Unraveling the Pathogenesis of Type 1 Diabetes with Proteomics

PRESENT AND FUTURE DIRECTIONS*

Thomas Sparre‡§, Martin R. Larsen¶¶, Peter E. Heding‡, Allan E. Karlsen‡, Ole N. Jensen¶¶, and Flemming Pociot‡

Type 1 diabetes (T1D) is the result of selective destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans. T1D is due to a complex interplay between the β-cell, the immune system, and the environment in genetically susceptible individuals. The initiating mechanism(s) behind the development of T1D are largely unknown, and no genes or proteins are specific for most T1D cases. Different pro-apoptotic cytokines, IL-1β in particular, are present in the islets during β-cell destruction and are able to modulate β-cell function and induce β-cell death. In β-cells exposed to IL-1β, a race between destructive and protective events are initiated and in susceptible individuals the deleterious events prevail. Proteins are involved in most cellular processes, and it is thus expected that their cumulative expression profile reflects the specific activity of cells. Proteomics may be useful in describing the protein expression profile and thus the diabetic phenotype. Relatively few studies using proteomics technologies to investigate the T1D pathogenesis have been published to date despite the defined target organ, the β-cell. Proteomics has been applied in studies of differentiating β-cells, cytokine exposed islets, dietary manipulated islets, and in transplanted islets. Although the studies have revealed a complex and detailed picture of the protein expression profiles many functional implications remain to be answered. In conclusion, a rather detailed picture of protein expression in β-cell lines, islets, and transplanted islets both in vitro and in vivo have been described. The data indicate that the β-cell is an active participant in its own destruction during diabetes development. No single protein alone seems to be responsible for the development of diabetes. Rather the cumulative pattern of changes seems to be what favors a transition from dynamic stability in the unperturbed β-cell to dynamic instability and eventually to β-cell destruction. Molecular & Cellular Proteomics 4:441–457, 2005.

Diabetes mellitus (DM) is a group of disorders of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both (1). Indian physicians were able in the 5–6th century to distinguish between two forms of diabetes, one affecting elderly obese people (type 2 diabetes (T2D)) and the other affecting thin people who did not survive long (type 1 diabetes (T1D)). In 1922, the first T1D patient was treated with insulin. Before that time, no regular treatment of diabetes existed, and in best cases patients survived 2 or 3 years, but usually only for a few months (2).

Based upon etiology, diabetes can be divided into four main groups (1) as described below.

T1D—Absolute insulin deficiency due to an autoimmune-associated destruction specifically of the insulin-producing β-cells in the islets of Langerhans (Fig. 1).

T2D—Relative insulin deficiency due to decreased effect of insulin in the target tissues e.g. muscles and adipose tissue (insulin resistance) or due to a secretory defect of insulin with or without insulin resistance.

Other Specific Types of Diabetes—For example, specific genetic defects of β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemically induced, infections, uncommon forms of immune-mediated diabetes, and other genetic syndromes sometimes associated with diabetes.

Gestational Diabetes—Carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy (includes gestational impaired glucose tolerance and gestational diabetes).

The metabolic state between normal glucose homeostasis and diabetes encompasses a state termed “impaired glucose regulation” (impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG)). Individuals with IGT or IFG may be normo-glycaemic in daily life with normal or near-normal glycated hemoglobin levels. IGT and IFG are not independent clinical entities, but are rather risk categories for future dia-

betes-prone BioBreeding; BB-DR, diabetes-resistant BioBreeding; HLA, human leukocyte antigen; IFG, impaired fasting glycaemia; IFN, interferon; IGT, impaired glucose tolerance; iNOS, inducible NO-synthase; LP, low protein; MHC; major histocompatibility complex; NO, and nitric oxide; NOD, nonobese diabetic; PM, plasma membrane; RIN, rat insulinoma; T1D, type 1 diabetes; T2D, type 2 diabetes; 2-DGE, two dimensional gel electrophoresis; WF, Wistar Furth.
Diabetes and/or cardiovascular disease (1, 4, 5).

Over the last two decades, the incidence of diabetes has increased in an epidemic-like fashion worldwide. The increasing number of patients relates to T2D in particular, and the total number of diabetics is estimated to increase from 151 million in 2000, 180 million in 2003, to ∼220 million in 2010 (6, 7). T1D is estimated to increase from 4.4 million in 2000 to ∼5.4 million in 2010 (7). The increasing number of diabetics is not only a reflection of an increased population, but reflects a true increase in the incidence of both T1D (8–10) and T2D (6, 7, 9, 11).

The overall age-adjusted incidence of T1D displays enormous differences world-wide with a variation in incidences of more than 350-fold. The highest incidence is seen among Caucasian populations, in Sardinia (36.8/100,000 per year) and Finland (36.5/100,000 per year), and the lowest is seen in the Zunyi province of China (0.1/100,000 per year) (8).

The etiology for the most diabetic cases (both T1D and T2D) is believed to be the result of interactions between environmental and genetic components in genetically predisposed individuals. In a few percent of the cases the etiology and pathogenesis are not only a reflection of an increased population, but reflects a true increase in the incidence of both T1D (8–10) and T2D (6, 7, 9, 11).

In this review, we will deal with the pathogenesis of the autoimmune T1D studied through proteomics and describe the active involvement of the β-cell itself in β-cell destruction.

**TYPE 1 DIABETES**

T1D is a clinical diagnosis characterized by an irreversible selective destruction of the insulin-producing β-cells in the islets of Langerhans with involvement of cytokines and T-lymphocytes (T-cells) specific for β-cell antigens (15), which only becomes symptomatic when the β-cell mass is reduced by ∼90%.

T1D is a complex genetic trait, with multiple genetic loci contributing to susceptibility, but also with environmental factors playing a role in determining risk. There is a significant familial clustering of T1D with an average prevalence risk in siblings of 6% compared with 0.4% in the general population (8, 16). At least one locus that contributes strongly to this familial clustering resides within the major histocompatibility (MHC) region on chromosome 6p21.3. Other established T1D risk genes include the insulin gene region on chromosome 11p15 and the cytotoxic T-lymphocyte-associated 4 gene (CTLA4) region on chromosome 2q33 (17).

Most attention to environmental factors has been paid to virus infections and early exposure to cow’s milk protein as triggers of autoimmunity in individuals genetically susceptible to T1D (18–20), although no consensus can be reached by evaluating all published studies.

Another epigenetic factor is the influence of the intrauterine environment on β-cell development and later development of diabetes. Fetal undernutrition may dispose to T2D and coronary heart disease later in life in both humans and rodents (21–24). Low-protein (LP) diet given to pregnant rats during gestation induces permanent changes in the offspring e.g. altered islet cell proliferation, islet size, pancreatic insulin, apoptotic rate, and increased sensitivity to IL-1β and nitric oxide (NO) (25–27). In the offspring of T1D mothers, it has been suggested that exposure to a diabetic environment in utero is associated with increased prevalence of IGT and defective insulin secretory response in adulthood (28).

**MODEL FOR THE PATHOGENESIS OF T1D**

A generally accepted model for the selective β-cell destruction in T1D is proposed by the Copenhagen Model. This model was suggested by Nerup and co-workers in 1988 (15), revised in 1994 (29), and again in 2003 (30). This model implicates cytokines as major effector molecules in the β-cell destructive process. Briefly, the model suggests two phases in the pathogenesis of T1D. First there is an initial non-antigen-
in vivo but not sufficient for the destruction of β-cells. The exact sequence of the pathogenic event in the β-cell destruction can only be studied in vitro and in animal models due to the rapid progression of clinical diabetes and the difficulty of studying diabetes development in the human pancreas.

Cytokines in T1D and Rationale for Studying IL-1β-Exposed Rat Islets

β-cells have receptors specific for IL-1β, tumor necrosis factor α (TNF-α), and interferon gamma (IFN-γ) that induce several signal transduction pathways and alterations in gene transcription and protein synthesis (31–34). In addition, increased expression of IL-1, TNF-α, and IFN-α/γ are observed in both animal models and in humans at the onset of diabetes (35–46). IL-1β is selectively cytotoxic to isolated rat β-cells (47–49), and the effects are potentiated by TNF-α and IFN-γ (44, 50–53). The effects of IL-1β can be inhibited both in vitro and in vivo by IL-1 receptor antagonists (33, 54).

One of IL-1β’s effects on the β-cell is enhanced transcription of inducible NO-synthase (iNOS), which is especially toxic to β-cells (34, 44, 55–64). Inhibitors of iNOS protect β-cells in vitro and in vivo from the cytotoxic effects of cytokines (58, 65, 66).

Thus, IL-1β alone or in combination with TNF-α and IFN-γ is cytotoxic to rat, mouse, and human islets in vitro through the production of NO in β-cells. NO is, however, necessary but not sufficient for the destruction of β-cells. Inhibition of NO only partially protects against IL-1-induced β-cell destruction, indicating that additional mechanisms must be involved (34, 44, 67).

Cell Lines and Animal Models for T1D

Ideally, all research in human diseases should be made in humans. This is, however, neither ethical nor possible for several obvious reasons. A number of islet-derived β-cell lines (68) and animal models (45, 46, 69–71) have contributed substantially to our understanding of the pathogenesis of T1D. In addition to chemically and virus-induced diabetes, two animal models developing spontaneous diabetes have been widely used: the nonobese diabetic (NOD) mouse and the diabetes-prone BioBreeding (BB-DP) rat. Both these widely used models develop a diabetic syndrome with both insulitis and diabetes closely resembling T1D in humans (45, 46, 69, 70). Diabetes in both the NOD mice and the BB-DP rat are multifactorial, involves MHC genes, but are not solely explained by effects of or alterations in MHC genes (69).

It is important to have in mind that phenomena studied in the inbred NOD mice and BB rats represent observations in one individual due to genetic homogeneity, whereas in humans, T1D cases are genetically heterogeneous. This implies that observations made in spontaneous animal models inbred for dozens of generations might probably only represent specific subgroups of T1D patients and should be interpreted with some caution (72).

Proteomics in T1D Research

In biological systems such as cell lines or whole organisms, cellular functions are carried out by molecular complexes rather than by single molecules or single reactions. Cellular functions and pathways are connected in cellular metabolic networks and interactive networks. The integrity of these networks depends upon crosstalk between different pathways to minimize single gene defects alone or in combination with other gene defects or environmental factors (73–75). In this context, proteomics is an obvious tool for T1D pathogenesis research. However, only few studies have used proteomics technologies to elucidate the T1D pathogenesis (76). Most of these studies have used cell lines and tissue obtained from animal models of T1D.

Today, no published proteomic data based on human material directly studying β-cell destruction and development of T1D are available. One study has used SELDI coupled with TOF-MS to identify autoantibodies directed against glial fibrillary acidic protein from the peri-islet Schwann cells, enveloping the islets of Langerhans, in both NOD mice and in diabetic patients (77). The authors results suggested that autoimmunity against the pancreatic nervous tissue could be involved in T1D pathogenesis. Another study has described the global protein expression in whole human pancreata and created a reference two-dimensional gel electrophoresis (2-DGE) map with 302 identified proteins (78). Because the proteins identified in that study were from both endocrine and exocrine tissue, it is difficult to use the data for T1D pathogenesis research. Another published protein database is “The Mouse SWISS-2D PAGE Database,” which is a descriptive analysis of the protein expression in normal mouse liver, liver nuclei, muscle, white and brown adipose tissue, and pancreatic islets accessible at the ExPASy molecular biology server (79). These protein databases may serve as reference 2-DGE maps. However, proteins that change expression cannot be identified on 2-DGE databases alone, but have to be further identified using MS because co-localization of proteins on 2-DGE is quite common.

The first studies using 2-DGE for protein separation in relation to islets, β-cell lines, and T1D were done in the 1980s describing changes in the protein expression pattern in islets from mice with virus-induced hyperglycemia (80). Other researchers investigated the expression of specific enzymes such as hexokinase, glucokinase, and other proteins in β-cells combining 2-DGE and specific antibodies for identification (81–83). In 1987, Nepom et al. used 2-DGE and immunoprecipitation to analyze human leukocyte antigen (HLA) molecules from T1D patients and demonstrated that hybrid HLA molecules are associated with heterozygosity (84). Others have used 2-DGE to describe insulin secretory granule biogenesis (85), exocytosis (86), and the effect of glucose on...
β-cells (87, 88). 2-DGE has furthermore been used to characterize autoantigenes and epitopes (89, 90).

More recent proteomic studies of mouse islets compared islet protein expression with proteins involved in Alzheimer’s disease. Several of the proteins identified in the islets are known to be related to Alzheimer’s disease suggesting common pathways of T2D and Alzheimer’s disease (91).

The studies described do not address or only superficially address issues of the pathogenesis of T1D. Proteome analyses investigating the pathogenesis of T1D have been the focus of our research group.

We have in our research focused on pancreatic islets of Langerhans as a whole organ, because most of the cells in an islet are β-cells, and that islet cells do work together. Our published studies have yielded a complex and detailed picture of protein expression changes associated with maturation into the β-cell phenotype, cytokine-mediated β-cell destruction, and islet destruction in vitro and in vivo during diabetes development. The picture that emerges from these analyses is complicated and far from complete. Still many questions remain to be answered. However, we are studying the ailing β-cell through a new window and the challenge is now to fully understand what we are seeing.

The purpose is to identify and describe the changes in protein expression specific for β-cells or of proteins induced by IL-1β exposure of Wistar Furth (WF) and BB-DP islets in vitro and in vivo during development of diabetes, respectively (Fig. 2). Furthermore, the aim is to test the hypothesis that the β-cell is not a passive bystander of its own destruction. In addition, we have demonstrated that an environmental factor (intrauterine protein restriction) may influence islet protein expression in ways unfavorable for long-term β-cell survival and that when a pre-β-cell becomes a β-cell it changes its cellular defense mechanisms.

**β-CELL DIFFERENTIATION**

The organogenesis of the pancreas is a complex and highly coordinated dynamic process initiated in the endodermal primitive gut tube at a very early stage from an endodermal pancreas-specified precursor cell. From these pancreas-specified precursor cells both exocrine and endocrine cells are developed dependent upon differential mesenchymal expression of signaling molecules such as Pdx1, Nkx6.1, Notch1, and NeuroD. During islet development, different cell types, each with their characteristic phenotype, are created (92). During this differentiation, the β-cell becomes a highly specialized insulin-producing cell and accounts for up to 80% of the islet cells partly surrounded by glucagon-producing α-cells. During differentiation of the α- and β-cells, their sensitivity to cytokines and toxins diverges in favoring increased β-cell sensitivity (93).

Studies of a glucagon-producing rat cell line (NHI-glu) that changes phenotype to an insulin-producing cell line (NHI-ins) after in vivo passage as a subcutaneous tumor in rats (93, 94) suggest that β-cell sensitivity to cytokines is acquired and reflected in the protein expression pattern (95). By comparative 2-DG analysis of the NHI-glu versus NHI-ins cells, 135 protein spots out of 2,239 detectable were significantly differentI expressed as a result of maturation into the cytokine-sensitive NHI-ins phenotype. Of these, 74 were down-regulated, 44 were up-regulated, 16 were suppressed, and 1 was expressed de novo in the insulin-producing cells compared with glucagon-producing cells. From 93 of the 135 protein spots, 97 different proteins were identified by MALDI-MS and revealed a complex pattern of alterations affecting many different cellular functions such as protein synthesis, cellular defense, and apoptosis. More than 30% of the identified proteins were present in more than one spot, indicating that posttranslational modifications are important for the change in phenotype. The data suggest that during differentiation the β-cell phenotype is altered in its ability to protect itself against hydrogen peroxide and other organic hydroperoxides through down-regulation of GST (95). Several of the identified proteins have also been identified in islets exposed to IL-1β (96, 97). Taken together, the study has identified proteins and probably modified proteins involved in β-cell maturation, insulin gene expression, and acquired β-cell IL-1β sensitivity (95).

**PROTEOME ANALYSIS OF IN VITRO IL-1β-EXPOSED RAT ISLETS**

When islets of Langerhans from the genetically susceptible BB-DP rat are exposed to 150 pg/ml IL-1β for 24 h 82 proteins out of 1,815 in total change expression (98). These proteins represent proteins involved in a) energy transduction and redox potentials, b) glycolysis and Krebs cycle, c) protein, DNA, and RNA synthesis, chaperoning, and protein folding, d) signal transduction, regulation, differentiation, and apoptosis, and e) cellular defense (97).

In comparison, islets from nondiabetes developing WF rats exposed to 150 pg/ml IL-1β for 24 h showed all out of ~1,500 protein expression changes (96, 99). Most of the proteins changed in the two studies were identical or affecting the same pathways. Fewer proteins are changed in the BB-DP islets under the same experimental conditions, suggesting that fewer changes are needed in the BB-DP islets to induce instability and destruction. The involvement of a limited number of genes/proteins in response to cytokine exposure is further supported by microarray analyses of sorted rat β-cells and rat insulinoma (RIN) cells, where a large number of mRNA transcripts change expression dependent upon cytokine and exposure time (100, 101). Many of the same genes and pathways were affected in all four studies, although different methods were used, and expression analyses of mRNA and protein are not directly comparable (102, 103).

Taken together, these studies indicate that cytokine exposure of islets and β-cells induces changes in the expression of both mRNAs and proteins involved in a variety of different functions comprising both primary and secondary changes. No single gene or protein has been held responsible for the
observed effects. These studies do not allow any discrimination between primary or secondary changes, nor do they describe the importance of the observed changes. Furthermore, the findings should be interpreted with some caution because they reflect the specific experimental conditions, e.g., islet isolation procedure, concentration of IL-1β, exposure time, labeling interval, and the general culture conditions as well as the limitations in the methods used.

In the described studies ~1,900 protein spots were found emphasizing the importance of the methods used, because under optimal experimental conditions in 2-DGE studies of mouse tissue more than 10,000 protein spots can be visualized (104). However, the proteins identified in the islet studies are all newly synthesized during the labeling period because we used a metabolic labeling method with sulfur-35 (\(^{35}\)S) methionine incorporation. However, even with these limitations, the complexity of the effects of IL-1β on islets and β-cell lines substantiates the theory that development of T1D is the result of a collective, dynamic instability, rather than the result of a single factor (105).

**IN VIVO ANALYSIS OF IL-1β-EXPOSED RAT ISLETS**

In order to extrapolate the *in vitro* findings mentioned above to an *in vivo* model for model for T1D, syngeneic islets are transplanted under the kidney capsule to prediabetic BB-DP rats to enable us to study the protein expression in islet...
transplants during development of diabetes (106). This study aims to describe the relevance of the proteins identified in vitro after IL-1β exposure for the development of diabetes in vivo in syngeneically transplanted prediabetic BB-DP rats and in diabetes-resistant BioBreeding (BB-DR) and WF rat islets. First, we demonstrated by in situ hybridization that mRNA for IL-1β was present in the syngeneic islet transplants and the highest concentration was found at onset of diabetes. Second, we were able to identify all 82 protein spots that changed expression in BB-DP rat islets in vitro in the syngeneically transplanted BB-DP, BB-DR, and WF rat islets. These 82 proteins could, in the transplants, be followed throughout the development of diabetes, in transplants from animals escaping diabetes in BB-DP rats, and to the corresponding time points in syngeneic BB-DR and WF transplants (Fig. 3).

Following the 82 in vitro IL-1β-influenced proteins in both BB-DP and BB-DR/WF transplants over time throughout the experiment, not all the 82 protein changes observed in vitro occurred. Ten of the 82 protein spots were not changed at any time point, suggesting no major role for these proteins in vivo but a potential role in diabetes development for the remaining 72 of the 82 proteins. Comparing the BB-DP transplants from day 48 after transplantation (nondiabetic) with those from the day of onset of diabetes (day 48 ± 5 days after transplantation), the highest number of changes was found and most of these (25 out of 32) were changed as by IL-1β in vitro. It was at the same time point that the highest expression of mRNA for IL-1β was found. The 82 proteins changed their expression in a complex pattern, and this study did not allow us to consider any small and statistically nonsignificant changes in protein expression levels or to decide which changes were primary or secondary. The transplanted rats all had normal blood glucose values unless immediately prior to and at onset of diabetes (107).

Taken together, this suggests that IL-1β is present in islets early in the prediabetic period and may participate in the initiation of diabetes in the BB-DP rat through the induction of changes in protein expression levels. These changes in protein expression may alter the stability of the β-cell to a state of instability and potential destruction. The role of each specific
protein in the disease process is difficult to determine, and no single protein seems to be responsible for the development of diabetes, but rather the cumulative number of changes seems to interfere with the stability of the β-cell, thereby pushing it toward destruction and development of diabetes.

When using spontaneous diabetes-developing animal models, such as the BB rat, it is important to keep in mind that during β-cell destruction in vivo a plethora of cytokines, including IL-1β, and other factors are involved (108) and that IL-1β exposure of islets in vitro is a simplified model for β-cell destruction. The BB-DP transplantation model reflects the spontaneous diabetes development with all its known and unknown influencing factors and their interactions in the BB-DP rat (46, 107). Using other cytokines or combinations of cytokines and exposure time in vitro might possibly give a different protein expression profile, as seen for example in mRNA expression studies of sorted β-cells and β-cell lines (100, 101), although many changes involve the same genes/proteins and pathways.

Following the expression of proteins over time might also reflect the dynamic changes in the β-cell mass changes dependent on age, function, and demand (109, 110) and, in the transplantation model, changes in the re-organization and re-vascularization taking place in the transplanted islets (111). Because the β-cell mass changes during life (112), a number of proteins are expected to change their expression level throughout the study period. Proteins changing expression over time in rats not developing diabetes, in casu BB-DR/WF transplants, may in part originate from the normal processes of aging and β-cell turnover. Hence, such proteins may be of minor interest for the disease process. Furthermore, this study does not allow us to consider any small and statistically nonsignificant changes in protein expression levels and to decide whether many minor changes together are what produce the diabetic phenotype, β-cell destruction, and diabetes.

However, in this study we have identified and followed IL-1β-induced changes in protein expression in vitro in syngeneically transplanted islets and have related these changes to the development of diabetes in the BB-DP rat. Furthermore, we have produced a rather detailed picture of protein expression during development of diabetes in the BB-DP rat and produced evidence to support that IL-1β-induced in vitro protein expression changes in islets also occur during autoimmune β-cell destruction.

In BB-DP rats escaping diabetes (day 174 after transplantation) and in age-matched BB-DR rats, only a few proteins (5 proteins) were expressed differently, the lowest number for all the comparisons between BB-DP and BB-DR/WF transplants. This suggested that very few differences are indicative of stability. These protein spots contained galectin-3 (gal-3), the progesterone receptor membrane component 1 (25-Dx), adenosine phosphoribosyltransferase/uridine monophosphate-cytidine monophosphate (UMP-CMP) kinase, and two yet unidentified proteins. Four of these are changed in expression as by IL-1β in vitro. Gal-3, an inhibitor of apoptosis (113–115), is less expressed in BB-DR transplants compared with BB-DP transplants escaping diabetes, which might suggest a lower need for inhibition of apoptosis in BB-DR transplants.

The transplant also contains other cell types than β-cells, e.g. α-cells, infiltrating mononuclear cells, endothelial cells, and kidney tissue. Non-islet tissues are sought identified and removed under a microscope immediately after retrieval from the rat. All these factors mentioned are supposed to be equal in age-matched groups, except for the mononuclear cell infiltration, which is primarily seen in the BB-DP transplants at onset of diabetes. Nevertheless, proteins that are changed in expression despite their origin are believed to play a role in changing the stability of the β-cell and are as such of potential interest.

**INTRAUTERINE PROTEIN RESTRICTION**

Fetal under-nutrition may result in intrauterine growth restriction and increased incidence of diseases such as T2D and coronary heart diseases in adult life in humans (21–23) as well as in rodents (24). When an isocaloric LP diet is given to rat dams throughout gestation, changes such as decreased islet cell proliferation, islet size, pancreatic insulin content and islet vascularization, and an increased apoptotic rate are seen in the offspring at birth (25, 26). The increased rate of apoptosis in the LP islets can be normalized by supplementation with the amino acid taurine to the LP diet given to pregnant rats (116). The function of the fetal endocrine pancreas is also influenced, because fetal islets in vitro after 7 days of culture secrete less insulin in response to various secretagogues (117, 118). LP islets are more sensitive to NO and IL-1β (27) and as such interesting for the pathogenesis of T1D as well. The functional changes induced by the LP diet in utero are persistent and adult offspring are insulinopenic and glucose-intolerant (117). The LP islets show increased susceptibility to cytokines despite a normal diet given after birth or after weaning (119) and NOD mice receiving taurine in the drinking water in early life reduces insulitis and delays onset of diabetes (120), indicating that phenotype changes can be induced by an environmental factor.

We have tested the hypothesis that the effects in utero of a LP diet on β-cell development and function are caused by intrauterine changes in the programming of β-cell gene expression and that this is reflected in the protein expression pattern (121). This study demonstrated that changes in the protein expression pattern occurred in fetal rat islets after protein restriction during gestation. Overall, the specific protein expression changes were compatible with the functional findings described above, and the study suggested that the changes in the islet protein expression pattern of expression were acquired (121).

The proteins identified comprise proteins from the following...
pathways and functional groups: 1) energy transduction, redox potentials; 2) glycolysis and Krebs cycle; 3) RNA and DNA metabolism; 4) protein synthesis and metabolism; 5) protein folding and chaperoning; 6) cell cycle, differentiation, signal transduction, and transcription; 7) cellular structure; 8) cellular defense, and 9) miscellaneous functions. The study showed that in Wistar rats, the intrauterine LP milieu may program islet gene expression in ways unfavorable for the future of the progeny (121). Lasting consequences, e.g. lower \( \beta \)-cell mass, lower plasma insulin levels, and lower insulin secretion after a glucose challenge and increased susceptibility to cytokines, are present also in adulthood (27). Theoretically, this may be of importance for our understanding of the development of both T1D and T2D and suggests that environmental factors such as the diet may change the susceptibility to cytokines and thereby change the stability of the \( \beta \)-cell toward a pathological phenotype.

Whether the lasting consequences induced by the LP diet, e.g. lower \( \beta \)-cell mass, lower plasma insulin levels, and lower insulin secretion and increased susceptibility to cytokines present in both fetal islets and in adulthood (27, 119, 122), can be directly related to changes in the fetal islet protein expression pattern previously described is likely but not definitely proven. The control (C) and the LP islets are comprised of about 90% \( \beta \)-cells and have proliferated and differentiated during 7 days in culture during which they were withdrawn from the maternal milieu. However, the C and the LP islets are kept under the same environmental influence during the in vitro culture period. Therefore, the difference in the phenotype of endocrine cells observed after 7 days following withdrawal from the abnormal metabolic milieu is of great interest, because it suggests maternal programming of gene expression. Similar dietary influence on phenotype and programming of islet function is seen in rat pups offered a high-carbohydrate milk formula during the suckling period. These animals develop chronic hyperinsulinemia in the post-weaning period and obesity in adulthood, despite a normal rat chow diet. This hyperinsulinemia phenotype is transferred to the next generation despite normal diet both during pregnancy and after pregnancy (123).

DISTURBANCES IN THE STABILITY-DYNAMIC INSTABILITY

Most diseases are complex entities to which genes make a necessary, but only partial, contribution (124). The disease phenotypes are controlled not only by genes but also by protein-to-protein, DNA-to-protein interactions in a self-organizing network that displays system-wide dynamics and regulates all cellular processes from metabolic pathways to signaling pathways. Considering proteins not only as individual proteins, but rather as participants in a network of protein-to-protein interactions, they do form parts of a context of cellular functions within functional entities. This implies that the robustness of a system is dependent upon the ability of the network to form and use alternative pathways to overcome perturbations (125). When perturbed, networks alter their output and energy which, depending on the environmental influence, can produce either a pathological, e.g. T1D, or a normal phenotype (126). The threshold between stable and unstable is dynamic, varying with the ever-changing quantitative relations among the parameters controlling the dynamics. In the case of initiation of \( \beta \)-cell destruction, the \( \beta \)-cell moves from a normal state to a pathological state and potential destruction (105) (Fig. 4). Realizing that it is the dynamics of these networks that might create a diabetic phenotype may provide new insights into the pathogenesis and treatment of T1D.

TRANSCRIPTOMICS VERSUS PROTEOMICS IN T1D RESEARCH

Processes involved in cytokine-mediated \( \beta \)-cell destruction requires de novo protein synthesis (99, 127–130). Despite many years of research, the signals initiating gene transcription and protein synthesis and alterations in these profiles during \( \beta \)-cell destruction are incompletely understood. Not all mRNAs present in a cell are translated into proteins, and mRNA encodes only for unmodified pre-forms of the proteins. Therefore analyses at the protein level yields important information not obtainable by transcriptome analysis.

To date, a number of studies using gene array technology in the study of \( \beta \)-cell physiology and pathogenesis of T1D have been published (100, 101, 131–138).

Proteins are the product of a highly specialized and regulated process encoded in the genetic information. The expression of a gene can be examined at the level of mRNA or at the corresponding protein. However, the relationship between the mRNA of a specific gene and the corresponding protein products is not necessarily linear. Proteins with high abundance have a generally better correlation to the corresponding mRNA than low-abundance proteins (102, 103, 139–145). Furthermore, proteins are often modified post-translationally or present in one or more isoforms in higher species. This is not necessarily encoded for in the mRNA sequence and the complexity of protein profiles therefore exceeds that of corresponding mRNA profiles (146). This is illustrated in the comparison of proteome and transcriptome data obtained from
the same β-cell line during maturation where only two proteins and transcripts were in common (137). In the proteome study, 37% of the proteins were posttranslationally modified. Further discrepancies between proteome and transcriptome studies can be explained by e.g. differences in detection sensitivity of the selected method, sample preparation, specific experimental conditions, translational regulation, alternative splicing of mRNA, posttranslational modifications, time difference in mRNA, and protein production and degradation. This further emphasizes the importance of cautiousness when comparing transcriptome and proteome studies studying the same phenomenon such as cytokine-exposed islets, β-cells, or β-cell lines.

Two studies have compared gene and protein expression in islets and β-cell lines, and both studies were able to suggest a close correlation between basal mRNA and protein expression (133, 138).

OBSTACLES

Large amounts of protein expression data have been generated from the studies described in this review. However, it is very important to keep in mind that the data are limited in their absoluteness because all studies are performed under specific controlled experimental conditions and with current technologies and knowledge available. Changes in specific experimental parameters such as exposure time to cytokines, temperature, growth media composition, and tissue used can all influence the outcome. Furthermore, the technologies used have a series of limitations arising from e.g. sample preparation, protein extraction, 2-DGE, protein visualization, protein expression analysis, and protein identification (104, 147–150), which all can influence the results. The results have to be regarded as a snapshot of narrow time windows of a highly dynamic process that constantly are influenced by both the internal and external environment.

Another important issue for present and future studies is to extract the important information from expression profiling studies with large numbers of data derived from both transcriptomics and proteomics and to understand the biological sense. Furthermore, the correlation between a gene and its gene products needs to be better elucidated because there is no consensus regarding this in the literature.

Not only studying a single linear biological/chemical reaction but the entire set of expressed genes or proteins in a biological sample, systems biology, requires advanced data mining analysis methods such as cluster analysis. These tools may identify groups or clusters of proteins relevant for the issue studied.

FUTURE STUDIES

Even in the studies presented in this review, it is difficult to fully interpret the data with traditional and conventional methods. Therefore, future studies will involve cluster analysis programs, neural networking, and data mining. Currently, we are applying cluster analysis on the global expression pattern of proteins during spontaneous development of diabetes in islet transplants from BB-DP and BB-DR rats. This will hopefully help us in understanding the complex pattern of interactions and cross-talks taking place in the β-cell during development of diabetes. In other research areas, we are currently examining the gene expression profiles in whole pancreata from animals treated with prophylactic insulin and further studies of LP islets using microarrays, and protein expression at different glucose concentrations in transplants. A future achievement will be also to create a database with all proteins we have identified in our rat proteome studies (at present more than 450) and combine them with their expression profiles during development of and protection from diabetes.

T1D is characterized by production of autoantibodies to β-cell antigens, and today approximately two dozen of β-cell antigens have been identified although their pathogenic role is unclear. The autoantibodies can be present years before onset of diabetes and can be used as predictors of later T1D development (151, 152). With autoantibodies and potentially also autoantigens present in the blood years prior to onset of diabetes, the ability to study the plasma proteome (153, 154) makes proteomics studies of blood samples from diabetes-disposed individuals an obvious issue for future studies and identification of new biomarkers for T1D. Other new and future tasks will be to study membrane proteins, secreted proteins, posttranslational modifications, and to have access to human material.

Hopefully, this will unveil new patterns of expression changes and bring new clues and ideas into the study of T1D pathogenesis and create new ideas for protection or even cure of T1D.

EMERGING PROTEOMIC TECHNOLOGIES

Future diabetes research needs exceedingly robust and sensitive quantitative proteomic technologies that will facilitate detailed and accurate studies of protein composition and the dynamics of protein expression and turnover, protein interactions, and posttranslational modifications.

To date, global proteomic studies of diabetes have been performed by 2-DGE, image analysis, and MS. As outlined in previous sections, such studies have provided insights into some of the cellular processes that are perturbed in this disease.

2-DGE-based proteomic technology has several advantages due to high protein resolving power, including separation of protein variants with altered isoelectric points due to amino acid substitutions, splice variants, or posttranslational modifications such as phosphorylation. In addition, identification of 2-DGE-separated proteins by MS is relatively straightforward, because a large number of peptides are usually matched to the amino acid sequence of the protein, providing high-confidence protein identification. However, standard 2-DGE technology has some disadvantages, which limit the
number of detectable proteins in a given sample. The main
disadvantage is that membrane proteins are poorly soluble in
standard IEF buffers, and they are therefore underrepresented
in most 2-DGE-based proteomic studies. More than one-third
of the proteins coded by the human genome are estimated to
be membrane proteins or membrane-associated proteins with
functions spanning from cell-cell communication/attachment
to cell signaling, maintenance of cell membrane potential,
mediation of the transport of ions and proteins, regulation of
vesicle transport within the cell, and many other functions.
Plasma membrane (PM) proteins are the first barrier to the
environment, and because of their central role in cell signaling
and transport, nearly half of the 15 most frequently prescribed
drugs target PM proteins. Thus they are considered important
potential drug targets and markers for the pharmaceutical
industry and therefore very important in the study of T1D.
Strong zwitterionic detergents may solubilize membrane pro-
TEINS so that they can be resolved by 2-DGE (155). However,
this requires a larger number of gels to be analyzed and a
significantly higher amount of starting material due to numer-
ous purification steps. Other groups of proteins like low-
abundant, small, or large proteins and basic proteins are also
underrepresented in current routine 2-DGE studies, but can
be enhanced using specialized techniques such as extreme-
range pH (156, 157). Nevertheless, 2-DGE-based proteomic
technologies are continuously improved to enhance handling,
throughput, and sensitivity (158) and will remain a key tool in
proteomics, particularly when combined with other biochem-
ic techniques for protein prefractionation and MS for protein
characterization.
Recent advances in MS instrumentation, peptide separa-
tion technology, and bioinformatics have had a major impact
on proteomics (159, 160). Whereas 2-DGE technology is ap-
plied to separate intact proteins, the new MS-driven proteo-
mic approaches are used to analyze complex peptide
mixtures derived by proteolytic digestion of protein extracts.
Nanoliter flow HPLC interfaced to ESI MS/MS and MALDI
tandem mass spectrometers enable the separation and anal-
ysis of exceedingly complex peptide mixtures derived from
cell or tissue lysates or from purified cellular organelles or
protein complexes. In contrast to the 2-DGE approach only
small amounts of cellular material is needed for analysis by
LC-MS/MS, typically on the order of 10–50 μg of protein
starting material and these peptide-oriented technologies are
also applicable to proteomic investigations of membrane pro-
TEINS (161–163) (Table I). Development of and improvements

### Table I

| Specification and comparison of 2-DGE and LC-MS for proteome analysis |
|-------------------------------------------------|-------------------------------------------------|
| **Analytic species** | Intact protein mixture | Complex peptide mixtures derived by enzymatic or chemical degradation of proteins |
| **Dynamic range** | Depends on protein detection method: radio labeling, 10⁴–10⁵; fluorescent labeling, >10³; dyes/silver staining, <10³ | Depends on protein and peptide separation methods and type of mass spectrometer used for peptide detection and fragmentation, typically 10⁴–10⁵ |
| **Sample consumption** | Typically 50–500 μg protein per gel | Typically 5–50 μg protein per experiment |
| **Heterogeneity due to splicing events, degradation, posttranslational modifications, or mutations** | Detected by altered protein migration during IEF and/or during SDS-PAGE | Modifications are identified by observation of a mass increment or deficits, relative to unmodified expected peptides |
| **Membrane/hydrophobic proteins** | Lost when using standard 2-DGE experimental conditions; strong zwitterionic detergents in the IEF buffer allow 2-DGE analysis of membrane/hydrophobic proteins | Peptide-based methods are suitable for membrane proteins; optimized protocols for preparation of cellular and organelle membrane and membrane protein digestion are available |
| **Quantification** | Based on imaging; spot density relative to the sum-density of all spots on the 2-DGE gel; differential staining efficiency of processed/modified proteins may affect quantization | Based on mass spectrometric peptide ion intensities: i) comparison of peptide ion intensities in parallel LC-MS experiments; ii) protein or peptide encoding by stable isotope labeling and determination of light/heavy peptide ratios |
| **Protein sequence coverage** | Usually rather high, because only one or few proteins are present in each 2-DGE spot; ranging from below 30% to over 90% depending on sample amount, number of cleavage sites, and sample complexity | Varies with complexity of protein sample; usually rather low (2–25%) but can be over 70% for highly abundant proteins, even in complex mixtures |
Protein quantization is central to achieve insights into dynamic cellular processes in health and disease. Thus, much effort has been directed toward development of accurate methods for protein and peptide quantization by MS (164). A highly promising approach is stable isotope labeling of proteins and peptides. In this approach, protein samples are typically compared in a pair-wise manner by measuring the peptide abundance ratio between a stable isotope-encoded “heavy” peptide species with the corresponding natural “light” peptide species. In such experiments the light form (experiment) and heavy form (control) of the proteins are mixed and then separated by standard biochemical, electrophoretic, or chromatographic methods. Standard protein separation methods cannot distinguish the light and heavy version of proteins or peptides leading to co-migration of the heavy and light versions of proteins throughout the experiment. The final analysis of peptides is achieved by MS, which is able to distinguish the light and heavy forms, thereby providing a readout of the relative change in abundance between experiment and control.

Stable isotope encoding of proteins in cell culture is achieved by metabolic labeling using growth media containing $^3$H-, $^{13}$C-, or $^{15}$N-substituted precursor molecules, e.g. $^{15}$N- (165) or $^{13}$C/$^{15}$N-encoded amino acids (166). The latter method (SILAC) is widely applicable to mammalian cell cultures for studies of protein expression changes and investigations of cell-signaling processes (167). However, metabolic labeling methods are not useful in studies of tissue samples or mammals (although a stable isotope-labeled rat has been reported) (168). Instead, chemical approaches to stable isotope labeling of proteins and peptides have been developed, including the ICAT method (cysteine-specific light/heavy reagents) (169) and the recently presented amine-reactive isobaric tagging reagents (iTRAQ) (170). The latter technology enables four-plex experiments, but relies on extensive MS/MS analysis of all peptide components in a sample. These methods and other emerging stable isotope-labeling techniques for protein quantization will have a major impact on MS-driven proteomics in the coming years and will extend our ability to address very complex issues in diabetes disease etiology by proteomics.

A majority of diseases including diabetes arise from malfunctioning proteins, including constitutively active or inactive protein species originating by mutations, by erroneous processing/maturation, or by altered posttranslational modifications. These molecular errors in turn lead to mis-location of proteins, wrong or missing interactions with key effector molecules, or simply to degradation of the altered protein. Sensitive quantitative proteomics methods have to be applied to investigate these intricate phenomena as they relate to diabetes. Posttranslational modifications represent a special challenge as they are often highly dynamic and of low abundance (e.g. phosphorylations) or of very complex structure (e.g. N-glycosylation) (171, 172). Proteomic approaches to investigate membrane proteins, posttranslational modifications, and cell-signaling pathways are being developed in numerous laboratories and will remain a key research theme in proteomics for years to come. Based on the concept of “modification-specific proteomics” (172), we have presented “shave-and-conquer” proteomic approaches to investigate plasma membrane phosphoproteins and glycosylphosphatidylinositol-anchored proteins and discovered a wealth of novel membrane proteins, including receptor kinases and ion pumps (161, 162, 173). Epidermal growth factor-stimulated
cell signaling processes in human cells have been studied using SILAC and MS (174), an approach that can be extended to study insulin signaling as well. We have recently demonstrated that G-protein-coupled signaling cascades in yeast may be elucidated by quantitative phosphoproteomics by combining efficient phosphopeptide affinity enrichment methods (IMAC) with SILAC and high-performance MS/MS (175). We identified phosphorylation of key protein molecules, from the 7TM receptor via the mitogen-activated protein kinase pathway to downstream effector molecules, including transcription factors. Comprehensive characterization of glycosylated proteins by MS have also been achieved in our laboratory and by others (176–178) and holds great promise for detailed glycoproteomic of diabetic tissues and organs.

CONCLUSION

Today it is beyond any doubt that T1D is a multifactorial polygenic disease of largely unknown etiology. This review demonstrates that, in an animal model for T1D, several proteins seem to be involved in the pathogenesis of diabetes and that no single protein can be accused of being “The Diabetes Protein.” This is in line with genetic studies in humans, where no single gene has been pointed out as “The Diabetes Gene” (17).

The studies reviewed strongly suggest that diabetes is the result of a wide range of minor changes in protein expression that collectively alter the stability of the healthy β-cell to a prediabetic phenotype of instability and possibly β-cell destruction. In other words, T1D is a multifactorial complex disease and can be regarded as the phenotype of the collective dynamic changes, involving both protective and destructive mechanisms in many cellular pathways acting together. The sum of these processes may lead to a pathological state. However, this process is not irreversible, and the instability of the β-cell can be changed into a stable state. For example, the environment can be manipulated, because biological systems are redundant and, if negatively influenced, alternative pathways through which normal level of activity may be restored and maintained for vital functions (74, 75, 126). Furthermore, changes in fetal environment are able to make the β-cell more sensitive to cytokines and free radicals through alterations in the protein expression in fetal islets. The number of proteins changed is limited and their known and putative functions can be directly related to changes observed in the altered phenotype. In other words, it is possible to change an expected phenotype, either from a stable to a more unstable state or in the opposite direction with regard to susceptibility to diabetes. This creates the hope for the future development of strategies for prevention and treatments of T1D, although a lot of experiments still need to be performed.

This review emphasizes that the use of proteomics is a useful and powerful method for identification of proteins and pathways involved in diabetes development. Today there are still no methods available that efficiently can isolate, separate, quantify, and identify all proteins within a specific proteome and ways to understand the gained information. Therefore many discoveries awaits their discovery in the future. One of the big challenges is to test the hypotheses generated from the data and to clarify the dynamics and specificity of the changes observed and finally to confirm it in β-cell lines and in vivo.

Diabetes is a complex disease, and much work lies ahead before we gain detailed insights at the molecular level into disease etiology. With the current pace in proteomics technology development and focus on biomedical applications, there is no doubt that rapid advance will provide answers about diabetes much sooner than we anticipate.

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§ To whom correspondence should be addressed: Steno Diabetes Center, Niels Steensensvej 8, DK-2820 Gentofte, Denmark. Tel.: 45-4443-9160; Fax: 45-4443-7872; E-mail: tspa@novonordisk.com.

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