Detection of β cell death in diabetes using differentially methylated circulating DNA

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In diabetes mellitus, β cell destruction is largely silent and can be detected only after significant loss of insulin secretion capacity. We have developed a method for detecting β cell death in vivo by amplifying and measuring the proportion of insulin 1 DNA from β cells in the serum. Using primers that are specific for DNA methylation patterns in β cells, we have detected circulating copies of β cell-derived demethylated DNA in serum of mice by quantitative PCR. Accordingly, we have identified a relative increase of β cell-derived DNA after induction of diabetes with streptozotocin and during development of diabetes in nonobese diabetic mice. We have extended the use of this assay to measure β cell-derived insulin DNA in human tissues and serum. We found increased levels of demethylated insulin DNA in subjects with new-onset type 1 diabetes compared with age-matched control subjects. Our method provides a noninvasive approach for detecting β cell death in vivo that may be used to track the progression of diabetes and guide its treatment.

Results

Methylation-Specific Primers Can Detect Differentially Methylated Insf Gene DNA from jTC3 and PMJ Murine Cell Lines. To identify differentially methylated CpG dinucleotides present in the Insf gene in β cells, we examined the methylation patterns of the Insf gene in the glucose-responsive murine insulinoma cell line jTC3 (23). As a non-β cell control, we used the PMJ macrophage cell line. DNA from both cell types was extracted and subjected to bisulfite treatment as described in Materials and Methods. We identified a differentially methylated CpG dinucleotide at position NUCL:52339278 (http://genome.ucsc.edu/cgi-bin/hgGateway, Feb 2009 GRCh37/hg19) on chromosome 19, corresponding to the CpG in position +177 downstream from the Insf transcription start site, which was demethylated in jTC3 cells and methylated in control PMJ cells (Fig. 1A). This CpG dinucleotide is located in the coding region of the insulin mRNA residing in the proinsulin protein and is evolutionarily conserved in mouse and human insulin genes.

To verify the tissue specificity of demethylation at this site, we determined the frequency of demethylated and methylated CpG sites in products of the methylation-insensitive PCR from bisulfite-treated DNA from sorted murine insulin-positive cells isolated from MIP-GFP mice and from liver (Fig. 1B). The majority of the sites were demethylated in DNA from β cells. The CpG site at +177 was demethylated in 13 of 15 clones isolated from β cells, but in 0 of 8 clones isolated from liver (P < 0.001). We found that 25% of the 105 sites, or 33% of the clones, showed methylated cytosines in at least one of the seven CpG sites analyzed. In contrast, 86% of the 56 sites analyzed from liver were methylated.

The relatively low amounts of circulating DNA in the serum posed a challenge for detecting cell-specific DNA species. Thus, we designed a nested PCR in which we amplified insulin DNA with methylation-insensitive primers between a region spanning the CpG dinucleotide of interest, followed by a second reaction with methylation-sensitive primers capable of differentiating β cell-derived and non-β cell-derived insulin DNA (Fig. 2 and Table S1). The first PCR generated a product of 204 bp that was.


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gel-extracted to improve real-time PCR efficiency. This first-step product was used as template in a second PCR with methylation site-specific primers. Real-time PCR analysis showed a 256-fold (eight-cycle) increase in demethylated DNA levels relative to methylated DNA from either the βTC3 or PMJ cell line, demonstrating the nucleotide modification of CpG dinucleotides owing to demethylation at position 523393278. (B) Sequence analysis of product amplified in the first-step PCR. The sequence of 15 clones from murine β-j cells and 8 clones from murine liver cells are shown (○ indicates demethylated cytosines; ●, methylated cytosines). The locations of the methylation sites from the transcription start site are indicated. The primers of the second-step PCR were specific for methylated/demethylated cytosine at bp +177, corresponding to nucleotide 52339278.

Demethylated Ins1 DNA Is Enriched in Primary Murine Islets and Cell-Sorted Insulin-Positive Cells. To test our assay’s ability to detect demethylation-specific modification of DNA from primary murine tissues, we collected kidney, liver, brain, and islet tissues from NOD/SCID mice, which, unlike WT NOD mice, do not develop insulin or β cell destruction. DNA was extracted and treated with bisulfite, followed by the nested PCR analysis described above. Methylation-specific primers demonstrated a >12-fold increase in demethylated DNA in the crude islet preparations compared with liver, kidney, and brain (Fig. 3A).

To confirm that β cells were the primary source of the demethylated insulin DNA in our nested PCR, we dissociated murine islets into single cells and stained them for insulin. Insulin-positive β cells and insulin-negative cells were sorted by FACS (Fig. 3B), and the DNA was isolated and treated as described above. There was a 45-fold increase in demethylated DNA in the insulin-positive cell fraction compared with insulin-negative cells from islets (Fig. 3C). Product sequencing revealed an identical demethylated modification in insulin-positive islet cells as in the βTC3 cell line, whereas the non-β cell fraction demonstrated a methylated CpG dinucleotide, as observed in the PMJ cell line.

We next analyzed the linearity of the ratio between the two DNA species by mixing demethylated DNA (derived from β cells) and methylated DNA (derived from non-β cells) and measuring the difference in cycle threshold (Ct) values detected (Fig. 3D). The difference in the Ct values of the methylated and demethylated products of the second-step PCR were characterized using the demethylation index as described in Materials and Methods, which corresponds to quantitative differences in the quantity of DNA. There was a linear relationship between the log ratio of β cell-derived and non-β cell-derived DNA and a demethylation index between 100:1 and 1:100 (r² = 0.957; P < 0.01), suggesting that it is possible to measure the quantitative differences in the DNA species over this wide range.

Circulating Demethylated Ins1 DNA Is Increased in Streptozotocin-Treated BALB/c Mice. To determine whether our assay can detect β cell death in vivo, we collected serum from BALB/c mice before and after treatment with high-dose (200 mg/kg) streptozotocin (STZ), and isolated, processed, and analyzed the DNA as described above. The STZ-treated mice demonstrated increased glucose levels at 24 h after STZ injection, indicating acute injury to β cells (P < 0.001) (Fig. 4A). Despite a modest decline in glucose levels at 8 h after treatment (P < 0.05), most likely reflecting loss of β cell membrane integrity and release of insulin granules, there was a 2.6-fold increase in the demethylation index at 8 h (P < 0.05) and a 3.8-fold increase at 24 h (P < 0.02) (Fig. 3C). We studied the percentage of nucleated cells in the islets after STZ treatment and found a reduced percentage of DAPI-positive, insulin-positive cells staining in the islets at 8 h after STZ treatment (UnTx = 55.1% vs. t8 = 41.3%; P < 0.002) (Fig. 3D). We also found a further reduction in the percentage of DAPI-positive, insulin-positive cells at 24 h after STZ treatment (Fig. 3C), which corresponded to the peak in circulating demethylated DNA and increased baseline glucose levels (UnTx = 55.1% vs. t24 = 32.8%; P < 0.0001) (Fig. 3B). Taken together, these data indicate the ability of methylaty-
could be detected in the NOD mouse model of spontaneous diabetes, a model of chronic autodestruct in human T1D. NOD mice were challenged with an i.p. glucose tolerance test (IPGTT) beginning at 7 wk of age, during which basal glucose levels were normal, and extending through the development of overt hyperglycemia (Fig. 5A). The IPGTTs revealed subtle changes in glucose tolerance beginning at 9 wk of age that showed a statistically significant difference from the 7-wk response only at 14 wk (P < 0.05) (Fig. 5B). The fasting glucose levels remained normal at all time points (Fig. 5A and B) (24). The demethylation index increased significantly before the decline in insulin levels and before the increase in fasting glucose levels (P = 0.0002) (Fig. 5C). At 14–15 weeks, the median demethylation index was increased by 21-fold (range, 3.2–211-fold; n = 12) compared with the average of 7-wk-old mice (P < 0.01) (Fig. 5C and D). Interestingly, in 16- to 24-wk-old mice with overt hyperglycemia, the index declined but was still elevated compared with that in the 7-wk-old NOD mice (P < 0.05).

The range of increase in demethylation indices in the prediabetic mice was broad, possibly related to individual differences. To understand the relationships between β cell mass and the demethylation index, we investigated the relationship between total pancreatic insulin content and the demethylation index in a separate experiment with prediabetic NOD mice. We found a decline in pancreatic insulin content with age that was statistically lower at 15 wk compared with 7-wk-old NOD mice (P < 0.05). At the same time, the demethylation index increased by 13-fold at 11 wk compared with 7 wk (P < 0.05), and by 14-fold at 15 wk (P < 0.01) (Fig. 5D). To analyze the relationship between pancreatic insulin content and the demethylation index in individual mice, we compared these two parameters and found that they were significantly negatively correlated (r² = 0.28; P < 0.05) (Fig. 5E). Taken together, these data show a link between an increased demethylation index and β cell loss.

**Demethylated Ins DNA Is Increased in Human Islets and in Serum from Patients with New-Onset T1D.** We used a similar strategy to analyze demethylated insulin DNA in human tissues. Primers for the first-step and nested PCR reactions were prepared from the analogous sequences in human INS on chromosome 11 (Fig. 6A and Table S1). Total DNA was isolated and used in the first-step PCR after bisulfite treatment.

We sequenced the products of the first-step PCR and identified two peaks in the CpG site at nucleotide 2182036 (http://genome.ucsc.edu/cgi-bin/hgGateway, Feb 2009 GRCh37/hg19) in position +399 downstream from the transcription start site in the DNA from human islets. This double peak corresponds to methylated and demethylated cytosines. Only a single peak, corresponding to methylated cytosine, was found in human kidney DNA (Fig. 6A and B).

We then sorted primary insulin-positive human β cells from dissociated islets by staining with the zinc-selective dye FluoZin-3-AM and cloned products of the first-step reaction from these cells, and compared the sequences with kidney cells (25). All of the clones (10 of 10) from purified β cells showed demethylated DNA at bp 273 and 399 in the insulin gene, compared with 0 of 12 clones from kidney (P < 0.001) (Fig. 6C). Moreover, CpG sites were rarely demethylated in kidney (<25% of clones), and none of the clones from kidney exhibited demethylation at all of the CpG sites, whereas all sites but one were demethylated in all 10 clones sequenced from human β cells.

We then compared the demethylation index in DNA isolated from islets, kidney, and liver as well as in unmethylated and methylated synthetic DNA (Fig. 6D). We found a significant increase in the demethylation index in islets (P < 0.001) compared with liver (57-fold) and kidney (91-fold). The demethylation index with islet DNA (0.729 ± 0.05) was similar to the demethylation index with synthetic unmethylated DNA (0.70 ± 0.03). The identity of the products was verified by sequencing. The average interassay coefficient of variation from three separate analyses of this tissue DNA was 21.7% ± 6.4%.

Finally, we compared the demethylation index in serum samples from patients with T1D (n = 5; mean age, 10.8 ± 1.02 y; range, 8–14 y) within the first year (mean duration of T1D, 7.0 ± 1.30 mo;
Discussion

We describe a unique method for the detection of β cell death in vivo in models of autoimmune and chemically induced diabetes in mice, in human tissues, and in serum from patients with T1D. This assay identifies methylated CpG dinucleotide in the insulin DNA that is derived exclusively from β cells. Our findings indicate that this method provides a biomarker for detecting β cell loss in prediabetic mice during progression of diabetes, and suggest it does the same in patients with new-onset T1D.

Kuroda et al. (26) previously identified demethylation of CpG sites in the insulin promoter, consistent with the notion that methylation of promoters is a mechanism for controlling tissue-specific gene expression. However, our analysis targeted differentially methylated CpG dinucleotides in the Ins1 gene in mice and the Ins gene itself in humans. The conservation of demethylation of this sequence across species suggests that its methylation may play an active role in regulation of insulin gene transcription. In addition, via sequencing, we showed that CpG sites both upstream and downstream of the CpG at +177 are also equally demethylated in β cell DNA, indicating that the entire region contributes to gene regulation.

Our sequence analysis revealed that unlike human Ins, which was completely demethylated in primary β cells, murine Ins1 was demethylated in 75% of the CpG sites studied from murine β cells isolated from MIP-GFP mice. Based on the available data, we are not sure whether this reflects allele-specific inactivation of insulin 1 in β cells that produce insulin 2 (and vice versa), possibly in response to metabolic stress, or whether there is some genomic imprinting and silencing of only one of the parental alleles (27). The MIP-GFP transgene may account for β cell stress, given that it has been known to result in β cell death in certain lines (28). A caveat of our approach is that the death of β cells in which insulin genes are methylated as a result of stress might go undetected.

We were able to detect acute β cell death in vivo, as indicated by the presence of β cell-derived demethylated DNA after STZ treatment. The fact that hyperglycemia was not observed at the 8 h time point demonstrates our method’s ability to detect β cell death before frank hyperglycemia occurs. This conclusion is supported by our histomorphic analysis of the percentage of nucleated cells in the islet, which revealed a drop in the percentage of DAPI-positive, insulin-positive cells, suggesting that DNA material can be released to the surrounding tissues and can be detected in the circulation. In the model systems that we have studied, toxin- and cytolysis-induced cell death were clearly detectable, but whether other forms of β cell death that might not result in release of nuclear contents into the circulation, such as apoptosis or autophagy, will be identified is unclear (29, 30).

In autoimmune diabetes, β cell death is considered a chronic process rather than an acute process such as occurs after STZ measurement of insulin content and demethylation index, respectively. The insulin content and demethylation index in 11- and 15-wk-old mice were compared with 7-wk-old mice. *P < 0.05; **P < 0.02 by post hoc analysis of ANOVA. (E) Relationship between pancreatic insulin content and demethylation index in individual mice. Two measurements from each mouse are plotted (r² = 0.28; P < 0.05).

Fig. 5. Serum-derived demethylated Ins1 DNA is increased in prediabetic NOD mice with impaired glucose tolerance. (A) IPGTT data for prediabetic NOD mice at various ages (n = 5 per group). Note that the fasting glucose (at t = 0) is similar at all time points. (B) Area under the curve of IPGTT data from A. *P < 0.05. (C) Demethylation index measured with DNA from the sera of prediabetic (wk 7–14) and diabetic NOD mice. P = 0.0002 by ANOVA; **P < 0.01; *P < 0.05; n = 5, 5, 5, 7, and 5 mice/group. The box-and-whisker plots show the minimum and maximum values. (D and E) In a separate experiment, pancreata and serum were harvested from mice at the indicated ages (n = 5 mice per time point) for range, 4–11 mo) after diagnosis with healthy control subjects who were age-matched, because demethylation might have been affected by islet growth in children (Fig. 6E). The demethylation index was significantly higher in the patients with T1D (P < 0.02); and the average demethylation index in the nondiabetic subjects was similar to the index with DNA isolated from liver or kidney. We also conducted a similar analysis with second-step PCR primers that target bp +329. Analysis with this primer pair resulted in overall lower demethylation indices, but we found a similar significant increase in the demethylation index (4.42 × 10⁻⁴ ± 2.07 × 10⁻⁴ vs. 2.37 × 10⁻⁶ ± 1.81 × 10⁻⁶) in this second cohort of subjects with recent-onset (i.e., first 1-1/2 y) T1D (n = 12) compared with healthy control subjects (n = 11; P = 0.015).
administration, and changes with chronic destruction may be more difficult to detect (1, 2). Nonetheless, we were able to detect a significant increase in β cell-derived demethylated DNA in the circulation of prediabetic NOD mice even when fasting glucose levels and overall glucose tolerance were not significantly impaired compared with young NOD mice. Just before the onset of hyperglycemia, however, a dramatic increase in the level of demethylated insulin DNA occurred. Our analysis of pancreatic insulin content and β cell-derived demethylated DNA in the serum revealed a statistically significant inverse correlation. In addition to the variability that is intrinsic to these biological measurements, another possible explanation for the discrepant data are that the demethylation index may rise before a decline in insulin content is apparent. As shown by our analysis of prediabetic NOD mice, this was clearly the case, with the greatest increase in the demethylation index seen before hyperglycemia was identified.

We have previously shown that β cells may fail to express insulin (i.e., are degranulated) at the time of onset of diabetes in NOD mice but are still viable and may recover with immunotherapy, such as anti-CD3 mAb (24). Moreover, β cell function might be affected by ambient glucose level, certain drugs, and other factors (31). With the caveat that stressed β cells may methylate insulin DNA and die without release of demethylated insulin, we do not expect our measurements to be affected by changes in β cell function, in contrast to measurements of circulating insulin mRNA in the serum of recipients of islet allotransplants (24, 31f, 32). Thus, our approach to analysis may be useful in distinguishing β cell death from impaired function. Further studies in other disease settings are needed, however.

Interestingly, our measure of β cell death demonstrated continued release of demethylated insulin DNA after the appearance of frank hyperglycemia, but at a reduced level compared with prediabetic (i.e., 14-wk-old) mice. The decline in β cell-derived DNA after the onset of hyperglycemia suggests that the relative abundance of demethylated insulin DNA in the circulation may be reduced because of a total loss of β cell mass. For example, a higher percentage of β cells may be destroyed after diagnosis with hyperglycemia than before diagnosis, but fewer β cells actually may be destroyed (24, 31, 32).

Our method developed in mice was also able to detect circulating β cell-derived DNA in humans. We found uniform demethylation of CpG sites within the insulin gene in human β cells and methylation in non-β cells. Our tissue analysis findings are consistent with this finding from the sequence analysis. Importantly, the average demethylation index was significantly greater in subjects with new-onset T1D, in whom β cell death occurs, than in healthy control subjects. Not all patients with T1D had an increased demethylation index, likely reflecting the heterogeneity of the disease process in individuals. Additional studies correlating the demethylation index with β cell function as well as with samples from nondiabetic subjects at high risk for T1D will help determine whether a similar relationship occurs during the progression of human disease.

Overall, our data demonstrate the usefulness of our application of methylation-specific PCR for detecting β cell death in vivo. The assay may provide insight into the progression of T1D, survival of β cells after transplantation, or turnover of β cells during pregnancy or growth. Further studies are needed to evaluate and validate our method’s general applicability in clinical settings such as after immune interventions or in prediabetes to identify individuals at risk for progressing to T1D. Such studies in these settings will be important in assessing the applicability of this method for human studies.

Materials and Methods

Mice. Female NOD/LtJ, MIP-GFP NOD, and BALB/c mice were obtained from The Jackson Laboratory and maintained under pathogen-free conditions. Seven-wk-old NOD mice were screened for hyperglycemia every 2 wk and were diagnosed with diabetes when two consecutive glucose levels >200 mg/dL were measured in whole blood from the tail vein using a Bayer Glucometer Elite XL. The animal care protocol was approved by Yale University’s Animal Use Committee.

Human Subjects. Tissues were obtained from the pathology laboratory at Yale New Haven Hospital. Serum was collected from healthy control subjects and from individuals with recent-onset (i.e., within the first 1–1/2 y) T1D participating in a clinical trial (NCT 00378508). Institutional Review Board approval was obtained for the collection of tissues and sera, and informed consent was obtained from subjects for the collection of sera.

STZ Treatment. Eight-wk-old BALB/c mice received a single i.p. injection of 200 mg/kg of STZ. Blood glucose levels were measured at 8 h and 24 h after STZ treatment. At designated time points, mice were killed and serum and pancreas were collected for further analysis.

Insulin Content of Pancreas. Whole pancreas was snap-frozen in liquid nitrogen (33). Insulin was extracted with precooled (−20 °C) acid-ethanol, and the insulin content was measured with a mouse insulin ELISA kit (Crystal Chem).
DNA Collection and Bisulfite Treatment. For isolation of purified β cells, islets were isolated from NODSCID mice, and single cell suspensions were prepared by collagenase digestion. The cells were stained intracellularly with guinea pig anti-insulin antibodies, followed by a secondary FITC-conjugated donkey anti-guinea pig antibody. The stained cells were then FACS-sorted to either insulin-positive or insulin-negative fractions. Other β cells were isolated from islets from NOD MIP-GFP mice, and insulin-positive cells were sorted on the basis of GFP fluorescence. Purified human β cells were isolated from dissociated islets that were permeabilized and stained with Fluozin-3-AM (25). The β cells were sorted by gating on the upper 16% of the stained cells.

DNA from tissue, cells, and serum samples was purified using the Qiagen QIAamp DNA Blood Kit following the manufacturer-recommended protocol. Synthetic unmethylated and methylated DNA was purchased from Millipore. Purified DNA was quantitated using a NanoDrop 2000 spectrophotometer. DNA was then subjected to bisulfite treatment and purified on a DNA binding column to remove excessive bisulfite reagent using the Zymo EZ DNA Methylation Kit.

First-Step PCR and Gel Extraction. A methylation-independent reaction was carried out to increase the DNA template for PCR analysis. The forward and reverse primers and melting temperatures for the murine and human genes are listed in Tables S1 and S2. For the reaction, bisulphate-treated DNA template was added from sorted β cells, pancreatic islet cells, and control tissue, either kidney or liver) were purified using a Qiagen PCR Purification Kit and ligated via TOPO-TA cloning into the pCR2.1-TOPO vector (Invitrogen). For the mouse experiments, primers outside the region in the nested PCR reactions are given in Tables S1 and S2. The PCR products were excised from a 3% agarose gel. Negative controls without DNA did not yield products in the first-step reaction. In certain experiments, the purified product was sequenced at Yale University’s Keck Biotechnology Research Laboratory.

Cloning and Sequencing of Insulin DNA. PCR products obtained using methylation-independent primers from sorted cells, pancreatic islet cells, and control tissue, either kidney or liver) were purified using a Qiagen PCR Purification Kit and ligated via TOPO-TA cloning into the pCR2.1-TOPO vector (Invitrotn). For the mouse experiments, primers outside the region in the nested PCR reactions are given in Tables S3. The PCR products were excised from a 3% agarose gel. Negative controls without DNA did not yield products in the first-step reaction. In certain experiments, the purified product was sequenced at Yale University’s Keck Biotechnology Research Laboratory.

First-Step PCR and Gel Extraction. A methylation-independent reaction was carried out to increase the DNA template for PCR analysis. The forward and reverse primers and melting temperatures for the murine and human insulin genes are listed in Tables S1 and S2. For the reaction, bisulphate-treated DNA template was added from sorted β cells, pancreatic islet cells, and control tissue, either kidney or liver) were purified using a Qiagen PCR Purification Kit and ligated via TOPO-TA cloning into the pCR2.1-TOPO vector (Invitrotn). For the mouse experiments, primers outside the region in the nested PCR reactions are given in Tables S1 and S2. The PCR products were excised from a 3% agarose gel. Negative controls without DNA did not yield products in the first-step reaction. In certain experiments, the purified product was sequenced at Yale University’s Keck Biotechnology Research Laboratory.

Statistical Analyses. Data are expressed as mean ± SEM. The differences between means and the effects of treatments were analyzed by one-way ANOVA with Tukey’s post hoc test using Prism 5 (GraphPad software) to identify the significance (P < 0.05) for all pairs of combinations. Nonnormally distributed data were analyzed using nonparametric tests.

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Table S1. Primer sequences and PCR conditions used for studies of murine Ins1

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Table S2. Primer sequences and PCR conditions used for studies of human Ins

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Table S3. Primer sequences used for cloning and sequencing of murine Ins1

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