Evaluation of Polyneuropathy Markers in Type 1 Diabetic Kidney Transplant Patients and Effects of Islet Transplantation

Neurophysiological and skin biopsy longitudinal analysis

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OBJECTIVE — The purpose of this study was to evaluate whether islet transplantation may stabilize polyneuropathy in uremic type 1 diabetic patients (end-stage renal disease [ESRD] and type 1 diabetes), who received a successful islet-after-kidney transplantation (KI-s).

RESEARCH DESIGN AND METHODS — Eighteen KI-s patients underwent electroneurographic tests of sural, peroneal, ulnar, and median nerves: the nerve conduction velocity (NCV) index and amplitudes of both sensory action potentials (SAPs) and compound motor action potentials (CMAPs) were analyzed longitudinally at 2, 4, and 6 years after islet transplantation. Skin content of advanced glycation end products (AGEs) and expression of their specific receptors (RAGE) were also studied at the 4-year follow-up. Nine patients with ESRD and type 1 diabetes who received kidney transplantation alone (KD) served as control subjects.

RESULTS — The NCV score improved in the KI-s group up to the 4-year time point ($P = 0.01$ versus baseline) and stabilized 2 years later, whereas the same parameter did not change significantly in the KD group throughout the follow-up period or when a cross-sectional analysis between groups was performed. Either SAP or CMAP amplitudes recovered in the KI-s group, whereas they continued worsening in KD control subjects. AGE and RAGE levels in perineurium and vasa nervorum of skin biopsies were lower in the KI-s than in the KD group ($P < 0.01$ for RAGE).

CONCLUSIONS — Islet transplantation seems to prevent long-term worsening of polyneuropathy in patients with ESRD and type 1 diabetes who receive islets after kidney transplantation. No statistical differences between the two groups were evident on cross-sectional analysis. A reduction in AGE/RAGE expression in the peripheral nervous system was shown in patients receiving islet transplantation.

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Chronic sensorimotor diabetic polyneuropathy (DPN) is a common long-term complication of type 1 diabetes, affecting >50% of patients (1). Electroneurographic studies, based on nerve conduction velocity (NCV) studies, represent an objective method for DPN assessment (2) and may also predict mortality (3). Among treatments that may prevent either DPN onset or progression by restoring normoglycemia, pancreas transplantation has been widely studied with NCV in the recent past (4,5). Until now, little has been known of the effect of islet transplantation on DPN.

Indications for allogenic pancreatic islet transplantation in type 1 diabetes have been expanding over the past few years (6) thanks to recent improvements in long-term graft survival rates (7) that depend on advances in islet isolation and purification and new immunosuppressive protocols (8). Compared with the whole organ transplant, which has a 5% mortality rate 1 year after surgery and severe surgical complications (9), islet transplantation is a minimally invasive therapeutic approach, allowing for long-term insulin independence and metabolic control (7,8).

However, the question of whether long-term diabetes complications may be halted or even reversed by transplantation is still under investigation. Immunosuppressive treatment may interfere with renal function (10); yet the protective role of islet transplantation on both long-term graft survival and function of the transplanted kidney in type 1 diabetic patients has been demonstrated (11,12). In type 1 diabetic patients who had received a kidney transplant, benefits for either macro-/microangiopathy or cardiovascular function (13,14) and also for retinal complications (15,16) were reported. Stabilization of peripheral neuropathy was reported after islet transplantation alone; i.e., it was not associated with kidney transplantation (15,16).

The aim of this study was to evaluate whether islet transplantation might stop

the progression of neuropathy in type 1 diabetic patients with end-stage renal disease (ESRD) bearing a kidney graft: thus, we performed longitudinal NCV studies in a group of uremic type 1 diabetic patients who had received kidney transplants throughout the 6 years after islet transplantation. Further, at the 4-year follow-up, we investigated cross-sectionally the skin expression of advanced glycation end products (AGEs) and their specific receptor (RAGE), which were reported to play a role in the pathogenesis of DPN (17).

RESEARCH DESIGN AND METHODS

Among all consecutive patients with ESRD and type 1 diabetes who had received a successful islet-after-kidney transplantation from 1991 until January 2004 (KI-s group), 18 (age 38.7 ± 5.7 years; male-to-female ratio 8:10) with sustained C-peptide secretion of >0.5 ng/ml for >6 months were referred to our laboratory for electroneurographic assessment of their polyneuropathy, which had been diagnosed previously on the basis of clinical findings (18). Nine patients with ESRD and type 1 diabetes were considered a control group (KD [kidney only] group: age 39.1 ± 2.2; male-to-female ratio 4:5). The efficacy of islet function in the KI-s group was assessed by fasting circulating C-peptide levels. KI-s patients were followed for an average of 53.4 ± 7.1 months after transplantation. Any patients who lost islet function early after transplantation (within 6 months) were enrolled in the KD group. If the transplanted islets produced >0.5 ng/ml C-peptide, the patient was considered a subject in the KI-s group.

Subanalysis of the KI-s group
Within the KI-s group, different patients appeared to have different degrees of metabolic control. Therefore, a subanalysis of the patients who reached full islet function (fasting C-peptide >1 ng/ml) was performed.

Diabetes management after kidney-islet transplantation
Insulin therapy was used after transplantation to maintain strict glycometabolic control in patients. In some patients oral hypoglycemic agents were used to improve islet function, particularly when insulin resistance was clearly evident on the basis of a requirement for very high doses of insulin.

Laboratory assessment
Fasting levels of cyclosporine, creatinine, A1C, serum C-peptide, total cholesterol, and triglycerides were assayed at baseline and 2, 4, and 6 years after transplantation (19). Serum C-peptide levels (intra-assay and interassay coefficients of variation both 3.0%) were assayed by radioimmunoassay using a commercial kit (Medical System, Genoa, Italy).

Islet transplantation
Both kidneys and islets came from cadaver donors; transplantation was performed according to HLA matching for kidney graft, whereas ABO compatibility was used for islet transplantation (13). The cross-match test was negative in all cases. The details of the procedure can be found in the online appendix (available at http://dx.doi.org/10.2337/dc07-0206).

Nerve conduction study
Nerve conduction study was performed after standard laboratory procedures by an operator who did not know which group the patient belonged to. The details of the procedure can be found in the online appendix.

NCV index
Besides standard conduction parameters, an NCV index was assessed for each patient, as already reported (20). Briefly, conduction velocities of each nerve segment (motor conduction velocities of deep peroneal and ulnar nerves; sensory conduction velocities of the sural nerve and either wrist-finger or elbow-wrist segments of the median nerve) were first considered to obtain five nerve NCV Z scores [(patient's NCV value – mean NCV value in control healthy subjects)/SD of the same nerve in control healthy subjects]. The patient's NCV index was the mean of each nerve NCV Z score. Age-related normative values (mean ± SD) from our electromyography laboratory were considered for calculation. The NCV index estimates to what extent individual NCV values deviate from the mean value of a reference population in terms of SD, limiting the specific intraindividual variability for each nerve trunk and allowing for an easier longitudinal evaluation. Because of the method of NCV index calculation, most negative NCV values identify the most severe polyneuropathies. In subjects with complete nerve unexcitability, the last available NCV value was considered for NCV index calculation.

Skin biopsy
Patients underwent skin punch biopsy on the internal surface of the arm 4.5 ± 1.2 years after transplantation as described elsewhere (13). The procedure is easy, minimally invasive, and well tolerated by patients. Samples were taken with the patient's consent and ethical review board approval.

AGEs and RAGE quantification
N^ε-(carboxymethyl) lysine (CML)-protein adduct content and RAGE expression were assessed in paraffin-embedded sections by immunohistochemical analysis. Immunoreactivity for CML and RAGE in nerves and perineural vessels was evaluated with a semiquantitative scale for staining (−, absent; +, mild; ++, moderate; +++, strong). The details of the procedure can be found in the online appendix.

Statistical analysis
Because of the limited sample size, statistical analysis was performed by means of nonparametric tests. We performed the following comparisons: *1*) NCV indexes at the different follow-up examinations (2, 4, and 6 years) were compared with basal values by means of the Wilcoxon signed rank-sum test for paired samples in the entire sample and in the KI-s and KD subgroups; and *2*) changes from baseline of NCV indexes at the different follow-up examinations (2, 4, and 6 year) were compared between the KI-s and KD groups by means of the Wilcoxon rank-sum test. We also performed a repeated-measures ANOVA to simultaneously explore the role of the time of evaluation, defined as a within-subject factor, and of the group of treatment (KI-s versus KD), defined as a between-subjects factor, and their interaction on the NCV index value. This type of analysis permits taking into account the correlation of measures performed on the same subject and exploring the role of the time of evaluation and the treatment on the NCV index.

RESULTS

Population and metabolic variables
The two groups of recipients had similar pretransplant and peritransplant characteristics, in particular the pattern of rejection episodes, cytomegalovirus infections, and kidney retransplantation (data not shown). The mean numbers of HLA matches for the kidney and plasma renin activity levels were similar in the

Table 1—NCV index and other electroneurographic findings (baseline and longitudinal)

Groups and variables	Basal	2 years	4 years	6 years
KI-s				
n	18	18	18	9
Age (years)	41.8 ± 6.2			
A1C (%)	8.0 ± 1.1	7.7 ± 1.8	7.4 ± 1.8	7.5 ± 0.4
Creatinine (mg/dl)	1.6 ± 1.5	1.3 ± 0.5	1.4 ± 0.7	1.1 ± 0.2
C-peptide (ng/ml)	0.1 ± 0.1	1.8 ± 1.0	1.1 ± 0.5	1.4 ± 1.1
NCV index	−2.9 ± 0.9	−2.8 ± 1.1	−2.6 ± 1.0*	−2.7 ± 0.9
Sural SAP$_{ampl}$	7.9 ± 3.2	9.0 ± 8.1	16.4 ± 25.3	13.7 ± 19.0
Median SAP$_{ampl}$	16.7 ± 9.0	19.6 ± 10.1	19.4 ± 11.1	20.0 ± 15.6
Peroneal CMAP$_{ampl}$	3.0 ± 4.2	2.5 ± 2.6	2.8 ± 3.9	4.4 ± 4.5
Ulnar CMAP$_{ampl}$	10.2 ± 4.0	9.5 ± 3.3	10.3 ± 3.0	11.6 ± 2.2
KD				
n	9	9	9	9
Age (years)	39.1 ± 2.2			
A1C (%)	11.1 ± 2.3	8.0 ± 0.4	8.6 ± 0.4	8.1 ± 0.4
Creatinine (mg/dl)	1.7 ± 0.1	1.9 ± 0.2	2.0 ± 0.3	2.5 ± 0.7*
C-peptide (ng/ml)	0.1 ± 0.1			
NCV index	−2.7 ± 1.2	−2.5 ± 0.9	−2.6 ± 1.0	−2.8 ± 1.1
Sural SAP$_{ampl}$	4.4 ± 2.8	3.6 ± 0.8	6.5 ± 0.7	6.3 ± 7.3
Median SAP$_{ampl}$	13.3 ± 6.8	15.7 ± 9.0	17.8 ± 5.0	11.8 ± 6.6
Peroneal CMAP$_{ampl}$	3.9 ± 3.2	3.5 ± 2.5	2.0 ± 1.8	3.2 ± 3.6
Ulnar CMAP$_{ampl}$	13.7 ± 4.6	10.6 ± 5.1	12.4 ± 3.3	9.7 ± 3.0*

Data are means ± SD. *$P < 0.05$ versus basal values. SAP$_{ampl}$, sensory action potential amplitude (expressed as microvolts); CMAP$_{ampl}$, compound motor action potential amplitude (expressed as millivolts).

two groups (data not shown), as were immunosuppression, lipid profile, and medications. After steroid withdrawal, no kidney or islet rejections were evident. At 6 months after islet transplantation, when almost all of the patients had completed steroid withdrawal, a significant reduction in A1C was evident (data not shown) in the whole group of patients.

No significant intergroup differences were found for baseline body weight (KI-s 61.3 ± 9.6 vs. KD 62.0 ± 2.2 kg), diabetes duration (KI-s 26.3 ± 10.4 vs. KD 22.2 ± 1.4 years), and dialysis duration (KI-s 42.5 ± 6.2 vs. KD 27.6 ± 4.1 years). Among follow-up data, the main result was the mean creatinine value in the KD group, which increased significantly over baseline at 6 years' follow-up ($P = 0.004$) (Table 1). These results confirm previous reports of a protective effect of transplanted islets on kidney grafts. C-peptide secretion was higher in the KI-s group and absent in the KD group (Table 1); the insulin requirement was lower in the KI-s group than in the KD group (data not shown), with better glycometabolic control (Table 1).

NCV index
No significant differences were observed in pretransplant NCV scores between KI-s and KD groups ($P = 0.6$). Both KI-s and KD groups were neuropathic at baseline according to electroneurographic findings, showing NCV index >2 (e.g., with an NCV score exceeding the mean normal value by >2 SD). The longitudinal NCV index study showed that at the 2-year follow-up both KI-s and KD groups scored a little better than at baseline; at the following time point (4 years after transplant), however, the NCV index continued to improve only in the KI-s group, reaching statistical significance in comparison with pretransplant values at 4 years of follow-up ($P = 0.01$), whereas NCV worsened toward baseline values in the KD group (NS). At the latest follow-up (6 years), the NCV score improvement in comparison to baseline values was maintained in the KI-s group, whereas it worsened further in the KD group (Table 1). When we compared the NCV changes from baseline across the two groups, despite the evidence of a statistically significant difference at the 4-year follow-up in the KI-s group, results were not statisti-

cally significant. In particular, the median change from baseline at each time point showed differences between the KI-s and KD groups that did not reach statistical significance (0.41 vs. −0.08, 0.55 vs. 0.17, and 0.11 vs. −0.03 at the 2-, 4- and 6-year follow-ups, respectively).

Compound motor action potential
In the KI-s group, the longitudinal trend of compound motor action potential (CMAP) mean amplitudes showed slight, although not significant, improvements of either peroneal or ulnar nerves at the 6-year follow-up compared with baseline values. On the contrary, CMAP amplitudes of both nerves progressively declined in the KD group over time; the worsening was statistically significant for ulnar CMAP mean amplitude 6 years after kidney transplantation (9.7 ± 3.0 vs. 13.7 ± 4.6 mV; $P = 0.03$) (Table 1).

Sensory action potential
A slightly improving trend of sensory action potential (SAP) amplitudes through the different time points up to the 6-year follow-up was also recognized in patients of the KI-s group even though statistical
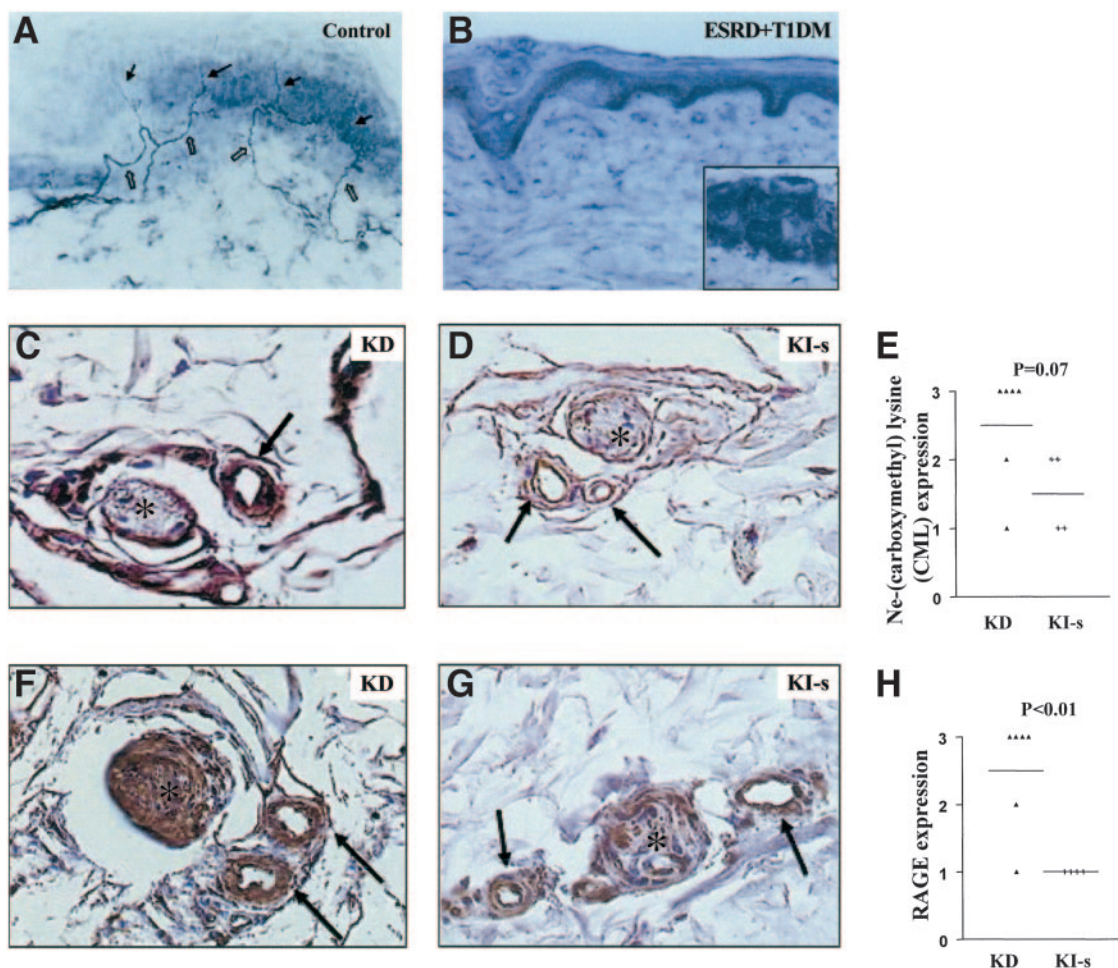
Figure 1— *Comparison with skin biopsies of control patients* (A) *dramatically showed the skin denervation in uremic type 1 diabetic patients* (ESRD+T1DM) (B). *Even sweat glands appeared to be completely denervated* (B, inset). *Immunohistochemical analysis for CML. Paraffin-embedded sections of skin biopsy specimens stained with immunoperoxidase technique. CML was detected by binding to anti-CML monoclonal antibody, biotin-conjugated. C: KD group: peripheral nerve* (∗) *with adjoining blood vessels (vasa nervorum, arrows) in dermis. There is strong* (+++) *staining of the perineurium and vasa nervorum. D: KI-s group: another skin biopsy section with only mild* (+) *staining of the same tissue structures. Original magnification,* ×400. *E: The score for CML expression showed a difference between the two groups despite no statistical significance* (P = 0.07). *Immunohistochemical analysis for RAGE. Paraffin-embedded sections of skin biopsy specimens stained with immunoper-oxidase technique. RAGE was detected by binding to anti-human RAGE goat polyclonal antibody followed by a biotinylated anti-goat IgG antibody. F: KD group: peripheral nerve* (∗) *with adjoining blood vessels (vasa nervorum, arrows) in dermis. There is a strong* (+++) *staining of the bundles of axons and vasa nervorum. G: KI-s group: another skin biopsy section with only mild* (+) *staining of the same tissue structures. Original magnification,* ×400. *H: The score for RAGE expression showed a higher expression of RAGE in the KD group* (P < 0.01).

significance was lacking. In general, this was true for both median and sural nerves, but only sural SAP amplitudes changed from neuropathic to normal values, whereas median SAP values started within the normal range at baseline. In the KD group, both median and sural SAP amplitudes fluctuated slightly through the different time points, but values always remained in a neuropathic spectrum (Table 1).

Regression model
Repeated-measures ANOVA showed that the type of treatment (between-subjects factor) did not influence the NCV index (P = 0.5); in addition, the time of evalu-ation (within-subject factor) and their interaction were not statistically significant (P = 0.4 and 0.4). It is also worthwhile to note that the 2-year response to treatment was maintained at the 4-year follow-up because all of the patients with an improv-ing 2-year NCV index had a further in-crease 2 years later.

Skin biopsy analysis and AGE/RAGE expression
An overall morphological analysis of skin biopsies dramatically showed the effect of diabetes and uremia on skin innervation (Fig. 1). Compared with healthy control subjects (Fig. 1A), the skin of uremic type 1 diabetic patients appeared to be com-pletely denervated. Even the sweat glands showed no evidence of residual innerva-tion (Fig. 1B, inset).

Paraffin-embedded sections of skin biopsy specimens were analyzed for CML and RAGE content by the immunoperox-idase technique. In the KD group (Fig. 1C), the perineurium of peripheral nerves (asterisk) and vasa nervorum (arrows) in dermis showed strong staining for CML, whereas the KI-s group (Fig. 1D) showed only mild staining. The score for CML ex-pression (Fig. 1E) differed between the two groups, although not significantly (P = 0.07).

Likewise, strong RAGE expression was observed in the bundles of axons and

vasa nervorum in the KD group (Fig. 1*F*), whereas only mild staining of the same tissue structures was evident in the KI-s group (Fig. 1*G*). The score for RAGE expression (Fig. 1*H*) confirmed the higher expression of RAGE in the KD than in the KI-s group ($P < 0.01$).

Subanalysis of the KI-s group according to the degree of metabolic control

Nine patients in the KI-s group experienced full function (average 52.0 ± 11.8 months) of transplanted islets, and five of them did not require insulin treatment for >2 years. Of interest, an improvement in NCV index was evident in the group of patients who achieved better glycometabolic control and the withdrawal of insulin therapy. In particular, the NCV index improved in the full function group from a baseline value of -2.9 ± 0.3 to -2.0 ± 0.4 at 4 years after islet transplantation. This tendency was confirmed for sural SAP amplitude too, which improved from 16.2 ± 13.3 at baseline in the full function group to 20.1 ± 17.1 at 4 years after islet transplantation, but was not evident in the group that did not experience full function of the transplanted islets (data not shown).

Limitations of the study

We acknowledge that a randomized trial would be the only way to determine whether islet transplantation can clearly improve diabetic neuropathy and be superior to intensive insulin treatment. The persistence of islet allograft function contributed to improved glycemic control and therefore to less severe diabetes complications. Similar results most probably will be obtained with better glycemic control in the control group, as demonstrated by the Diabetes Control and Complication Trial (5).

CONCLUSIONS — This study provided several indications, coming from both physiological and pathological sources, that islet transplantation may induce long-lasting stabilization of DPN. In further cross-sectional analysis, however, no statistical differences were evident between the two groups. It is likely that the reason for the lack of significance is the small number of patients and the low statistical power for NCV differences over time.

This evidence is first supported by an objective method, electroneurography, which is the most sensitive method for assessing DPN (21). In fact, the NCV index increased in the KI-s group from the first control (2 years), reaching maximum improvement at the 4-year follow-up, before stabilizing at the latest time point (6 years). On the other hand, the NCV index showed some improvement in the KD group as well, but it was short-lived (2 years), as experienced previously (20); then, the NCV index declined toward pretransplant values after the 4-year follow-up and further worsened by 6 years. Because pretransplant variables of age, sex, laboratory values, and electroneurographic findings did not differ at baseline between groups, we can conclude that this result is not affected by a bias in the selection of the groups but is probably due to the efficiency of islet function.

There are several reasons for this favorable trend of DPN in our KI-s group. First, islet function may prevent the well-known nephropathy of the transplanted kidney (11,12), as suggested by longitudinal behavior of creatinine levels (stable in KI-s group and significantly worsening in KD group). Thus, islet transplantation would eliminate an important neuropathic noxa such as chronic renal disease (22). An additional benefit may result from higher levels of C-peptide in the KI-s group, which were reported to improve nerve function in both experimental and clinical settings (23). The feature we want to highlight is that, despite wide and dramatic skin denervation at baseline, a significant reduction in vasa nervorum RAGE expression was found in patients with islet function at the 4-year follow-up, which clearly demonstrates that islet function reverses a primary pathogenetic mechanism specifically related to DPN (2,17).

Intensive insulin treatment was in fact shown to significantly improve peripheral nerve function, both autonomic and sensorimotor, in type 1 diabetic patients, compared with conventional therapy. In the secondary intervention cohort patients, i.e., those who had neuropathy at baseline (as in our population), although less severe, intensive therapy reduced the appearance of clinical neuropathy at 5 years by 57% (5). The injurious effect of chronic hyperglycemia on vessels and nerves has been attributed to various biochemical consequences of intracellular metabolism of excess glucose, including nonenzymatic glycation with formation of AGEs (24). AGEs are heterogeneous compounds originating from precursors formed both nonoxidatively and oxida-

tively; the latter group includes the monolysyl adduct CML, which has been detected in peripheral nerves from diabetic patients (25). In addition to direct, physicochemical effects, such as trapping and cross-linking of macromolecules, AGEs exert indirect, biological effects, mediated by cell surface receptors. RAGE, whose expression is positively regulated by AGEs (26), is the prototypic AGE receptor mediating AGE-induced tissue injury via induction of reactive oxygen species formation and activation of redox-sensitive signaling pathways, and CML is a major RAGE ligand (27). The finding that, despite a comparable, dramatic degree of skin denervation, patients in the KI-s groups showed a significant reduction in RAGE expression (associated with a nonsignificant decrease in CML content) in nerves and vasa nervorum compared with KD patients, supports a role for the downregulation of the AGE-RAGE pathway as a molecular mechanism underlying the improvement of neuropathy observed after successful islet transplantation.

Previous works have already reported beneficial effects of islet transplantation on DPN (24,25), though there are relevant differences from our study. First, both of the above-mentioned studies included type 1 diabetic patients and not type 1 diabetic patients with ESRD. Hence, neuropathy was supposed to be even more severe in our study, and the positive role of islet transplantation is strengthened by our study. Second, peripheral nerve function was assessed previously with NCVs only by Lee et al. (16), but the follow-up period was no longer than 2 years; in the article by Vàrkonyi et al (14), the follow-up was as long as 9.5 years on average, but they evaluated DPN only with a perceptive test of sensory threshold. Therefore, this is the first study based on both nerve conduction and skin biopsy to demonstrate that islet transplantation may induce long-lasting stabilization or even improvement of polyneuropathy in type 1 diabetic patients who received kidney transplants.

Looking at other electroneurographic variables such as the amplitude of both SAPs and CMAPs, which are currently recognized as indicators of axon integrity in peripheral neuropathies (27), we found that CMAP amplitude remained stable throughout the follow-up period in the KI-s group. On the contrary, the amplitude of both peroneal and ulnar nerve CMAPs declined progressively through

the follow-up period in the KD group (with significant differences for the 6-year ulnar nerve CMAP from baseline) (Table 1), as though axonal damage were still progressing after isolated kidney transplant. The amplitude of both sural and median nerve SAPs increased slightly through the different time points in the KI-s group, whereas the same parameter showed a fluctuating pattern in KD patients, suggesting that islet transplant positively affected even sensory nerve fibers; however, this conclusion must be made with caution because of the lack of statistical significance and somewhat heterogeneous baseline values.

Hence, changes of each electroneurographic variable depict distinct aspects of DPN influenced by islet transplantation. In fact, because NCV changes are related to glycemic control (20), improvements in the NCV index indicate an overall improvement of patient nerve function. As NCV mainly reflects pathological processes of large-diameter axons (27), we also included, among electroneurographic variables, the longitudinal analysis of either SAP or CMAP amplitudes, which were suggested as a means of assessing the contribution of smaller slow-conduction nerve fibers (2). These latter data are in keeping with evidence of RAGE expression in vasa nervorum and small skin nerve terminals in our patients. Taken together, electroneurographic and skin biopsy studies allowed longitudinal, long-term assessment of patients with DPN and served as surrogate markers of the restored glycemic control.

In light of the advantages suggested in our article, islet transplantation could become an option for improving quality of life for diabetic patients with brittle diabetes, for those who are unaware of life-threatening hypoglycemia, and even for those with severe, often painful forms of polyneuropathy. More studies with a larger number of patients are required to definitely clarify whether the positive trend observed in our preliminary study can ultimately result in a strong positive association.

In our small group of patients there were no differences with kidney-only transplantation. Islet transplantation in addition to kidney transplantation makes no difference in nerve function compared with kidney transplantation only.

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