Extrapancreatic Proinsulin/Insulin-expressing Cells in Diabetes Mellitus: Is History Repeating Itself?

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Abstract. Insulin is a key regulator of life. Until 25 years ago, the pancreatic β-cell was thought to be the only organ that produces insulin in the body. Insulin deficiency, whether absolute (in type 1) or relative (in type 2 diabetes), underlies the metabolic derangements in diabetes mellitus, and investigations on insulin have concentrated on pancreatic insulin production, its regulation and the metabolic consequences of insulin deficiency. The thymus was the next organ that was found to also produce insulin, a process that may tolerize the body to the molecule, protecting the host from developing autoimmune β-cell destruction and (type 1) diabetes. However, now and then there were descriptions of promiscuous insulin production outside the pancreas. During our investigations on diabetes gene therapy in rodents, we serendipitously came across the presence of mysterious cells marked by proinsulin production in unexpected organs, some of which cells may underlie certain chronic diabetic complications. Starting with a historical perspective on insulin expression in brain and thymus, this review focuses mainly on unraveling the mystery of extrapancreatic extrathymic proinsulin/insulin expression in diabetes mellitus.

Key words: Insulin, Diabetes mellitus, Islets, Gene therapy, Extrapancreatic

In this review, we will examine, in a historical context, the discovery of insulin outside the pancreas. The first part of the article deals with the brain and thymus, the first organs outside the pancreas that were discovered to produce proinsulin/insulin. We will then go on to discuss in some detail the discovery of insulin expression in other extrapancreatic organs and tissues and the possible implications of these findings.

I. Insulin in Brain

1. Historical debate on brain insulin expression

Havrankova et al. at the US National Institutes of Health (NIH) first reported that the normal rodent brain expresses insulin in 1978 [1]. They further advanced the extraordinary hypothesis of extrapancreatic ubiquitous insulin production in mammals, including humans [2, 3]. Their hypothesis was based on the presence of immunoreactive insulin as revealed by a difference in insulin concentration between different extrapancreatic tissues and blood as measured by radioimmunoassay, a technique first developed by Berson and Yalow, for which Yalow received the Nobel Prize in 1977 [4]. However, in 1981, Eng and Yalow questioned this finding, presenting evidence against the presence of immunoreactive insulin in guinea pig brain [5]. In 1982, they further claimed that insulin was undetectable in the guinea pig brain by comparing antisera used in their laboratory and that used by the NIH group [6]. The disagreement between the two groups continued unabated [7, 8]; the discrepancies between them might have originated from the different extraction procedures and not the radioimmunoassay antisera used. Was brain insulin for real?
In 1984, Birch et al. in New Zealand extracted immunoreactive insulin from whole rat brain by using the procedures of Havrankova et al. and Eng & Yalow, and confirmed the difference in the concentration of immunoreactive insulin by the two procedures [9]. They further characterized the extracted insulin by HPLC, and concluded that the insulin antibody immunoreactive material in each case was authentic (pancreatic) insulin; they estimated that the concentration of rat brain insulin was at least three times that of plasma by the extraction procedure of the NIH group [1]. Despite their negative conclusion on brain insulin, Eng & Yalow proposed that the ultimate proof of insulin production depends on the demonstration of its mRNA in the brain [5]. Though it was not what Eng & Yalow had expected, the presence of insulin mRNA in brain was finally reported by Devaskar et al. in 1994 [10].

II. The thymus

1. Insulin in the thymus: role in autoimmunity

Recent literature on immune tolerance documents that a large number of self-molecules with tissue-restricted expression are expressed in the thymus, both at the mRNA and the protein level, in both rodents and humans [15–17]. Many of these proteins are known autoantigens in autoimmune disease, and reduced expression in the thymus may play a key role on the selection of autoreactive T cells that are deleted in thymic medulla and peripheral lymphoid tissues [18–21], a negative selection aiming at removal of autoreactive T cells. Insulin mRNA expression in thymus was first reported in mice in 1994 [22], and subsequently in humans [23, 24] and rats [25]; expression of insulin in thymus have been cited as an example of the role of autoreative T cell formation in type 1 diabetes mellitus (T1D).

Direct evidence that level of thymic insulin expression affects development of self-tolerance to insulin was found in non-obese diabetic (NOD) mice [26], a model of spontaneous autoimmune diabetes that resembles human T1D [27]. NOD mice showed islet inflammation (insulitis) with lymphocytic infiltration, leading to β-cell destruction and diabetes [27]. In this model proinsulin/insulin is a key autoantigen for diabetes development [28] and immune responses against proinsulin epitopes have been found to be strongly correlated with diabetes [15, 29].

2. Low thymic insulin expression in T1D

Unlike humans who have only one form of insulin
encoded by a single gene on chromosome 11 [30], rodents have two non-allelic insulin genes, \( \text{Ins1} \) located on chromosome 19, and \( \text{Ins2} \) on chromosome 7; the two insulin gene products differ in two locations (amino acid positions 9 and 29 on the B chain [31, 32]). \( \text{Ins1} \) and \( \text{Ins2} \) are both expressed in significant amounts in pancreatic \( \beta \)-cell [33, 34]. However, in the thymus, \( \text{Ins2} \) is the one that is predominantly expressed, while \( \text{Ins1} \) is reported to be expressed at a negligible to very low levels [35–37]. Chentoufi et al. have produced a mouse model that expresses either one or two copies of the two insulin genes, and demonstrate that mice lacking \( \text{Ins2} \) express low thymic insulin and display peripheral autoreactivity to insulin, whereas mice lacking \( \text{Ins1} \) express normal thymic insulin levels and display no significant autoreactivity to insulin. Thus, normal level of thymic insulin, especially that involving insulin 2, plays a pivotal role in insulin-specific T-cell self-tolerance [38, 36]. Thebault-Baumont et al. showed a more direct role of thymic insulin expression for the onset of diabetes. They produced \( \text{Ins2} \) knockout mice in NOD background, which developed enhanced production of insulin autoantibodies and accelerated diabetes, an observation consistent with absence of thymic \( \text{Ins2} \) expression being directly linked to T1D progression [39]. Interestingly, Nakayama et al. recently showed that \( \text{Ins1} \) was also a target for autoreactivity in mice [40]. They generated NOD mice lacking both \( \text{Ins1} \) and \( \text{Ins2} \), and rescued them with a modified insulin gene to produce an insulin that was hormonally active but not recognized by lymphocytes. Mice with at least a single copy of \( \text{Ins1} \) gene developed diabetes, while mice lacking both natural forms of insulin showed no signs of any immune response against \( \beta \)-cells, and did not develop diabetes. These results indicate that not only \( \text{Ins2} \) but also \( \text{Ins1} \) could play a primary role for the development of autoimmune T1D in mice.

In humans, studies on the relation of thymic insulin expression and onset of T1D have demonstrated analogous results as in rodents. The \( \text{IDDM2} \) susceptibility locus maps to a variable number of tandem repeats (VNTR) in the promoter region of the insulin gene on chromosome 11 [41]; allelic variations at the INS VNTR-IDDM2 locus correlate with the level of insulin mRNA expression in the thymus [23, 24]. Moreover, parent-of-origin effects, known as imprinting, at the \( \text{IDDM2} \) locus, modulate the effect of the VNTR variation by reducing the level of insulin transcripts in the thymus [42, 43]. These results indicate that reduction of thymic insulin expression may lead to suppression of negative selection of insulin-specific autoreactive T cells, or to impaired selection of regulatory T cells, and facilitate the development of autoimmune T1D in humans.

3. Cellular origin of insulin expression in thymus?

Selected antigen-presenting cells (APCs) have the ability to synthesize and express self-molecules, a process essential for self-tolerance [21]. Smith et al. reported that the mouse thymus contains specialized cells expressing pancreatic genes including insulin and somatostatin [44]. They named these cells peripheral antigen expressing (PAE) cells. To study the role of PAE cells in immune tolerance, they transplanted a thymus from transgenic mice that harbors a rat insulin promoter (RIP)-driven SV40 T antigen (Tag) (that showed Tag expression in their thymus) into athymic non-transgenic hosts. They found that the transplanted mice showed tolerance to the Tag. Importantly, absence of thymic RIP-Tag gene expression in another line of transgenic mice was associated with absence of self-tolerance and susceptibility to autoimmunity. These data indicate that expression of insulin in PAE cells of thymic medulla limits development of autoimmune T cells to pancreatic \( \beta \)-cells.

Throsby et al. characterized the cell type of the PAE cells expressing pancreatic hormones in normal mice, and found that insulin-expressing cells were dendritic cells (DCs) and macrophages derived from the bone marrow [35]. Pugliese et al. confirmed the similar characters of the cells by a study using human samples obtained both from thymus and peripheral lymphoid organs [45]. We note, however, that Derbinski et al. reported that significant amounts of insulin and other tissue-restricted self-molecules were expressed only in medullary thymic epithelial cells (mTEC) but not in DCs or macrophages in normal C57BL/6 or NOD mice [46]. Although additional studies are needed to unequivocally establish the identity of these cells, Garcia et al. found proinsulin-expressing cells not only in thymus, but also in spleen and circulating blood cells where mTEC might not exist [47]. They proposed that these are specialized DC cells that promote self-tolerance not only in the thymus, but also in peripheral lymphoid tissues.
III. Proinsulin expression in multiple organs in diabetes

1. Proinsulin/insulin-expressing cells in STZ-diabetic liver

As the initial controversial report on the presence of extrapancreatic (especially brain) insulin was beginning to fade from memory a quarter of a century later, our laboratory stumbled onto an unexpected and strangely similar finding, though this time it involved the liver. In a project developing a novel strategy for induced islet neogenesis as a treatment for diabetes, we compared the histology of the liver of STZ-diabetic mice treated with a therapeutic vector containing a transcription factor (NeuroD/Beta2) with animals treated with an empty vector. The therapeutic vector elicited the appearance of islet-like clusters in the liver that produce proinsulin/insulin and other islet hormones leading to a complete amelioration of diabetes. However, to our dismay, we found that the diabetic mice treated with an empty vector that did not carry any therapeutic gene was associated with the appearance in the liver of small numbers of isolated cells that stained positive for proinsulin/insulin [48]. Repeat experiment using diabetic STZ-mice that had not been received any vector (therapeutic or empty) again revealed the appearance of these proinsulin-producing cells in the liver within days of diabetes development. These cells occurred in different parts of the liver but were more frequent in the vicinity of the portal triads. Similar cells were, however, not observed in liver sections of nondiabetic mice. Moreover, we detected the mRNA for insulin and other islet hormones and islet-specific proteins and transcription factors in diabetic but not in nondiabetic liver [48], indicating that diabetes induces the appearance of extrapancreatic proinsulin-producing cells in the liver. History does seem to repeat itself with a twist!

2. Proinsulin-expressing cells from the bone marrow

There are many unanswered questions on the proinsulin-expressing cells outside the pancreas and thymus. However, we do know some of their properties. First, the cells are present only in STZ-diabetic but not in nondiabetic mouse liver. Second, the insulin gene in these extrapancreatic cells retains to some extent a transcriptional response to hyperglycemia. Third, unlike in β-cells, it produces almost exclusively proinsulin and little if any mature insulin, and the presence of such cells does not have any detectable impact on the hyperglycemia of the diabetic animals.

We screened for the presence of extrapancreatic proinsulin-expressing cells in multiple organs in a type 1 model, STZ-diabetic mice and rats, and two type 2 models, ob/ob mice and mice with diet-induced obesity/diabetes. These types of cells appear in the liver, adipose tissue, spleen and bone marrow in addition to thymus in both hypoinsulinemic and hyperinsulinemic diabetes models [49]. In a parallel experiment, STZ induction of diabetes in transgenic mice that express green fluorescent protein (GFP) driven by the mouse insulin promoter (MIP-GFP mice) [50] led to the appearance of GFP-positive cells in the liver, adipose tissue and bone marrow. Moreover, we detected the presence in nondiabetic mice extrapancreatic, extrathympic proinsulin-expressing cells within three days of hyperglycemia induced by daily intraperitoneal glucose injections. Finally, use of carefully designed bone-marrow transplantation (BMT) experiments allowed us to show that most of the extrapancreatic proinsulin-expressing cells (in the liver and adipose tissue) have originated from the bone marrow [49].

Shortly after we published our observations [49], two other laboratories documented the induction of Ins1 and Ins2 gene expression when bone marrow cells are incubated in a high glucose medium [51, 52]. These authors transplanted the insulin-producing cells into the STZ-mice leading to significant amelioration of the hyperglycemia, though plasma insulin concentration was not reported. The character of the isolated bone marrow cells in these experiments differed significantly from the proinsulin-expressing cells that we observed in diabetic rodents in vivo. The bone marrow-derived cells in diabetic animals did not contribute to significant insulin production or β-cell formation in vivo [49], a conclusion that was corroborated by Taneera et al. [53] who found that, in contrast to the report of Ianus et al. [54], transplanted bone marrow cells did not assume a β-cell fate. In addition, we found that most, if not all, of the bone marrow-derived proinsulin-producing cells co-express TNF-α, a proinflammatory cytokine, a finding that prompted us to hypothesize that, rather than “fighting” hyperglycemia, these cells may mediate the ill effects of hyperglycemia, i.e., they may contribute to chronic diabetic complications.
3. Abnormal cell fusion underlie diabetic peripheral neuropathy

Diabetes causes devastating complications, including the “triopathies”, peripheral neuropathy, nephropathy and retinopathy. We have focused on peripheral neuropathy as a possible complication produced, or worsened, by the abnormal bone-marrow derived cells, since preliminary screening revealed the presence of proinsulin-producing cells in the dorsal root ganglion (DRG) and sciatic nerve of STZ-diabetic mice and rats with documented diabetic neuropathy [55]. Immunohistochemical analyses showed that the most of the proinsulin-expressing cells were DRG neurons and nerve fibers. Furthermore, diabetic mice that received BMT from MIP-GFP donors displayed GFP signals mostly overlapping those of neuronal markers. To determine the origin of the proinsulin-expressing cells, we transplanted bone marrow cells from MIP-GFP donors to β-galactosidase-transgenic (ROSA) mice and induced diabetes in the recipients by STZ after BMT. Surprisingly, the same proinsulin-expressing DRG neurons that expressed GFP also expressed β-galactosidase, indicating that these were fusion cells formed between bone-marrow-derived proinsulin-expressing cells and DRG neurons [55].

We isolated DRG neurons from diabetic and non-diabetic rats, and examined their morphology and function in vitro. We found that the absence or presence of proinsulin expression determines whether the DRG neuron shows normal or abnormal calcium homeostasis and apoptosis. The data indicate that bone marrow-derived cells marked by proinsulin expression have fused with nerve cells leading to accelerated cell death and abnormal nerve function, both features of diabetic peripheral neuropathy [55] (Fig. 1).

Recently, the fusogenicity (the ability of a cell to fuse with another cell) of bone marrow-derived cells was reported to play a role in the regenerating process in various organs recovering from injury [56]. However, the frequencies of the fusion event in normal organs were reported to be extremely low, being less than 1% among cardiomyocytes, and less than 0.1% among hepatocytes or Purkinje cells [57]. In contrast, we observed that the fusion cells marked by proinsulin expression made up approximately 10% of all DRG neurons in diabetic rats 12 weeks after STZ. Functional abnormalities and premature apoptosis occur almost exclusively in these fusion cells. We do not believe that proinsulin expression per se contributes significantly to the development of diabetic complications; rather, we believe that co-expression of other potentially harmful molecules such as TNF-α by these abnormal cells may play an important pathogenic role in diabetic neuropathy.

Fig. 1. Schematic view of bone marrow-derived Proins/TNF-α-expressing cells in the pathogenesis of diabetic peripheral neuropathy. In diabetes mellitus, abnormal Proins/TNF-α-expressing cells are appeared in the bone marrow by hyperglycemia. The abnormal cells migrate and fuse with neurons in the sciatic nerve and dorsal root ganglion (DRG), resulting in neuronal dysfunction and accelerated apoptosis.
IV. Future directions

Insulin production is widely distributed in the central nervous system, but their cellular origin and functional roles are still being unraveled. Thymic insulin expression is an important negative regulator for autoimmunity and T1D, though it is still unclear if these cells have originated directly in the thymus or from the bone marrow. By fusing with nerve cells, abnormal bone marrow-derived proinsulin-expressing cells in diabetes may contribute to neuronal apoptosis and dysfunction in diabetic neuropathy. At this time, we do not know the cell lineage origin of these abnormal cells and their relationship, if any, to insulin-expressing cells in the thymus. In the last few years, we have been peeling off layers of mystery covering the extrapancreatic and extrathymic proinsulin/insulin-expressing cells, reviving old ideas and creating new paradigms. The deeper we dig, the more surprises we uncover. We look forward to continue to unravel the marvels and mysteries behind these peculiar cells in diabetes.

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