Tracing the Pathogenesis of Type 1 Diabetes:
A Report on the 44th Annual Meeting of the European Association for the Study of Diabetes (EASD)

Liang-Hao Guo and Peter Achenbach

Diabetes Research Institute, Munich, Germany.
Address correspondence to: Peter Achenbach, e-mail: peter.achenbach@lrz.uni-muenchen.de

■ Abstract

Clinical type 1 diabetes is preceded by autoimmune destruction of the pancreatic beta-cells. However, progression to disease is not uniform. One challenge facing current diabetes research is therefore to identify biomarker profiles that accurately reflect the individual stage of type 1 diabetes pathogenesis and develop new techniques to distinguish between these profiles and associated diabetes risks. This report highlights some of the recent studies on diabetes biomarkers, with a particular focus on zinc transporter ZnT8, presented at the EASD meeting in September 2008 in Rome, Italy.

Keywords: type 1 diabetes · prediction · biomarker · autoantibody · zinc transporter · ZnT8 · SLC30A8 · metabolism · insulin resistance

Introduction

Much of our current understanding of the pathogenesis of type 1 diabetes (T1D) has derived from prospective studies in individuals with increased genetic susceptibility, such as relatives of patients with T1D. These studies have demonstrated that progression to diabetes may vary considerably between individuals and that, in order to optimize risk assessment, demographic, genetic, immune and metabolic markers should be combined. Today's best validated and most widely used predictive markers for T1D are autoantibodies directed against the biochemically defined target antigens: insulin (IAA), GAD65 (GADA), and IA-2 (IA-2A). Further T1D-associated biomarkers have been reported, and it is likely that some of these and potentially also some markers of type 2 diabetes (T2D) may complement and improve predictive profiles in subgroups of relatives.

Zinc Transporter ZnT8: relevance in diabetes development

Zinc transporter 8 (ZnT8) has recently been identified as a major target antigen in the autoimmune pathogenesis of human T1D [1]. Moreover, genome-wide association studies have linked a variation of the ZnT8 encoding gene *SLC30A8* with an increased risk of human T2D [2]. The rs13266634 single nucleotide polymorphism (SNP) in *SLC30A8* induces a non-synonymous transition from arginine to tryptophan at position 325 of ZnT8, and impaired beta-cell function has been reported for carriers of the arginine encoding "at risk" genotype [3]. ZnT8 is therefore interesting

from an immunological and functional point of view, raising the question whether it is possible to identify common mechanisms in the pathogenesis of T1D and T2D and, more particularly, how genetic variations in ZnT8 affect diabetes risk.

With respect to function, ZnT8 belongs to a family of specialized zinc transporter proteins that control intracellular zinc homeostasis. Beta-cells require zinc to form insulin hexamers within the secretory granules, which is necessary for efficient insulin secretion. ZnT8 is a beta-cell secretory granule membrane protein, but is also expressed in pancreatic alpha-cells. Interesting new data have now been reported from *SLC30A8* gene expression studies suggesting that ZnT8 is not only critical for insulin secretion in beta-cells but also plays a complementary role in inhibiting glucagon secretion in alpha-cells [4]. In these experiments, over-expression of the ZnT8 R325 (arginine) and W325 (tryptophan) variants in MIN-6 insulinoma cells resulted in a glucose dose-dependent stimulation of insulin secretion without affecting cellular insulin content and mRNA levels, whereas 50% knockdown of ZnT8 expression reduced basal insulin secretion associated with an increase in cellular insulin content. On the other hand, over-expression of either ZnT8 variant in αTC1-9 glucagonoma cells decreased glucagon secretion and cellular glucagon content, whereas ZnT8 knockdown led to an increase of glucagon mRNA and secretion. ZnT8 deficiency could therefore adversely affect both insulin and glucagon metabolism.

Another study was able to demonstrate that the R325W SNP affects the zinc transport efficiency of ZnT8 [5]. Here, MIN-6 cells over-expressing the ZnT8 R325 (T2D-risk) variant displayed elevated cytosolic Zn^{2+} uptake rates but decreased Zn^{2+} accumulation in secretory granules. In contrast, Zn^{2+} uptake into granules was more efficiently catalyzed by the W325 variant, suggesting that functional differences between the two ZnT8 variants may result in altered beta-cell intracellular Zn^{2+} homeostasis and may potentially explain the less efficient processing of proinsulin described in carriers of the T2D-risk C-allele. A third study reported that high levels of ZnT8 confer protection against cytokine-induced beta-cell death [6], which is of particular interest, since beta-cell function and mass is severely affected by the presence of cytokines, in particular IL-1β, in both T1D and T2D. ZnT8 expression in INS-1 insulinoma cells and neonatal rat islet was shown to be affected by cytokine treatment and reduced in presence of IL-1β and/or INF-γ. Conversely, over-expression of ZnT8 in INS-1 cells reduced cytokine-induced apoptosis. In conclusion, data from all three studies suggest that functional deficiency of ZnT8 could contribute to an increased risk of diabetes development.

With respect to autoimmunity, it has been shown that ZnT8 autoantibodies (ZnT8A) are directed against epitopes expressed in the cytosolic domains in the COOH-terminal and, less frequently, within the NH_2-terminal part of the protein. Moreover, the common non-synonymous polymorphism at position 325 is located in a COOH-terminal region, where more than 60% of new-onset T1D patients exhibit ZnT8A binding [1]. This has posed the question whether the R325W SNP may influence autoantibody responses. Three studies have now concordantly reported an association between *SLC30A8* genotype and ZnT8A reactivity [7-9]. Autoantibody responses in new-onset T1D patients from the US [7] and Japan [8], as well as in non-diabetic children with a first-degree family history of T1D from Germany [9], showed remarkable restriction to the ZnT8 R325 or W325 isoforms, depending on the presence of corresponding C or T-alleles of SNP rs13266634. A strong gene dosage effect was also evident such that the frequency of R325 or W325-restricted ZnT8A and autoantibody levels were much higher in homozygous than in heterozygous individuals. These data indicate that the variant residue at aa325 is a key determinant of humoral autoreactivity to ZnT8, and *SLC30A8* genotype is an important determinant of autoantibody specificity. Screening for ZnT8A should, therefore, consider both the R325 and W325 isoforms of the molecule.

Autoantibody screening in children from affected families showed that ZnT8A directed against the COOH-terminal part of the molecule can appear early in the pathogenesis of childhood T1D. These antibodies are associated with high diabetes risk [9]. Moreover, a strong association between *SLC30A8* R325W genotype and T1D risk was reported for ZnT8A-positive children, suggesting higher risk in carriers of homozygous polymorphisms. This is remarkable, since no association between *SLC30A8* genotype and T1D risk was found in two previous studies [10, 11]; nor was one observed in islet autoantibody-positive/ZnT8A-negative children in the current study. However, one paper does report an increased prevalence of the R325 encoding C-allele and the homozygous CC genotype in patients with T1D onset before age 5 years [12], which may be consistent with early ZnT8A development and rapid progression to disease. Genotyping at SNP rs13266634 could therefore be an effective secondary screening step to stratify T1D risk in ZnT8A-positive individuals.

Furthermore, autoantibodies against the COOH-terminal ZnT8 region also appear to be a valuable additional marker of adult-onset autoimmune diabetes, since they confirmed autoimmunity in a subgroup of single GADA-positive patients, and identified additional patients who were previously considered islet autoantibody-negative [13]. Finally, the first evaluation of ZnT8A in an international workshop organized by the Diabetes Antibody Standardization Program (DASP) demonstrated remarkable consistency in sensitivity and specificity. High levels of concordance between participating laboratories confirmed the validity of ZnT8A as diabetes-associated marker, and suggested general implementation of ZnT8A assays in the classification and prediction of T1D [14].

In support of the humoral studies, data from ELISPOT analyses indicated that the COOH-terminal region of ZnT8 also harbors epitopes, including an aa325 spanning peptide, which are more frequently recognized by INF-γ secreting CD4$^+$ T cells from newly diagnosed T1D patients than from healthy age and HLA matched controls [15]. Thus, disease-specific T cell responses to ZnT8 appear to be focused in the same region as the autoantibody response.

In conclusion, autoimmunity against the COOH-terminal region of ZnT8 is a relevant prognostic feature of T1D development, particularly in homozygous carriers of the R325 or W325 ZnT8 variants, and epitopes within this part of the molecule may be potential targets for antigen-specific immunotherapies designed to prevent or ameliorate the course of diabetes.

Improving screening techniques

Screening for multiple biomarkers can identify profiles associated with different diabetes risks and improve prediction. However, several single target assays may have to be run concurrently to determine profiles and achieve the highest sensitivity, which is not always feasible on a large-scale. It is therefore desirable to increase the amount of informative signals obtained by single assays.

Promising data have been reported from a study that investigated the potential of multimerizing epitopes and combining epitopes from different autoantigens into single vectors [16]. Model systems were developed using heterodimeric constructs of the two major polymorphic variants of ZnT8 (R325 and W325), and the two related autoantigens IA-2 and IA-2β. Autoantibody assays that used dimer antigens showed increased sensitivity without loss of specificity, as compared to testing monomeric antigens. The study suggested substantial advantage in assaying multimeric

autoantigen constructs through an increase in the number of major epitopes and improved assay sensitivity as a result of higher antibody avidity.

New array-based technologies may provide an alternative approach and also take into account autoantibody characteristics that are not associated with T1D development in order to distinguish disease-relevant from less or non-relevant profiles. For example, one study reported that persistent IAA in children, which is usually associated with high T1D risk, can be cross-reactive to non-insulin proteins, and specifically to casein, suggesting that in some cases, IAA could be secondary to immunization against food proteins. However, these cases do not show progression of islet autoimmunity to diabetes [17].

Metabolite profiles may anticipate progression to diabetes

Prognostic differences in serum metabolite profiles at a very early stage of pathogenesis have been found in a large cohort of prospectively followed children at T1D risk from Finland [18]. Children who progressed to diabetes showed reduced serum levels of succinate, citrate and phosphatidylcholine at birth, of multiple antioxidant ether phospholipids throughout follow-up and increased levels of proinflammatory lysophosphatidylcholines several months prior to islet autoantibody positivity, as compared to children who did not develop disease. Some changes in metabolite levels were associated with antibody responses to specific autoantigens, such as decreased levels of ketoleucine prior to seroconversion to IAA, or elevated levels of glutamate prior to the appearance of GADA. These data suggest that dysregulation of metabolism precedes islet autoimmunity and diabetes, and that metabolic interventions prior to islet autoimmunity may be used as a potential strategy for T1D prevention.

Insulin resistance is not associated with islet autoimmunity in children

It has been speculated that insulin resistance may contribute to the development of islet autoimmunity and diabetes in childhood. Recent data from a large cohort of children with a first-degree family history of T1D from Germany did not provide evidence that islet autoantibody-positive children are insulin resistant soon after seroconversion and during follow-up [19]. Increased HOMA-IR was significantly associated with female gender, older age and higher BMI, but was not associated with a faster progression rate to diabetes in islet autoantibody-positive children. Moreover, autoan-

tibody-positive children tended to have lower HOMA-IR than autoantibody-negative children, and did not have increased BMI. These results are inconsistent with the notion that insulin resistance is a risk factor for islet autoimmunity. However, insulin resistance and/or other T2D-related characteristics may superimpose on existing islet autoimmunity in some adolescents and (young) adults, leading to an accelerated onset of diabetes, and may therefore be useful secondary screening markers in autoantibody-positive overweight/obese individuals.

Concluding Remarks

Recruitment into T1D prevention trials should be based on careful selection of participants who are best suited to the individual therapeutic approaches. Accurate risk assessment is a prerequisite for selection. With at least four autoantigen clusters and multiple epitope targets in place, efforts must be directed to developing islet autoantibody profiling and signature assays using array technology. Such assays would facilitate the tracking of changes in pre-diabetes that may help predict disease progression, and could also be of value in monitoring the immune efficacy of intervention treatments.

■ References

1. **Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, et al.** The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci U S A* 2007. 104:17040-17045.

2. **Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, et al.** A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 2007. 445:881-885.

3. **Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, Kerr-Conte J, Van Lommel L, Grunwald D, Favier A, et al.** In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. *J Cell Sci* 2006. 119:4199-4206.

4. **Souza SC, Qiu L, Inouye KE, Roix JJ, Chen H.** Zinc transporter ZnT-8 regulates insulin and glucagon secretion in Min6 and alphaTC1-9 pancreatic cell lines. *Diabetologia* 2008. 51(Suppl 1):501.

5. **Chimienti F, Wheeler MB, Nicholson TJ, Bellomo EA, Wijesekara N, Tarasov AI, Taneja T, Sladek R, Baldwin SA, Baldwin J, et al.** Single nucleotide polymorphism rs13266634 modifies the zinc transport activity of SLC30A8/ZnT-8 in clonal pancreatic beta cells. *Diabetologia* 2008. 51(Suppl 1):101.

6. **Egefjord L, Petersen AB, Bang-Berthelsen CH, Pociot F, Karlsen AE, Schmidt K, Chimienti F, Brock B, Schmitz O, Rungby J, et al.** Toxic cytokines interleukin-1beta, tumor necrosis factor-alpha and interferon-gamma regulate zinc transporter gene expression in beta cells and Slc30A8 protects against cytokine-induced cell death. *Diabetologia* 2008. 51(Suppl 1):521.

7. **Wenzlau JM, Moua O, Liu Y, Yu L, Frisch LM, Fowler KT, Rangasamy S, Eisenbarth GS, Davidson HW, Hutton JC.** The common non-synonymous SNP in the Slc30A8 gene at aa325 determines ZnT8 autoantibody specificity in type 1 diabetes. *Diabetologia* 2008. 51(Suppl 1):583.

8. **Kawasaki E, Uga M, Kuriya G, Satoh T, Fujishima K, Moriuchi A, Fukushima K, Ozaki M, Abiru N, Yamasaki H, et al.** Association between anti-ZnT8 autoantibodies and SLC30A8 Arg325Trp variant. *Diabetologia* 2008. 51(Suppl 1):582.

9. **Achenbach P, Landherr U, Koczwara K, Lampasona V, Krause S, Ziegler AG, Bonifacio E.** Autoantibody responses to zinc transporter 8 in children at risk for type 1 diabetes. *Diabetologia* 2008. 51(Suppl 1):330.

10. **Qu HQ, Grant SF, Bradfield JP, Kim C, Frackelton E, Hakonarson H, Polychronakos C.** Association analysis of type 2 diabetes loci in type 1 diabetes. *Diabetes* 2008. 57:1983-1986.

11. **Brorsson C, Bergholdt R, Sjögren M, Eising S, Meden Sørensen K, Hougaard DM, Orho-Melander M, Groop L, Pociot F.** A non-synonymous variant in SLC30A8 is not associated with type 1 diabetes in the Danish population. *Mol Genet Metab* 2008. 94:386-388.

12. **Gohlke H, Ferrari U, Koczwara K, Bonifacio E, Illig T, Ziegler AG.** SLC30A8 (ZnT8) polymorphism is associated with young age at type 1 diabetes onset. *Rev Diab Stud* 2008. 5:25-27.

13. **Lampasona V, Buzzetti R, Bazzigaluppi E, Petrone A, Capizzi M, Tiberti C, Songini M, Giorgino F, Bonifacio E, Bosi E.** Zinc transporter 8 antibodies complement GAD and IA-2 antibodies in the identification of adult-onset autoimmune diabetes. *Diabetologia* 2008. 51(Suppl 1):584.

14. **Schlosser M, Mueller PW, Achenbach P, Bingley PJ.** First international proficiency testing of novel autoantibodies against Zn Transporter-8 and IA-2beta - results of the Diabetes Antibody Standardisation Program. *Diabetologia* 2008. 51(Suppl 1):335.

15. **Davidson HW, Rockell J, Wagner R, Wenzlau JM, Zipris D, Hutton JC, Gottlieb PA.** Cd4 T cell reactivity to the major diabetes autoantigen Slc30A8 in newly diabetic subjects. *Diabetologia* 2008. 51(Suppl 1):233.

16. **Hutton JC, Liu Y, Patel C, Yu L, Eisenbarth GS, Wenzlau JM, Davidson HW.** Engineering single chain multi-component autoantigens for enhanced detection of autoantibodies in type 1 diabetes. *Diabetologia* 2008. 51(Suppl 1):585.

17. **Koczwara K, Mueller D, Achenbach P, Krause S, Ziegler AG, Bonifacio E.** Insulin autoantibodies that cross-react with casein. *Diabetologia* 2008. 51(Suppl 1):344.

18. **Oresic M, Simell S, Sysi-Aho M, Nantö-Salonen K, Seppänen-Laakso T, Parikka V, Hekkala A, Mattila I, Katajamaa M, Keskinen P, et al.** Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. *Diabetologia* 2008. 51(Suppl 1):51.

19. **Winkler C, Zwilling M, Ziegler AG.** Relationship of insulin resistance to islet autoimmunity in children. *Diabetologia* 2008. 51(Suppl 1):338.