Swine model of heterotopic heart transplantation

The selected transplant donors and recipients were anesthetized using Zoletil (tiletamine plus zolazepam, 5 mg/kg, Virbac, Taiwan), succinylcholine (1.1 mg/kg), and atropine (0.6 mg/kg, Taiwan Biotech Co., Ltd., Taiwan), and they were maintained under anesthesia using isoflurane (3%/1.5 L/min, Halocarbon Laboratories Inc., USA) administered through a ventilator after intubation. The recipient was placed in the left decubitus position, and vascular access was established for the administration of immunosuppressive drugs. A right flank incision was created, and through a retroperitoneal approach, the infrarenal aorta and inferior vena cava were isolated (Figure S1). Next, the donor was heparinized (300 IU/kg intravenous injection (i.v.), Bodene (Pty) Limited, South Africa), and the donor heart was harvested after cardiac standstill was achieved using cold (4°C) cardioplegic solution (Plegisol, Abbott Laboratories, North Chicago, Ill, USA). An atrial septal defect was created in each donor heart, and the mitral valve was defunctionalized to minimize left ventricular atrophy and intracavitary thrombus formation (Figure S1). The recipient was heparinized (300 IU/kg i.v.), and the donor’s pulmonary artery was anastomosed end-to-side to a 1 to 2 cm venotomy in the inferior vena cava with a continuous 5-0 polypropylene suture (Prolene; Ethicon, Inc., Somerville, NJ, USA). Subsequently, the ascending aorta of the donor heart was anastomosed to the recipient’s abdominal aorta in a similar manner, followed by the administration of protamine (1.5 mg/kg; Leo Pharmaceutical Products, Denmark) to stop bleeding. Neither cold nor warm ischemic times exceeded 45 min. In most cases, removal of the aortic cross clamp resulted in spontaneous conversion to a normal sinus rhythm. However, in some cases, internal electrical defibrillation (10 to 20 J) or the administration of isoproterenol (initially, 0.02–0.06 mg; subsequently, 0.01–0.2 mg; Kowa Co., Ltd) was necessary. Iron supplementation (Fe-Back Injection, Nang Kuang Pharmaceutical Co., Ltd) was intravenously administered to alleviate blood loss during surgery.

Assessment of histocompatibility through 1-way mixed lymphocyte reaction

We utilized a mixed lymphocyte reaction (MLR) assay to determine major histocompatibility complex incompatibility. Peripheral blood mononuclear cells from 2 unrelated minipigs were cultured in a 96-well round-bottom plate (BD Bioscience). Responder (1 × 10⁵) and mitomycin-C (25 μg/mL)-treated stimulator (1 × 10⁵) cells were each plated in a well. To assess the allogeneic antigen-stimulated proliferation, cocultured cells were pulsed on the 4th day with 1 mCi/well [³H]-thymidine (Perkin Elmer, USA) for 18 h. Radioactivity was determined using a MicroBeta FilterMate-96 Harvester and a 1450 MicroBeta TriLux (both from PerkinElmer, Waltham, Massachusetts, USA). Responder cell proliferation was measured by [³H]-thymidine uptake and expressed as counts per minute (cpm). Each responder-stimulator pair was tested in triplicate. The stimulation index (SI) was calculated through the following formula: (Mean cpm of allogeneic MLR)/(Mean cpm of autologous MLR).

Measurement of mycophenolic acid through liquid chromatography and tandem mass spectrometry analysis

Plasma levels of mycophenolic acid (MPA), the active metabolite of MMF, was determined through a combination of high-performance liquid chromatography (HPLC) with tandem mass spectrometry (LC-MS/MS) using validated methods. For the measurement of the MPA predose concentration (C₀), blood was collected on days 0, 7, and 28 at 9:00 am before MMF administration; thus, MPA-C₀ was detected 16 h after the last dose of MMF. Blood samples were collected and stored at 4°C until centrifugation (<60 min after collection). After
centrifugation, the plasma samples were stored at −20°C until the MPA assay was performed. Plasma samples were extracted through protein precipitation using an acetonitrile solution (ACN, Duksan Pure chemicals, Korea). HPLC was performed using an Agilent 1260 Infinity Quaternary LC System equipped with an Agilent 6470 Triple Quadrupole LC/MS system multiple-reaction monitoring detection mode with precursor product ion pairs of 321.1 → 207.0. A stock solution of MPA (Sigma-Aldrich) at a concentration of 1000 µg/mL was prepared in 72% of ACN and stored at −20°C. Working stock solutions were diluted in drug-free pig plasma to obtain calibration standards of 0.312, 0.625, 1.25, 2.5, 5, and 10 µg/mL MPA. Calibration was accomplished through linear regression of the peak area of the analyte. A reverse-phase HPLC column was used for the separation of the analyte. The elution was performed in an isocratic mode with a mobile phase that consisted of distilled water and CAN (60:40, v/v) containing 0.1% formic acid. After being subjected to vortex mixing for 3 min, the samples were centrifuged at 12 000 rcf for 10 min. The extract was 5-fold diluted with water containing 0.1% formic acid and transferred for LC-MS/MS analyses.

**Definition of acute rejection and relative risk of acute rejection**

Based on autopsy findings of the minipigs at our preliminary study, each new onset of allograft bradycardia (<60 beats per minute) was defined as an episode of acute rejection. Minipigs that exhibited allograft bradycardia, in the absence of endomyocardial biopsy, were treated with 500 mg doses of methylprednisolone daily for 3 consecutive days. Minipigs experiencing resistant rejection episodes were treated with repeated courses of methylprednisolone pulse therapy until recovery from bradycardia was achieved. To examine whether person-time periods in which burixafor was administered were associated with a decreased incidence rate of acute rejection, the number of rejection episodes was recorded and divided by the total observed pig-days in each group. The ratio of the risk in the burixafor-treated group to the risk in the control group was calculated to assess the relative risk of acute rejection using the equation \[(E_{burixafor}/D_{burixafor}) / (E_{control}/D_{control})\], where E represents acute rejection episodes and D represents the total observed pig-days.

**Monitoring of graft survival**

The beating rate of cardiac allograft was monitored daily through palpation, and electrocardiography (Prucka CardioLab; GE Medical Systems, USA) was performed twice per week. When the beating rate of the allograft decreased, echocardiography (M7Vet Ultrasound Systems, Mindray, Australia) was performed to assess systolic function. Follow-up was continued to the time of allograft arrest or the study end date (150 days). In addition, the recipient that died prior to cardiac graft arrest was excluded from the survival analysis because the mortality was unrelated to the heterotopic cardiac allograft.

**Histological analysis**

Formalin-fixed cardiac specimens were embedded in paraffin, cross-sectioned, deparaffinized, rehydrated, and then subjected to hematoxylin and eosin (HE) or orcein staining. Intimal hyperplasia of the vascular grafts was examined using a Zeiss Axiophot 2 microscope and determined from computer images of orcein-stained cross sections (AxioVision, Version 4.7, Zeiss, Oberkochen, Germany). The area surrounded by the internal elastic lamina (IELA) and the luminal area (LA) were calculated using an image analysis program (Image J, Version 1.46r, NIH Image). The severity of intimal hyperplasia was calculated using the following formula: \([(IELA – LA)/IELA] × 100\%. After calculation, the severity of intimal hyperplasia for each graft was evaluated in 3 randomly chosen fields per coronary section for 5 cross sections in a blinded manner, and the evaluated severity levels were averaged for statistical analysis. For immunohistochemical analysis, tissue sections
were subjected to antigen retrieval by heating in 10 mM sodium citrate buffer (pH 7.2) for 10 min in a microwave, then treated for 5 min in 3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were incubated with antibodies to CD3 (Vertana) or C4d (Cell marque) overnight at 4°C. The application of isotype antibodies to tissue sections was used in negative controls. Signals were developed in a 30-min incubation step with the polymer-HRP detection kit QD420-YIK (Biognex) followed by 3,3’-diaminobenzidine detection. Slides were counterstained with hematoxylin.

**CRP, TNF-α and IL-6 measurements**
The plasma levels of C-reactive protein (CRP), TNF-α, and IL-6 at specified times were measured using commercially available ELISA kit (Immunology Consultants Laboratory for CRP; R&D for TNF-α, and IL-6). The detection limits of the ELISA kits were 6.25 ng/mL for CRP, 23.4 pg/mL for TNF-α, and 18.8 pg/mL for IL-6.

**Statistical analysis**
All data are expressed and plotted as the mean ± standard deviation. Statistical analyses were performed using Prism 5 software (GraphPad Sotware, San Diego, CA, USA). Survival curves were established using the Kaplan–Meier method and compared through a log-rank test, and the relative risk of acute rejection was compared using a chi-square test. Group differences in the intimal hyperplasia and inflammatory cell infiltration were assessed through a Wilcoxon rank-sum test or Wilcoxon signed-rank test, whichever was appropriate. A P value of < 0.05 was considered statistically significant.
Figure S1. Porcine model of heterotopic heart transplantation. (A) The donor heart was harvested after cardiac standstill achieved by using cold cardioplegic solution (Plegisol). (B) The graft was maintained in the ice-saline slurry and prepared for implantation by creating an atrial septal defect and defunction the mitral valve to minimize left ventricular atrophy and intracavity thrombus formation. (C) The recipient’s inferior vena cava (IVC) and the infrarenal aorta were isolated. (D) The graft heart was implanted by anastomosing the donor pulmonary artery to the recipient’s IVC and the donor ascending aorta to the abdominal aorta of the recipient. Graft function was monitored by using (E) electrocardiography (ECG) and (F) echocardiography (UCG). Arrows indicate electrical spikes attributed to heterotopic cardiac allograft.
Figure S2. Time course of cardiac allograft vasculopathy in miniature swine. Representative photomicrographs of H&E-stained (40×) cross-sections from allografts harvested at different time points after heart transplantation in the control groups. Scale bars indicate 100 μm. POD = postoperative day.
Figure S3. Pathology examination of cardiac allograft. (A) Transverse sections at apical- and mid-ventricular levels of left ventricle and (B) histological pictures (100 ×) of coronary artery of cardiac allograft at posttransplant day 77 selected from a representative case in the controls. Yellow and white arrows mark the internal and external elastic laminae, respectively. Inflammatory cell infiltrations are denoted by red arrows. Scale bars indicate 1 cm in (A) and 100 μm in (B). HE: hematoxylin and eosin.
Figure S4. Lesions of intimal hyperplasia in cardiac allografts from the controls and burixaor2D+B group. Representative photomicrographs of H&E-stained whole (40×) or partial (100× and 200×) cross-sections of vascular allografts at 133 and 134 days after transplantation revealing lesions of CAV. Scale bar, 100 μm.
Figure S5. Evaluation of potential adverse effects of multiple doses of burixafor on renal and liver functions. BUN = blood urea nitrogen; AST = aspartate aminotransferase; ALT = alanine aminotransferase.
Figure S6. Therapeutic potential of burixafor and immunomodulatory effects of burixafor-mobilized MSCs. The major findings across the continuum of our previous reports support that burixafor is effective in mobilizing bone marrow-derived MSCs, which have therapeutic potential to attenuate inflammation and control transplant arteriosclerosis.