recombinant human interleukin-1 receptor antagonist has been reported to improve glycemia and markers of beta-cell function and reduce the level of inflammatory markers in subjects with type-2 diabetes treated daily over a period of 13 weeks (118). This treatment is based on the concept that, in the islet in type-2 diabetes, there is a proinflammatory environment with imbalance between elevated interleukin-1β (76) and reduced expression of interleukin-1 receptor antagonist (119) leading to the induction of apoptosis. The beneficial effects of a short-term treatment with a human interleukin-1

receptor antagonist on beta-cell function parameters suggests that this principle might be effective to prevent beta-cell destruction and promote beta-cell regeneration in type-2 diabetes when administered over longer periods.

Beta-cell death in type-1 diabetes mellitus is caused by a T-cell mediated autoimmune attack. Immunosuppression is able to prevent further beta-cell loss in type-1 diabetes but is associated with unacceptable side effects (120). Intervention with a human IgG1 antibody directed against CD3 in subjects with new-onset type-1 diabetes over six consecutive days improved residual beta-cell function during the follow-up period of 18 months (121). The CD3 molecule is expressed on the majority of T-cells and application of a CD3-specific antibody results in a persistent decrease of the $CD4^+/CD8^+$ ratio. The therapeutic response was most prominent among patients with high residual beta-cell function, suggesting that intervention would be most beneficial early in the disease process.

Another factor that regulates beta-cell apoptosis in experimental models is estrogen. Estrogens might be the underlying reason for the observation that type-2 diabetes prevalence is lower in women than in men (122). Beta-cells in estrogen receptor knockout mice are vulnerable to oxidative stress and undergo apoptosis, which can be reversed by the treatment with estrogen (123). The effect of estrogens on glucose metabolism can also be reproduced in humans, since in women with postmenopausal hormonal therapy the incidence of diabetes mellitus is lower compared to women without estrogen therapy (124, 125). However, it is unlikely that estrogens have a relevant therapeutic benefit in the context of diabetes, since postmenopausal hormonal therapy might be associated with the induction of breast cancer (126) and selective estrogen receptor modulators (*e.g.* tamoxifen) have an antagonistic effect on beta-cells and reduce beta-cell survival (123).

Antioxidants are another approach for beta-cell protection, because beta-cells have a low expression rate of intrinsic antioxidants and are exposed to chronic oxidative stress in the hyperglycemic diabetic environment (84, 127). In clonal beta-cells, Zucker diabetic fatty rats and GK rats antioxidants (*e.g.* N-acetylcysteine, vitamin E) are beta-cell protective and improve glucose metabolism (128, 129). Studies in humans documenting beneficial effects of antioxidants on clinically relevant endpoints are missing.

Inhibition of human IAPP induced beta-cell apoptosis may be a target for future developments. It requires that the mechanism of apoptosis induction is fully understood, presently it has to be hypothesized that induction of endoplasmic reticulum (ER) stress is one important signaling component (79, 130). Therapeutic approaches for the stabilization of the ER (*e.g.* chemical chaperones) have not been tested in a model of IAPP-toxicity with primary human beta-cells. Recent reports suggest that oligmeric species of the IAPP aggregation process might be targets to inhibit the induction of ER stress, because these soluble oligomers interact with and destabilize ER membranes and represent the primary toxic principle of many amyloid forming peptides (*e.g.* Alzheimers beta protein, α-synuclein, tau) (79, 131). They share a common conformation-dependent structure that induces antibody production when injected into other species. These antibodies have been used in *in vitro* experiments to block human IAPP toxicity (131). However, in mice transgenic for human IAPP, production of these antibodies by vaccination with protofibrillar oligomeric Alzheimers beta protein did not inhibit beta-cell apoptosis and the progressive loss of beta-cell mass (132). Additional studies need to address whether the specific inactivation of human IAPP oligomers by other techniques might be effective to protect beta-cells from apoptosis. However, the transient existence of these oligomers during aggregation is a challenge for the efforts to make them a therapeutic target. A different approach for cytoprotection from human IAPP toxicity was reported in a study with activation of PPARγ by rosiglitazone which protects human islets from human IAPP induced apoptosis by activation of PI3-kinase (133). Taken together, more data is required about the role and molecular mechanisms of human IAPP induced beta-cell loss in diabetes mellitus until it may become a specific therapeutic target.

Inhibition of beta-cell apoptosis has also been studied by directly targeting apoptotic signaling factors of the extrinsic (*e.g.* cFLIP, A20) or intrinsic (Bcl-2, Bcl-xL) activation pathways (134). However, these molecules have been found to be less effective than targeting of unspecific effector molecules of the execution pathway of programmed cell death (*e.g.* X-linked inhibitor of apoptosis protein, XIAP). XIAP binds to the active site of the effector caspases-3, -7 and -9 and inhibits apoptosis induced by various factors. This is an example that enhanced protection from apoptosis by an intervention directed at more downstream factors of the apoptotic signaling pathways may occur at the expense of specificity. Since caspases are an important component of the apoptotic machinery in all cell types it is likely that their inactivation is associated with significant side effects unless caspase inhibitors can be delivered and confined to specific cell types.

7. CONCLUSIONS AND PERSPECTIVES

Islet morphology in diabetes mellitus is characterized by beta-cell deficiency and dysregulation of islet turnover. Humans have a much lower capacity for beta-cell regeneration than rodents, which is even further declining with aging. Data from studies with human autopsy and human donor pancreata suggest that, after the age of 15-20 years, beta-cell growth by replication (probably the primary mechanism for regeneration) is minimal (25, 135). Since most humans requiring antidiabetic therapy are older than 20 years it will require long-term studies to identify which treatment option might induce beta-cell regeneration in the clinical setting. Due to the coupling of beta-cell proliferation and death for tissue plasticity therapeutic approaches regulating both processes will be more effective. Regeneration of beta-cell mass will presumably be more successful if the proapoptotic stimulus on the beta-cell is not as substantial as in overt diabetes

mellitus. This would be the case in prediabetic states before the development of hyperglycemia multiplies the toxic stimuli on beta-cells. To take advantage of this early therapeutic window, it will be necessary to clearly identify subjects at risk, *e.g.* by application of sensitive analysis techniques of residual beta-cell function. Although early intervention is plausible from a pathophysiological point of view, prediabetic conditions (impaired fasting glucose, impaired glucose tolerance) have not been classified as disease states yet that require drug treatment. Clinical studies need to clarify whether diabetes prevention by beta-cell regeneration reduces relevant clinical endpoints and is cost-effective. Similarly, clinical trials have to analyze the benefit of preservation of beta-cell mass for disease progression. Incretin hormones, which enhance new beta-cell formation and concomitantly reduce beta-cell apoptosis, are already available for therapy of type-2 diabetes mellitus. In type-1 diabetes endogenous regeneration of beta-cell mass may be achieved if the autoimmune process is suppressed with drugs that do not inhibit beta-cell regeneration themselves. Since therapeutic targeting of autoimmune-mediated beta-cell death is associated with immune modulation and suppressive side effects decisions for interventions need to be based on the individual risk for disease, particularly in children. Combination therapies with agents that directly promote beta-cell regeneration and inhibit apoptosis (*e.g.* GLP-1) may enhance efficacy and lower risks. Due to the low beta-cell turnover in humans, it will probably require years until direct effects on beta-cell mass might be expected. However, one fundamental problem is that there are no sensitive techniques to quantify beta-cell mass *in vivo*. With elegant imaging techniques (*e.g.* PET, MRI) some progress has been reported in animal models (136, 137) but not in clinical trials. Therefore the clinical evaluation of cytoprotective therapies for the islets of Langerhans will initially be realized by the application of metabolic tests (*e.g. i.v.* glucose or arginin stimulation) (138). In animal and clinical studies, these tests reproducibly correlate (coefficient of correlation ~0.8.) with beta-cell mass, which was manipulated by beta-cell toxins, partial or total pancreatectomy or islet transplantation. Safety issues also have to be considered, since the therapeutic manipulation of regeneration and apoptosis may interfere with physiological tissue remodeling in other organs. This may be of concern because experimental studies usually last weeks to months, while clinical application would require treatment over many years, particularly in the context of diabetes prevention.

In conclusion, the development of therapeutic strategies for the preservation and/or regeneration of beta-cell mass requires detailed knowledge about the molecular mechanisms involved. Many signaling molecules, transcription factors, hormones and growth factors have been identified as potential targets. However, many of these factors have been examined in cell lines or animal models and await confirmation in humans. Strategies involving molecular biological techniques or gene therapy need to make significant progress before clinical application may be safe. Long-term clinical trials will be required to investigate whether therapeutic utilization of the regenerative capacity of the human pancreas provides the perspective for long-term stabilization or even cure of diabetes mellitus.

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