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KRP-203 Is a Desirable Immunomodulator for Islet Allotransplantation

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Background. The current standard immunosuppressive regimens, calcineurin inhibitors, have diabetogenic and antivascularization effects on islet grafts. KRP-203, a sphingosine-1-phosphate functional antagonist, exerts its immunomodulatory function through lymphocyte sequestration. However, the effect of this antagonist on islets is unclear. We examined the effect of KRP-203 on the islet function and vascularization and sought a calcineurin-free regimen for islet allotransplantation. **Methods.** KRP-203 was administered for 14 d to mice, then diabetogenic effect was evaluated by blood glucose levels and a glucose tolerance test. Static glucose stimulation, the breathing index, and insulin/DNA were examined using isolated islets. Islet neovascularization was evaluated using a multiphoton laser scanning microscope. After islet allotransplantation with either KRP-203 alone, sirolimus alone, or both in combination, the graft survival was evaluated by blood glucose levels and immunohistochemical analyses. A mixed lymphocyte reaction was also performed to investigate the immunologic characteristics of KRP-203 and sirolimus. **Results.** No significant differences in the blood glucose levels or glucose tolerance were observed between the control and KRP-203 groups. Functional assays after islet isolation were also comparable. The multiphoton laser scanning microscope showed no inhibitory effect of KRP-203 on islet neovascularization. Although allogeneic rejection was effectively inhibited by KRP-203 monotherapy (44%), combination therapy prevented rejection in most transplanted mice (83%). **Conclusions.** KRP-203 is a desirable immunomodulator for islet transplantation because of the preservation of the endocrine function and lack of interference with islet neovascularization. The combination of KRP-203 with low-dose sirolimus may be promising as a calcineurin-free regimen for islet allotransplantation.

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INTRODUCTION

Islet transplantation is a promising therapy for type 1 diabetes. In cell therapy, neovascularization of the graft after transplantation has decisive influence over the outcome. Current immunosuppressive regimens commonly include calcineurin inhibitors because of their efficiency in preventing graft rejection. However, both tacrolimus and cyclosporin A (CsA) have well-known diabetogenic effects in both small animal and clinical studies.[1,2] In addition, it has been shown that tacrolimus inhibits islet revascularization in a dorsal skinfold chamber (DSC) model.[3] Therefore, more islet-friendly regimens are necessary.

Fingolimod (FTY720), a nonselective sphingosine-1-phosphate (S1P) receptor agonist (functional antagonist)

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has previously shown promising results in preventing rejection of porcine fetal islet-like cell cluster xenotransplants in rat models, in combination with CsA. This combination, unlike other regimens, allowed adult porcine islets to maintain normoglycemia after xenotransplantation into a diabetic rat model for 53 ± 15.8 d.[4] Considering that adult porcine islets are extremely fragile and CsA is well-known to have detrimental effects on islet grafts, FTY720 may be useful for not only regulating immune reactions but also maintaining the islet function. However, the influence of this novel immunomodulator on the diabetogenic effect and islet neovascularization is unclear.

KRP-203 is an S1P agonist that shares molecular similarity with FTY720. Its selective action on S1P1 and S1P5 decreases the adverse effects in comparison to FTY720. For example, a 10-fold higher dose of KRP-203 was required in rats to cause transient bradycardia than that needed for FTY720.[5-7] In a rat model, KRP-203 alone prolonged the skin and cardiac graft survival after transplantation across minor histocompatibility antigen disparity, and it prolonged the cardiac and renal allograft survival after transplantation across major histocompatibility antigen disparity in combination with a subtherapeutic CsA dose.[5,7] However, its application for islet transplantation has been limited. Only 1 study showed that postgrafting short-term exposure to KRP-203 for 7 d resulted in the long-term survival of the renal subcapsular islet graft for >200 d in 5 of 12 Balb/c recipients (low-responder model) when a 3 mg/kg dose was administered.[8]

A recent study showed synergism between FTY702 and sirolimus for ameliorating inflammation and clinical scores in a murine model of experimental autoimmune encephalomyelitis.[9] Therefore, the potential utility of this combination regimen for preventing islet allograft rejection should be examined.

The present study therefore evaluated the effect of KRP-203 on the islet function and vascularization in search of a calcineurin-free regimen for islet allotransplantation (Figure 1).

MATERIALS AND METHODS

Animals

Male C57BL/6J, Balb/c, and Balb/c nu/nu mice were purchased from Japan SLC Inc., (Shizuoka, Japan). For all experiments, recipients were 8–10 wk old, and donors were 10–14 wk old. The recipient and follow-up mice were housed separately. All animals had free access to a standard diet and water. The experiments were approved by the local ethics committee (approved protocol ID: 2018 Medical-Animal-129) and performed in accordance with national and institutional regulations. Animals were maintained in a pathogen-free environment.

All surgeries were performed under anesthesia, and all efforts were made to minimize suffering. Male C57BL/6-Tg (CAG-EGFP) mice (9–12 wk old; Japan SLC Inc.) were used as islet donors for neovascularization experiments.

KRP-203 and Sirolimus Preparation

KRP-203 (Kyorin Pharmaceuticals Co., Ltd., Tokyo, Japan) was dissolved in sterile 0.5% methylcellulose (MC; Wako Pure Chemical Industries, Osaka, Japan) at 0.3 mg/mL by repeated vortex-mixing and sonication, and was further diluted to 0.1 mg/mL for some experiments. The solution was preserved for 1 wk at 4 °C. MC of 0.5% was used as a control vehicle.

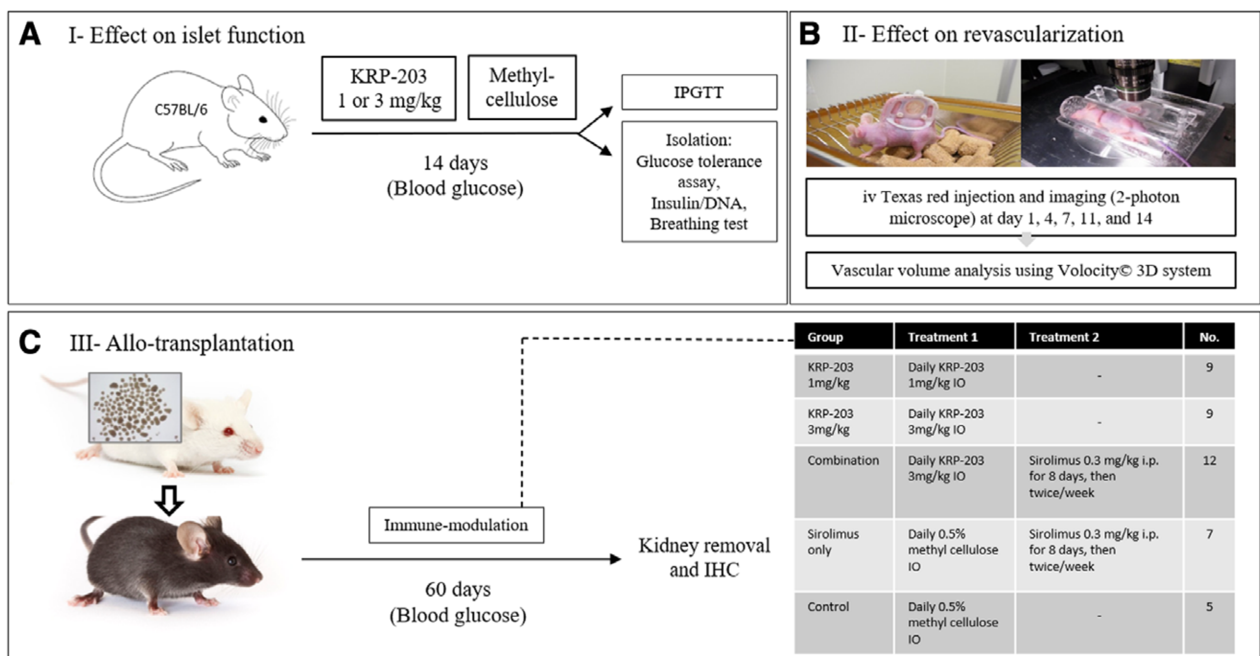


FIGURE 1. A schematic representation of the study methodology. A, Effect of KRP-203 on islet function, in comparison to vehicle (0.5% methylcellulose), was examined after daily administration to C57BL/6 mice for 14 d, through IPGTT and islet isolation and in vitro analysis. B, Effect of KRP-203 on islet revascularization was examined using a dorsal skinfold chamber model, in comparison to vehicle. C, In vivo immune-modulatory effect of KRP-203, alone or in combination with low-dose sirolimus, was investigated using Balb/c to C57BL/6 islet allotransplantation model. The different treatment regimens are summarized in the associated table. IHC, immunohistochemistry; IO, intraosseous infusion; i.p., intraperitoneal; IPGTT, intraperitoneal glucose tolerance test.

Sirolimus (LC Laboratories, MA) was dissolved in 70% ethanol to a concentration of 2 mg/mL. Aliquots were prepared and stored at −20 °C. On the day of injection, aliquots were diluted to 0.3 mg/mL using sterile distilled water.

Evaluating the Effect of KRP-203 on the Islet Function

C57BL/6J mice received KRP-203 (1 or 3 mg/kg) or vehicle (0.5% MC) daily through intragastric gavage (n = 7 per group). The blood glucose level (BGL) was monitored using a portable glucometer (Freestyle, Abbott, Tokyo, Japan) for 14 d, after which either the intraperitoneal glucose tolerance test (IPGTT) or islet isolation and subsequent in vitro functional analyses using isolated islets were performed.

Intraperitoneal Glucose Tolerance Test

The IPGTT was performed on day 15 of treatment as described previously[10] (n = 6 per group). In brief, after fasting for 14 h with free access to water, the BW and BGL were measured, and 1 g/kg glucose was injected intraperitoneally (i.p.). The blood glucose was measured at 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min, and the blood glucose curve was generated, and the area under the curve was used for the comparison. Blood samples were collected at 0 and 20 min in a separate group of mice undergoing IPGTT, and serum was separated by centrifugation for 1.5 min at 15 000*g*. Serum samples were stored at −80 °C until analyses. The C-peptide levels were measured using a mouse C-peptide ELISA kit according to the manufacturer's instructions (n = 7/group; Morinaga, Yokohama, Japan).

Islet Isolation for In Vitro Functional Analyses

Islet isolation was performed as previously described.[11] On day 15, KRP-203-treated (3 mg/kg) and control mice were anesthetized by isoflurane inhalation (Abbott Japan Co., Ltd., Tokyo, Japan). The bile duct was identified and clamped at the papilla of Vateri. Cold Hank's balanced salt solution (2 mL) containing 1 mg/mL collagenase (Sigma type V; Sigma Chemicals, St. Louis, MO) was injected into the common bile duct leading to the pancreas under a stereomicroscope. The pancreas was removed and incubated in a water bath at 37 °C for 12 min before being digested, and the cell suspension was washed 3 times in cold Hank's balanced salt solution and centrifuged for 1 min. Density-gradient centrifugation was performed for 10 min using a Histopaque-1119 (Sigma Diagnostics, St. Louis, MO) and Lymphoprep (Axis-Shield, Oslo, Norway) to isolate pancreatic islets. The islets were cultured in RPMI-1640 containing 5.5 mmol/L glucose and 10% fetal bovine serum at 37 °C in 5% CO_2 and humidified air overnight before the examination.

Static Glucose Stimulation Test

A static glucose stimulation test was performed on the cultured islets as described previously with some modifications.[12] Twenty islets were hand-picked, washed, and incubated for 150 min in a Krebs-Ringer bicarbonate solution containing 1.67 mmol/L glucose at 37 °C. Thereafter, the solution was changed and the islets were incubated for 90 min in 16.7 mmol/L glucose at 37 °C. The supernatant was collected, centrifuged, and stored at −80 °C. Subsequently, the insulin content in the supernatants was analyzed using an ELISA (Mercodia Mouse Insulin ELISA kit; Mercodia AB, Uppsala, Sweden) and expressed as pmol/20 islets (1 pmol = 5.56 ng of insulin). The stimulation index was defined as the ratio of the total amount of insulin secreted during high glucose stimulation (16.7 mmol/L) and that released during low glucose stimulation (1.67 mmol/L) (n = 4 per group).

Insulin/DNA

Twenty hand-picked islets were subjected to sonication in 200 μL of DNA buffer, and their insulin and DNA content were determined. Briefly, 50-μL samples were transferred to tubes containing 125 μL of acid ethanol (0.18 M HCl in 95% [v/v] ethanol), and insulin was extracted overnight at 4 °C. Insulin levels (pmol) were then measured using an insulin ELISA kit (Mercodia). The DNA content (μg) was measured in duplicate 50-μL samples of the aqueous homogenate using a fluorescence DNA quantification kit (AK-06; Cosmo Bio, Tokyo, Japan), and absorbance was measured using a Glomax 20/20 luminometer (Promega; n = 5 per group). The test was performed on paired samples on 2 occasions.

Islet Respiratory Activity

We evaluated the islet respiratory activity using scanning electrochemical microscopy as described previously.[11,13] In brief, the respiratory activity of 5 hand-picked islets in each group was calculated by evaluating the difference in the reduction current around the samples using 2–4 μm platinum-coated microelectrodes. The glucose-stimulated respiratory activity was indicated by the stimulation index of the respiratory activity, defined as the ratio of the respiratory activity at a high glucose concentration (16.7 mmol/L) against that at the basal glucose concentration (1.67 mmol/L) (n = 4 per group).

Measurement of Trough Levels of KRP-203

Twenty-three hours after the last dose of KRP-203 (1 or 3 mg/kg) or vehicle (n = 8, 7, and 4, respectively), blood samples from randomly selected mice were collected from the inferior vena cava. Plasma was separated and the analysis was performed using liquid chromatography with tandem mass spectrometry, [See **Methods S1,** http://links.lww.com/TP/C258, for detailed methodology].

Evaluating the Effect of KRP-203 on Islet Neovascularization

DSC Implantation

A DSC, composed of polyacetal resin (generally known as Duracon; grade M90-44, Polyplastics Co., Ltd., Tokyo, Japan), was introduced into the recipient mice as described previously.[3] A DSC was implanted into male Balb/c nu/nu mice (8–12 wk old; Japan SLC Inc.). This model, unlike wild-type Balb/c mice, supported a relatively long observation period and the assessment of the effect of KRP-203 on engraftment and vascularization without any interference from the specific immune responses.

Islet Transplantation into the DSC

Pancreatic islets from C57BL/6-Tg (CAG-EGFP) mice were isolated as described above and cultured for 24 h. Balb/c nu/nu mice underwent islet transplantation into the

DSC without anesthesia 1 d after DSC implantation. Seven to 12 islets were transplanted into each DSC by hand-picking. The space between the skin flap and cover glass was filled with saline to remove air bubbles.

Experimental Groups

The recipient mice with transplanted pancreatic islets were divided into 2 groups: the control group (0.5% MC) and the KRP-203-treated groups (3 mg/kg/d). KRP-203 or vehicle was administered daily to mice via the intragastric route for 14 d starting from the day of transplantation. Plasma samples were collected as described above from both groups to measure the trough levels of KRP-203 at day 15.

In Vivo Imaging of Islets Transplanted into the DSC

Imaging and image analyses were performed as described previously[3] (control group: n = 7, KRP203 group: n = 8) (See **Methods S1, SDC,** http://links.lww.com/TP/C258, for detailed methodology).

Evaluating the Effect of KRP-203 for Preventing Islet Allogenic Rejection

In Vivo Transplant Model

The pancreata of donor Balb/c mice were digested and islets were isolated as described before. Six hundred islet equivalents of overnight cultured islets were transplanted under the left kidney capsule of diabetic C57BL/6J recipients that had been injected with intravenous streptozotocin (170 mg/kg; Sigma-Aldrich, St. Louis, MO) 7 d earlier. Diabetes was defined by glucose levels of ≥400 mg/dL on 2 consecutive measurements. BGLs between 100–250 mg/dL were considered normal. BGLs were measured using tail vein samples for the first 3 d then twice a week using a portable glucometer (Freestyle). Nephrectomy was performed at day 60 posttransplant or at the time of rejection in rejected grafts. Graft-bearing kidneys were fixed in 4% formaldehyde and embedded in paraffin. Sections were prepared from the graft site followed by hematoxylin–eosin staining and immunohistochemical staining for the microscopic analysis. Two consecutive glucose measurements of >300 mg/dL were considered the criterion for rejection.

Immunomodulation Protocol

KRP-203 groups: KRP-203 was administered by oral gavage at 1.0 or 3.0 mg/kg daily starting 1 d before transplantation (n = 9 per group).

KRP-203 control group: received 0.5% MC alone as a vehicle (n = 5).

Combination group: for the combination calcineurin-free regimen, 3.0 mg/kg of KRP-203 was administered daily starting 1 d before transplantation in addition to a short course of low-dose sirolimus (0.3 mg/kg) via i.p. injection from day −1 to day 7 (n = 12), followed by twice-weekly administration.

Sirolimus-only group: 0.5% MC was given instead of KRP-203, in addition to the same sirolimus regimen as the combination group (n = 7).

Measurement of Trough Levels of KRP-203 and Sirolimus

Representative plasma samples were collected for assessing KRP-203 trough levels at the study end point or at the time of rejection. Whole-blood samples for assessing sirolimus trough levels were collected around day 30 posttransplantation in EDTA-coated tubes, and the trough levels were measured using liquid chromatography/electrospray ionization tandem mass spectrometry as described previously.[14]

Immunohistochemical Analyses

To analyze the infiltrating cell groups, graft-bearing kidneys with rejected and nonrejected grafts were stained with hematoxylin–eosin and anti-insulin (ab181547; Abcam, Tokyo, Japan), anti-CD4 (ab183685; Abcam), anti-CD8 (ab203035; Abcam), anti-CD11b (LS-C141892-100; LSBio, WA), anti-CD20 (ab64088; Abcam), and anti-CD56 (ab220360; Abcam) antibodies. EnVision+ System- HRP Labeled Polymer Anti-Rabbit (4003; DAKO, Glostrup, Denmark) was used as a secondary antibody except for cases with anti-CD8 antibodies, wherein goat anti-rabbit H&L (HRP; 401353; Calbiochem, CA) was used instead. Stained samples were viewed using a Leica DM 4-B microscope and imaged with the Leica application suite, version 4.6 (Leica, Wetzlar, Germany).

Mixed Lymphocyte Reaction

A mixed lymphocyte reaction (MLR) was performed to investigate the effect of KNF-451 (active metabolite of KRP-203) and sirolimus alone or in combination on the lymphocyte proliferation in vitro (n = 5 per group) (See **Methods S1, SDC,** http://links.lww.com/TP/C258, for detailed methodology).

Statistical Analyses

Statistical analyses were performed using IBM SPSS software (version 22). Data are presented in the form of mean ± SD. Graft survival was represented using Kaplan-Meier graph and a Log-rank (Mantel-Cox) test was used for pairwise comparison over strata. A 1-way ANOVA test with post hoc (Bonferroni) comparison was used to compare means in MLR assay groups. Two-tailed Student t-test was used to compare means in the other parts of the study. Shapiro-Wilk test was used to assess normal distribution. In the case the data did not follow normal distribution, Mann-Whitney U test was performed. A $P \leq 0.05$ was considered significant.

RESULTS

Effects of KRP-203 on the Islet Function

Blood Glucose

There was no significant difference in the BGL at day 14 between the control and KRP-203 groups (1 or 3 mg/kg; $P = 0.186$ and 0.611, respectively) (n = 10, 12, and 12, respectively).

Intraperitoneal Glucose Tolerance Test

The IPGTT showed a normal curve pattern for the 3 groups (n = 6 per group). The area under the curve showed no significant difference between the control and KRP-203 groups (1 or 3 mg/kg) ($P = 0.295$ and 0.310, respectively), although it was slightly higher with 3 mg/kg KRP-203 (Figure 2). The C-peptide measurements at 0 and 20 min after glucose injection showed no significant difference among the 3 groups, except that the C-peptide level at 20 min was significantly higher in the KRP-203 3 mg/kg group than in the control group ($P = 0.02$). The C-peptide

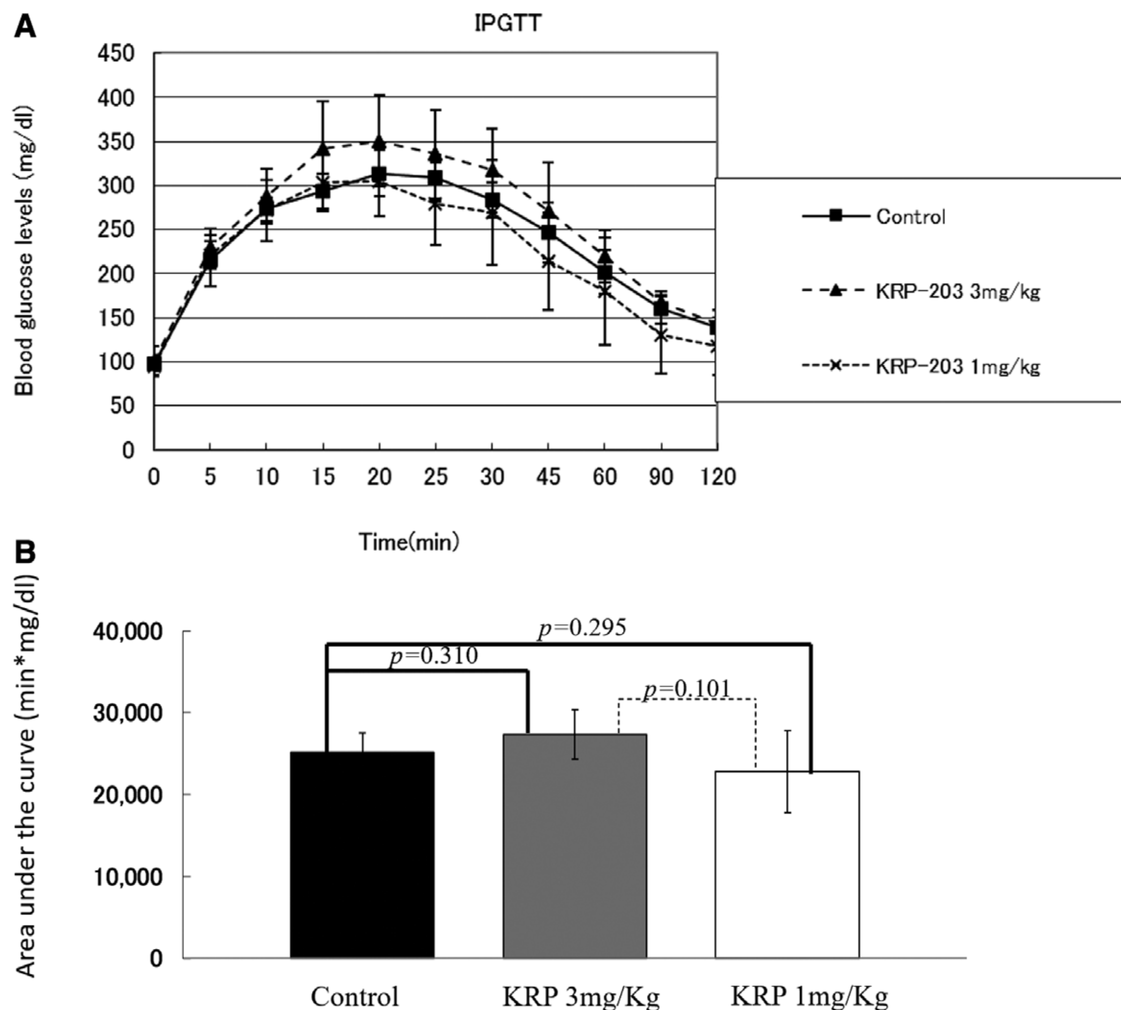


FIGURE 2. Effects of KRP-203 on the IPGTT. A, Blood glucose levels (mg/dL) after the intraperitoneal administration of 100 mg/kg glucose to mice 14 d after daily administration of KRP-203 or vehicle (n = 6 per group). B, Area under the curve in the IPGTT. IPGTT, intraperitoneal glucose tolerance test.

values (n = 7 per group) were 0.15 ± 0.07, 0.15 ± 0.05, and 0.15 ± 0.09 ng/mL at time 0 and 0.22 ± 0.08, 0.29 ± 0.13, and 0.33 ± 0.01 at 20 min for the control, 1 mg/kg, and 3 mg/kg groups, respectively.

In Vitro Functional Analyses

The results of the static glucose stimulation (n = 4), islet respiratory activity assay (n = 4) and insulin/DNA (n = 5) assessments are shown in Figure 3. The results were comparable between the KRP-203 (1 and 3 mg/kg) and control groups (*P* = 0.813, 0.481, and 0.640, respectively).

Trough Levels of KRP-203 in Treated Mice

The average trough level of KRP-203 was 2.00 ± 0.4 (n = 8) and 6.42 ± 0.9 (n = 7) ng/mL in mice treated with 1 or 3 mg/kg, respectively, whereas it was not detectable in the control group (n = 4).

Effects of KRP-203 on Islet Neovascularization

DSC model

Capillary sprout formation could be visualized at 4 d after transplantation and in some cases on day 1, and these sprouts were interconnected at day 7 (Figure 4). The newly formed microvascular network gradually enlarged. The rate of increase in the newly formed vascular volume surrounding the islets in the KRP-203-treated group (n = 8) showed no significant difference from the control group (n = 7) at any time point (*P* = 0.613, 0.951, 0.970, and 0.097 at 4, 7, 11 and 14 d, respectively, Figure 4).

Trough Level of KRP-203 in Treated Mice

The average trough level of KRP-203 was 8.49 ± 1.6 ng/mL (n = 5) in mice treated with 3 mg/kg, whereas it was not detectable in the control group receiving vehicle (n = 2).

KRP-203 as an Immunomodulator for Islet Allotransplantation

KRP-203 Monotherapy

All mice were cured of diabetes by day 5 posttransplantation. KRP-203 monotherapy prevented graft rejection for 60 d (study endpoint) in 4 of the 9 mice with either dose (1 or 3 mg/kg) (Figure 5).

Combination Regimen

Combining KRP-203 (3 mg/kg, intragastric route) and low-dose sirolimus (0.3 mg/kg, i.p.) effectively prevented

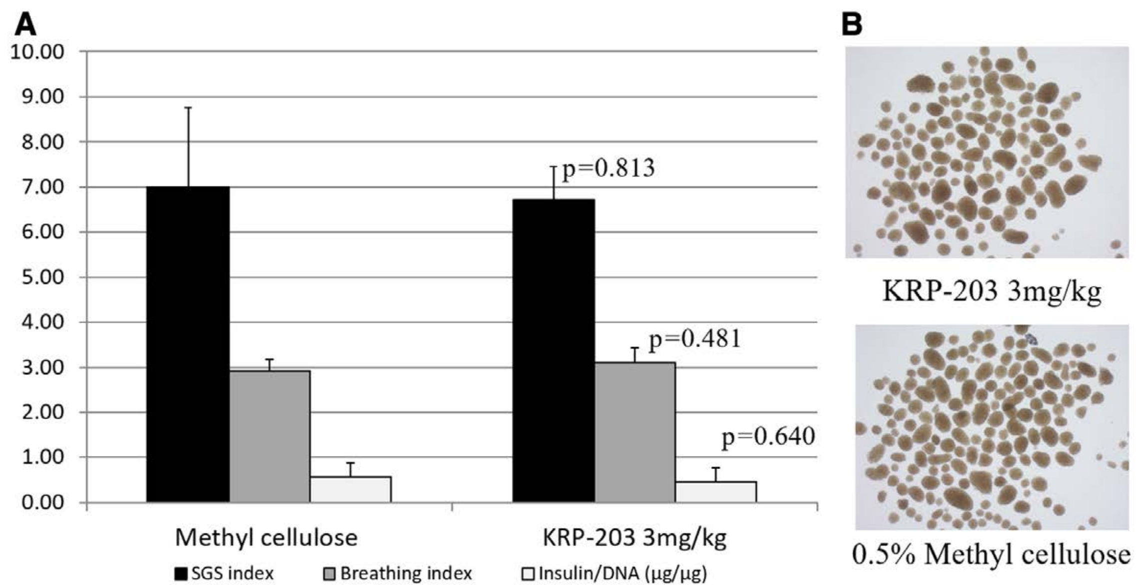


FIGURE 3. Effects of KRP-203 on islet in vitro functional assays. A, Mean values of the functional assays in the test and vehicle groups (n = 4 per group, insulin/DNA: n = 5 per group). B, Representative photos of isolated islets in both groups (40×). SGS, static glucose-stimulated insulin release.

graft rejection for 60 d in 10 of 12 mice (83%) compared to 2 of 7 mice (29%) when sirolimus was used alone (Figure 5).

All medications significantly improved graft survival in comparison to the control group ($P \leq 0.05$). KRP-203 and sirolimus combination regimen significantly improved graft survival in comparison to monotherapy groups ($P \leq 0.05$). On the other hand, there was no significant difference in graft survival between sirolimus and KRP-203 monotherapies (Figure 5). See **Table S1, SDC,** http://links.lww.com/TP/C258, for the Log-rank test results.

Trough Levels of Sirolimus in Treated Mice

The average trough level of sirolimus was 12.38 ± 0.11 ng/mL in the sirolimus-only group (n = 3) and 11.53 ± 2.86 ng/mL in the combination group (n = 5).

Mixed Lymphocyte Reaction

A MLR analysis (n = 5 per group) showed that sirolimus at 12 ng/mL concentration completely inhibited lymphocyte proliferation, while the effect of KRP-203 (as shown by the active metabolite) was minimal (12.6±17.5%, significantly lower than all other treatment groups, $P \leq 0.05$). This finding is consistent with what we know concerning the mechanism of action of S1P inhibitors. There was no significant difference between low and high concentrations of sirolimus or between sirolimus groups and combined treatment groups (Figure 6).

Immunohistochemistry

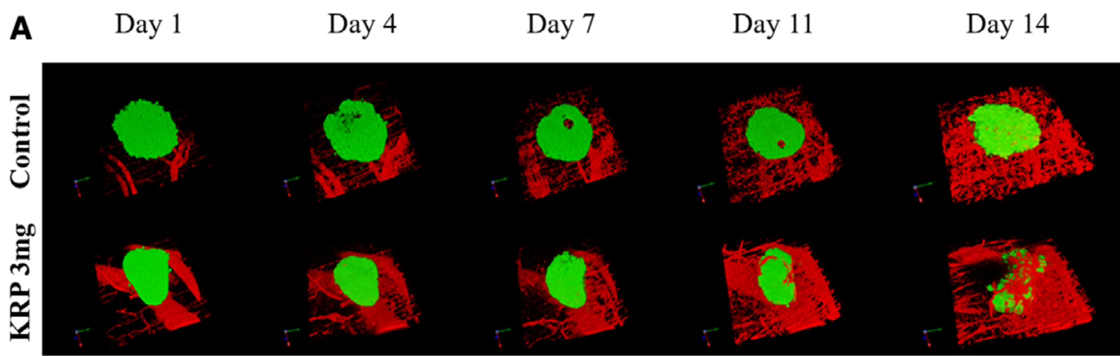
Intact islets with well-preserved morphology were demonstrated using anti-insulin staining in nonrejected grafts, whereas in others, rejection was confirmed with destroyed islets. In the rejection groups, CD4+ cells were observed more frequently than CD8+ subclass at the time of graft-bearing kidney retrieval for analyses. Interestingly, a relatively higher infiltration with CD4+ cells and considerable infiltration with B lymphocytes in the nonrejected grafts were noted when sirolimus was used, whereas almost no infiltrating cells were detected in the KRP-203-only group (Figure 7).

DISCUSSION

Despite promising experimental results,[15,16] clinical immunosuppression-free islet transplantation is not yet possible. Current immunosuppressive regimens for islet allotransplantation, commonly containing calcineurin inhibitors, have detrimental effects on the islet engraftment and function. However, the complete omission of calcineurin inhibitors has not been possible because of the risk of graft rejection.

KRP-203 is a selective S1P receptor agonist (functional antagonist) that desensitizes lymphocytes to the S1P gradient by inducing the internalization of the S1P1 receptor, thus interfering with lymphocyte egress from peripheral lymph nodes. This mechanism is thought to be the main immunomodulatory pathway of this class, although other mechanisms have been proposed to act in conjugation.

Several studies investigated the utility of KRP-203 for preventing organ allo-rejection in small animals, including skin,[7] heart,[7] and kidney[5] transplantation models. However, in all of these studies, combined with a subtherapeutic dose of CsA was necessary to effectively prevent allo-rejection. Khattar et al[8] showed that a short course of KRP-203 alone was not effective for inducing tolerance for an islet allograft, but it significantly improved tolerance induction when donor-specific regulatory T lymphocytes (Treg) were transplanted into low-responder recipients (Balb/c), resulting in a long-term graft function. The nonselective structural analog FTY720 has shown promising results by significantly prolonging the adult porcine islet xenograft function when combined with CsA.[4] However, in limited clinical trials, FTY702 failed to show a clear benefit over MMF in kidney transplantation despite occasional promising results,[17-20] in addition to the inferior safety profile due to the nonselective action of FTY702.[20]



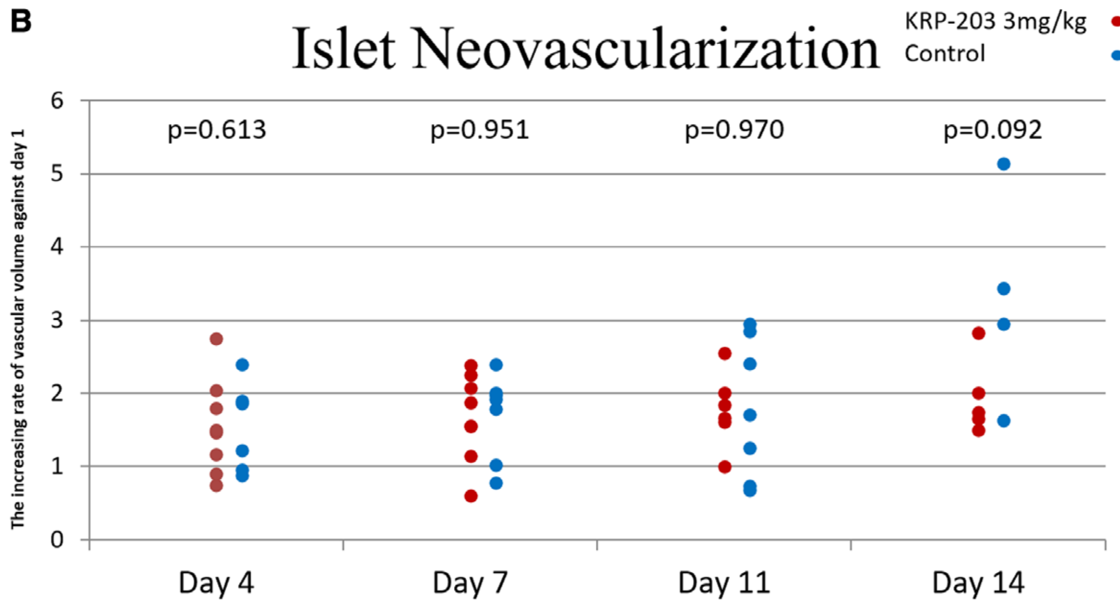


FIGURE 4. Effects of KRP-203 on islet neovascularization. A, Representative 2-photon laser scanning microscope images during the follow-up time points in the test and control groups showing green fluorescent protein positive-donor islets and the vascular network after intravenous injection of Texas Red. B, Individual data showing the change in vascular volume during the follow-up period in each group (KRP-203: n = 8, control: n = 7).

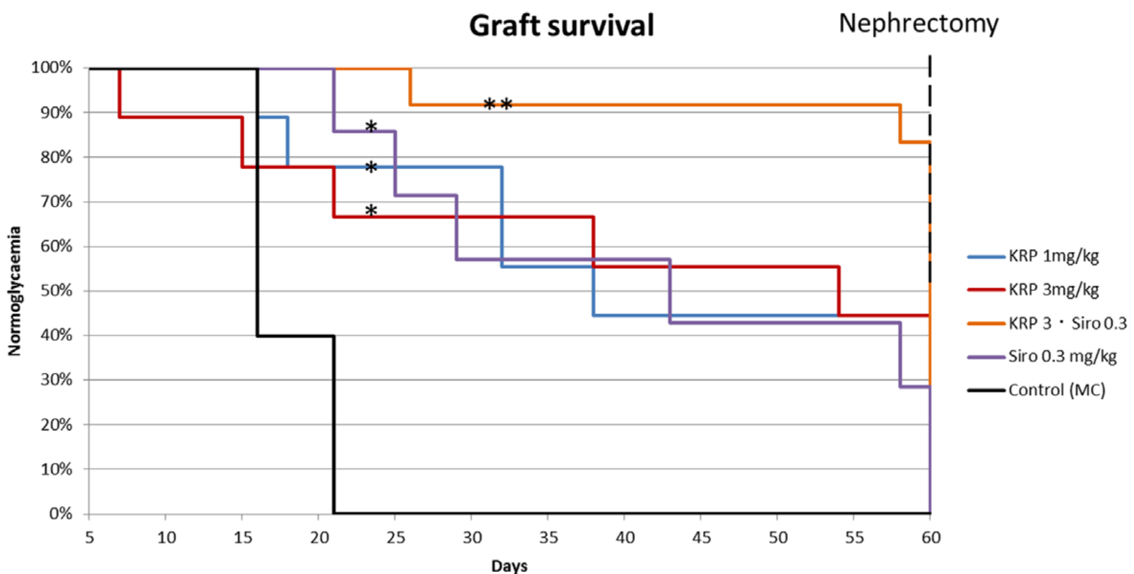


FIGURE 5. An allograft survival graph showing the effect of the different immunomodulatory regimens (n = 9, 12, 7, and 5 for KRP-203 doses, combination regimen, sirolimus-only, and control groups, respectively). MC, methylcellulose; Siro, sirolimus.

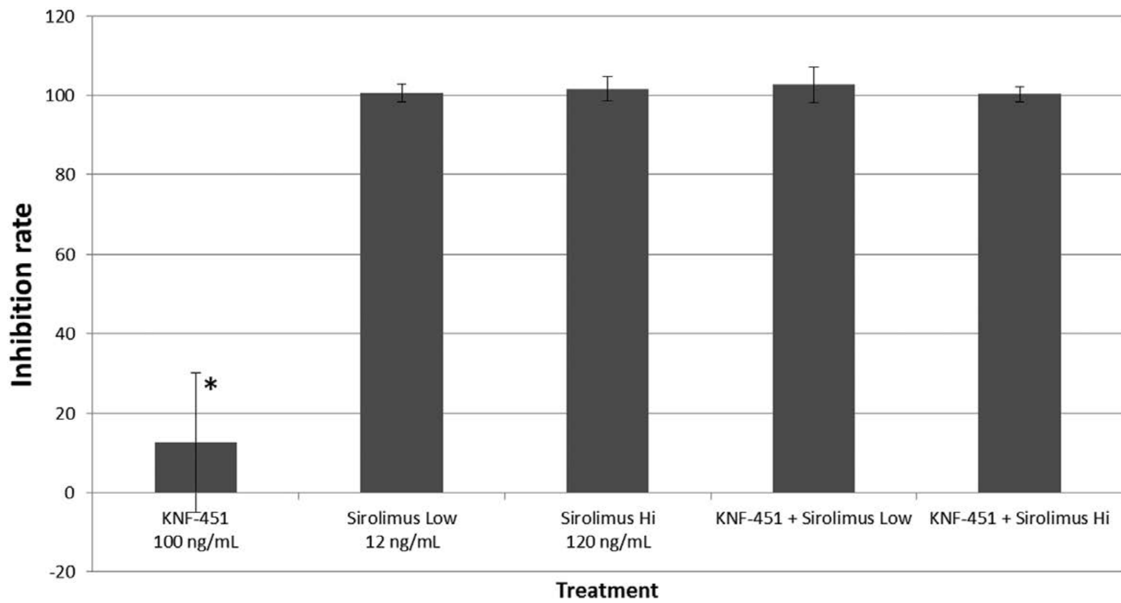


FIGURE 6. Inhibition rate of lymphocyte proliferation compared to the control after the mixed lymphocyte reaction assay (n = 5 per group). *KNF-451 addition resulted in significantly less inhibition in comparison to all other groups ($P < 0.05$).

NON REJECTED
REJECTED
KRP 3mg/kg
KRP + Siro 0.3mg/kg
Sirolimus 0.3mg/kg
KRP 3mg/kg
KRP+ Siro 0.3mg/kg
H&E
Insulin
CD4
CD8
CD20
CD11b

FIGURE 7. Immunohistochemical staining of the allograft-bearing kidneys in mice receiving KRP-203, sirolimus, or a combined regimen. H&E, hematoxylin and eosin; Siro, sirolimus.

Nevertheless, as islet transplantation represents a specific entity that differs from organ transplantation regarding the antigenic load, investigating more delicate immunomodulatory regimens based on the selective antagonist KRP-203 seems attractive.

Although the ability of KRP-203 to desensitize the S1P1 receptor is dose-dependent, we did not detect any marked difference in the graft survival between the 1 and 3 mg/kg doses. Interestingly, the surviving grafts showed very minimal cellular infiltration as evidenced by immunohistochemical analyses. In cases of rejection, although the samples were collected 2 d after rejection as evidenced by hyperglycemia, the predominance of CD4+ compared to CD8+ lymphocytes was noted. A previous report[21] showed that CD4+ lymphocytes were sufficient for inducing rejection of pancreatic allografts in the absence of CD8+ cells. S1P-receptor blockade was shown to decrease both CD4+ and CD8+ lymphocytes in peripheral blood.[22,23] Our MLR results also confirmed that KRP-203 had minimal inhibitory effect on lymphocyte proliferation.

Sirolimus has been investigated for utility in islet transplantation since the establishment of the Edmonton protocol. Sirolimus monotherapy at 0.3 mg/kg was shown to significantly prolong the islet graft survival when Balb/c mice were used as recipients,[24] but in B/6 recipients, twice-weekly sirolimus alone was not effective (rejection in 5 of 7 mice). In addition to the efficiency of sirolimus when combined with calcineurin inhibitors for preventing rejection in clinical islet transplantation, the experimental evidence of its role in preventing recurrent autoimmune diabetes and its compatibility with tolerance induction protocols has positioned sirolimus as a very useful tool in organ and cell transplantation.[25] However, the significant side effects of sirolimus have been always a concern.[25,26] Corroborating the above-mentioned reports, in the present study, we noticed a tendency toward a delayed islet function in the sirolimus group compared with the other groups, although a previous report[24] showed no influence on the in vitro islet function at similar trough levels as in this study (average 11.53 ± 2.86 ng/mL). Therefore, we sought a way to minimize the dose and frequency of sirolimus administration. Using body surface area for calculating the equivalent doses for human,[27] the doses used in the present study would be equivalent to 0.024 mg/kg for sirolimus (≈1.5 mg dose for a 60 kg individual) and 0.24 mg/kg for KRP-203 (14.5 mg dose for a 60 kg individual). For reference, a daily dose of 0.2 mg/kg of sirolimus is commonly used for maintenance in clinical islet transplantation (target trough level equal 5–15 ng/mL)[25,28] in combination with tacrolimus or mycophenolate mofetil.

A recent study demonstrated synergism between sirolimus and FTY720 in a mouse model of experimental autoimmune encephalomyelitis.[9] However, such a combination for organ transplantation was not previously investigated.

In the present study, the use of short-course sirolimus (8 d starting from day −1) followed by twice-weekly injection of low-dose sirolimus (0.3 mg/kg) resulted in rejection in 5 of 7 grafts. Adding daily KRP-203 (3 mg/kg) resulted in significant improvement (2 of 12 were rejected). An MLR analysis showed no interference with the sirolimus function by the addition of KRP-203, but at a concentration similar to the in vivo trough level of sirolimus, sirolimus alone completely inhibited lymphocyte proliferation. When sirolimus was used (either alone or in combination with KRP-203), infiltration of the graft site with CD4+ and B lymphocytes was evident both in the rejected and nonrejected grafts. The significance of this infiltration in the nonrejected grafts is unclear. Several studies have demonstrated the allo-immune regulatory function of a B-lymphocyte subset, suggesting that B lymphocytes act as both enhancers and regulators of the allo-immune responses.[29,30] One study showed that host B lymphocytes were necessary for achieving tolerance in a cardiac transplantation model,[31] and another showed that Treg were crucial for tolerance induction in an islet allotransplantation model.[32] Given these findings, the interactions between a certain type of lymphocytes and islet grafts in the transplant site seem important for achieving immunologic tolerance. As neither CD4+ nor B lymphocytes can be located at the graft site due to the main mechanism of KRP-203, the local infusion of Treg[8] and/or type 2 innate lymphoid cells[33] into the graft site together with the systemic administration of KRP-203 is logical and may be useful for further prolonging the graft survival.

S1P signaling is required for vascular integrity and angiogenesis.[34] Deletion of S1P1 results in improper vascular maturation in neonatal animals that is evidenced by defects in smooth muscle cells/pericytes layer and abnormal migration response.[35] Further investigations on the mechanism of action showed that S1P signaling acts as a negative feedback to maintain the integrity of blood vessels, thereby preventing excessive permeability and VEGF-induced vascular sprouting.[36] Indeed, a retinal vascularization model showed that S1P1 blocking resulted in increased vascular sprouting and vascular density.[36] However, these vascular sprouts are thought to be disorganized and nonfunctional, resulting in tumor disruption and regression. On the other hand, the roles of S1P2 and S1P3 in vascular development were shown to be partially redundant and cooperative to S1P1 signaling.[34,37] Therefore, examining the effect of S1P1 blocking by KRP-203 on islet neovascularization was necessary. In contrast to the effects of tacrolimus, the present study showed no significant inhibition of islet neovascularization by KRP-203 compared to the control group. In our model, which previously showed a significant inhibitory effect of tacrolimus on islet revascularization,[3] vascular density was used for the comparison, but endothelial junctions were not investigated. Although the DSC model has long been used to evaluate the in vivo vascularization, its limitations should also be considered when evaluating these outcomes. Notably, we did not notice the leakage of injected dye in any of the imaged islets. In addition, the early graft function was observed in all allografts in the allotransplantation model receiving daily KRP-203 irrespective of dose, suggesting that islet neovascularization was well established under the use of KRP-203. However, a direct comparison with tacrolimus was not performed.

In summary, KRP-203 may be a useful immunomodulator for pancreatic islet transplantation, mainly because of absence of major impact on endocrine function and lack of any interference in islet neovascularization. Although KRP-203 alone was partially successful in preventing islet allograft rejection, regardless of the dose (1 or 3 mg/kg), the combination of KRP-203 with low-dose sirolimus effectively prevented rejection in the majority of transplanted mice. Therefore, this combination may be a promising

candidate calcineurin-free regimen for islet allotransplantation and should be investigated further using large animal models.

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