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The Effect of Composite Pig Islet–Human Endothelial Cell Grafts on the Instant Blood-Mediated Inflammatory Reaction

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Instant blood-mediated inflammatory reaction (IBMIR) causes rapid islet loss in portal vein islet transplantation. Endothelial cells are known to protect against complement-mediated lysis and activation of coagulation. We tested composite pig islet–human endothelial cell grafts as a strategy to overcome IBMIR. Porcine islets were cocultured with human endothelial cells in specially modified culture medium composed of M199 and M200 for 1–9 days. A positive control group, negative control group, and the endothelial cell-coated group were examined with an in vitro tubing loop assay using human blood. The endothelial cell-coated group was subdivided and analyzed by degree of surface coverage by endothelial cells (≤50% vs. >50%) or coculture time (<5 days vs. ≥ 5 days). Platelet consumption and complement and coagulation activation were assessed by platelet count, C3a, and thrombin–antithrombin complex (TAT), respectively. After 60-min incubation in human blood, the endothelial cell-coated group showed platelet consumption inhibition and low C3a and TAT assay results compared to uncoated controls. When the endothelial cell-coated group was subdivided by degree of surface coverage, the ≤50% coated group showed less platelet consumption and less activation of complement and coagulation compared with the positive control (uncoated) group. On analysis by coculture time, only the subgroup cocultured for <5 days showed the same protective effect. Human endothelial cell-coated pig islets, especially the partially coated and short-term cocultured pig islet–human endothelial cell composites, reduced all components of IBMIR. If the optimal endothelial cell–islet coculture method could be identified, human endothelial cell coating of pig islets would offer new strategies to improve xenogenic islet transplantation outcomes.

Key words: Endothelial cells; Humans; Islets of Langerhans; Swine; Transplantation; Heterologous

INTRODUCTION

In the clinical setting, pancreatic islet transplantation is performed via the portal vein. The majority of transplanted human islets are destroyed before engraftment (11) by instant blood-mediated inflammatory reaction (IBMIR) (3), which may even be stronger in the setting of xenografts because of species barrier (2,4).

Many strategies have been developed for overcoming IBMIR. Among these, heparin treatment is the most commonly used agent in clinical islet transplantation. However, administration of heparin may cause complications such as bleeding (19) and lacks the ability to inhibit complement activation of xenogeneic IBMIR (4).

Coating of pig islets with human endothelial cells might have several advantages to overcome IBMIR. First, the endothelial lining of blood vessels can endure direct contact with blood under normal conditions (15). In addition, human endothelial cells coating of pig islets may hide cross-species molecular incompatibilities. Sec-

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ond, endothelial cells have potent effects on angiogenesis (9,13), which should provide a synergistic effect on islet engraftment. Third, inhibition of the interaction between pig islets and human blood will result in decreased inflammation, reducing the severity of rejection later in the course (5). Previously, composite human islet–endothelial cell grafts showed beneficial effect in an in vitro model for investigating allogeneic IBMIR (8). Thus, we investigated whether coating of pig islets with human endothelial cells can inhibit IBMIR in xenograft environment.

MATERIALS AND METHODS

Islet Isolation and Culture

The pancreases of adult market pigs were harvested at the local slaughterhouse. The splenic lobe of the gland was rapidly dissected ex situ, immersed in UW solution, and transported to the islet-processing laboratory. Pancreatic islet isolation and purification were performed as previously described (10). The islets were cultured free-floating in standard culture medium (M199; GIBCO BRL, Grand Island, NY, USA) supplemented with Earle's salts, L-glutamine, 2200 mg/L sodium bicarbonate, 25 mM HEPES buffer, and 10% pig serum. Culture medium was changed every second day. Modified islet culture medium was manufactured for coculture, and was made up of M199 and M200 (Cascade Biologics, Portland, OR, USA) supplemented with the low serum growth supplement (LSGS) kit (Cascade Biologics).

In Vitro Test of Islet Function

Glucose-stimulated insulin release was measured and expressed as the stimulation index (10), calculated as the ratio of stimulated (16.7 mM glucose) to basal (1.67 mM glucose) insulin release during 60 min of static incubation in Krebs' Ringer bicarbonate HEPES buffer. Insulin levels were determined with an insulin immunoradiometric assay kit (Biosource, Nivelles, Belgium).

Endothelial Cell Preparation

Human aortic endothelial cells (HAEC; Cascade Biologics) were cultured at 37°C in 5% CO_2 in M200 medium containing 10% FCS, 100 μg/ml penicillin/streptomycin, and 15 mg/ml of LSGS. Cells were grown to confluence in 100-mm petri dishes. Medium was changed every second day. HAEC were used from passages 4–7. The HAEC were stained with the PKH26 Red Fluorescent Cell Linker kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

Endothelial Cell Coating of Pig Islets

Endothelial cell coating was performed as previously described (8), with some modifications. HAEC were harvested using 1× trypsin-EDTA (GIBCO BRL, Invitrogen). The HAEC were washed with M200 medium, centrifuged at $180 \times g$ for 5 min using a 14-ml Falcon tube (Becton-Dickinson Labware, Lincoln Park, NJ, USA), and counted in a Burker chamber. After washing, the HAEC were suspended in M200 medium to 3×10^6 cells/ml. Ten thousand islet equivalents (IEQ) were mixed together with 3×10^6 HAEC in 300 μl modified culture medium. The mixture was incubated for 1.5 h at room temperature and mixed gently with pipetting every 15 min. Coated islets were transferred to 150-mm petri dishes before transplantation and cultured for 1–9 days. The coverage of each islet with endothelial cells was examined under fluorescent microscopy and calculated with an image analysis program (i-solution ver7.3, IMT, Korea).

In Vitro Tubing Loop System

Blood donors were healthy without specific disease and had no medication history for 1 month before donation. The protocol was approved by the Institutional Review Board of Seoul National University Hospital and each subject submitted written informed consent.

A modified tubing loop model (3,7) was used for in vitro simulation of portal flow. Using an 18-gauge needle, fresh human blood was collected in a surface-heparinized 60-ml syringe (Corline Systems AB, Uppsala, Sweden). This blood was transferred to a polyvinyl chloride (PVC) tube heparinized on the inner surface (Corline Systems AB) (diameter 6.3 mm, length 390 mm) via a surface heparinized silicon tube. Each PVC tube was filled with 7 ml of fresh human blood; 100 μl of PBS was added as a negative control, 5000 IEQ of uncoated islets in 100 μl of PBS were added as a positive control, and 5000 IEQ of endothelial cell coated islets were added as the treatment group. The loops were then closed and placed in a 37°C incubator on a rocking apparatus. Blood was sampled at 0, 5, 15, 30, and 60 min. For the zero time samples, the blood was transferred directly to EDTA-containing tubes and 1 ml of blood acquired from the tubing loop was placed in an Eppendorf tube containing 40 μl of 0.2 M EDTA. About 100 μl of blood was used for full blood count analysis, and the remaining blood was centrifuged at $4000 \times g$ for 20 min at 4°C. After 1-h incubation, all the loop contents were filtered through 70-μm diameter filters (Filcons, Cuptype; DAKO, Glostrup, Denmark). The remaining filtered blood was collected in EDTA tubes for hematologic analysis (platelets, lymphocytes, monocytes, and granulocytes), assays of complement activation (C3a), and coagulation activation (thrombin–antithrombin (TAT)).

Hematologic analyses were performed by Coulter ACT-diff analyzer (Beckman Coulter, Miami, FL,

USA). C3a and TAT were quantified using ELISA assay kits (C3a EIA kit: Quidel, San Diego, CA, USA; Enzygnost-TAT kit: Behringwerke, Marburg, Germany).

Statistical Analysis

For comparison between different blood donors, platelet counts were transformed to a percentage of the zero time value. C3a and TAT assay results were transformed to a percentage of the positive control value. Data are presented as mean ± SEM. Mean values were compared using the paired Student *t*-test. Bonferroni correction was used for subgroup analysis by multiplying the *p*-value obtained by the number of comparisons. Differences with values of $p < 0.05$ were considered significant.

RESULTS

In Vitro Tests of Islet Function in Various Culture Conditions

The stimulation index of uncoated pig islets in M199 medium was 1.98 ± 0.19 (mean ± SEM, $n = 5$). Uncoated islets in modified culture medium showed similar glucose-stimulated release values (1.95 ± 0.27, $n = 5$) to those in M199 medium and the endothelial cells coating

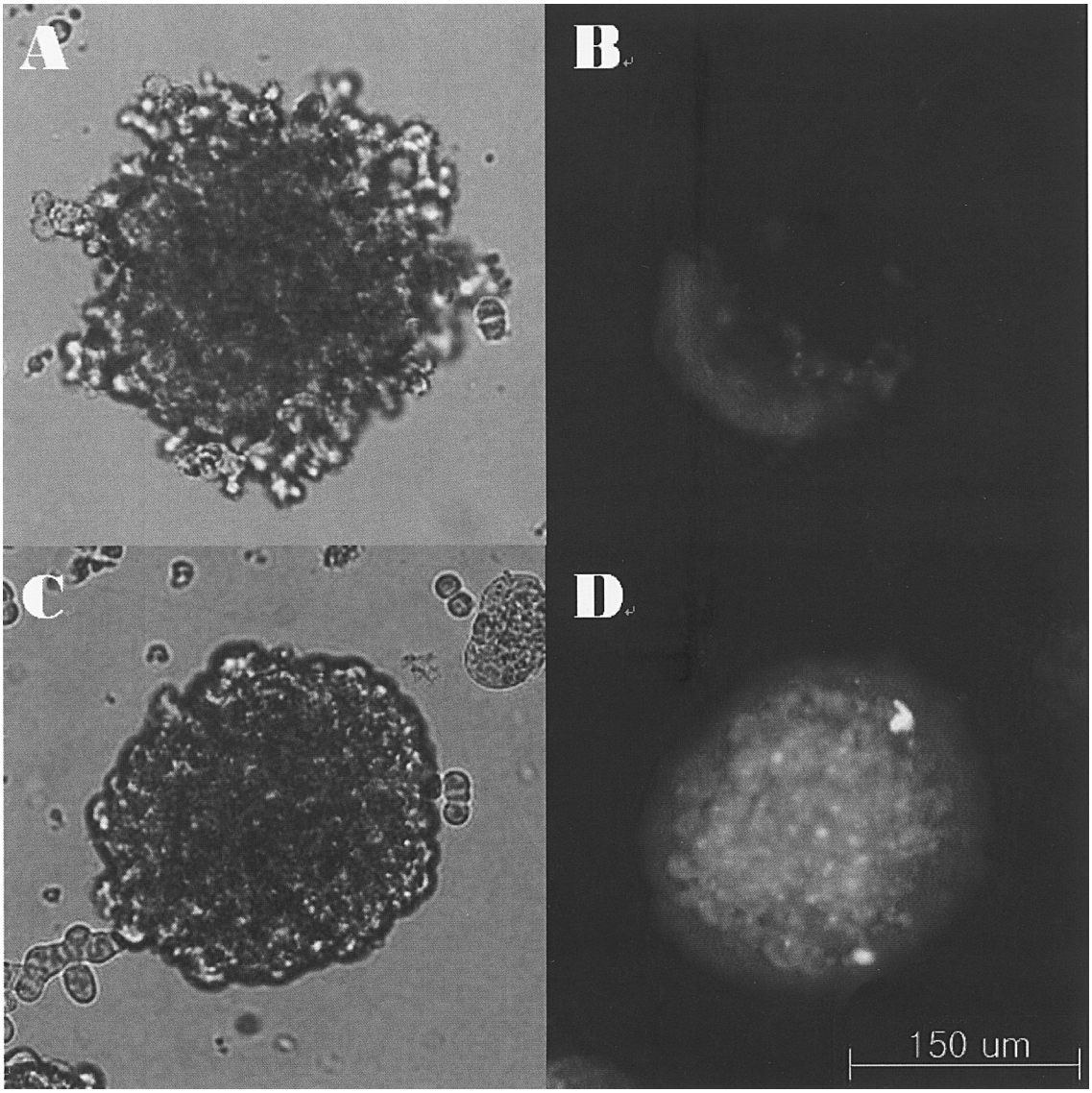

Figure 1. Pig islets coated with human endothelial cells. Endothelial cells were labeled with the fluorescent dye PKH26. An early phase endothelial cell coated islet under light (A) and fluorescence (B) microscopy. Endothelial cell growth after 1–9 days of coculture under light (C) or fluorescence (D) microscopy. Magnification ×400.

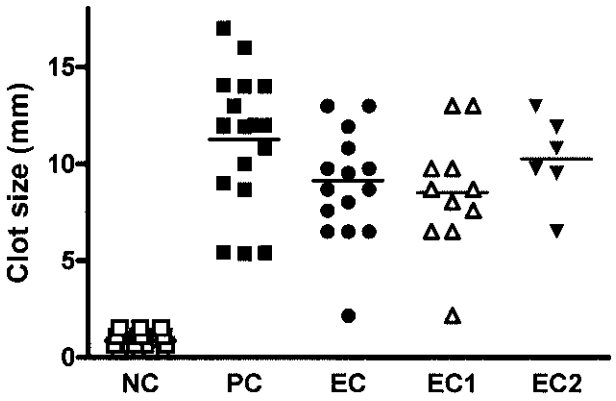

Figure 2. Differences between groups in the volume of blood clots after incubation. After the loop contents were filtered, islets were left on the filter with different levels of blood clot formation. Horizontal bars represent means. NC: negative control tube without islets; PC: positive control tubes with untreated islets; EC: endothelial cell coated islets; EC1: endothelial cell coated islets with ≤50% coverage; EC2: endothelial cell coated islets with >50% coverage.

procedure had no harmful effect on insulin secreting capacity of pig islets (1.96 ± 0.29, $n = 5$).

Endothelial Cell Coating of Pig Islets

In a single-species model (8) using composite human islet–endothelial cells grafts, the endothelial cells grew until they reached confluence. However, in our two-species model, human endothelial cells grew very slowly on pig islets. This necessitated increase of coculture time and the ratio of endothelial cells to islets in the mixing procedure. The number of endothelial cells used to coat pig islets was 10 times higher than that used to coat human islets. After 1–9 days of coculture, 3.5–94.5% of coverage was observed (Fig. 1). The degree of coverage was well correlated with the duration of coculture.

Blood Clot Formation

Blood clot formation as a result of IBMIR was observed after 1-h incubation (Fig. 2). The volume of blood clot correlated with decreased platelet, monocyte, and granulocyte counts. The clot diameter was 11.25 ± 0.80 mm in the positive control ($n = 18$), 0.86 ± 0.1 mm in the negative control ($n = 18$), and 9.12 ± 0.69 mm in the endothelial cell coated group ($p = 0.06$, $n = 17$). The endothelial cell-coated group was subdivided and analyzed by degree of surface coverage by endothelial cells (≤50% vs. >50%) or coculture time (<5 days vs. ≥ 5 days). Unexpectedly, clot formation was more inhibited in the subgroup of coated islets with ≤50% coverage (8.51 ± 0.91 mm, $p = 0.390$, $n = 11$) than in those with >50% coverage (10.25 ± 0.92 mm, $p = 0.328$, $n = 6$), although this difference was not significant (Fig. 2). Because the majority of the subgroup of coated islets with ≤50% coverage belonged to the subgroup cocultured for <5 days, clot formation was more inhibited in the subgroup cocultured for <5 days compared to the subgroup cocultured for ≥ 5 days.

Hematologic Analyses

The positive control group showed exhausted platelets and decreased lymphocyte, monocyte, and granulocyte counts (Table 1). The endothelial cell coated group as a whole showed a protective effect on the platelet

Table 1. Blood Cell Counts, Complement, and Coagulation Analysis

	Initial Count (0 min, $n = 18$)	Negative Control (No Islets) (60 min, $n = 18$)	Positive Control (Untreated Islets) (60 min, $n = 18$)	Total Endothelial Cell-Coated Group (60 min, $n = 18$)	Subgroup With ≤50% Coverage (60 min, $n = 11$)	Subgroup With >50% Coverage (60 min, $n = 7$)
Lymphocytes	100%*	95.2 ± 1.7%	82.9 ± 3.4%	89.1 ± 2.6%	93.6 ± 1.7%	81.9 ± 5.2%
Monocytes	100%*	105.1 ± 7.3%	21.4 ± 4.3%	40.6 ± 8.9%§	44.8 ± 13.4%	34.0 ± 9.5%
Granulocytes	100%*	98.2 ± 0.8%	34.3 ± 2.5%	56.0 ± 5.2%§	58.9 ± 6.7%§	51.5 ± 8.4%
Platelets	100%*	56.7 ± 2.0%†	2.1 ± 0.4%	14.5 ± 4.7%§	22.5 ± 6.8%¶	8.9 ± 4.4%
C3a	1.16 ± 0.65%	42.5 ± 3.8%	100%‡	83.1 ± 4.3%§	80.1 ± 5.3%§	88.7 ± 7.7%
TAT	0.01 ± 0.00%	5.7 ± 1.5%	100%‡	56.2 ± 8.0%§	46.6 ± 6.8%§	71.4 ± 16.7%

Values are mean ± SEM. C3a, complement C3a; TAT, thrombin–antithrombin complex.
*The initial baseline count was defined as 100%. Subsequent values were expressed as a percentage of this value.
†Decreased by air and tube contact interaction with blood.
‡The final result of the positive control was defined as 100% and other values were expressed as a percentage of this value.
§Significant difference ($p < 0.01$) by Student paired t-test compared with positive control.
¶Significant difference ($p < 0.05$) by Student paired t-test compared with positive control.

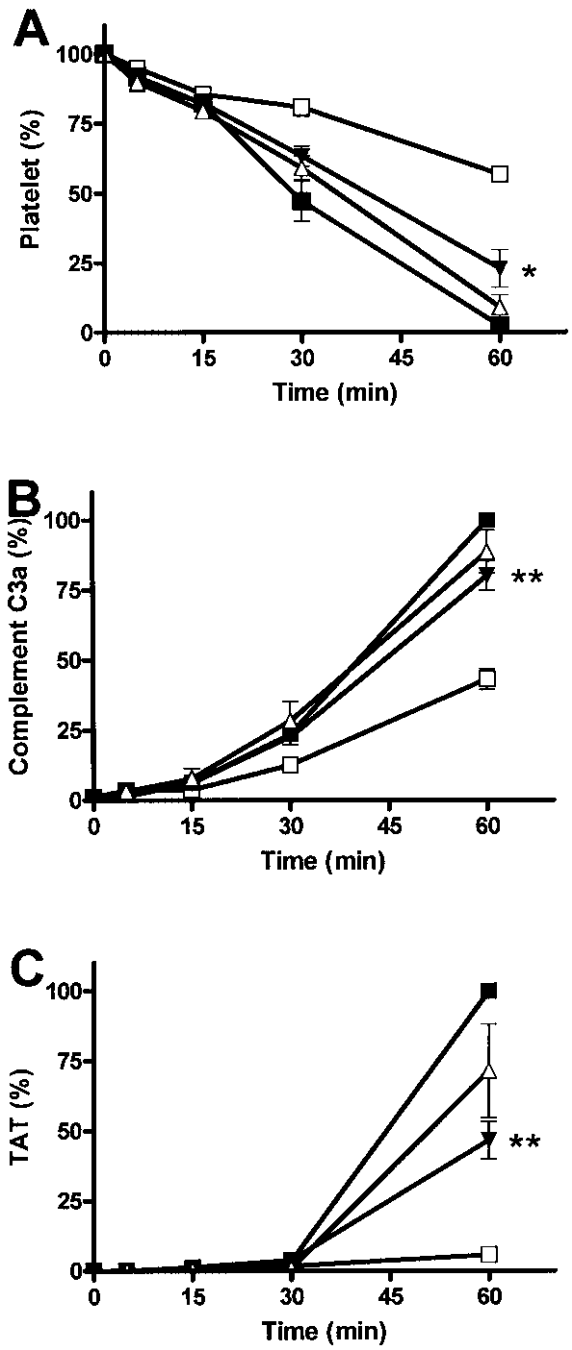

Figure 3. The effect of coating pig islets with human endothelial cells on platelet, complement and coagulation. Samples were taken at 0, 5, 15, 30, and 60 min. (A) Platelet (B), complement and (C) thrombin–antithrombin analysis was performed. Only the subgroup of endothelial cell coated islets with ≤50% coverage shows significant inhibition of platelet consumption, complement, and coagulation activation. Negative control (open boxes), positive control (filled boxes), endothelial cell-coated subgroup with >50% coverage (open triangles), and endothelial cell-coated subgroup with ≤50% coverage (filled triangles). *$p < 0.05$, **$p < 0.01$.

count compared with the positive control ($p < 0.01$), and the ≤50% coated subgroup also showed a statistically significant protective effect on the platelet count ($p < 0.05$), whereas the >50% coated subgroup did not (Fig. 3A). Similar results were observed only in the subgroup of cocultured for <5 days.

C3a and TAT ELISA Assays

The endothelial cell-coated group showed significantly decreased complement ($p < 0.01$) and coagulation ($p < 0.01$) activation compared to the uncoated group (Table 1). Subgroup analysis showed that the ≤50% coated subgroup also showed a significant decrease in both assays, whereas the >50% coated subgroup did not (Fig. 3B, C). Similar results were observed in the subgroup analysis of coculture time.

DISCUSSION

Whereas complement activation mainly occurs secondary to the activation of the coagulation system in allogeneic IBMIR (16), porcine islets in human blood trigger direct complement activation (4,7). Because endothelial cells express various surface protectants such as complement regulatory protein, heparan sulfate, antithrombin III, tissue factor pathway inhibitor, and thrombomodulin, it prevents activation of complement cascade and coagulation cascade (15). In addition, endothelial cells prevent platelet aggregation by the release of prostacyclin, ectoADPases, thrombomodulin, nitric oxide, and platelet-derived ADP (15). Furthermore, human endothelial cells coating of pig islets may reduce thrombotic and inflammatory reaction affected by cross-species molecular incompatibilities in the pig to human islet xenotransplantation, because human endothelial cells express human specific surface protectants such as complement regulatory protein, tissue factor pathway inhibitor, and thrombomodulin (2,6). In this study, we found human endothelial cell coating of pig islet reduced all components of xenogeneic IBMIR. Furthermore, there was no harmful effect of modified culture medium and endothelial cell coating of pig islets on insulin-secreting capacity of islets.

The size of blood clot may reflect therapeutic efficiency of the specific strategy chosen for islet protection. In this study, blood clot size showed significant correlation with platelet consumption, and with decreases in monocytes and granulocytes. Furthermore, clot size must be regarded as an indicator of thrombus formation in clinical applications. Because portal vein thrombosis is a critical life-threatening complication, some new therapeutic approach should be evaluated to inhibit thrombus formation before practical application.

In this study, the protective potency of the subgroup of islets with ≤50% endothelial cell coverage was simi-

lar to that of human islets covered to 50% with endothelial cells in the previous allogeneic IBMIR (8). However, the protective effect of coated islets did not correlate with the degree of surface coverage with endothelial cells in this study. The subgroup of islets with ≤50% endothelial cell coverage (or the coculture group <5 days) was more effective in inhibition of IBMIR than those with >50% coverage (or coculture group ≥ 5 days). This paradoxical phenomenon could be explained by endothelial cell activation. Endothelial cell activation causes changes including loss of surface heparin sulfate, enhanced tissue factor expression, low thrombomodulin, and increased expression of von Willebrand factor and cell surface adhesion molecules (14). The culture substrate and culture medium may account for this activation (11,13). We minimized manipulation of the endothelial cells for covering the islets to prevent endothelial cell activation and ruled out a detrimental effect of modified culture medium and endothelial cell coating on pig islets, but not on endothelial cells. Therefore, we could not exclude the possibility that endothelial cells of the long-term coculture group were activated by suboptimal culture condition, although endothelial cells could grow in modified culture medium. Hence, monitoring of endothelial cell activation should be included in future experiments for more effective endothelial cell coating. In addition, wide variation of HAEC coverage (ranging between 4% and 95%) between different experiments indicates that the coating procedure needed further optimization.

It is well known that the choice of source of endothelial cells for specific applications is very important, as there is considerable phenotypic variation among endothelial cells from different organs (17), different individuals (1), and different vessel sizes (12). Endothelial progenitor cells (18) may be promising candidates for future coating materials because of their anticoagulant effect and promotion of angiogenesis.

In conclusion, human endothelial cell-coated pig islets, especially the subgroup composites showing ≤50% endothelial cell coverage and cocultured for <5 days, showed a marked reduction of IBMIR compared to the uncoated islets. This result can be explained on the basis that the procoagulant effect of endothelial cells dominated over their anticoagulant effect during long-term coculture. If the optimal coculture method could be identified, human endothelial cell coating of pig islets would offer new strategies to improve xenogeneic islet transplantation.

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