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Type 1 diabetes (T1D) results from immune-mediated destruction of insulin-producing β-cells. The killing of β-cells is not currently measurable; β-cell functional studies routinely used are affected by environmental factors such as glucose and cannot distinguish death from dysfunction. Moreover, it is not known whether immune therapies affect killing. We developed an assay to identify β-cell death by measuring relative levels of unmethylated INS DNA in serum and used it to measure β-cell death in a clinical trial of teplizumab. We studied 43 patients with recent-onset T1D, 13 nondiabetic subjects, and 37 patients with T1D treated with FcR nonbinding anti-CD3 monoclonal antibody (teplizumab) or placebo. Patients with recent-onset T1D had higher rates of β-cell death versus nondiabetic control subjects, but patients with long-standing T1D had lower levels. When patients with recent-onset T1D were treated with teplizumab, β-cell function was preserved (P < 0.05) and the rates of β-cell were reduced significantly (P < 0.05). We conclude that there are higher rates of β-cell death in patients with recent-onset T1D compared with nondiabetic subjects. Improvement in C-peptide responses with immune intervention is associated with decreased β-cell death. Diabetes 62:1676–1680, 2013

Immune Therapy and β-Cell Death in Type 1 Diabetes


Type 1 diabetes (T1D) is initiated years before clinical onset and continues until nearly all insulin-producing cells have been destroyed by autoimmune processes (1). It is estimated that at presentation 10–30% of β-cells remain and that β-cell death continues over the next 3–5 years until the majority of patients lose clinically significant levels of insulin production (2).

It has heretofore not been possible to directly measure β-cell destruction in patients. The measurements that are used actually measure β-cell function: they do not gauge the pathologic process and can be affected by metabolic factors such as ambient glucose levels. Moreover, newer immune therapies, such as FcR nonbinding anti-CD3 (teplizumab) and an anti-CD20 monoclonal antibodies (rituximab) or CTLA-4 Ig (abatacept), have been shown to decrease the rate of decline in β-cell function, but owing to the lack of a more direct measure of β-cell destruction, their effects on the primary cause of the disease have not been assessed (3–5). As suggested by our previous studies, immune modulatory agents may cause functional recovery of degranulated β-cells even without eliminating β-cell killing (6).

We recently developed a method for detecting β-cell death in vivo by measuring the relative amount of β-cell–derived INS DNA in serum (7). This approach is based on the understanding that insulin is primarily transcribed by β-cells, which epigenetic modifications of the INS gene such as unmethylation of CpG sites occur in the INS gene to achieve this task (8,9). When β-cells die and release their DNA into the circulation, the released DNA exhibits these modifications. Therefore, measurement of the amount of unmethylated INS DNA in the peripheral circulation may reflect the amount of β-cell death. We designed and validated a two-step nested PCR reaction to measure the levels of unmethylated Ins1 DNA in mice with diabetes induced by streptozotocin and during development of diabetes in nonobese diabetic mice. We presented preliminary data in humans with TID (10).

In this study, we modified the assay and used it to evaluate β-cell destruction in nondiabetic subjects, patients with new-onset T1D, and those treated with an immune modulator (teplizumab) that is known to preserve β-cell function (4,13,14). We show that individuals with new-onset T1D have increased rates of β-cell death compared with nondiabetic control subjects but individuals with long-standing disease have lower levels than healthy control subjects. We found a decreased rate of β-cell death in patients who were treated with teplizumab, suggesting that the drug may work by decreasing β-cell death.

RESEARCH DESIGN AND METHODS

Sera were collected from nondiabetic control subjects, participants with T1D in a clinical trial of teplizumab (Delay), and patients with long-standing TID. The Delay trial was a randomized placebo-controlled trial testing whether a course of treatment with teplizumab would reduce the decline in C-peptide after 12 months in patients with T1D for 4–12 months’ duration (15). From the total of 58 subjects, baseline samples were available on 43 subjects and paired samples (at baseline and 1 year) on 37 subjects. Samples from the other subjects were either not available or did not meet quality-control standards. Serum was obtained from age-matched nondiabetic control subjects from the clinical laboratory at Yale New Haven Hospital. Institutional review board approval was obtained for the collection of serum for these studies.

DNA collection and bisulfite treatment. DNA was purified from 200 μL serum using QIAamp DNA Blood kits as suggested by the manufacturer (Qiagen, Valencia, CA), with a modified incubation period of 20 min at 45°C in the final step. DNA was bisulfite treated using an EZ DNA Methylation kit (Zymo Research, Irvine, CA).

PCR reactions. The assay design is shown in Fig. 1A. A methylation pattern-independent first-step reaction was performed with 8–20 ng bisulfite-treated DNA (10). The product of the first-step PCR reaction was used as template in a second reaction with methylation site-specific primers (Supplementary Fig. 1A). The threshold cycle (Ct) was determined for reactions with the unmethylated and methylated primer pairs. The relative abundance of
DNA was added at a 1:2 ratio to serum-derived DNA, DNA within mixtures of human cells. When islet-derived kidney confluent (Fig. 1 and Supplementary Fig. 1). Sequencing studies of DNA, and evaluated the interassay reproducibility (10) studies to determine the limit of detection of islet derived performance.

RESULTS

Detection of unmethylated INS DNA: assay performance. We modified our previous reported assay to improve the specificity of detection, performed recovery studies to determine the limit of detection of islet derived DNA, and evaluated the interassay reproducibility (10) (Fig. 1 and Supplementary Fig. 1). Sequencing studies of fluorescence-activated cell sorter–sorted β-cells, islets, or kidney confirmed the identification of unmethylated INS DNA within mixtures of human cells. When islet-derived DNA was added at a 1:2 ratio to serum-derived DNA, the difference in the Ct values of the methylated and unmethylated products was 5.5 (or ~45-fold) compared with methylated DNA. However, below a concentration of islet DNA of 16 pg/μL, the Δ was indistinguishable from the sample without islet DNA added. We determined the reproducibility of the measurements with repeated blood draws in healthy control subjects (1.27%) and the interassay coefficient of variation (with samples from patients with new-onset T1D, 6.43%) (Supplementary Fig. 1C).

Circulating levels of unmethylated insulin DNA in patients with T1D. The Delay trial was a randomized placebo-controlled study of teplizumab in subjects with recent-onset (i.e., 4–12 months’ duration) T1D, which tested whether a single course of the drug would attenuate the decline in C-peptide after the new-onset period. We first compared the levels of unmethylated INS DNA in serum from patients at study entry with age-matched nondiabetic individuals (Table 1). The levels of unmethylated INS DNA were significantly higher in the patients (Δ = −11.9 ± 0.63) compared with control subjects (−15.9 ± 0.54 [Fig. 2A]; P = 0.001). In addition, there was an inverse relationship between the relative level of unmethylated insulin and are under the curve (AUC) of the C-peptide responses to a mixed meal (Fig. 2B) (r = −0.34, P = 0.03).

To determine whether β-cell mass affected the level of INS DNA, we compared the levels of unmethylated INS DNA in patients with long-standing T1D, with and without residual C-peptide responses to a mixed meal (Table 1),

![Diagram](image-url)
with levels in age-matched nondiabetic control subjects. Subjects with long-standing T1D with or without significant levels of insulin production (i.e., <0.2 pmol/mL) had undetectable levels of unmethylated INS DNA (not shown).

**Effects of treatment with teplizumab on the levels of unmethylated insulin DNA.** Paired samples for analysis of the level of unmethylated INS DNA were available from 39 subjects in the Delay trial. Drug-treated subjects showed a significantly reduced decline in C-peptide 1 year after treatment (17.7 ± 6.39 vs. 40.2 ± 7.69%, \( P = 0.03 \) [Fig. 3A]) and reduced change in insulin requirements (change in insulin dose from baseline of 0.06 ± 0.05 vs. 0.25 ± 0.04 units/kg/day, \( P = 0.009 \) [Fig. 3B]), but the HbA1c levels were not significantly changed (not shown). Teplizumab- and placebo-treated subjects had a similar level of unmethylated INS DNA at baseline (Fig. 3C). In the teplizumab-treated subjects but not in the placebo-treated subjects, the relative level of unmethylated INS DNA declined significantly at the 1-year end point (\( P = 0.005 \) and \( P = \text{ns} \), respectively), suggesting a reduced level of \( \beta \)-cell death. The decline in the level of unmethylated INS DNA was significantly greater in the teplizumab-treated subjects (\( P = 0.04 \)) (Fig. 3D).

**DISCUSSION**

These studies show that the levels of unmethylated INS DNA are elevated in subjects with new-onset T1D compared with nondiabetic control subjects and suggest active \( \beta \)-cell destruction at the time of onset of disease. The levels of INS DNA do not simply reflect \( \beta \)-cell mass because they were elevated in the subjects with new-onset disease versus age-matched nondiabetic control subjects even though their \( \beta \)-cell mass is clearly reduced. Nonetheless, the quantitative measure of \( \beta \)-cell death is affected by the total \( \beta \)-cell mass because in long-standing patients, with reduced mass, the levels were lower than those even in nondiabetic control subjects.

The mechanism of improvement in \( \beta \)-cell function after immune therapy is not known: our preclinical studies in NOD mice showed that there was functional recovery of degranulated \( \beta \)-cells (9). This is the first evidence indicating that immune therapy that reduces the decline in \( \beta \)-cell functional responses in T1D does so by decreasing \( \beta \)-cell death. We cannot, however, be certain that teplizumab treatment alone accounts for all of the improvement in C-peptide responses in the drug-treated group. The average HbA1c in the drug-treated group was lower than in the placebo group at baseline (6.28 ± 0.15 vs. 7.02 ± 0.42%, \( P = 0.04 \)), which may have affected the C-peptide responses. However, the HbA1c levels were not affected by drug treatment in either group, and the levels of unmethylated INS DNA in the two groups were similar at baseline. Therefore, the effects of the imbalance of the HbA1c levels did not account for differences in the rates of \( \beta \)-cell death.

While the levels of unmethylated INS DNA decreased in teplizumab-treated subjects together with reduced decline in C-peptide AUC and reduced need for exogenous insulin, we did not find a direct relationship between the changes in INS DNA and these clinical parameters. Several factors may affect this relationship. At diagnosis, there may be

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**TABLE 1**

Clinical characteristics of patients with recent-onset T1D and long-standing T1D

<table>
<thead>
<tr>
<th></th>
<th>Delay subjects (T1D)</th>
<th>Nondiabetic control subjects</th>
<th>Long-standing T1D</th>
<th>C-peptide ( \geq 0.2 ) pmol/mL</th>
<th>C-peptide (&lt; 0.2 ) pmol/mL</th>
<th>Nondiabetic control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>24/19</td>
<td>7/6</td>
<td>2/3</td>
<td>5/1</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>12.51 ± 0.618</td>
<td>16.68 ± 0.538</td>
<td>21.6 ± 2.25</td>
<td>22.6 ± 1.49</td>
<td>20.8 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Duration (years)</td>
<td>0.61 ± 0.031</td>
<td>6.15 ± 0.056</td>
<td>8.8 ± 2.31</td>
<td>7.2 ± 1.06</td>
<td></td>
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</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.64 ± 0.157</td>
<td>6.44 ± 0.23</td>
<td>6.95 ± 0.07*</td>
<td></td>
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</tr>
<tr>
<td>Average insulin use (units/kg/day)</td>
<td>0.379 ± 0.026</td>
<td>0.754 ± 0.252</td>
<td>0.897 ± 0.083</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C-peptide AUC (pmol/mL)</td>
<td>0.829 ± 0.069</td>
<td>0.824 ± 0.29</td>
<td>0.039 ± 0.023**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SEM. AUC, area under the curve. *\( P = 0.04 \), **\( P = 0.015 \) vs. subjects with C-peptide \( \geq 0.2 \) pmol/mL.
a functional component to the impaired metabolic response that may or may not have reversed after study entry. Moreover, the kinetics of \( \beta \)-cell death is not known. Cell death may be greater before diagnosis, and the levels that we found may represent a decline from those peak levels (10). Our findings also imply that \( \beta \)-cell death may continue for an extended period of time after the diagnosis of T1D. The study subjects had been diagnosed with diabetes for an average of 7 months prior to entry, and the placebo-treated subjects still had an increased level of unmethylated \( \text{INS} \) DNA 1 year later compared with the nondiabetic control subjects (\( P = 0.013 \)) (Fig. 3). We showed that reduced \( \beta \)-cell mass may affect the level of \( \beta \)-cell \( \text{INS} \) DNA, suggested by our analysis of subjects with long-standing T1D. The levels we measured may also vary considerably between individuals based on age as well as the absolute \( \beta \)-cell mass as suggested by our studies in patients with long-standing disease.

One limitation of this approach is that \( \beta \)-cell death can only be detected if \( \beta \)-cells release their DNA into the serum. This is likely to occur during necrotic cell death associated with immune destruction by cytolytic T cells or by cytokines, but we do not know how \( \beta \)-cells die in human T1D (16–18). Other mechanisms of cell death, such as autophagy, or clearing of dying \( \beta \)-cells by phagocytic cells may not be identified with this approach (19). Therefore, it will be important to confirm our finding in other clinical studies that may affect \( \beta \)-cell destruction.

In summary, we identified higher rates of \( \beta \)-cell death in patients with recent-onset T1D versus nondiabetic control subjects and have shown that teplizumab treatment is associated with reduced level of \( \beta \)-cell death. The method that we have developed may help investigators understand the pathogenesis and treatment of human T1D including the relationships between functional and pathologic changes in \( \beta \)-cells. Moreover, this tool may be useful for decisions regarding for whom and when immune intervention would be most appropriate.

ACKNOWLEDGMENTS

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J.A.B. has a patent application for teplizumab. E.M.A. and K.C.H. have a patent application for the assay of unmethylated insulin DNA and are members of the scientific advisory board of Islet Sciences, Inc. No other potential conflicts of interest relevant to this article were reported.

J.L. designed, modified, and performed the assay; analyzed data; and wrote the manuscript. S.D. designed the clinical trial, carried out the clinical trial, collected samples, and wrote and reviewed the manuscript. S.G. carried out the clinical trial, collected samples, and wrote and reviewed the manuscript. E.M.A. wrote and reviewed the manuscript. J.A.B. wrote and reviewed the manuscript and designed the clinical trial. K.C.H. analyzed data, wrote the manuscript, and designed the clinical trial. K.C.H. is the guarantor of this work and, as...
such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES