Running title: Beta cell hormones and autoimmunity

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Neonatal and infant beta cell hormone concentrations in relation to type 1 diabetes risk

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Abstract

Type 1 Diabetes is preceded by autoimmunity identifiable as islet autoantibodies. Seroconversion to islet autoantibodies is greatest around 1 year of age and is more frequent in children born to fathers with type 1 diabetes as compared to children born to mothers with type 1 diabetes. Here we asked whether beta cell function changes in the neonate and infant reflect variations in islet autoantibody seroconversion frequency. Insulin, proinsulin and c-peptide concentrations were measured in multiple serum samples taken from birth to age 2 years in 101 children who had a first degree relative with type 1 diabetes and who had been followed for islet autoantibody seroconversion. Serum insulin and proinsulin concentrations were highest at birth declining by age 3 months and stable thereafter until age 2 years. C-peptide concentrations, proinsulin/insulin, and proinsulin/c-peptide ratios were stable from age 3 months. No differences were observed between children who developed islet autoantibodies and children who remained islet autoantibody negative. Children born to a mother with type 1 diabetes had higher birth concentrations of insulin ($p=0.005$) and proinsulin ($p=0.014$) as compared to children of non-diabetic mothers. Increased insulin concentrations in children of type 1 diabetes mothers persisted until age 6 months. In conclusion, we could not relate excursions in beta cell hormone products to autoantibody development, but suggest that the higher exposure to insulin and proinsulin in the neonate may be linked to the reduced relative frequency of islet autoantibody seroconversion observed in children of mothers with type 1 diabetes.

Keywords

Type 1 diabetes; Insulin; Proinsulin; Autoantibodies

Abbreviations

HLA, Human leukocyte antigen; AAb+, islet autoantibody positive; AAb-, islet autoantibody negative.

Introduction

Type 1 diabetes is an autoimmune disease characterized by the appearance of autoantibodies in early childhood, and subsequent loss of beta cells and beta cell function until insulin therapy is required. The incidence of islet autoantibody seroconversion varies with age. A peak incidence is observed around 1 year of age (1, 2), and the magnitude of incidence is affected by several factors, including a protective effect by maternal diabetes (3). Of interest, incidence of seroconversion with respect to age is not the same for autoantibodies associated with other endocrine autoimmune diseases. In particular, we have observed that the incidence of thyroid autoimmunity increases around puberty and adolescence (1). While the variation in incidence may be due to changes in exposure to environmental factors specifically associated with disease, it is also possible that it may reflect changes at the target organ. For example, in the case of thyroid autoimmunity, adolescence is a period when the thyroid gland exhibits strong activity due to body growth and morphological changes (4). It is possible that in the process of heightened activity, the presentation of self antigens is increased and susceptible subjects are at higher risk to develop autoantibodies during that time. In the case of islet autoimmunity, early remodeling of islets is reported (5). Beta cells are functional soon after birth, but changes in diet after introduction of foods will likely influence beta cell activity and insulin demand (6). It is also clearly established that beta cells of neonates born to mothers with type 1 diabetes have a different workload than those from neonates born to non-diabetic mothers (7). Thus, it is conceivable that such changes may directly determine the incidence of islet autoantibody seroconversion in infancy. Here, we examined serum concentrations of beta cell hormone and prohormone from birth until the age of 2 years in children with a first degree relative with type 1 diabetes with

the aim to find evidence for this hypothesis. We specifically asked whether there were changes in beta cell workload as reflected by proinsulin/insulin ratio that may explain the increased islet autoantibody incidence around 1 year.

Methods

Study material: Samples were obtained from children participating in the BABYDIET study (8). The BABYDIET study was an intervention study to determine the effect of delaying gluten introduction into the diet of infants on the risk of developing type 1 diabetes. In short, newborn children were eligible if they were younger than 3 months and were offspring or siblings of patients with type 1 diabetes and also had HLA genotypes that conferred a high diabetes risk (DRB1*03-DQA1*0501-DQB1*0201/DRB1*04-DQA1*0301-DQB1*0302; DRB1*04-DQA1*0301-DQB1*0302/DRB1*04-DQA1*0301-DQB1*0302 or DRB1*03-DQA1*0501-DQB1*0201/ DRB1*03-DQA1*0501-DQB1*0201). Children (n=150) of consenting parents were randomized to two dietary intervention groups, one that introduced gluten-containing cereals at 12 months (late exposure) and one that introduced gluten at 6 months of age in line with national feeding guidelines (control group). Children were monitored intensively with 3-monthly collection of venous blood, urine and stool, and 3-day dietary records of weighed food intake for 3 years and yearly thereafter. Children were not required to be fasting at blood draw. The endpoint of the study was the development of persistent antibodies to one or more of the antigens Insulin, GAD65 or IA-2. Persistence was defined as being positive in at least two consecutive samples, and all venous blood samples were tested. From the available time points, samples were used at birth and 3, 9, 15 and 24 months. A total of 329 samples from 103 of the children were selected on the basis of sample availability at -80 degree. Of these 103 children, 55 were born to a mother who had type 1 diabetes, and the remainder were born to a mother without diabetes, including gestational diabetes. Birth weight and gestational age were obtained from obstetric records. Breast-feeding duration was recorded by the parents of all children ().

Additional cord blood samples from 86 children were obtained from the BABYDIAB study (9). Autoantibodies were measured using radioligand binding assays as previously described. (9, 10)

Hormone measurements: Proinsulin was determined by ELISA (Millipore Human total proinsulin ELISA Kit; Merck Millipore (Darmstadt, Germany) analyzed on a Tecan reader infinite 200pro according to the manufacturers guidelines. The limit of detection was 1.9pM. The between assay coefficient of variation stated by the manufacturer is 2.9% for a proinsulin concentration of x. Insulin and c-peptide were determined using the Millipore Milliplex Map kit “human endocrine” analyzed on a Luminex 200. The limit of detection was 4 pM for both insulin and c-peptide. Between assay CVs are stated by t he manufacturer as 4.74% for an insulin concentration of x and 3.5% for a c-peptide concentration of x. Assays were performed on stored frozen samples that had not been previously thawed. For each of these analytes, all samples were measured in three assays using a single batch of assay kits.

Statistical analysis: To assess the longitudinal behavior after birth, we calculated first-order to third-order polynomial growth models (11) for each parameter and ratio in each group, performing model selection according to the Akaike Information Criterion (12).This enabled us to take account of the exact time points when each measurement was taken and therefore to use the full information of the data. Model curves between groups were compared by Gaussian tests on estimated means. The Mann-Whitney-U Test was used to compare the concentrations at birth of insulin, proinsulin, c-peptide and the ratios proinsulin/insulin and proinsulin/c-peptide between islet autoantibody seroconverters (AAb+) and non-seroconverters (AAb-) and between children of mothers with type 1 diabetes and children of mothers without diabetes, respectively. Further, birth weight, gestational age and mode of delivery

were associated with hormone concentrations at birth and age 3 months and additionally compared by maternal diabetes status using Pearson correlations, Mann-Whitney-U tests and Chi-Square tests (as appropriate). For all analyses, a two-tailed P value of <0.05 was considered significant. P values were not corrected for multiple comparisons.

Results

Serum insulin and proinsulin concentrations were highest at birth declining by age 3 months and remaining relatively stable thereafter until age 2 years. Concentrations at birth varied markedly between children (insulin, median 73; range 4 -430 pM; proinsulin, median 16.5; range 2- 127 pM). The proinsulin/insulin ratio was stable over the first 2 years of life, but again there was substantial variation between children. Serum c-peptide concentration and proinsulin/c-peptide ratio were measured from age 3 months, and were stable until age 2 years (Figure 1).

No differences in serum insulin, proinsulin, and c-peptide concentrations, and proinsulin/insulin and proinsulin/c-peptide ratios were observed between children who developed islet autoantibodies and children who remained islet autoantibody negative (Figure 2). Of note, insulin and proinsulin concentrations at birth were low with relatively small variation in children who developed islet autoantibodies.

As expected, children born to a mother with diabetes had higher serum insulin (median 125 pM, IQR 94-215) and proinsulin (median 29pM, IQR 19-33) concentrations at birth than children born to a mother without diabetes (Insulin median 57pM, IQR 29-72; proinsulin 13pM, IQR 10-16; P=0.0047 and 0.014, respectively; figure 3). No difference in the proinsulin/insulin ratio was observed. The findings were similar in a second cohort obtained from the BABYDIAB study (insulin P=0.0015, proinsulin P=0.0003; data not shown). The estimated longitudinal curves from the data (Figure 4) indicated that higher insulin concentrations in children born to mothers with type 1 diabetes remained until age 6 months (P<0.0001) and that c-peptide concentration was increased until age 9 months in this group. The hormone concentrations at birth or 3 months were not significantly associated with birth weight, gestational age or mode of delivery (data not shown). Children of diabetic mothers

had a significantly lower gestational age at birth (median age: 38.0 weeks vs 39.0 weeks for children born to non-diabetic mothers; p=0.021) a higher birth weight (median birth weight: 3665 g vs 3375 g for children of non-diabetic mothers; p=0.024) and were more frequently delivered by cesarean section (45.5% vs 18.8% for children from non-diabetic mothers; p=0.004).

Discussion

We sought to determine whether increased demands on the beta cell, and in particular evidence of beta cell stress, could be related to the increased incidence of islet autoimmunity found at around one year of age in genetically susceptible children. This study found increased concentrations of the insulin hormone and prohormone at birth, but failed to see considerable fluctuations in concentrations of these hormones from age three months to two years. Moreover, the ratios of proinsulin to insulin or proinsulin to c-peptide, which are potential measures of beta cell hyperactivity (7, 13, 14), were also stable during this age period. Thus, we found no support from these data for our hypothesis that beta cell stress may contribute to an increased incidence of islet autoimmunity in early childhood.

While the main findings of the study did not support our *a priori* hypothesis, there are several limitations to be considered. First, measurements were not performed on fasting samples since it was not feasible to schedule study visits around the feeding times in the first two years of life of the children. Thus it remains possible that differences with respect to age may have been observed if fasting samples were analysed. Second, the measurements are static concentrations and do not reflect variation in beta cell function or demand on function in a continuous manner. Third, the numbers of children studied, even if substantially more than previously reported, remain relatively small. We, therefore, hesitate to conclude that the demand on the beta cell has no influence on the risk of islet autoantibody seroconversion. Indeed, it is very likely that changes in the diet seen after six months of age will affect beta cell function, especially with the introduction of insulinogenic foods to infant diets (6).

No differences were found in the children who developed islet autoantibodies as compared to those who remained islet autoantibody negative. There were, however, striking differences between children who were born to mothers with type 1 diabetes as compared to children born to non-diabetic mothers that may be relevant to pathogenesis of islet autoimmunity. As previously reported (7) children born to mothers with type 1 diabetes had increased concentrations of insulin and proinsulin at birth. We further found increases up to age 3 months. It is noted that mothers with type 1 diabetes have increased glucose and insulin concentrations in their breast milk (15, 16) which may further increase insulin concentration in the first months of life consistent with this finding.

Children of mothers with type 1 diabetes have a reduced risk for type 1 diabetes compared to children of fathers with type 1 diabetes (17-19). It is therefore tempting to speculate on whether and how increased neonatal insulin and proinsulin concentrations may influence type 1 diabetes risk. We expect that protective mechanisms act at reducing islet autoimmunity risk since we have demonstrated that children of mothers with type 1 diabetes have a decreased incidence of seroconversion to islet autoantibodies (3). It is also noteworthy that this reduced incidence in children of mothers with type 1 diabetes is restricted to the peak period found at around one year of age (1, 3). We postulate, therefore, that increased exposure to insulin and proinsulin in these early months may lead to more efficient deletion of autoreactive T cells in the thymus, and/or the generation of insulin-specific T regulatory cells in the periphery, and that this protects children from the wave of islet autoimmunity seen in early infancy. These hypotheses can be tested, and if validated, would provide indirect evidence that beta cell function contributes to the development of islet autoimmunity.

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Figure legends

Figure 1: Serum concentrations of insulin (A), proinsulin (B), the proinsulin/insulin ratio (C), c-peptide concentration (D), and proinsulin/c-peptide ratio (E) over time in children who have a first degree relative with type 1 diabetes. Results are from 329 samples obtained from 103 children. The solid line shows the estimated mean and the shaded area the respective confidence intervals determined from longitudinal modeling of the data.

Figure 2: Longitudinal modeling of the concentrations of insulin (A), proinsulin (B), the proinsulin/insulin ratio (C), c-peptide concentration (D), and proinsulin/c-peptide ratio (E) in serum from children who developed islet autoantibodies (n=84; red line) or remained islet autoantibody negative (n=245; blue line). The shaded areas show the respective 95% confidence intervals around the mean (solid lines).

Figure 3: Comparison of serum concentrations at birth of insulin (A), proinsulin (B), and the proinsulin/insulin ratio (C) in children born to mothers with type 1 diabetes (n=13; filled circles) and children born to non-diabetic mothers (n=12; open circles). The horizontal line marks the median. Statistical analysis by Mann-Whitney-U test.

Figure 4: Longitudinal modeling of the concentrations of insulin (A), proinsulin (B), the proinsulin/insulin ratio (C), c-peptide concentration (D), and proinsulin/c-peptide ratio (E) in blood samples of children born to mothers with type 1 diabetes (n=171; red line) and to non-diabetic mothers (n=158; blue line). The solid line shows the estimated mean and the shaded area the respective 95% confidence intervals. Differences between groups were found for Insulin until age 6 months ($p=0.005$) for c-peptide until age 9 months ($p=0.0015$). Statistical analysis by Gaussian tests on estimated means.