Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity

Razvan Gheorghe Radu, Shimpei Fujimoto, Eri Mukai, Mihoko Takehiro, Dai Shimono, Koichiro Nabe, Makiko Shimodahira, Rieko Kominato, Yo Aramaki, Yuichi Nishi, Shogo Funakoshi, Yuihiro Yamada, and Yutaka Seino. Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. Am J Physiol Endocrinol Metab 288: E365–E371, 2005. First published October 12, 2004; doi:10.1152/ajpendo.00390.2004.—Tacrolimus is widely used for immunosuppressant therapy, including various organ transplantations. One of its main side effects is hyperglycemia due to reduced insulin secretion, but the mechanism remains unknown. We have investigated the metabolic effects of tacrolimus on insulin secretion at a concentration that does not influence insulin content. Twenty-four-hour exposure to 3 nM tacrolimus reduced high glucose (16.7 mM)-induced insulin secretion (control 2.14 ± 0.08 vs. tacrolimus 1.75 ± 0.02 ng/islet⁻¹·min⁻¹, P < 0.01) without affecting insulin content. In dynamic experiments, insulin secretion and NAD(P)H fluorescence during a 20-min period after 10 min of high-glucose exposure were reduced in tacrolimus-treated islets. ATP content and glucose utilization of tacrolimus-treated islets in the presence of 16.7 mM glucose were less than in control (ATP content: control 9.69 ± 0.99 vs. tacrolimus 6.52 ± 0.40 pmol/islet, P < 0.01; glucose utilization: control 103.8 ± 6.9 vs. tacrolimus 74.4 ± 5.1 pmol/islet⁻¹·90 min⁻¹, P < 0.01). However, insulin release from tacrolimus-treated islets was similar to that from control islets in the presence of 16.7 mM α-ketoisocaproate, a mitochondrial fuel. Glucokinase activity, which determines glycolytic velocity, was reduced by tacrolimus treatment (control 65.3 ± 3.4 vs. tacrolimus 49.9 ± 2.8 pmol/islet⁻¹·60 min⁻¹, P < 0.01), whereas hexokinase activity was not affected. These results indicate that glucose-stimulated insulin release is decreased by chronic exposure to tacrolimus due to reduced ATP production and glycolysis derived from reduced glucokinase activity.

Tacrolimus (FK-506) is an immunosuppressant widely used in human organ transplantation. Immunosuppression by the agent is due to blocking of antigen-stimulated expression of genes, including interleukin-2 in T lymphocytes, which is required for T-cell proliferation (34). Interleukin-2 gene transcription is activated by dephosphorylation and nuclear translocation of a transcriptional cofactor, the nuclear factor of activated T cells (NFAT). Tacrolimus binds specific intracellular proteins, FK-506-binding proteins (FKBPs), and inhibits calcineurin (protein phosphatase-2B), a Ca²⁺/calmodulin-dependent Ser/Thr phosphatase (7, 28) that dephosphorylates and translocates NFAT to nuclei (20, 37). Thus tacrolimus suppresses interleukin-2 gene transcription by inhibiting the calcineurin/NFAT pathway.

The main adverse reaction in tacrolimus-treated patients is hyperglycemia due to reduced insulin release (6, 16, 26). The mechanism of the inhibitory effect of tacrolimus on insulin release has been described. Tacrolimus markedly reduces the number of endocrine secretory granules in human pancreatic β-cells, which correlates with the blood concentration of the agent and is reversible by reduction of the dose (5, 15). The agent decreases insulin release, insulin content, and insulin mRNA concentration dependently in experiments using cell lines, rat islets, and human islets (27, 30, 31, 32). Tacrolimus also suppresses insulin gene expression, which is induced by intracellular Ca²⁺ elevation via the calcineurin/NFAT pathway (17). Thus reduced insulin content due to reduced insulin synthesis plays an important role in the inhibitory effect of tacrolimus on insulin release.

The mechanism of glucose-stimulated insulin release from pancreatic β-cells has been well documented. Glucose stimulates insulin secretion by both triggering and amplifying signals in pancreatic β-cells (14). The triggering pathway includes entry of glucose into β-cells, acceleration of glycolysis in cytosol and glucose oxidation in mitochondria, an increase in ATP content and ATP-to-ADP ratio, closure of ATP-sensitive K⁺ (KATP) channels, membrane depolarization, opening of voltage-dependent Ca²⁺ channels (VDCCs), an increase in Ca²⁺ influx through VDCCs, raised intracellular Ca²⁺ concentration ([Ca²⁺]), and exocytosis of insulin granules. It has been reported that glucose also enhances insulin secretion by KATP channel independently. The KATP channel-independent amplifying action of glucose has been confirmed by treatment of β-cells with diazoxide, which prevents KATP channels from closing, and with a depolarizing concentration of extracellular K⁺ that restores Ca²⁺ influx (1, 12). Because glucose does not increase [Ca²⁺], but nevertheless augments insulin release under these conditions, glucose may well exert its effects by increasing Ca²⁺ efficacy in stimulation-secretion coupling, due at least in part to the direct effect of increased ATP derived from glucose metabolism on exocytosis (14). Thus glucose metabolism in β-cells plays a crucial role in glucose-induced insulin release. However, the effect of tacrolimus on glucose-induced insulin release and glucose metabolism has not been examined in detail.

Address for reprint requests and other correspondence: S. Fujimoto, Dept. of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto Univ., 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan (E-mail: fujimoto@metab.kuhp.kyoto-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We have investigated the effects of chronic exposure to tacrolimus on insulin release by functional analysis of the metabolic responses of tacrolimus-treated islets.

MATERIALS AND METHODS

Materials. Tacrolimus (FK-506) was donated by Fujisawa Pharmaceutical (Tokyo, Japan). RPMI 1640 and glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Sigma (St. Louis, MO). Luciferin-luciferase was obtained from Turner Designs (Sunnyvale, CA). \(^{1} \text{H}_2 \text{O}\) and \([5-\text{H}^{3}]\text{glucose}\) were obtained from Amersham (Buckinghamshire, UK). All other agents were obtained from Nacalai Tesque (Kyoto, Japan).

Animals. Male Wistar rats weighing 180–230 g were obtained from Shimizu (Kyoto, Japan). The animals were fed standard lab chow ad libitum and allowed free access to water in an air-conditioned room with a 12:12-h light-dark cycle until the experiments. All experiments were carried out with rats aged 8–12 wk. The animals were maintained and used in accordance with the Guidelines for Animal Experiments of Kyoto University.

Islet isolation and culture. Islets of Langerhans were isolated from Wistar rats by collagenase digestion, as described previously (10). Isolated islets were cultured for 24 h in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5.5 mM glucose with or without tacrolimus at 37°C in humidified air containing 5% CO₂.

Measurement of insulin release from isolated rat pancreatic islets. Insulin release from intact islets was monitored using either batch incubation or a perfusion system described previously, using Krecs-Ringer bicarbonate buffer (KRBB) supplemented with 0.2% bovine serum albumin (BSA; fraction V) and 10 mM HEPES, adjusted to pH 7.4 (KRBB medium) (8).

For static incubation experiments, cultured islets were preincubated at 37°C for 30 min in KRBB medium supplemented with 2.8 mM glucose. Groups of five islets were then batch incubated for 30 min in 0.7 ml of KRBB medium with test materials. At the end of the incubation period, the islets were pelleted by centrifugation, and aliquots of the buffer were sampled.

For perfusion experiments, groups of 20 cultured islets were placed in each of the parallel chambers of a perfusion apparatus and perfused with KRBB medium at a rate of 0.7 ml/min at 37°C. The medium was continuously gassed with 95% O₂-5% CO₂. The islets were perfused for 30 min to establish a stable insulin secretory rate at a basal level of glucose and then exposed to the stimulating levels of glucose.

The amount of immunoreactive insulin was determined by RIA with rat insulin as standard.

Measurement of insulin and DNA content. Insulin contents and DNA contents of islets were measured as previously described (10). Briefly, after an aliquot of incubation medium for insulin release assay was taken, the islets remaining were lysed. Insulin content was determined by RIA using rat insulin as standard.

Measurement of DNA contents of islets was measured as previously described (10). Briefly, after groups of cultured islets were preincubated at 2.8 mM glucose for 30 min, groups of 15 islets were batch incubated in 0.5 ml of KRBB medium containing 2.8 or 16.7 mM glucose at 37°C for 30 min. Incubation was stopped by the addition of 0.1 ml of 2 M HClO₄. The tubes were immediately mixed with vortex and sonicated in ice-cold water. They were then centrifuged, and a fraction (0.4 ml) of the supernatant was mixed with 0.1 ml of 2 M HEPES and 0.1 ml of 1 M Na₂CO₃. The ATP concentration was measured by adding 0.2 ml of luciferin-luciferase solution to a fraction sample (0.1 ml) in a bioluminometer (Luminometer model 20e; Turner Designs). To draw a standard curve, blanks and ATP standards were run through the entire procedure, including the extraction steps.

Measurement of glucose utilization. Glucose utilization was measured, using a previously described method (2) with a slight modification. Cultured islets were preincubated in KRBB medium with 2.8 mM glucose at 37°C for 30 min. Batches of 30 islets for each condition were incubated at 37°C for 90 min in 150 μl of medium containing 1.5 μCi of \([5-\text{H}^{3}]\text{glucose}\). Aliquots of the incubation medium (100 μl) and 20 μl of 1 M HCl were transferred into small tubes and placed into a glass vial containing 0.5 ml of H₂O. The capped vials were incubated overnight at 37°C to vaporize H₂O from the solution. The inner tube was then lifted out, and the disintegrations per minute of water-melting H₂O in the vial were counted. In a parallel incubation, the recovery ratio of H₂O was measured using 3H₂O. After subtracting blank disintegrations per minute from sample disintegrations per minute, glucose utilization was calculated using the disintegrations per minute, specific radioactivity of [5-\text{H}^{3}]\text{glucose}, and recovery ratio of H₂O.

Measurement of glucokinase and hexokinase activity. Glucokinase activity was measured by a fluorometric assay according to a method reported previously (25, 38). After cultured islets were preincubated with KRBB medium with 2.8 mM glucose, 100 islets were homogenized in 250 μl of solution containing 20 mM KH₂PO₄, 100 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5% glycerol, and 1 mM DT (pH 7 by KOH), and the supernatants (islet extracts) were obtained from the homogenates by centrifugation (10,000 g, 15 min) at 4°C. The glucose phosphorylation rate was estimated as the increase in NADH through the following reaction: glucose-6-phosphate + NAD → 6-phosphoglucono-6-lactone + NADH by NAD-dependent G6PDH. The enzyme reaction was performed using 25 μl of islet extracts in a 160-μl solution consisting of 50 mM HEPES (pH 7.4 by NaOH), 100 mM KCl, 8 mM MgCl₂, 0.5 mM NAD, 5 mM ATP, 1 mM DT, and 1 U/ml G-6-PDH supplemented with two concentrations (50 mM and 0.5 mM) of glucose at 37°C for 1 h; it was stopped by adding 290 μl of stopping solution (300 mM Na₂HPO₄, 0.46 mM SDS, pH 8.0). NADH concentration was measured by fluorometry (Shimazu RF-5000, Kyoto, Japan) at a 340-nm excitation and a 450-nm emission. Blanks in the absence of ATP were incubated in a parallel experiment and were subtracted from the total fluorescence of the corresponding complete reaction mixtures. Glucokinase activity was determined by subtracting hexokinase activity measured at 0.5 mM glucose from the activity measured at 50 mM glucose.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by unpaired Student’s t-test. P < 0.05 was considered significant.

RESULTS

Effect of chronic exposure to tacrolimus on glucose-induced insulin release, insulin content, and DNA content in islets. Chronic (24 h) exposure to tacrolimus (3–30 nM) concentrations dependently suppressed 16.7 mM glucose-induced insulin release but did not affect basal insulin release in the presence of 2.8 mM glucose (Table 1). Insulin content was significantly reduced by 24-h exposure to 10 and 30 nM tacrolimus (Table 2). However, 24-h exposure to 3 nM tacroli-
Table 1. Concentration-dependent effect of chronic exposure to tacrolimus on glucose-induced insulin release from pancreatic islets

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Basal (2.8 mM glucose)</th>
<th>Stimulated (16.7 mM glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.02</td>
<td>2.14±0.08</td>
</tr>
<tr>
<td>3 nM Tacrolimus</td>
<td>0.26±0.03</td>
<td>1.75±0.02*</td>
</tr>
<tr>
<td>10 nM Tacrolimus</td>
<td>0.24±0.02</td>
<td>1.53±0.04*</td>
</tr>
<tr>
<td>30 nM Tacrolimus</td>
<td>0.21±0.02</td>
<td>1.49±0.03*</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 16 determinations from several experiments. Inslets were cultured under the conditions indicated for 24 h. After cultured islets were preincubated with 2.8 mM glucose for 30 min, insulin release in the presence of 2.8 and 16.7 mM glucose for 30 min was determined. *P < 0.01 vs. control (cultured without tacrolimus).

Table 2. Effect of chronic exposure to tacrolimus on insulin content and DNA content

<table>
<thead>
<tr>
<th>Experimental Condition During Culture</th>
<th>Insulin Content, ng/islet</th>
<th>DNA Content, ng/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.0±0.7</td>
<td>19.3±0.7</td>
</tr>
<tr>
<td>3 nM Tacrolimus</td>
<td>26.2±1.0</td>
<td>21.1±0.8</td>
</tr>
<tr>
<td>10 nM Tacrolimus</td>
<td>22.4±1.1*</td>
<td>20.1±1.0</td>
</tr>
<tr>
<td>30 nM Tacrolimus</td>
<td>20.4±0.8*</td>
<td>19.9±1.0</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 16 determinations from several experiments. At the end of the experiments indicated in Table 1, insulin content and DNA content were determined, using islets in a randomly selected half of the batches. *P < 0.01 vs. control (cultured without tacrolimus).

Fig. 1. High (16.7 mM) glucose-induced biphasic insulin release in control and tacrolimus-treated islets. Islets were cultured with or without 3 nM tacrolimus for 24 h. After cultured islets were perfused with 2.8 mM glucose (G2.8) for 30 min, they were stimulated with 16.7 mM glucose (G16.7) at time 0 for 30 min. A: time course of high glucose-induced biphasic insulin release in control and tacrolimus-treated islets. Values represent means ± SE of 6 determinations. All values in tacrolimus-treated islets after 10 min of exposure to high glucose were significantly less than the corresponding values in control (P < 0.01). B: average values calculated from the data in A. Average values during indicated time period after high-glucose exposure in control (open bar) and tacrolimus-treated islets (solid bar) are shown. *P < 0.01 vs. corresponding control.

Table 3. Reversible effect of tacrolimus on glucose-induced insulin release from islets

<table>
<thead>
<tr>
<th>Experimental Condition During Culture</th>
<th>Basal (2.8 mM glucose)</th>
<th>Stimulated (16.7 mM glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (24-h no tacrolimus)</td>
<td>0.26±0.02</td>
<td>2.15±0.09</td>
</tr>
<tr>
<td>24-h Tacrolimus</td>
<td>0.26±0.02</td>
<td>1.65±0.06*</td>
</tr>
<tr>
<td>12-h No Tacrolimus + 12-h tacrolimus</td>
<td>0.24±0.01</td>
<td>1.72±0.07*</td>
</tr>
<tr>
<td>12-h Tacrolimus + 12-h no tacrolimus</td>
<td>0.28±0.03</td>
<td>2.24±0.09†</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 8 determinations from several experiments. Two groups of islets were cultured with or without 3 nM tacrolimus for 24 h (control and 24-h tacrolimus group). One group of islets was cultured without tacrolimus for the first 12 h and with 30 nM tacrolimus for the second 12 h (12-h no tacrolimus + 12-h tacrolimus group). Another group of islets was cultured with 30 nM tacrolimus for the first 12 h and without tacrolimus for the second 12 h (12-h tacrolimus + 12-h no tacrolimus group). After cultured islets were preincubated with 2.8 mM glucose for 30 min, insulin release in the presence of 2.8 and 16.7 mM glucose for 30 min was determined. *P < 0.01 vs. control (24-h no tacrolimus). †P < 0.01 vs. 24-h tacrolimus.
high glucose in both groups of islets and reached a plateau within 10 min after exposure (Fig. 2A). A significant attenuation of NAD(P)H fluorescence was observed from 10 min in tacrolimus-treated islets (at 10 min: tacrolimus 124.9 ± 1.3, n = 11, vs. control 129.3 ± 1.1%, n = 12, P < 0.05; Fig. 2A).

Mean NAD(P)H fluorescence during the first 10 min after high-glucose exposure was not affected, whereas that during the second and third 10-min period was suppressed in tacrolimus-treated islets [mean NAD(P)H fluorescence from 20 to 30 min: tacrolimus 124.5 ± 1.2, n = 11, vs. control 133.9 ± 1.4%, n = 12, P < 0.01; Fig. 2B].

Effect of tacrolimus on ATP content. ATP content was greater in islets incubated with 16.7 mM glucose than in islets incubated with 2.8 mM glucose in both control (2.8 mM glucose, 5.22 ± 0.51, vs. 16.7 mM glucose, 9.69 ± 0.99 pmol/islet, n = 10, P < 0.01) and tacrolimus-treated islets (2.8 mM glucose, 4.75 ± 0.50, vs. 16.7 mM glucose, 6.52 ± 0.40 pmol/islet, n = 10, P < 0.05). ATP content in the presence of 16.7 mM glucose was significantly reduced in tacrolimus-treated islets (P < 0.01) but in the presence of 2.8 mM glucose was not affected by tacrolimus (Fig. 3A).

Effect of tacrolimus on glucose utilization. Glucose utilization was greater in islets incubated with 16.7 mM glucose than in islets incubated with 2.8 mM glucose in both control (2.8 mM glucose, 35.1 ± 6.1, vs. 16.7 mM glucose, 103.8 ± 6.9 pmol/islet−1·90 min−1, n = 9, P < 0.01) and tacrolimus-treated islets (2.8 mM glucose, 24.6 ± 3.7, vs. 16.7 mM glucose, 73.4 ± 5.1 pmol/islet−1·90 min−1, n = 9, P < 0.01). Glucose utilization in the presence of 16.7 mM glucose was significantly reduced in tacrolimus-treated islets (P < 0.01) but in the presence of 2.8 mM glucose was not affected by tacrolimus (Fig. 3B).

Effect of tacrolimus on glucokinase and hexokinase activity. Glucokinase activity was significantly decreased by the tacrolimus treatment (tacrolimus 49.9 ± 3.4 vs. control 65.3 ± 3.4 pmol-islet−1·60 min−1, n = 10, P < 0.01), whereas hexokinase activity was not affected (tacrolimus 35.5 ± 2.8 vs. control 34.1 ± 2.1 pmol-islet−1·60 min−1, n = 10, not significant; Fig. 3C).

DISCUSSION

In the present study, we show that a low concentration of tacrolimus suppresses high glucose-induced insulin secretion from pancreatic islets without affecting insulin content. This inhibitory effect of tacrolimus is the result of reduced ATP production and glycolysis due to decreased glucokinase activity.

We found that 3 nM tacrolimus, a lower concentration than used in previous reports (31, 32, 41), significantly decreased glucose-induced insulin release after 24-h exposure without affecting insulin content, which indicates that reduced insulin release by tacrolimus is not necessarily derived from reduced insulin content. To investigate the mechanism of reduced insulin release by tacrolimus independent of reduced insulin content, we used 3 nM tacrolimus-treated islets. Recommended trough concentrations of tacrolimus in blood are 3–6 ng/ml (3.6–7.2 nM) and 5–15 ng/ml (6.1–18.2 nM) in islet transplantation (36) and in liver transplantation (3), respectively.
EFFECT OF TACROLIMUS ON INSULIN RELEASE

The mitochondrial membrane generated by transport of high-energy electrons in the respiratory chain. These electrons are derived from NADH and FADH₂, which are derived from the TCA cycle in the matrix and/or transferred from the cytosol by the shuttle system. A reduced supply of substrates to mitochondria results in a decreased H⁺ gradient across the mitochondrial membrane. Glucose utilization reflects the velocity of glycolysis (24), and NAD(P)H autofluorescence dominantly reflects the redox state of mitochondria (29). Because both glucose utilization and NAD(P)H fluorescence in the presence of high glucose were decreased in tacrolimus-treated islets, the reduced ATP content in these islets may well be attributable to a decreased supply of reduced equivalents to mitochondria.

In pancreatic islets, KIC is oxidized, enhancing ATP production and triggering insulin release (21). However, the mechanism of KIC-induced insulin release is not fully understood, and two distinct mechanisms are proposed. First, KIC, which is converted to acetyl-CoA via a branched-chain α-ketoacid dehydrogenase (BCKDH)-dependent pathway, enters into the TCA cycle and is oxidized (18, 19). Second, KIC, together with endogenous glutamate, is converted to α-ketoglutarate via glutamate-ketoacid transaminase (GKAT), which enters into the TCA cycle and is oxidized (11). Because both BCKDH and GKAT are mitochondrial enzymes, KIC should be metabolized within mitochondria without affecting cytosolic glycolysis. Our results show that mannoheptulose, a glucokinase inhibitor (39), and iodoacetate, a glycoldehyde-3-phosphate dehydrogenase inhibitor (35), both of which inhibit glycolysis, decreased glucose-induced insulin release but did not affect KIC-induced insulin release. These results are compatible with the notion that KIC is metabolized within mitochondria without affecting cytosolic glycolysis. Accordingly, KIC-induced insulin release was examined to clarify the effect of tacrolimus on mitochondrial metabolism independent of glycolysis. Because tacrolimus reduced glucose-induced insulin release but did not affect KIC-induced insulin release, the decreased glucose metabolism should be derived from reduced glycolysis.

Glucokinase is a rate-limiting enzyme in glycolysis and serves as glucose sensor in pancreatic β-cells (22). Regulation of the enzyme has a central impact on β-cell function, as demonstrated in studies of glucokinase mutations identified in patients with maturity onset diabetes of the young (42). The activity of the enzyme is reduced in tacrolimus-treated islets and presumably plays a primary role in reducing glucose-induced insulin release without a decrease in insulin content by the agent.

Recently, Uchizono et al. (41) reported an effect of chronic exposure to tacrolimus (mainly 100 nM) on the mechanism of insulin release under reduced insulin content. Interestingly, glucose-induced insulin release was found to be more profoundly suppressed than mitochondrial fuel-induced insulin release, a phenomenon clarified by our results. They assert that tacrolimus impairs glucose-induced insulin release and has effects distal to the rise in intracellular Ca²⁺, which could be explained by a reduced ATP level by the agent, as ATP directly enhances Ca²⁺ efficacy in the exocytotic system of insulin release (9, 33, 40).

Glucokinase activity in pancreatic β-cells is regulated on both translational and posttranslational levels, but the mechanism remains largely unknown (23). Further investigation of suppression of glucokinase activity by tacrolimus may clarify the regulation of glucokinase activity in pancreatic β-cells.
ACKNOWLEDGMENTS

We thank S. Akagi for technical assistance.

GRANTS

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grants for Leading Project for Bioimaging from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Health and Labor Sciences Research Grants for Research on Human Genome, Tissue Engineering Food Biotechnology, from the Ministry of Health, Labor and Welfare of Japan; Health and Labor Sciences Research Grants for Comprehensive Research on Aging and Health from the Ministry of Health, Labor and Welfare of Japan; and Establishment of Core of Excellence (COE) for Integration of Transplantation Therapy and Regenerative Medicine (COE program of the Ministry of Education, Culture, Sports, Science and Technology of Japan).

REFERENCES


