Analysis of β-Cell Death in Type 1 Diabetes by Droplet Digital PCR

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Type 1 diabetes (T1D) and other forms of diabetes are due to the killing of β-cells. However, the loss of β-cells has only been assessed by functional studies with a liquid meal or glucose that can be affected by environmental factors. As an indirect measure of β-cell death, we developed an assay using a novel droplet digital PCR that detects INS DNA derived from β-cells. The release of INS DNA with epigenetic modifications (unmethylated CpG) identifies the β-cellular source of the DNA. The assay can detect unmethylated DNA between a range of approximately 600 copies/μL and 0.7 copies/μL, with a regression coefficient for the log transformed copy number of 0.99. The assay was specific for unmethylated INS DNA in mixtures with methylated INS DNA. We analyzed the levels of unmethylated INS DNA in patients with recent onset T1D and normoglycemia subjects at high risk for disease and found increased levels of unmethylated INS DNA compared with nondiabetic control subjects (P < .0001). More than one-third of T1D patients and one-half of at-risk subjects had levels that were more than 2 SD than the mean of nondiabetic control subjects. We conclude that droplet digital PCR is a useful method to detect β-cell death and is more specific and feasible than other methods, such as nested real-time PCR. This new method may be a valuable tool for analyzing pathogenic mechanisms and the effects of treatments in all forms of diabetes.

Type 1 diabetes (T1D) is caused by the immune-mediated destruction of pancreatic β-cells. The time of presentation with hyperglycemia, up to 80% of β-cells are thought to have been destroyed (1). Therapies to prevent or reverse the disease have the goal of preventing β-cell destruction or replacing lost β-cells, but there have not been ways to detect β-cell death in a quantitative manner. Therapies to prevent diabetes and β-cell killing are most valuable before onset of clinical symptoms, the time when cells are being destroyed but the pathologic processes leading to demolition of β-cells are silent. Therefore, a method that can detect β-cell death would improve assessment of progression of diabetes and permit intervention at a time when therapies would have the greatest benefit.

The INS (insulin) DNA is methylated and not transcribed in most cells in the body. β-Cells in the islets of Langerhans are the only significant source of unmethylated INS DNA (2). Because dying cells may release nuclear DNA into the circulation, Akirav et al developed a nested real time (RT)-PCR assay to detect β-cell death by measuring the levels of unmethylated INS DNA from dying β-cells (3). The need for a nested PCR was due to the extremely low concentration of unmethylated INS DNA in serum samples. However, this methodology may introduce artifacts due to the preferential amplification of the methylated DNA resulting from its high copy number in serum along with the risk of amplicon contamination. It is also not well suited for wide use. We therefore used a new
technology called droplet digital PCR (ddPCR) to detect unmethylated INS DNA in a quantitative manner. Droplet digital PCR allows the quantification of DNA without the use of standard curves (4). The sample is dispersed into droplets that behave as individual PCRs. It is assumed that the distribution of the template in the droplets adheres to a Poisson distribution, given the number of droplets generated. Therefore, the qualitative endpoint (positive/negative) of the reaction is converted into an absolute quantification of the number of templates. This improves the sensitivity of finding rare gene targets significantly: from 5% of target by RT-PCR to 0.001% by ddPCR (5). In this study, we describe the use of this new technology to detect β-cell death in individuals with recent onset T1D and in normoglycemic relatives of patients at high risk for the development of the disease. We found that there are elevated levels of unmethylated INS DNA in the serum of patients and individuals at very high risk for T1D.

Materials and Methods

Study subjects and samples

Serum samples were obtained from 39 (27 children) nondiabetic control subjects (Yale New Haven Hospital Clinical Laboratory), 43 subjects within the first year after diagnosis of T1D (Yale New Haven Hospital Clinical Laboratory and Barbara Davis Center), and 26 “at-risk” subjects. The patients with new onset disease included 22 females and 21 males, age 11.0 ± 0.65 years, with an average duration of diabetes of 4.04 ± 0.67 months (range 0–12 mo) and average Hemoglobin A1c level of 8.6 ± 0.41%. The 26 at-risk subjects (12 females, 14 males; age 18.6 ± 2.11 y) were relatives of patients with T1D enrolled in the Type 1 Diabetes TrialNet Pathway to Prevention study (TN-01). These subjects had at least 2 biochemical autoantibodies and metabolic abnormalities during a glucose tolerance test but had not been formally diagnosed with diabetes and had normal HbA1c levels. Their risk of diabetes over 5 years has been estimated to be approximately 70%. The study protocol was approved by the Institutional Review Boards of Yale University, the University of Colorado, and the Ancillary Studies Committee of Type 1 Diabetes TrialNet.

Islet DNA was prepared from human islets (Diabetes Research Institute, Miami, Florida). Human kidney and liver samples were the gift of Dr David Rimm (Department of Pathology, Yale University). We designed plasmids with synthetic DNA sequences identical to bisulfite-treated methylated (L2_M) and unmethylated sequences (L2_UM) of the INS DNA segment (Figure 1, A and B). Plasmids containing the cloned target sequences L2_UM and L2_M were used for optimization of PCR conditions.

Isolation and bisulfite treatment of DNA

DNA was isolated from 200 μL of serum and tissues using the QIAGEN DNA blood and tissue kit. Isolated DNA was treated with bisulfite using the EZ DNA methylation kit (Zymo Research).

Droplet digital PCR primer and probes

The probes targeted 2 methylation-sensitive sites of the human insulin gene (hg19_knownGene_uc021qcd.1 range, chr11: 2181009–2182439) at nucleotides 21814010 and 21814012, which are +396 and +399 from the Transcription Start Site (Figure 1, A and B, and Supplemental Table 1) (http://genome.ucsc.edu/cgi-bin/hgGateway, Feb 2009 GRCh37/hg19).

Droplet digital PCR

The assay design is shown in Figure 1C. Each 25-μL volume consisted of Droplet PCR Supermix (Bio-Rad), 900nM primer, 250nM probe, and 5 μL of sample. The mixture and droplet generation oil were loaded onto a droplet generator (Bio-Rad). The generated droplets were transferred to a 96-well PCR plate and sealed. The PCR was run on a thermal cycler with: 10 minutes of activation at 95°C, 40 cycles of a 2 step amplification protocol (30 s at 94°C denaturation and 60 s at 58°C), and a 10-minute inactivation step at 98°C. The PCR plate was transferred to a QX100 Droplet Reader (Bio-Rad), and products were analyzed with QuantaSoft (Bio-Rad) Analysis software. Discrimination between droplets that contained the target (positives) and those which did not (negatives) was achieved by applying a fluorescence amplitude threshold based on the amplitude read from the negative template control. For each sample, the ratio of unmethylated INS DNA:methylated INS DNA was calculated.

Statistical methods

Unless indicated, the mean ± SEM is shown. Groups were compared either by ANOVA (Kruskall-Wallace statistic) or Mann-Whitney test using GraphPad Prism 5 software.

Results

Assay optimization specificity, sensitivity, and recovery

The multiplexed ddPCR assay could specifically discriminate methylated insulin DNA from unmethylated insulin DNA. The assay was sensitive, allowing us to detect 0.7 copies/μL of target DNA and specific, as it was able to detect unmethylated INS DNA in the presence of a 10 000-fold excess of methylated INS DNA (Supplemental Figure 1). The ddPCR response was linear over 4 orders of magnitude (Figure 2). The linear regression correlation coefficient (r²) for the log transformed copy number between ddPCR and the plasmid dilution series was 0.99, with a slope of 0.99 ± 0.017 (P < .002).

To address the specificity over a wide range of total DNA, we tested bisulfite-treated DNA from kidney and liver (which is almost entirely methylated) with islets DNA to dilutions as low as 0.2 ng. The ratio from kidney and liver DNA was in the range of 0.1 to 0.2, whereas the ratio for islets was between 2.8 and 3.5, approximately 30-fold higher than liver/kidney DNA. The ratios remained consistent with serial dilution, demonstrating that the mea-
urea concentration (Supplemental Figure 2).

Analysis of unmethylated INS DNA in patients with T1D, subjects at risk for T1D, and nondiabetic subjects

The ratios of unmethylated INS DNA were significantly higher in patients with recent onset T1D compared with controls ($P < .0001$) (Figure 3A). We also compared the ratios from 2 sets of biologic serum replicates (ie, samples from patients with T1D drawn within 24 h from the same individual) and found good agreement between the ratio values: the coefficients of variation were 1.87% and 1.37% (data not shown). To further determine the sensitivity and specificity of our assay to distinguish between nondiabetics and T1D and at-risk using a threshold ratio of 0.26 (mean $\pm$ 2 SD of nondiabetic controls), a receiver operating characteristics (ROC) curve analysis was done. The area of the ROC curve was 0.834 ($P < .0001$) (Figure 3B). At a threshold ratio of 0.26, the assay had 38% sensitivity and 95% specificity for discrimination of individuals with T1D. A total of 38% of the patients had levels above this threshold.

In addition to detecting ongoing \( \beta \)-cell death in patients with T1D, the assay would have even greater value in identifying \( \beta \)-cell death in normoglycemic individuals who are at risk for developing the disease. In order to determine whether the measurements were elevated in at-risk individuals who were progressing to disease, we studied the unmethylated/methylated INS DNA ratio in samples from 26 normoglycemic subjects who were at very high risk for development of T1D. These participants, who were relatives of patients with T1D and participating in the TrialNet Pathway to Prevention trial, were identified as having at least a 70% risk of developing T1D over 5 years on the
basis of having positive titers for at least 2 biochemical autoantibodies and an abnormal glucose tolerance test. However, these individuals had not been diagnosed with T1D and had normal glycosylated hemoglobin A1c levels. The at-risk subjects also had higher ratios than the non-diabetic control subjects ($P < .0001$) (Figure 3A). The area of the ROC for the at-risk subjects vs nondiabetic controls was 0.897 ($P < .0001$) (data not shown). Using a threshold ratio of 0.26, 58% of at-risk subjects tested positive.

The average insulin secretory response to the oral glucose used to study these patients was $52951 \pm 5796$ pmol/mL, but we did not find a relationship between insulin secretion and the ratio (data not shown). Interestingly, when directly compared, the at-risk subjects had a higher ratio than those with recent onset T1D ($P = .02$ by Mann-Whitney).

We then compared the results of this assay with the previously described method of measurement of unmethylated INS DNA by RT-PCR (Figure 4) in a subgroup of the subjects with new onset T1D (3). We found a significant relationship between the 2 measures performed on the same samples ($P = .005, r = 0.48$). The ddPCR showed low detectable signals in samples from 39% of subjects in whom the level of unmethylated INS DNA was undetectable in the RT-PCR assay (ie, $\Delta Ct < -23$, where $\Delta Ct$ [cycle threshold] is the difference in Ct value for methylated INS DNA $\text{−} \text{Ct}$ value for unmethylated INS DNA).

**Discussion**

Studies of the natural history of T1D have been limited to analyses of $\beta$-cell function, which do not identify the primary pathologic process that causes the disease. Moreover, measurement of $\beta$-cell function may be affected by environmental factors. To address this issue, we have developed an assay to measure $\beta$-cell-derived INS DNA in serum, which is identified by the methylation status of CpG dinucleotide of the INS DNA. Our previous work indicates that, in mice, the level of unmethylated Ins1 DNA reflects $\beta$-cell death as verified by histomorphic staining of islet cells (3). In this report, we have used a novel technique, ddPCR, which significantly improved this approach over the analysis by RT-PCR (3, 6, 7). This new method is able to detect approximately 1 copy/2 $\mu$L in a 25-$\mu$L PCR. The new method allowed us to multiplex the reaction but also improved the specificity, reproducibility, and feasibility of the assay.

We found increased levels of unmethylated INS DNA in the serum of more than one-third of subjects with recent onset T1D and in more than 50% of nonhyperglycemic subjects at high risk of T1D. Not all of the at-risk subjects and patients studied showed elevated levels, which likely reflects the heterogeneity of the disease kinetics in these 2 populations. The rate at which the at-risk subjects progress to hyperglycemia varies, some may be diagnosed within a month, whereas in others, it may take 5 years or more. Nonetheless, the levels of INS DNA are higher in the at-risk subjects than those with recent onset T1D ($P = .02$ by Mann-Whitney).
The lower limit of the cycle threshold of unmethylated INS DNA) and ratio in at-risk subjects. Further studies of larger cohorts may help to establish the utility of this method for identifying β-cell killing in clinical settings, such as over time in the at-risk subjects, after immune therapy of T1D, after islet and pancreas transplants, and even in other forms of diabetes where the loss of β-cells is a pathogenic mechanism.

Acknowledgments

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This work was supported by National Institutes of Health (NIH) Grants DK095639, DK085466, and U1LRR024139; the Juvenile Diabetes Research Foundation Grant 17-2012-546; the State of Connecticut Research Grant 2012-0222; NIH Grants DK057846, DK094400, DP3 DK101122-01; a gift from the Howalt family and Seraph Foundation; and by a grant from the Riva Foundation.

Disclosure Summary: The authors have nothing to disclose.

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