Detection of insulin mRNA in the peripheral blood after human islet transplantation predicts deterioration of metabolic control

BERNEY, Thierry, et al.

Abstract
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Detection of Insulin mRNA in the Peripheral Blood after Human Islet Transplantion Predicts Deterioration of Metabolic Control


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Recent updates of the Edmonton trial have shown that insulin independence is progressively lost in approximately 90% of islet transplant recipients over the first 5 years. Early prediction of islet graft injury could prompt the implementation of strategies attempting to salvage the transplanted islets. We hypothesize that islet damage is associated with the release and detection of insulin mRNA in the circulating blood. Whole blood samples were prospectively taken from 19 patients with type 1 diabetes receiving 31 islet transplants, immediately prior to transplantation and at regular time-points thereafter. After RNA extraction, levels of insulin mRNA were determined by quantitative reverse transcriptase-polymerase chain reaction. All patients exhibited a primary peak of insulin mRNA immediately prior to transplantation and at regular time-points thereafter. After RNA extraction, levels of insulin mRNA were determined by quantitative reverse transcriptase-polymerase chain reaction. All patients exhibited a primary peak of insulin mRNA immediately after transplantation, without correlation of duration and amplitude with graft size or outcome. Twenty-five subsequent peaks were observed during the follow-up of 17 transplantations. Fourteen secondary peaks (56%) were closely followed by events related to islet graft function. Duration and amplitude of peaks were higher when they heralded occurrence of an adverse event. Peaks of insulin mRNA can be detected and are often associated with alterations of islet graft function. These data suggest that insulin mRNA detection in the peripheral blood is a promising method for the prediction of islet graft damage.

Key words: Graft function, islet transplantation, monitoring, reverse transcriptase PCR

Introduction

Islet of Langerhans transplantation has come of age as a therapeutic method for type 1 diabetes. This is largely the result of the impact of the ‘Edmonton protocol’ that allowed for the first time consistent achievement of insulin independence after islet transplantation. The main reasons for this success are the use of a steroid-free, islet-sparing sirolimus/tacrolimus immunosuppressive combination and the implementation of sequential islet infusions in order to increase the transplanted islet mass (1). Unfortunately, figures of 80% insulin independence at 1 year have not been sustained, since the latest update of the Edmonton experience reported insulin independence rates of approximately 10% at 5 years, although graft function (C-peptide positivity) was retained in a vast majority of patients (2). Several hypotheses could explain this late graft loss, such as allogeneic rejection (3), recurrence of autoimmunity (3,4), islet toxicity of the immunosuppressive drugs (5–7), lack of beta-cell regeneration due to the anti-proliferative properties of sirolimus, or ‘exhaustion’ of the islet graft. These alleged mechanisms of islet graft loss are not mutually exclusive, and occur on a terrain of suboptimal beta-cell functional reserve, as suggested by markedly decreased insulin responses to stimulation in islet transplant recipients as compared to controls (8). A low rate of engraftment is thought to be the result of early islet loss during the isolation procedure or in the graft microenvironment within the liver, secondary to ischemia-reperfusion-like injury and to nonspecific inflammatory phenomena (9–13).

We have recently reported the detection of circulating beta cells in the peripheral blood, by RT-PCR for insulin mRNA, early after islet transplantation, and proposed that this apparent release of beta-cell material could be a marker of injury to the islet graft (14). In the present study, we have measured insulin mRNA by real-time quantitative RT-PCR in the peripheral blood of islet transplant recipients during post-transplant follow-up. We have tested the hypothesis that the extent of early islet graft loss as measured by the amplitude and duration of early peaks of circulating insulin mRNA could be a predictor of long-term islet graft...
function. We have also explored the possibility that a decrease of islet graft function over the course of follow-up could be heralded by a subsequent peak of circulating insulin mRNA, in an attempt to identify a surrogate marker of islet graft rejection/autoimmune injury.

Methods

Islet isolation

Pancreata were procured from brain-dead multiorgan donors as described (15), and shipped on ice in preservation solution to the islet isolation facilities at the University of Geneva, Switzerland, or at the University of Alberta, Edmonton, Canada. Islet isolation was performed using local modifications of the automated method (1,16,17). Briefly, pancreata were perfused intraductally with Liberase (Roche-Boehringer-Mannheim, Indianapolis, IN, USA) or Collagenase NB1 (SERVA, Uetersen, Germany). Pancreatic tissue was then dissociated by enzymatic and mechanical actions in a Ricordi chamber. Islets were separated from the rest of the pancreatic digest on continuous density gradients in a COBE 2991 cell processor (COBE BCT, Lakewood, CO, USA). Islets were counted and scored for size on samples taken from the preparations. The islet mass was calculated as number of islet equivalents (IEQ) of a standard size of 150 μm using a routine algorithm (18).

Islet transplantation

Only islet preparations meeting release criteria (islet count > 5000 IEQ/kg, viability > 70%, purity > 30%, packed tissue volume < 10 mL, absence of microorganisms on Gram staining) were used for transplantation into type 1 diabetic patients. Islets were transplanted either immediately within 4 h of the end of the isolation procedure (N = 17) or after short-term culture (≥ 10 h; N = 20). In most instances, islets were infused into the portal vein using a transhepatic percutaneous approach under radiological control as described (19). In two cases of simultaneous islet-kidney (SIK) transplantation, islets were infused intraportally by catheterization of a colonic vein by a surgical open approach (20).

Patients

Thirty-seven islet transplantation procedures were performed in 19 patients (10 women and 9 men) with type 1 diabetes, with a mean age of 46 ± 10 years and a mean duration of diabetes of 35 ± 8 years. Procedures were performed between July 2002 and August 2004 at three different locations (Geneva, Grenoble, Edmonton) under protocols reviewed and approved by the Institutional Review Boards of Geneva University Hospitals, Grenoble University Hospital Center, or the University of Alberta. Procedures were 25 islet-transplant-alone (ITA), 10 islet-after-kidney (IAK) and 2 SIK transplantations. Mean size of the infused islet preparations was 412 000 ± 102 000 IEQ or 6100 ± 1300 IEQ/kg. Patients received from 1 to 3 islet infusions in order to reach a minimum of 10 000 IEQ/kg. Of the 19 patients entering the study, 13 received a second islet infusion and 5 received a third infusion. Mean follow-up after first transplant was 414 ± 151 days (range: 210–598) and mean follow-up after each transplant was 216 ± 163 days (range: 8–564). Patient characteristics are shown in Table 1.

All patients received an immunosuppressive regimen according to the Edmonton protocol (1). Induction was done with daclizumab (Zenapax; Roche-Pharma, Basel, Switzerland) at a dose of 1 mg/kg every 14 days for a total of 5 doses. Maintenance immunosuppression was done with sirolimus (Rapamune; Wyeth-Ayerst, Zug, Switzerland) aiming for trough levels of 12–15 ng/mL for the first 3 months, 8–10 ng/mL thereafter, and tacrolimus (Prograf; Fujisawa, Villars-sur-Glâne, Switzerland) aiming for trough levels of 4–6 ng/mL. Induction was repeated for each islet infusion.

Patient follow-up

Patients were seen every day until hospital discharge after transplantation. Patients were then seen at the outpatient clinic weekly until 6 weeks post-transplantation, bimonthly until 3 months post-transplantation and monthly thereafter. As a rule, insulin weaning was stopped or insulin therapy was resumed whenever one of the following cut-off values were consistently reached: fasting blood glucose > 8 mM/L for 3 days per week, 2-h postprandial blood glucose > 10 mM/L for 3 days per week. Resuming insulin was also recommended if HbA1c was > 7% consistently (2). Blood samples were taken on each occasion. The following metabolic events were recorded and correlated with detection of circulating insulin mRNA: achievement or loss of graft function (C-peptide positivity), achievement or loss of insulin independence, increase in daily insulin requirements, increase in HbA1c and acute rejection. Increase in HbA1c was considered in comparison to the most recent previous value as a baseline. Only islet infusions with a minimum follow-up of 50 days were considered for analysis.

Sample recovery, RNA isolation and RT-PCR

Samples of venous blood (2.5 mL) were collected before (t = 0) and at various time-points (up to 17 months) after transplantation. They were drawn into vacutainer PAXgene blood RNA tubes (PreAnalytiX; Qiagen, Garstligweg, Switzerland) and kept at −20°C until processed. RNA isolation was performed using the PAXgene blood RNA kit (PreAnalytiX; Qiagen) including optional DNAse treatment. Reverse transcription (RT) was performed in a total volume of 20 μL using a maximum of 3 μg of total RNA, oligoDT primers and SuperScriptII reverse transcriptase (Life Technologies, Basel, Switzerland). Insulin cDNA (138 bp fragment) was amplified using one-tenth of the RT reaction with the Quantitect™ SYBR Green PCR kit (Qiagen) and 0.75 μM of primers sense (5′CTCT TCT ACA CAC CCA AGA CC) and antisense (5′GT TCCA AAG TGC CAC GCT TC). PCR cycles were 15 min at 95°C, 45× 15 s 95°C, 30 s 58°C, 11 s 72°C, followed by a melting curve and cooling steps in a LightCycler (Roche Diagnostics, Basel, Switzerland). For all samples, two independent amplifications were performed. Computerized quantification was done using an imported external standard curve (obtained with purified PCR products and quantified by molecular cloning).
**Table 1**: Patient and graft characteristics after each islet transplant procedure

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>BMI (kg/m²)</th>
<th>Diabetes duration (years)</th>
<th>Transplant number</th>
<th>Type of transplant*</th>
<th>Time since kidney transplant</th>
<th>IEQ†</th>
<th>IEQ/kg†</th>
<th>Follow-up (days)‡</th>
<th>Institution**</th>
<th>Outcome††</th>
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<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>F</td>
<td>21.0</td>
<td>39</td>
<td>1</td>
<td>SIK</td>
<td>4 months</td>
<td>282,000</td>
<td>5,036</td>
<td>116</td>
<td>GVA</td>
<td>II → d565 ↓ 85%</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>F</td>
<td>26.0</td>
<td>42</td>
<td>1</td>
<td>IAK</td>
<td>23 years</td>
<td>366,833</td>
<td>4,753</td>
<td>53</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>F</td>
<td>21.0</td>
<td>25</td>
<td>1</td>
<td>IAK</td>
<td>4 years</td>
<td>367,333</td>
<td>5,651</td>
<td>239</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>F</td>
<td>25.0</td>
<td>43</td>
<td>1</td>
<td>IAK</td>
<td>13 years</td>
<td>387,316</td>
<td>5,523</td>
<td>65</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>M</td>
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<td>32</td>
<td>1</td>
<td>IAK</td>
<td>12 years</td>
<td>380,000</td>
<td>6,333</td>
<td>15§</td>
<td>GRE</td>
<td>II</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>M</td>
<td>25.0</td>
<td>37</td>
<td>1</td>
<td>IAK</td>
<td>15 years</td>
<td>510,083</td>
<td>6,370</td>
<td>28§</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>F</td>
<td>20.5</td>
<td>53</td>
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<td>ITA</td>
<td>4 months</td>
<td>564,083</td>
<td>8,997</td>
<td>564</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>M</td>
<td>21.5</td>
<td>27</td>
<td>1</td>
<td>ITA</td>
<td>4 months</td>
<td>476,999</td>
<td>7,739</td>
<td>77</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>M</td>
<td>19.0</td>
<td>45</td>
<td>1</td>
<td>ITA</td>
<td>360,790 9,620</td>
<td>367,912</td>
<td>6,731</td>
<td>283</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>M</td>
<td>24.0</td>
<td>25</td>
<td>1</td>
<td>ITA</td>
<td>311,998 5,054</td>
<td>454,791</td>
<td>7,580</td>
<td>210</td>
<td>GVA</td>
<td>II</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>M</td>
<td>24.5</td>
<td>23</td>
<td>1</td>
<td>ITA</td>
<td>475,750 7,707</td>
<td>385,591</td>
<td>6,247</td>
<td>425</td>
<td>GVA</td>
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</tr>
<tr>
<td>12</td>
<td>58</td>
<td>F</td>
<td>24.0</td>
<td>32</td>
<td>1</td>
<td>SIK</td>
<td>266,150 4,753</td>
<td>387,912</td>
<td>6,731</td>
<td>283</td>
<td>GVA</td>
<td>II</td>
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<tr>
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<td>41</td>
<td>M</td>
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<td>1</td>
<td>SIK</td>
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<td>75</td>
<td>GVA</td>
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<tr>
<td>14</td>
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<td>30.1</td>
<td>32</td>
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<td>ITA</td>
<td>477,263 6,035</td>
<td>454,791</td>
<td>7,580</td>
<td>210</td>
<td>GVA</td>
<td>II</td>
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<tr>
<td>15</td>
<td>49</td>
<td>F</td>
<td>26.9</td>
<td>39</td>
<td>1</td>
<td>ITA</td>
<td>389,374 9,655</td>
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<td>425</td>
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<td>II</td>
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<tr>
<td>16</td>
<td>31</td>
<td>F</td>
<td>29.7</td>
<td>30</td>
<td>1</td>
<td>ITA</td>
<td>350,473 4,061</td>
<td>466,833</td>
<td>6,142</td>
<td>8§</td>
<td>GVA</td>
<td>II</td>
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<tr>
<td>17</td>
<td>63</td>
<td>M</td>
<td>27.2</td>
<td>36</td>
<td>1</td>
<td>ITA</td>
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<td>467,333</td>
<td>6,143</td>
<td>515</td>
<td>GVA</td>
<td>II</td>
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<tr>
<td>18</td>
<td>42</td>
<td>F</td>
<td>25.1</td>
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<td>ITA</td>
<td>497,472 6,258</td>
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<td>4,772</td>
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<td>GVA</td>
<td>II</td>
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<tr>
<td>19</td>
<td>49</td>
<td>M</td>
<td>28.9</td>
<td>25</td>
<td>1</td>
<td>ITA</td>
<td>596,330 6,950</td>
<td>431,007</td>
<td>7,183</td>
<td>106</td>
<td>GVA</td>
<td>II</td>
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</tbody>
</table>

*SIK: simultaneous islet kidney; IAK: islet after kidney; ITA: islet transplant alone.
†IEQ: number of islet equivalents infused; IEQ/kg: number of islet equivalents per kilogram body weight.
‡Number of days until next transplant or until end of study for the last transplant.
§Follow-up < 50 days: Transplant not considered in analysis.
**Institution where transplant was performed (GVA: Geneva University Hospitals; GRE: Grenoble University Hospital Center; EDM: University of Alberta, Edmonton).
††Outcome of islet transplantation: II = insulin independence. Duration of insulin independence from first islet infusion, and insulin independence or reduction in insulin requirements at the end of follow-up after final islet infusion are indicated.

optical density measurement) by the ‘Fit Point’ calculation method. All RT reactions were controlled by amplification of G protein subunit (Gs), a low-expressed gene in blood cells, for 30 cycles (1 min 94°C, 1 min 50°C, 1 min 72°C) in a semi-quantitative manner with 0.25 μM of primers sense (5' ACT TCT GGA ATC TTT GAG ACC AAG) and antisense (5'TTA AAG GCT TTA ATT AAT TTG GGG GTT CC). Results were corrected when less than 3 μg was used for RT and expressed as number of copies of mRNA by sample of blood (2.5 mL). Peak amplitudes were calculated by multiplying the mean value of insulin mRNA peaks by the number of days of mRNA positivity.

**Insulin mRNA quantification**

In order to precisely quantify the number of copies of insulin mRNA, we calibrated the assay using human islets.
Human islets were dissociated with trypsin (0.05%, Gibco BRL Invitrogen, Basel, Switzerland). Live single cells were counted and added to whole blood obtained from healthy volunteers in increasing amounts (0, 5, 10, 20, 50 and 100 cells per mL of blood). On an average, about 20% of cells in the suspension were not beta cells (other endocrine, exocrine or ductal cells). RNA isolation and RT-PCR were carried out as described above. Results were corrected by islet purity. Numbers of insulin mRNA copies measured by LightCycler were plotted against numbers of islet cells added to blood, and linear regression analysis was performed. The resulting equation allowed direct calculation of number of beta cells according to measured copy numbers. In this set of experiments, quantification by the LightCycler was done both with the ‘Fit Point’ and the ‘2nd Derivative’ methods. In the Fit Point method, specificity is improved at the expense of sensitivity because fluorescent background is largely not considered. With the 2nd Derivative method, where a small increase of fluorescence marking double-stranded cDNA during the PCR process is considered as positive, sensitivity rather than specificity is improved. With both methods, a correction for RT efficiency was applied, because of the efficiency rate of only 25% of the method (supplier communication).

Statistical analysis
All statistical analyses were done using the Statistica software package (StatSoft, Tulsa, OK, USA). Nonparametric Mann-Whitney U-test was used for comparison of continuous variables. Nonparametric Spearman Z-test was used for statistical analysis of linear regressions. p values < 0.05 were considered significant.

Results

Correlation between detected circulating mRNA and circulating beta cells
Using the Fit Point method, a coefficient of correlation (R²) of 0.7 was obtained by linear regression analysis with the related equation \( y = 3436.2x \), where \( y \) is the number of mRNA copies and \( x \) the number of beta cells. When using the 2nd Derivative method, we obtained an R² of 0.59 and a related equation \( y = 25482x \). After application of the correction factor for RT efficiency, a value of \( 1.4 \times 10^4 \) insulin mRNA copies per beta cell was obtained with the Fit Point method, and of \( 10^5 \) copies per cell with the 2nd Derivative method. This is in accordance with previous results where a mean of \( 5 \times 10^4 \) to \( 1.5 \times 10^5 \) insulin mRNA copies were found for rat beta cells and \( 10^5 \) copies/cell for murine beta cells (21).

Islet graft function
Graft function was observed after all transplants, as demonstrated by C-peptide positivity and a decrease in daily insulin requirements (Figures 1A and 1B). Thirteen of 19 patients achieved insulin independence, 10 were insulin independent at 1 year and 9 were still off insulin at the end of the follow-up period. Outcome of each patient is shown in Table 1. Improvement of glycemic control was demonstrated by a normalization of HbA1c (Figure 1C).

Detection of circulating insulin mRNA
Insulin mRNA could not be measured in the peripheral blood of any patient immediately prior to islet transplantation. Circulating mRNA for insulin was detected in all patients immediately after transplantation, as early as 6 h after the end of islet infusion ('primary peaks'), indicating a release of beta cells in the peripheral blood after the procedure. Mean duration of primary peaks was 4.2 days (range 1–13 days), and their mean amplitude was 510 copies/2.5 mL (range 20–6695 copies). The highest mean amplitude was recorded 6 h after islet infusion. Twenty-five subsequent peaks of insulin mRNA were detected...
in the peripheral blood during the follow-up of 17 islet transplantations (‘secondary peaks’). Mean duration of secondary peaks was 12.6 days (range 1–63 days), and mean peak amplitude was 932.5 copies/2.5 mL (11–6780 copies). Secondary peaks were observed with a mean time lag of 62 days after last islet infusion (range 7–294 days). Numbers of insulin mRNA copies measured during follow-up are shown in Figure 2. Using the equation obtained by linear regression analysis, these numbers of copies corresponded to a range of $10^{-4}$–$7 \times 10^{-2}$ cells/2.5 mL or $8 \times 10^{-4}$–$5 \times 10^{-1}$ cells/2.5 mL depending on the PCR quantification method used.

**Significance of primary insulin mRNA peaks**

Primary peaks were observed after each islet infusion, and amplitude and duration of the primary peaks were similar after first, second or third infusion (1st infusion: 671 ± 1536 copies/2.5 mL, 4.7 ± 3.5 days; 2nd infusion: 292 ± 179 copies/2.5 mL, 3.9 ± 1.7 days; 3rd infusion: 261 ± 387 copies/2.5 mL, 2.8 ± 2.5 days; $p > 0.25$). No correlation was observed between amplitude of the primary peak of insulin mRNA and transplanted islet mass, in terms of IEQ ($R^2 = 0.09$) or IEQ/kg ($R^2 = 0.16$), or islet graft function, in terms of decrease in insulin requirements ($R^2 = 0.05$). Similarly, no correlation was observed between duration of the primary peak and transplanted islet mass, in terms of IEQ ($R^2 = 0.11$) or IEQ/kg ($R^2 = 0.25$), or islet graft function, in terms of decrease in insulin requirements ($R^2 = 0.16$). Amplitude and duration of primary peaks was similar after transplantation of fresh or cultured islets (Fresh islets: 609 ± 1631 copies/2.5 mL, 4.7 ± 3.5 days; cultured islets: 377 ± 333 copies/2.5 mL, 3.7 ± 2.2 days; $p > 0.3$).

**Significance of secondary insulin mRNA peaks**

Fourteen of 25 secondary peaks (56%) were simultaneous to or closely followed by at least 1 event related to islet graft function. Events were kidney graft rejection ($N = 2$), a doubling of exogenous insulin requirements ($N = 3$), restart of insulin therapy ($N = 7$) and a significant increase in HbA1c > 0.7% ($N = 11$). Mean time lag between the detection of insulin mRNA peak and the event was 17 days (range 0–54 days). Importantly, there was no event occurrence that was not preceded by a secondary peak of insulin mRNA. Duration of peaks was significantly higher when they heralded occurrence of an adverse metabolic event ($19 \times 1$ day, $p = 0.02$). Amplitude of peaks was higher with a trend toward significance prior to an event ($341 \text{ vs. } 32$ copies/2.5 mL, $p = 0.06$). An overview of all insulin mRNA secondary peaks is shown in Table 2. A cut-off value of 7 days for peak duration was associated with a sensitivity of 64%, a specificity of 90%, a positive predictive value (PPV) of 90% and a negative predictive value (NPV) of 67%. A cut-off value of 70 copies/2.5 mL for peak amplitude was associated with a sensitivity of 79%, a specificity of 73%, a PPV of 79% and a NPV of 73%. Defining test positivity with either peak duration $> 7$ days OR peak amplitude $> 70$ copies/2.5 mL gave a sensitivity of 85%, a specificity of 73% and a PPV and NPV of 80%.

There was no correlation between absolute or relative basal C-peptide levels and occurrence of an mRNA peak or a metabolic event. Indeed, mRNA peaks and metabolic events were indifferently followed by either an increase or a decrease in basal C-peptide levels.

**Discussion**

There are currently no early markers of islet graft dysfunction available that predict islet graft rejection or recurrence of autoimmunity on the graft, and prompt for rapid and adequate antirejection therapy. Hyperglycemia and the need to resume or significantly increase exogenous insulin occur late in the immune islet destruction process, when the major part of the islet graft has already been lost. Serial liver biopsies are possible, but cumbersome, especially in the absence of an appropriate trigger, and the challenge of obtaining representative islet samples when scattered diffusely throughout the liver make this approach impractical for routine graft monitoring. For these reasons, there is a critical need for markers of islet cell damage, to be routinely used for the monitoring of the islet graft during follow-up (22,23).

Several methods of islet graft monitoring have been advocated or explored. On the immunological standpoint, some correlation has been shown between increased titers of anti-HLA circulating antibodies or GAD-65 and IA-2 autoantibodies and islet graft failure, indicating probable rejection or recurrence of autoimmunity (24,25), but these observations are not transferable to clinical monitoring, mostly...
Table 2: Characteristics of secondary peaks of insulin mRNA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tx number</th>
<th>Time after Tx (days)</th>
<th>Peak amplitude (copies/2.5 mL)</th>
<th>Peak duration (days)</th>
<th>Event</th>
<th>Time between peak and event (days)</th>
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</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>43</td>
<td>92</td>
<td>1</td>
<td>Renal graft rejection</td>
<td>19</td>
</tr>
<tr>
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<td>1</td>
<td>12</td>
<td>24</td>
<td>1</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>29</td>
<td>1322</td>
<td>21</td>
<td>HbA1C + 2%/Restart Insulin</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>294</td>
<td>36</td>
<td>21</td>
<td>Restart insulin, HbA1C + 0.8%</td>
<td>simultaneous</td>
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<tr>
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<td>2</td>
<td>7</td>
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<td>HbA1C + 1.6%</td>
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because of lack of patient-to-patient consistency in the observations. Direct detection of cytotoxic alloreactive or autoreactive T-cell responses in mixed lymphocyte cultures or in proliferation tests in the presence of autoantigens was also reported to herald islet graft failure (26). Detection of HLA- or autoantigen-specific T-cells using the novel MHC class II tetramer technology is a promising and elegant method (27,28). However, these are cumbersome and time-consuming methods.

Islet imaging, using magnetic resonance or positron emission tomography technologies for example, is gaining interest as an approach for islet graft monitoring, and preliminary data on animal models are promising (29,30).

Last, detection of gene expression in the peripheral blood by RT-PCR was recently shown to be of value for the prediction of islet graft failure. Detection of circulating mRNA for the cytotoxic lymphocyte genes of granzyme B, perforin and Fas-ligand was demonstrated to herald the loss or decrease in islet graft function, both in nonhuman primates and in human recipients of islet transplants (31,32). These markers of cytotoxic T-cell activity are likely to appear not only in the presence of an immune phenomenon such as rejection or recurrence of autoimmunity, but also in response to infectious or inflammatory processes, as reported in the study, accounting for a relative lack of specificity.

We have previously reported that circulating mRNA for insulin could be detected immediately after islet transplantation using a qualitative RT-PCR assay, and we proposed that this observation was a reflection of early islet damage in the engraftment period, with ensuing release of beta cells in the peripheral blood (14). This was comforted by the observation that circulating insulin mRNA was detectable for a much longer time (up to 10 weeks) in patients on a steroid-containing immunosuppressive regimen known to be toxic to the islets than in patients on a steroid-free regimen (up to 2 weeks). Therefore, we decided to test the hypothesis that monitoring of circulating insulin mRNA could be a valuable tool for the prediction of injury to the islet graft. In contrast to our previous study, we used a real-time quantitative RT-PCR with the aim to correlate the amplitude of the mRNA peaks with the presence or absence of a subsequent event signaling islet damage.

It is interesting to note that the numbers of circulating beta cells measured in each peak of insulin mRNA corresponded to less than 1 cell per sample of 2.5 mL of whole blood. It is unlikely that such small numbers represent the effect of a sampling error. It is tempting to extrapolate the numbers of circulating beta cells measured in 2.5 mL to the whole circulating blood volume. However, even with this extrapolation, 40% of secondary peaks would represent less than 10 beta cells circulating in the whole blood. Therefore, it is probable that, rather than intact whole beta cells, we in fact...
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detected altered circulating beta cells, or more likely beta-cell degradation products within circulating phagocytes.

We observed a primary peak of insulin mRNA after each islet infusion, confirming our previous observation (14). The early appearance of the primary peaks (6 h) and their high amplitude indicated an important release of circulating beta-cell material and possibly reflect the immediate destruction of a sizeable proportion of the islet graft at the time of transplantation. An instant blood-mediated inflammatory reaction (IBMIR) injuring the islets has been described in the minutes that follow the contact of isolated islets with blood and might at least partly account for this observation (33). Local release of cytotoxic factors or pro-inflammatory mediators during isolation or at the site of implantation undoubtedly also explain this early islet destruction (9–12,34). Finally, hypoxia in islets freshly disconnected from their vascular supply and not yet revascularized could play an added role in early islet injury (35).

Although we confirmed the rather short duration (4 days in average) of the initial peak in this series of patients transplanted on a steroid-free immunosuppressive regimen, neither the amplitude, nor the duration of this primary peak were indicative of subsequent islet graft function. It is possible that higher and longer peaks could be observed in the event of graft primary nonfunction, which did not occur in this series of patients. It is also of interest to note that the magnitude of the initial peak did not correlate with the total islet mass infused, and that larger islet grafts apparently do not suffer from initial damage proportional to graft size.

Interestingly, the occurrence of secondary peaks of circulating insulin mRNA showed a good correlation with the subsequent observation of metabolic events indicative of islet graft dysfunction, such as marked increases in HbA1c or insulin requirements. Although secondary peaks were not always followed by such events, they were preceded by significantly higher and longer peaks. With appropriate cut-off levels, negative and positive predictive values of 80% were obtained in this preliminary experience. This makes our assay a valuable tool for the monitoring of islet grafts to drive the decision to start antirejection therapy. However, one should point out that one limitation in this pilot study resides in the rather long intervals between insulin mRNA determinations (monthly after the third month post-transplant), which might have impacted on the accuracy of the measure of peak duration.

Our assay is indicative of beta-cell shedding in general and is not specific for allorejection. Since islet cell damage can also result from recurrence of autoimmunity, which could arguably also be treated with a boost of immunosuppression, such a discriminating ability is not required. Discriminating between autoimmune destruction of beta cells in recurring type 1 diabetes and other causes of islet cell damage could theoretically be done by concomitant detection of mRNA coding for glucagon or somatostatin in the peripheral blood. One would expect to detect only mRNA for insulin in case of an autoimmune phenomenon, but messengers for all three hormones in case of islet cell damage secondary to rejection or inflammatory events. The fact that islets can also theoretically be damaged in the long term by nonspecific inflammatory mechanisms or lose function to progressive exhaustion is more problematic, since it might prompt the initiation of unnecessary antirejection treatment. In this regard, coupling of our assay with the granzyme B assay described by Han et al. (31,32) might improve their specificity and enable to discriminate between immune and nonimmune islet damage on one hand, and between immune islet destruction and infectious/inflammatory events on the other hand. Such discriminating ability could provide an accurate trigger for the appropriate initiation of antirejection therapy.

Acknowledgments

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References