CHRONIC DIABETIC COMPLICATIONS: THE BODY'S ADAPTIVE RESPONSE TO HYPERGLYCEMIA GONE AWRY?

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ABSTRACT

In the last two decades, diabetes mellitus has reached epidemic proportions in the United States. Most of the recent increase in prevalence of diabetes involves type 2 disease and is a result of an alarming increase in prevalence of obesity, although type 1 diabetes may also be on the rise. Advances in treatment have essentially eliminated ketoacidosis as a cause of death and led to better glycemic control than ever before. Consequently, diabetics now live a longer life, allowing many of them to develop the chronic complications of the disease. Here we review the evidence that a maladaptive response to hyperglycemia contributes to diabetic neuropathy, a major microvascular complication. We postulate that the same response may also be the culprit for the other chronic complications of diabetes.

Introduction

Diabetes mellitus was an invariably fatal illness in the pre-insulin era. In 1889, Oskar Minkowski first demonstrated that removal of the pancreas led to diabetes in dogs, documenting that diabetes is caused by the absence of a factor produced by the pancreas. In 1921, Frederick Banting and Charles Best, with the help of James Collip, purified the factor from the pancreas and named it "insulin"; their discovery revolutionized the treatment of diabetes. Although the expectation that insulin would be the long awaited cure for diabetes has remained unfulfilled, over the years, insulin therapy has essentially eliminated ketoacidosis as the cause of death among diabetics. This monumental discovery in the history of medicine has enabled patients to be actively employed and to stay out of the hospital most of the time. In the last two decades, recombinant DNA technology led to efficient production of insulin, and the development of insulin variants and formulations with widely varying durations of action, advances that, in conjunction with the development of incretins and insulin-sensitizers and improved protocols for islet transplantation, have led to better glycemic

control of type 1 and type 2 diabetes than at any time in history. Unfortunately, none of these measures represent a cure. Moreover, the longer lifespan of diabetics resulting from improvements in therapy has led to the emergence of chronic diabetic complications as the major cause of morbidity and mortality among people afflicted with the disease.

Recently, our laboratory has developed an *in vivo* induced islet neogenesis approach as a new therapy for diabetes and has established the feasibility of the regimen in laboratory animals (1). During the course of our investigation we discovered that hyperglycemia *per se* causes insulin-expressing cells to appear in multiple tissues and organs outside the pancreas (1,2). This review describes how control experiments that did not work as anticipated led to some totally unexpected discoveries. The observations form the basis of our hypothesis that chronic diabetic complications may be the result of the body's adaptive response to hyperglycemia gone awry.

Experimental Gene Therapy for Type 1 Diabetes

Type 1 diabetes mellitus is caused by absolute insulin deficiency, usually the result of destruction of the pancreatic β cell by autoimmunity. Cure of type 1 diabetes would require the reversal of the autoimmunity (3) and the restoration of normal insulin production by the body (4). Our research focus has been on the reinstatement of insulin production in diabetic animal models. Islet transplantation, a strategy to restore insulin production for type 1 diabetes, has been under development for almost three decades (5); however, it was only with the introduction of the Edmonton protocol in 2000 (6) that the procedure has been met with a high enough success rate to justify its clinical application in specialized centers and then only in highly selected patients. When carried out in the best centers, the majority of islet transplant recipients can be taken off insulin therapy during the first year after the experimental procedure. However, long-term studies show that most if not all treated patients need to be restarted on insulin injections a few years later (7). Another drawback of islet transplantation is the requirement for lifetime immunosuppressive therapy with its associated side effects. Finally, the availability of pancreatic islet donors is severely limiting; as a result, only a few hundred patients have received islet transplants per year, though tens of thousands have met the clinical criteria for the procedure. With these limitations in islet transplantation, recent research has focused

on developing new methods for restoring normal insulin production by cell and gene therapy (4,8).

The construction of biomechanical artificial pancreas and of glycemia-responsive insulin-producing cells *in vitro,* the taming of insulinoma cells to strip them of their malignant potential, the encapsidation of insulinoma or other β cells or engineered insulin-producing cells to allow them to evade immune attack after implantation, the induced differentiation of embryonic stem cells into β cells *in vitro,* and the amplification of β cells *in vitro* to increase the yield to circumvent donor shortage are major avenues of research in the cell therapy area (8). In the gene therapy arena, a popular strategy has been the hepatic delivery of insulin transgenes driven by glycemia-regulatable promoters to diabetic animals to reverse the hyperglycemia (4). Proinsulin is normally processed to mature insulin (consisting of α and β chains connected by disulfide bonds) in the β cell before the hormone is secreted. Proinsulin processing does not happen in the liver which lacks the processing enzymes. Researchers get around this problem either by creating insulin transgenes encoding a single-chain insulin (A and B chains in the same polypeptide) which has about 20% normal insulin activity, or by engineering proinsulin transgenes with built-in cleavage sites for furin (a ubiquitous protease), which allows the proinsulin to be processed into mature insulin in the liver (see Ref. (4) for details). These partial solutions to proinsulin processing notwithstanding, the Achilles' heal for any approach using an insulin transgene is that the kinetics of insulin secretion in response to changes in blood glucose cannot be mimicked by glycemia-responsive promoter-driven insulin transgene expression (Table 1, middle column). The insulin secretory response in animals treated by this method lags behind by hours the minute-to-minute fluctuations in blood glucose that occur

TABLE 1
Blood Glucose Regulation of Insulin Secretion

Insulin Response to Blood Glucose	Insulin Gene Therapy	Induced Islet Neogenesis
Glucose-sensing mechanism	Liver-type	β-cell type
Proinsulin processing	Single chain insulin or add sites for furin	Yes*
Control mainly posttranscriptional and posttranslational	Transcriptional	Yes*
Regulated exocytosis: insulin granules	No*	Yes*
Role of non-β cells	No*	Yes*

* "Yes" and "no" refer to the *Insulin Response to Blood Glucose* pathways depicted in the left column.

with normal eating, drinking and exercise. Insulin secretion in normal β cells is mediated by regulated exocytosis of preformed insulin granules packaged inside vesicles that happens almost instantaneously after the β cell senses any change in the ambient blood glucose concentration (9).

As the current knowledge and technical capability do not allow us to duplicate the sophisticated insulin secretory apparatus of pancreatic β cells using insulin transgene therapy, we have adopted an alternative strategy—induction of β cell formation in the liver by delivering transcription factors that regulate islet development—to treat diabetes. We used a helper-dependent adenovirus to deliver Neurod (also known as beta2), a transcription factor (10) that regulates islet and β cell morphogenesis (11), together with betacellulin, an islet growth factor (12), to the liver of mice with streptozotocin (STZ)-induced diabetes (1). Within three weeks of treatment, the blood glucose of the mice returned to normal and stayed normal for the duration of the experiment (four months). Treated animals displayed normal plasma insulin levels under basal conditions and during an intraperitoneal glucose tolerance test. Immunohistochemical analysis of the liver revealed the presence of islet-like cell clusters underneath the liver capsule that produced the major islet hormones, insulin, glucagon, somatostatin and pancreatic polypeptide (for details see Ref. (1)). Immuno-electron microscopy revealed that the insulin-producing cells in the liver did not produce albumin or glycogen and morphologically resembled β cells, a phenotype that was supported by the presence of insulin granules inside secretory vesicles and the expression of β cell-specific genes including the pancreatic-type glucokinase, the proinsulin processing enzymes (prohormone convertases) 1/3 and 2, the sulfonylurea receptor (SUR1) and the K-ATP channel Kir6.2 (1) (Table 1, right column).

The data observed with Neurod/betacellulin gene therapy summarized above was "marred" initially by an unexplained finding—the presence of a low, but detectable, level of insulin gene transcripts in the liver of untreated diabetic mice and those treated with an empty vector, though such transcripts were not detected in nondiabetic controls. In other words, the untreated diabetic control liver samples "did not work" as we had expected!

Extrapancreatic Proinsulin-Producing Cells in Multiple Organs in Diabetes

The finding that insulin transcripts are detected in the liver of untreated STZ-diabetic mice by RT-PCR was unexpected but quite reproducible. It led us to reexamine the immunohistochemistry of the

liver sections from nondiabetic and diabetic mice. It turned out that for more than a year we had missed the presence of proinsulin-positive cells in liver sections of STZ-diabetic mice. Since the number of such cells is quite small, our anticipation that these sections "should be" negative had blinded us to the presence of small numbers (about 3–4 per mm^2) of proinsulin-positive cells in these sections. Despite an extensive search, we did not find such cells in sections of nondiabetic mice, an observation consistent with the absence of proinsulin transcripts in RNA isolated from these samples. Additional investigation showed that proinsulin-positive cells are also present in diabetic rats and in two type 2 diabetic mouse models, the *Ob*/*Ob* mice and the high fat diet-induced diabetic mice (2). Initial screening revealed that such cells are present not just in the liver, but also in the adipose tissue and bone marrow of diabetic animals. Bone marrow transfer experiments using genetically marked bone marrow cells indicated that hyperglycemia *per se* induces proinsulin expression in bone marrow cells, which then populate other tissues, including the liver and adipose tissue, of diabetic animals (2).

The bone marrow origin of proinsulin-producing cells was supported by a study by Oh et al. (13) who found that incubation of isolated rat bone marrow cells in high glucose medium induced insulin gene expression in about fifty percent of these cells within a week. The authors showed further that subcapsular renal transplantation of the bone marrow cells that underwent the *in vitro* manipulation led to partial amelioration of diabetes in STZ-diabetic mice. Thus, although the amount of proinsulin produced by the bone marrow cells exposed to hyperglycemia *in vivo* is extremely small, if there is a way to tweak the glycemia-induced insulin production, high glucose medium-treated bone marrow cells are a potential target for cell therapy for diabetes.

Current Understanding of the Pathogenesis of the Chronic Complications of Diabetes

Chronic diabetic complications are the major cause of morbidity and mortality among patients with diabetes. As alluded to earlier, the longer lifespan of patients with both type 1 and type 2 diabetes resulting from improvements in diabetes management has allowed the chronic complications to manifest as patients age. Microvascular (neuropathy, nephropathy, and retinopathy) and macrovascular complications (accelerated atherosclerosis, heart attacks and stroke) are a major cause of loss of life and productivity in these patients. Many biochemical abnormalities have been implicated in the pathogenesis of

diabetic complications (14–16). Hyperglycemia results in oxidative stress (14,17–20), hypoxia and ischemia (21), activation of the polyol pathway (22), increased advanced glycation end products (23,24), activation of protein kinase C (25,26) and mitogen-activated protein kinases (27), and growth factor deficiency (28). Despite the complex and multifactorial nature of the pathogenesis of the micro- and macrovascular complications of diabetes, we have made considerable progress in this area since the first biochemical studies on the subject about thirty years ago. During this time reports from multiple laboratories have led to the consensus that the biochemical perturbations summarized above may account for all the mechanisms involved; future research is generally relegated to exploration of additional details (further "*splitting*" the known pathways involved (29)) and synthesizing unifying hypotheses that will explain all the known facts (by "*lumping*" the pathways (29) together as a hypothetical hierarchical response to hyperglycemia). In his 2004 Banting Lecture, Michael Brownlee proposed an elegant unifying mechanism (30), which states that "intracellular hyperglycemia. . . causes increased mitochondrial production of ROS (*reactive oxygen species*). The ROS causes strand breaks in nuclear DNA, which activate PARP (*poly[ADP-ribose] polymerase*). PARP then modifies GAPDH (*glyceralde-3-phosphate dehydrogenase*), thereby reducing its activity. Finally, decreased GAPDH activates the polyol pathway, increases intracellular AGE (*advance glycation end product*) formation, activates PKC (*protein kinase C*) and subsequently NFκB, and activates hexosamine pathway flux" and "subsequent modification of proteins by *N*-acteylglucosamine" (30) (Notations in *Italics* are added by us).

The appearance of proinsulin-producing cells outside the pancreas represents the body's futile attempt to reverse the hyperglycemia. We note that the insulin gene products are present in the ectopic organs in minute quantities in the form of insulin transcripts and extremely low levels of immunoreactive proinsulin that never leaves the cell. We hypothesized that these proinsulin-positive cells may be involved in the evolution of diabetic complications, though before we embarked on the next series of experiments we had no idea whether such cells would be a bane or a boon for patients who harbor them.

Bone Marrow-derived Proinsulin-Producing Cells Underlie Diabetic Neuropathy

Mice and rats 8–12 weeks after STZ-induced diabetes develop peripheral neuropathy as evidenced by a significantly impaired motor

nerve conduction velocity and compound muscle action potential (measured on the tibial nerve), as well as impaired sensory nerve conduction velocity and sensory nerve action potential (plantar nerve). The ouabain-sensitive Na^+K^+-ATPase activity of the sciatic nerve was also reduced in these rats (31). Immunohistochemical screening showed that sciatic nerve cells and dorsal root ganglion (DRG) neurons in these diabetic rats and mice harbor proinsulin-expressing cells. Twelve weeks after STZ-induced diabetes, approximately 10% of DRG neurons stain positive for immunoreactive proinsulin. The same cells also stain positive for tumor necrosis factor-α (TNF-α) and CD45, a bone marrow cell marker. RT-PCR confirmed that insulin1 and insulin2 transcripts are present in these cells. Furthermore, laser capture microdissection of tissue sections showed the coexpression of these transcripts in the same DRG neurons (31). Bone marrow transplantation experiments using genetically marked donors showed that the proinsulin-positive cells are derived from the bone marrow, and further, they represent fusion cells between bone marrow derived (BMD) cells and neuronal cells in the DRG, and neuronal and Schwann cells in the sciatic nerve (31). An additional piece of evidence that they represent fusion cells is that essentially all the proinsulin-positive cells are polyploid whereas the nonproinsulin producing cells in the diabetic animals, like those in nondiabetic animals, remain diploid (31).

Are the fusion cells interesting curiosities of no functional or pathological significance? Are they involved in the pathogenesis of diabetic neuropathy, or do they represent an adaptive/protective response to the neuropathy? To address these questions, we examined the potential involvement of proinsulin-producing BMD neuronal cells in two pathways implicated in diabetic neuropathy—programmed cell death and depolarization-induced changes in intracellular calcium concentration. Programmed cell death, or apoptosis, is a major cause of neuronal degeneration in diabetic neuropathy (32,33,17,18). Apoptotic cells can be detected by the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assay. A screen of 1435 DRG neurons from nondiabetic rats failed to reveal any TUNEL-positive cells, indicating the rarity of neuronal apoptosis in the absence of diabetes. In contrast, histochemical examination of DRG neurons in diabetic rats showed 6 TUNEL-positive cells among 1513 neurons screened. Importantly, all 6 TUNEL-positive cells occurred in proinsulin-positive neurons. Therefore, diabetes leads to increased apoptosis in DRG neurons of rats which affects exclusively neuronal cells that have fused with proinsulin-positive BMD cells. Similarly, a significantly higher proportion of proinsulin-positive than proinsulin-nega-

tive DRG neurons from diabetic rats express annexin V, another marker of apoptosis; furthermore, when they were maintained in culture, proinsulin-positive neurons displayed shorter survival than nonproinsulin-producing neurons from the same animals (31). Next we examined the calcium homeostasis of neurons isolated from diabetic and nondiabetic rats. DRG neurons from STZ-diabetic rats have been shown to display upregulated resting intracellular calcium concentration ($[Ca^{2+}]_i$) (34) and a prolonged phase of recovery of KCl-induced depolarization (Δ time) (34,35). We measured these parameters in isolated DRG neurons and found that proinsulin-positive neurons from diabetic rats display the same disturbed calcium homeostasis as that reported previously for diabetic neurons, i.e., increased resting $[Ca^{2+}]_i$ and prolonged Δ time after KCl-induced depolarization, whereas nonproinsulin-producing neurons isolated from the same diabetic rats display relatively normal calcium dynamics that is not significantly different from that of neurons isolated from nondiabetic animals (31). Therefore, the presence and absence of abnormal calcium homeostasis is correlated with the presence and absence of proinsulin expression (and polyploidy, another marker for cell fusion), despite the fact that, as both nonproinsulin-producing and proinsulin-producing DRG neurons were isolated from the same STZ-diabetic animals, they have been exposed to the same degree of hyperglycemia.

The evidence summarized above indicates that the fusion of proinsulin-expressing BMD cells with nerve cells underlies diabetic neuropathy (31). It is important to point out that this mechanism occurs in parallel with perturbations in the other biochemical pathways reported previously in hyperglycemia as summarized above under *Chronic Complications of Diabetes Mellitus.* It is not our intent to ignore or trivialize these well documented biochemical mechanisms. As noted by Michael Brownlee, these mechanisms represent important "pieces of the puzzle" in the pathogenesis of diabetic complications (30). In demonstrating the fusion of proinsulin-producing BMD cells with neuronal cells in animals with diabetes and the downstream events that manifest as abnormal calcium homeostasis and accelerated apoptosis, we have simply uncovered a new, and possibly essential, piece of the puzzle in the pathogenesis of diabetic neuropathy.

Chronic Diabetic Complications: the Body's Adaptive Response to Hyperglycemia Gone Awry?

It appears that diabetes leads to the appearance of BMD proinsulin-producing cells as an adaptive response to the hyperglycemia. Here we

show that these cells contribute to diabetic neuropathy. In this brief review, we suggest that the pathogenesis of diabetic neuropathy may be the result of a futile adaptive response to hyperglycemia gone awry. Importantly, it is our belief that proinsulin serves only as a marker of hyperglycemia-induced inappropriate gene expression and plays little or no direct pathogenic role in the development of diabetic neuropathy. We found that in STZ-diabetic rats and mice, bone marrow cells that express proinsulin also coexpress TNF-α (31), and possibly other harmful molecules. As a diffusible molecule, TNF-α can travel some distance away from its site of production, thereby amplifying the harmful effects of the BMD cells. This interpretation would explain why a relatively low proportion, e.g., 10%, of such cells, could wreck havoc among nerve cells and fibers in the general neighborhood. Is there evidence that the presence of BMD cells marked by proinsulin production is the etiological mechanism underlying the other chronic diabetic complications? Pilot experiments indicate that BMD proinsulin-producing cells may have a wide-spread distribution, affecting multiple tissues and organs in diabetes. We are in the process of examining this hypothesis in diabetic mice and rats by asking the question whether these cells directly cause damage to the structure or function of these other organ systems affected by diabetes. Two years ago we discovered the presence of proinsulin-positive BMD cells in diabetes by serendipity (1,2). We believe that the odds are good that they play a role in the initiation, evolution or progression of at least a subset of the chronic micro- and macrovascular complications of diabetes.

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DISCUSSION

Boxer, Ann Arbor: Have you identified in the bone marrow the source of the cells that you think are responsible?

Chan, Houston: No, we have not yet. We are trying to purifying them. They are very difficult to purify. We thought we could use the insulin promoter-driven GFP. But the amount of insulin produced is so small so that the signal overlaps with the background when we tried to do flow cytometry. That's in the works. We are working on that.

Boxer: I would just make a comment. There have been a few children with acute lymphoblastic leukemia reported in the literature that have had sustained hypoglycemia, and the mechanism has totally been unknown. So that observation may leave room for further studies.

Chan: Actually, we have examined humans with exactly that. Some of them may have proinsulin though we need additional studies.

Tweardy: Larry, that's a remarkable follow up of an unexpected result in a control. You are to be congratulated for pursuing that to this end, because it has such important implications. The question I have for you is: what is it about the fusion of the pro-insulin

producing cells that leads to apoptosis of those cells that are unlucky to be their partners?

Chan: Obviously, we don't know the mechanism, but I think pro-insulin just happens to be a marker. I think we have to clearly keep that in mind. It's a marker of inappropriate gene expression, because we saw co-expression of TNF-alpha in the bone marrow in all the cells that expressed proinsulin. So when you have hyperglycemia, somehow it activates some transcriptional program. That leads to the expression of multiple genes and TNF-alpha is one of them. For example, in rats and mice 12 weeks after induction of diabetes, only 10% of the neurons stained positive for proinsulin—only about 10%. But if these cells also produce TNF-alpha, it could amplify the effect. So that's why we think it's not just the proinsulin. Probably proinsulin may not even play a role in it. It's just a marker that led us to that particular mechanism.

Frohman: Larry, these are beautiful studies. I have two questions. One is: how wide-spread are these cells in other tissue types? Have you been able to identify the pro-insulin staining cells in kidney, heart, other tissues that have a relationship to the macro-vascular complications in diabetes. The other question is: have you attempted to see whether you can shut these cells down with insulin therapy in these animals?

Chan: To answer the second question first—we don't know if we can shut these cells down, because we really haven't done that. But we have treated the STZ mice early with insulin and showed that treatment prevents the appearance of these cells indicating that it is the hyperglycemia and not STZ itself causing the phenomenon. We have not really treated well-established disease and looked at the reversal—we haven't done that. And the first question was the tissue distribution of the proinsulin-positive cells—I can tell you that they are fairly widely distributed—they are present in multiple organs. Kidney—is a good question. RT-PCR is positive for proinsulin, but we have not yet found definitively insulin-positive cells by staining. But we saw the message, I don't know why. We're still looking. After we first saw the message in liver, it took us four months before we were sure we had positive cells by staining. You really have to look hard for them. They're very difficult to find, because there are so few of them.