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Beneficial effects of the transgenic expression of human sTNF-αR-Fc and HO-1 on pig-to-mouse islet xenograft survival

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ABSTRACT

Both human soluble tumor necrosis factor-α receptor-Fc (sTNF-αR-Fc) and heme oxygenase-1 (HO-1) transgenic pigs have been generated previously for xenotransplantation. Here, we investigated whether overexpression of sTNF-αR-Fc or HO-1 in pig islets prolongs islet xenograft survival. Adult porcine islets were isolated from human sTNF-αR-Fc or HO-1 transgenic and wild type pigs, and were transplanted into diabetic nude mice. Effects of the expression of both genes on islet apoptosis, chemokine expression, cellular infiltration, antibody production, and islet xenograft survival were analyzed. Human sTNF-αR-Fc transgenic pigs successfully expressed sTNF-αR-Fc in the islets; human HO-1 transgenic pigs expressed significant levels of HO-1 in the islets. Pig-to-mouse islet xenograft survival was significantly prolonged in both the sTNF-αR-Fc and HO-1 groups compared with that in the wild type group. Both the sTNF-αR-Fc and HO-1 groups exhibited suppressed intragraft expression of monocyte chemoattractant protein-1 (MCP-1) and decreased perigraft infiltration of immune cells. However, there was no difference in the anti-pig antibody levels between the groups. Apoptosis of islet cells during the early engraftment was suppressed only in the HO-1 group. Porcine islets from both sTNF-αR-Fc and HO-1 transgenic pigs prolonged xenograft survival by suppressing islet cell apoptosis or secondary inflammatory responses following islet death, indicating that these transgenic pigs might have applications in successful islet xenotransplantation.

1. Introduction

Xenotransplantation of porcine islets is expected to solve the problem of donor-organ shortage and is considered a promising treatment for diabetes mellitus [1]. However, significant loss of islet cells during isolation and engraftment, and strong xenogeneic rejection limit its clinical application [2–4]. To overcome these barriers, several genetically modified pigs have been generated to enhance successful engraftment or to suppress xenogeneic rejection without heavy immunosuppression. Local expression of cytoprotective or immune-modulating molecules in porcine islets through the utilization of genetically modified pigs could improve islet engraftment or suppress islet xenograft rejection without the need for increased systemic immunosuppression, thereby avoiding related side effects.

Previous studies have demonstrated that expression of human tumor necrosis factor (TNF-α) contributes to islet xenograft rejection [5,6] while soluble TNF-α receptor (sTNF-αR) inhibits TNF-α-induced apoptosis and inflammation in porcine endothelial cells [7]. Recently, transgenic (TG) pigs expressing human soluble TNF-α receptor type I-IgG1 Fc fusion protein (sTNF-αR-Fc) under the cytomegalovirus (CMV) promoter were generated [7]. Serum from sTNF-αR-Fc TG pigs suppressed proinflammatory responses and apoptosis of porcine endothelial cells [7].

Heme oxygenase-1 (HO-1), which has anti-oxidant, anti-apoptotic, and anti-inflammatory effects [8], improves the outcomes of islet xenografts [9,10]. Because of these beneficial effects of HO-1, TG pigs for HO-1 under the Simian virus (SV) 40 or CMV promoter have been generated [11,12]. Aortic endothelial cells from HO-1 TG pigs showed relative resistance against apoptosis and inflammation, and kidneys from HO-1 TG pigs exhibited resistance against rejection in an ex-vivo perfusion

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model [12]. Fibroblasts isolated from HO-1 TG pigs also exhibited resistance against oxidative stress- or cytokine-induced apoptosis and inflammatory response [11].

In this study, we investigated whether porcine islets isolated from human sTNF-αR-Fc TG pigs or human HO-1 TG pigs exhibit decreased apoptosis of islet xenograft in the early engraftment period, suppressed inflammatory responses, and allowed better islet xenograft survival in pig-to-mouse islet xenotransplantation.

2. Materials and methods

2.1. Animals

Human sTNF-αR-Fc TG pigs overexpressing sTNF-αR-Fc under the CMV promoter were generated on White Yucatan miniature pig background, as described previously [7]. Human HO-1 TG pigs overexpressing HO-1 with the hemagglutinin (HA) tag under the CMV promoter were also generated on White Yucatan miniature pig background, as described previously [11]. Wild type White Yucatan miniature pigs were purchased from Optifarm Solution (Cheonan, Republic of Korea), and were used as controls. BALB/c nude mice were obtained from Orient Bio Inc. (Seong Nam, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committees of the Seoul National University College of Medicine. The animal studies were conducted in accordance with the 'Principles of laboratory animal care.

2.2. Islet isolation and confirmation of HO-1 and sTNF-αR-Fc expression

Porcine pancreata were injected intraductally with Liberase PI (Roche, Basel, Switzerland). Digestion and islet isolation were performed by using Ricordi's automated isolation technique and a COBE® 2991 centrifuge (Cobe BCT Inc., Lakewood, CO, USA), as described previously [13].

Human sTNF-αR-Fc was detected using a human IgG enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, MN, USA), as described previously [7]. Human HO-1 was identified by Western blotting, where rabbit anti-human HO-1 antibody (1:200; Abcam, MA, USA) and anti-rabbit IgG coupled with horseradish peroxidase were used as the primary and secondary antibodies, respectively [11].

2.3. Pig-to-mouse islet transplantation

Diabetes was induced by an intraperitoneal injection of 50 mg/kg streptozotocin (Sigma-Aldrich, MO, USA) to BALB/c nude mice for five consecutive days. Mice whose blood glucose levels were higher than 400 mg/dL for two consecutive days were used as transplant recipients. Adult porcine islets were cultured overnight after isolation, and 5000 islet equivalents (IEQ) were transplanted under the unilateral renal subcapsular space of diabetic mice that had been injected with streptozotocin one week before transplantation. Blood glucose levels were monitored once daily for 1 week after transplantation, and then three times a week during the observation period. When the blood glucose level in the transplanted mice increased again after an initial normalization, and remained higher than 350 mg/dL for more than two consecutive days, islet xenografts were considered as failed grafts. Islet xenograft survival was assessed in eleven to fourteen mice for each group. Six and nine islet xenografts were harvested and used in each group for histologic study and tissue cytokine analysis, respectively. Nephrectomy was executed in recipients with functional graft for up to 100 days to ensure that normal glucose levels had been maintained by the islet xenografts.

2.4. Histological analysis

Islet xenografts were harvested on day 2, 8, and 28 after transplantation, and were embedded in paraffin. Immunohistochemical staining was performed using the following primary antibodies: guinea pig anti-insulin (DAKO), rat anti-mouse F4/80 (BM8; eBioscience, San Diego, CA, USA), rat anti-mouse neutrophil (NIMP-R14; Abcam), rat anti-mouse/human CD45R (B220) (14-0452; eBioscience). Tissue sections were incubated with primary antibodies at 4 °C overnight. The next day, the slides were treated using ZytoChem Plus HRP One-Step Polymer anti-mouse kit (ZUC053, Zytomed, Berlin, Germany) with the NovaRED substrate kit (SK-4800, CA, USA). Goat anti-human IgG-HRP (SC-2453, Santa Cruz Biotechnology, TX, USA) and rabbit anti-HA (3724, Cell Signaling Technology, MA, USA) were used to stain human sTNF-αR-Fc and HA-tagged HO-1, respectively. Apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Millipore, MA, USA). TUNEL-positive cells were counted automatically with the Image J software [14] and were confirmed by manual counting by two independent researchers.

2.5. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from homogenized islet xenografts by using an RNeasy Mini Kit (Qiagen, Boston, MA, USA) according to the manufacturer's instructions. A total of 1 μg RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Mouse mRNA expression levels were determined by real-time PCR using an Applied Biosystems Prism 7300 system, with the SYBR green master mix (Applied Biosystems) and specific primers (Table 1). All samples were normalized to mouse GAPDH expression levels.

2.6. Assessment of anti-pig humoral response

To assess anti-pig-specific antibody response to the xenografts, MPN3 (immortalized porcine aortic endothelial cells) cell lines were incubated with five-fold diluted sera that were isolated from the mice at 37 °C for 2 h, and then stained with anti-mouse IgM-APC or IgG-PE (eBioscience) [15]. Mean fluorescence intensity (MFI) ratio was calculated by dividing the MFI of experimental serum with that of the negative control.

2.7. Statistical analysis

Student's *t*-test or analysis of variance test was used to analyze the differences between groups, as appropriate. Data were presented as mean ± standard error of mean, and P values < 0.05 were considered significant. All the analyses were performed using STATA 12 (StataCorp, TX, USA).

3. Results

3.1. Expression of sTNF-αR-Fc and HO-1 in porcine islets isolated from transgenic pigs

The characteristics of source pigs and isolated islets are summarized in Table 2. There were no significant differences in their characteristics, including islet morphology, among the different groups of porcine islets. The sTNF-αR-Fc TG pigs expressed sTNF-αR-Fc in a tissue-nonspecific manner. Significant levels of human IgG were detected in both the serum and islet-culture supernatant, and the level was higher in the serum than in the islet-culture supernatant (Fig. 1A). Western blotting confirmed successful expression of HO-1 in islet lysates from HO-1 TG pigs (Fig. 1B).

When the tissue expression of sTNF-αR-Fc and HO-1 was assessed on day 28 after pig-to-mouse islet transplantation, islet xenografts from sTNF-αR-Fc TG pigs and HO-1 TG pigs were found to express sTNF-αR-Fc and HO-1, respectively (Fig. 1C–D). Together, these results