Pancreatic Islets Activate Portal Vein Endothelial Cells In Vitro

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Abstract. This study assessed the active role of the portal vein endothelium in the functional loss of pancreatic islet (PI) grafts, considered in the context of PI transplantation for treatment of type I diabetes mellitus. We hypothesized that PI engraftment may be jeopardized by portal vein endothelial cell-induced activation of host T cells. We designed an in vitro system using portal vein endothelial cells (PVEC) from Lewis (Lew) rats and PI from Brown-Norway (BN) and Lew rats. PI were co-cultured with Lew PVEC for three days. The PI were removed and trypsinized PVEC were divided into three groups: (A) PVEC not exposed to PI; (B) PVEC exposed to syngeneic PI; and (C) PVEC exposed to allogeneic PI. The groups were analyzed by flow cytometry for ICAM-1 and MHC Class I and Class II molecules. Functional assays of lymphocytes (ie, lymphocyte proliferation assay, 51Cr release assay, and cytokine release assay) were also performed. We observed MHC Class I and ICAM-1 upregulation on PVEC after PI contact. MHC Class II molecule was not upregulated on PVEC after PI exposure. IL-6 production was increased non-specifically following PI coculture with PVEC. TNF-α was increased only after allogeneic PI-PVEC coculture. PVEC exposed to either allogeneic or syngeneic PI could not stimulate naïve splenocyte proliferation or cytotoxicity. We conclude that PVEC allo-PI interaction results in increased ICAM-1 and MHC Class I expression on the PVEC. Neither lymphocyte proliferation nor cytotoxicity could be enhanced in response to enhanced MHC Class I and ICAM-1 expression, which was associated instead with non-specific inflammatory cytokine release. (received 26 April 2002; accepted 8 June 2002)

Keywords: Pancreatic islets, portal vein endothelium, antigen presentation, inflammatory cytokines

Introduction

Insulin-dependent diabetes mellitus type 1 (IDDM-I) can now be successfully reversed by transplanting pancreatic islets of Langerhans into the portal venous tract of the liver, where the islets sustain insulin-production. This new therapeutic approach for the treatment of IDDM-I was announced on July 27, 2000 by Shapiro et al [1]. They reported 100% success in achieving insulin-independence for more than one year in 12 consecutive patients with type I diabetes. These patients received multiple islet grafts in conjunction with a novel steroid-free immunosuppressive regimen. The requirement for multiple islet transplants to reverse diabetes in the 12 patients was due to a significant loss of functional islets during the peritransplant period. Multiple factors, such as ischemic injury, immunologic response, and non-immunologic inflammatory response, may be responsible for islet graft loss [2]. Improvements are therefore needed to maintain islet cell viability and function, to promote long-term engraftment, and to minimize the loss of islet mass.

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The first step toward this goal is to understand better the mechanisms of islet cell loss during the peritransplant period. In our laboratory, we focused on understanding the interaction between the portal vein endothelium and the pancreatic islet graft. We studied the activation of portal vascular endothelial cells (PVEC) in vitro in contact with isolated pancreatic islets (PI), and we hypothesized that the portal vein endothelium interacting with PI graft could play the role of an antigen-presenting cell and induce host T cell activation. The current study examines PVEC activation after MHC Class I and II as well as ICAM-1 flow cytometric analysis and tests the associated lymphocyte response using proliferation and cytotoxicity assays as well as cytokine release assay.

**Methods**

**Animals.** Lewis (Lew) and Brown Norway (BN) rats from Harlan Sprague Dawley (Indianapolis, IN) were fed and watered ad libitum. All studies were performed in accordance with guidelines set by the American Council on Animal Care and were approved by the Animal Care and Use Committee of the University of Illinois at Chicago.

**Endothelial cell isolation and characterization.** Endothelial cells were harvested according to McGuire and Orkin [3]. The full length of the portal vein was removed from adult Lew rats using sterile techniques. The vein was then rinsed twice with cold (4°C) Hanks balanced salt solution (HBSS). The vein was cleaned of periadventitial fat and connective tissue by sharp dissection and cut into 2-mm rings. The ring segments were placed, endothelial side down, on plastic Petri dishes and incubated overnight in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere. Just enough complete medium was used to keep the explants moist (Dulbecco's Modified Eagles Medium [DMEM] supplemented with L-glutamine, Heps buffer, 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1% gentamicin). Twenty-four hr later, 1 ml of complete medium was added per 10 cm² of the tissue culture dish. Culture continued for 5-7 days until the endothelial cells migrated from the explants onto the tissue culture dish. The explants were then

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**Fig. 1.** An in vitro system of LEW portal vein endothelial cell culture was exposed to LEW pancreatic islets (syngeneic) (left), or to BN pancreatic islets (allogeneic) (right). One set was unexposed to islets as a control (bottom center).
removed in a sterile fashion and discarded. The endothelial cells were examined by a binocular microscope and photographed at various stages of cellular outgrowth. Immunofluorescence staining of Factor VIII was used to identify the rat portal vein endothelial cells [4].

**Rat pancreatic islet isolation.** Rat islets were isolated from the donor pancreas by a modification of a method previously described [5]. Briefly, donor Lew rats were anesthetized with ether inhalation. Through a midline incision, the distal bile duct was ligated at the point of entry into the duodenum and above the bifurcation of the right and left hepatic ducts [6]. The bile duct was incised and cannulated; 20 ml of collagenase solution (1 mg/ml, Boehringer Mannheim, Indianapolis, IN) in Krebs Heps buffer (KHB) (containing 10% HBSS, 10% 0.1M Heps buffer, 10% sodium bicarbonate, and 2% bovine serum albumin in distilled water) was infused by a pulsed infusion technique. The distended pancreases were surgically harvested, and the donors were killed under general anesthesia. Islets were isolated by a two-step digestion process, with first an incubation at 37°C for 8 min with intermittent shaking, and second with purification of the islets on a Eurocollins-Ficoll (Ficoll 400- DL, Sigma Chemical Co., St. Louis, MO) discontinuous gradient (densities: 1.108, 1.096, 1.069, and 1.037). Purified islets, recovered from the first and second interface of the gradient, were hand-picked and counted under inverted microscopy.

**PVEC-PI coculture.** We designed an in vitro system for culturing PVEC from Lew rats and PI from BN and Lew rats, using Dulbecco’s Modified Eagles Medium (DMEM) supplemented with L-glutamine, Heps buffer, 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1% gentamicin. DMEM was preferred because it favors the growth of PVEC over PI, which played the role of an antigen in this system (Fig. 1). PI were co-cultured with Lew PVEC (at 1:100). This dose-ratio of islet cell/PVEC is comparable to the dose of islet cells normally injected into the portal vein during islet cell transplantation. PI were then removed after 3 days. Trypsinized PVEC were divided into 3 groups: PVEC not exposed to PI (Group A); PVEC exposed to syn-PI (Group B); and PVEC exposed to allo-BN PI (Group C). Flow cytometric studies were performed on PVEC to identify expression of ICAM-1 and MHC Class I and Class II.

**PVEC FACS Vantage Flow cytometry analysis.** PVEC were gently scraped from the tissue culture dish, using a rubber policeman, and the cells were then washed once and suspended in 2 ml at 10^8 cells/ml in PBS and 0.2% BSA. In order to block the Fc receptors, heat inactivated non-specific rat IgG was added at 1 mg/ml. The cell suspension was split into 4 samples, and to each was added an IgG FITC (control), anti-RT1A-FITC to label MHC CLASS I, anti-RT1B-FITC to label MHC CLASS II, and anti-ICAM-1-FITC at 1 mg/10^6 cells. After incubation for 30 min, the cells were washed in PBS with 0.2% BSA (400 x g, 7 min) and resuspended in 0.3 ml PBS with 0.2% BSA, containing 1 mg/ml propidium iodide (PI).

Ten thousand cells were analyzed per sample, gated according to light and side scatter, and gated for viability based on PI staining. The fluorescent profiles were measured using a Coulter Epics Elite ESP Flow Cytometer (Hialeh, FL). Fresh Lew splenocytes (isolated as described below) served as positive controls of MHC Class I and MHC Class II expression. In a separate positive control experiment, interferon-γ (IFNγ) was added to PVEC from group B, and subsequently MHC Class II was upregulated (data not shown).

**Lymphocyte proliferation assay.** Lymphocytes were isolated from fresh Lew spleens, following sterile removal in anesthetized rats. The spleen was minced with forceps and scissors in cold (4°C) RPMI medium and pressed between sterile frosted glass slides. The splenic tissue was then suspended in cold RPMI and centrifuged (400 x g, 5 min). The supernatant was discarded; the pellet was resuspended in fresh RPMI, from which the lymphocytes were separated using lymphocyte separation medium (LSM, ISN Biomedicals, Chicago, IL) and washed twice. Lymphocytes were suspended in 2 ml of cold RPMI and cell count and viability was determined using a hemocytometer and 0.5% trypan blue.
Fig. 2. PVEC were stained with FITC bound class-I MHC mab (solid signal), and nonspecific IgM for control (dotted signal). An increase of fluorescence intensity was noted in PVEC (group B) and PVEC (group C), which had both been exposed to PI, compared to PVEC (group A), which were not exposed to PI.

Fig. 3. PVEC were stained with FITC bound class-II MHC mab (solid signal) and nonspecific IgM FITC bound for control (dotted signal). No significant class-II MHC expression was noted in either group.
PVEC cultures were trypsinized and scraped from the tissue culture dish. They were washed once, resuspended in 2 ml of cold RPMI and counted. Thereafter, lymphocytes (2x10⁶/ml) and PVEC (4x10⁶/ml) were suspended in RPMI containing 1% L-glutamine, 1% penicillin/streptomycin, 5% fetal bovine serum, and 5x10⁻⁵ M 2-mercaptoethanol. Then, 100 µl of each suspension was plated together in triplicate wells of a 96 well microtiter plate in the following combinations: PVEC from each group (A, B, or C) with either lymphocytes alone, irradiated lymphocytes (2000 Gray), or medium alone. Control wells contained lymphocytes with either 100 mg/ml of phytohemagglutinin (PHA), irradiated lymphocytes, or medium alone.

The plates were then incubated at 37°C in an atmosphere of air/5% CO₂. After 96 hr, 2 mCi ³H-TdR (Amersham, Arlington Heights, IL) was added to each well. Using a Skatron TiterTek cell harvester (Flow Laboratories, Rockville, MD), the cultures were harvested onto glass-fiber filter paper after 20 hr of additional incubation. [³H]-thymidine incorporation, measured by liquid scintillation counting (Packard Tri-carb Model 3380), was expressed as counts per min (cpm) and used to quantify the lymphocyte proliferative responses.

**Lymphocyte cytotoxicity assay.** CRL-2057 insulinoma cells (ATCC, Manassas, VA) were grown in-vitro in RPMI containing 1% L-glutamine, 1% penicillin/streptomycin, and 5% fetal bovine serum. Complete medium was renewed twice weekly, and subcultures were obtained every 6 to 8 days. After 2-5 cell passages, cells were trypsinized, washed once, and resuspended in complete medium (10⁷ cells/ml). Na₂⁵¹CrO₄ (Amersham Pharmacia, Amersham, UK) was added to the cell suspension to reach 100 mCi ⁵¹Cr/10⁷ cells. The mixture of ⁵¹Cr-labeled target cells was incubated at 37°C for 1 hr and the cells were then washed once in HBSS for 30 min. The radioactive supernatant was discarded, and the cells were resuspended in complete medium at 10⁶ cells/ml.

Fresh lymphocytes harvested from Lew rats were cocultured with PVEC at a 1:5 ratio in supplemented RPMI for 4 days in 5 combinations of lymphocytes: with (#1) PVEC group A, (#2) PVEC group B, (#3) PVEC group C, (#4) with medium

Fig. 4. PVEC were stained with FITC bound ICAM-1 mab (solid signal) and non-specific IgG for control (dotted signal). An increase of fluorescence intensity was noted in PVEC (group B) and PVEC (group C), which had both been exposed to PI, compared to PVEC (group A), which were not exposed PI.
alone (negative control), and (#5) with Interleukin-2 at 10 IU/ml (positive control).

The lymphocytes were separated from PVEC using lymphocyte separation medium (LSM, ICN Biomedicals), washed once in PBS, and resuspended in complete medium. Three serial dilutions of effector lymphocytes were obtained and 0.1 ml of each added in triplicate to wells in a 96 well plate. These dilutions resulted in effector to target ratios ranging from 1:100, 1:50, 1:25 when they were added to 0.1 ml of $^{51}$Cr labeled target cells/well ($10^5$ cells per well). Control wells for total $^{51}$Cr release received labeled target cells and 0.1 ml of 1% NP40. The culture plate was incubated at 37°C for 4 hr and centrifuged once (250 x g, 15 min). Then 80 ml of supernatant was harvested from each well and transferred in a LumaPlate-96, containing solid scintillator, to be processed in a $\gamma$-counter. The specific cytotoxicity was calculated by the formula:

\[
\text{cytotoxicity (\%)} = \left( \frac{\text{experimental cpm - background release cpm}}{\text{total release cpm - background release cpm}} \right) \times 100.
\]

**Determination of cytokine levels in “activated PVEC”- PI cocultures.** The same groups of PVEC, PVEC not exposed to PI (A), PVEC exposed to syn-PI (B), and PVEC exposed to allo-BN PI (C), were cocultured with lymphocytes. Tissue culture supernatant was then harvested respectively at time 0, day 1, day 2, and day 3 of coculture from each of the three groups, A, B, C, and from control groups as follows: (#1) lymphocytes alone, (#2) islets alone, and (#3) PVEC + lymphocytes alone. Supernatant samples were then processed through a sandwich enzyme linked-immuno-sorbent assay (ELISA) (Biosource International, Camarillo, CA) to measure rat IL-6 and TNF-\(\alpha\). Standards of known rat IL-6 content were diluted from 1000 pg/ml to 500, 250, 125, 62.5, 31.2 and 0 pg/ml. Their absorbance values were plotted against concentration, generating a standard curve. The detection threshold was 0.1 pg/ml for both IL-6 and TNF-\(\alpha\).

Then, 100 \(\mu\)l of each sample (standard, control, unknown) was added to each well and incubated for 2 hr at 37°C. The wells were washed 4 times. One hundred \(\mu\)l of biotinylated primary antibodies were then added to each well and incubated for an hr at room temperature. The wells were washed again 4 times, and 100 \(\mu\)l of streptavidin-peroxidase enzyme was added and incubated for 30 min at room temperature. After 4 additional washes, 100 \(\mu\)l of the enzyme substrate chromogen was added and incubated for 30 min at room temperature in the dark. The intensity of colored product was directly proportional to the concentration of rat cytokine in the original sample. The absorbance of each well was read by a plate reader at 450 nm and compared to the standard curve.

**Statistics.** Results were expressed as mean ± SD; \(p\) values were calculated by unpaired t-test (\(p <0.05\) was considered statistically significant).

**Results**

**PVEC cultures.** The cells that migrated from the portal vein explants onto the tissue culture dish were examined under the inverted microscope at different time points, spreading apart at day 6 and progressively forming a cobblestone pattern, each cell having a polygonal shape. When these cells were treated with FITC-bound von Willebrand Factor VIII antibody, a fluorescent granular perinuclear reaction was observed, which confirmed a PVEC-culture purity of 85%. Between 6 and 10 days of culture, this pattern of staining is characteristic of endothelial cells, and if these cultures are maintained for more than 15 days, they may become contaminated with fibroblasts [4].

**Pancreatic islet characterization.** Pancreatic islets were obtained from Lew and BN rats and purified by Ficoll gradient separation. A representative sample was stained red with dithizone. About 250 islets were obtained from each 350-g rat. As a control, these purified islets from acinar tissue were transplanted into streptozotocin-induced diabetic rats, achieving a marked reduction in mean serum glucose levels. Serum glucose levels fell from a mean of 357 mg/dl on post-transplant day 0 to 119, 135, and 188 mg/dl on post-transplant days 1, 2, and 3 respectively (\(n = 4\)). In the in vitro system, maintaining PI function was unnecessary, because PI primarily played the role of an antigen; its presentation to lymphocytes by PVEC was tested in this system.
VEC expression of MHC class I, MHC class II, and ICAM-1 molecules. PVEC were found to constitutively express MHC class I and to upregulate MHC class I in response to both syngeneic and allogeneic PI exposure (Fig. 2). The bimodal histograms for groups B and C were likely due to a contaminating population of fibroblasts. In addition, since a greater number of events was counted in PVEC from group C, the percentage positive cells were as follows: PVEC group A 48.9% ± 0.4; PVEC group B 45.9% ± 0.8; PVEC group C 59.4 ± 0.8. MHC class II however was neither constitutively expressed, nor upregulated by PI contact (Fig. 3). PVEC constitutively expressed ICAM-1, which were upregulated in response to both syngeneic and allogeneic PI exposure (Fig. 4). In Fig. 3 bimodal expression in the panel for PVEC from group B was also noted.

Lymphocyte proliferation assay. Lymphocyte proliferation was not significantly increased after coculture with PVEC that had been exposed to either allogeneic or syngeneic islets versus PVEC that were non-exposed to islets (3 separate experiments, data not shown).

Lymphocyte cytotoxicity assay. Lymphocytes previously cocultured with the 3 groups of PVEC did not show any significant destruction of β cells from the insulinoma cell line (2 separate experiments, data not shown).

Cytokine release in “activated” PVEC-lymphocyte co-cultures. IL-6 secretion, though increased, was not significantly different for the PVEC cocultures exposed to either syngeneic or allogeneic islets, with a peak of IL-6 secretion observed at 24 hr in 2 separate experiments, each performed in duplicate wells (Fig. 5). There was increased TNF-α release at 24 hr with PVEC cocultures exposed to allogeneic islets, compared to syngeneic islets, in 2 separate experiments (p <0.05) (Fig. 6).

Discussion

The portal venous tract of the liver is the preferred site of islet implantation for a diabetic patient to receive a pure form of insulin. However, recent studies indicate approximately 30% loss of pancreatic islet mass during the early phase of engraftment. The reason for this loss remains an enigma. The present study, in which portal vein endothelial cells were exposed to pancreatic islets in vitro, attempts to assess the alterations of the portal vein endothelial surface that take place during the infusion of the pancreatic islet graft. A nonspecific inflammatory event was found to occur, rather than a specific immune lymphocyte response.

Interestingly, the FACS Vantage flow cytometry analysis does not show the MHC-Class II molecule to be upregulated after either allogeneic or syngeneic PI exposure in our in vitro system, suggesting that PVEC have no role as antigen presenting cells in the PVEC–PI interaction. Our results confirm that MHC class II molecules are not constitutively expressed on the surface of rat PVEC, a feature of rat endothelial cells in general [7]. But we found that MHC-class II molecules could be expressed on rat PVEC exposed to IFNγ directly (Fig. 2b). Other investigators showed that MHC–Class II is expressed on human endothelial cells cocultured with allogeneic T cells, perhaps as a consequence of IFNγ secretion by the T cells [7]. The work of Pober and Cotran [7] suggests that rat endothelial cells usually remain MHC-class II negative until late in the course of an inflammatory response. This change in phenotype, referred to as activation, involves complex molecular interactions that may also occur in response to various forms of stress such as inflammation. Other cell types such as macrophages and Kupfer cells in the liver could also potentially activate PVECs.

However, surface markers (i.e., MHC-Class I and ICAM-I) become upregulated after either syngeneic or allogeneic PI exposure. The bimodal histogram patterns may be due to contaminating fibroblasts, so further purifying experiments are needed. Enhanced nonspecific expression of MHC- Class I on PVEC surfaces theoretically makes PVEC a potential antigen presenting cell (APC) for class I restricted T cells. ICAM-1 (CD54), a surface molecule expressed on endothelial cells, macrophages, and dendritic cells, binds to its ligand, LFA-1, expressed on activated T cells at the site of
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**Fig. 5.** IL-6 was measured in the supernatant of lymphocyte-PVEC coculture after either no PI exposure (group A), syngeneic PI exposure (group B), or allogeneic PI exposure (group C). The mean values of duplicated wells from 1 of 2 experiments are shown. Peak production of IL-6 was observed for both groups B and C at day 1 of the coculture.

**Fig. 6.** TNF-α was measured in the supernatant of lymphocyte-PVEC coculture after either no PI exposure (group A), syngeneic PI exposure (group B), or allogeneic PI exposure (group C). The mean values of duplicated wells from 1 of 2 experiments are shown. Peak production of TNF-α was observed for group C at day 1 of the coculture.
inflammation, where it plays a role in T-cell adhesion and costimulation [8]. Therefore, non-specific expression of the molecule ICAM-1 on PVEC, potentially acting as a ligand for the leukocyte integrin LFA-1, can facilitate lymphocyte diapedesis and migration into tissues [9]. To assess further the expression of these surface molecules, ICAM-1 and MHC-Class-I, on “activated” PVEC after PI contact, we explored the functional response of naïve syngeneic splenocytes cocultured with the “activated” PVEC. No significant differences in lymphocyte proliferative or cytotoxic responses were shown when naïve syngeneic splenocytes were incubated with PVEC previously exposed to either syngeneic or allogeneic PI. We concluded that ICAM-1 and MHC-Class I upregulation were not associated with significant lymphocyte activation. While it is apparent in some model systems that endothelial cells might be able to stimulate allogeneic lymphocytes [10], the lack of a lymphocyte response in our in vitro system suggests that PVEC activation may be associated with a nonspecific cytokine release instead. ELISA assays measuring cytokine levels in the cocultures of “activated PVEC” with naïve splenocytes showed increased concentrations of both IL-6 and TNF-α.

We conclude that the reactivity of the portal vein endothelium with increased ICAM-1 and MHC-Class I expression is associated with a release of IL-6 in a non-specific manner, because it occurs after exposure to either syngeneic or allogeneic PI. Interestingly, the increased release in TNF-α in the coculture of splenocytes and “activated PVEC” with allogeneic PI, as opposed to no increase with syngeneic PI, may initiate a more specific immune response. Therefore we hypothesize that the PI-PVEC interaction may trigger a nonspecific cytokine-mediated inflammatory cascade, which in
the allograft situation with MHC mismatch is augmented by an immune rejection response in the host, leading to islet graft damage (Fig. 7); the absence of this latter response in syngeneic PI transplants allows these grafts to survive as the inflammatory response wanes.

A review of previous studies has shown that TNF-α can cause vascular endothelial cells to express new surface receptors and to release inflammatory cytokines such as IL-6 [11]. Furthermore, cytokines such as IL-6, and TNF-α suppress islet function with decreases of insulin content and secretion [12,13].

Our study contributes to the understanding of the loss of 30% of pancreatic islet function during the first period of PI engraftment and disproves the hypothesis that the portal vein endothelium interacting with PI graft plays the role of an antigen-presenting cell. Other investigators who have studied islet cell graft survival have also described a cytokine release during the post-islet isolation phase as suggested in our in vitro system [14]. Furthermore Ricordi and Herring [2] state that apoptosis and other non-immune inflammatory pathways trigger islet cell damage during the early post transplant phase. We are pursuing our investigation by assessing islet cell survival and function in vitro and testing our current hypothesis, which is summarized in Fig. 7. Our next set of studies will attempt to modulate the inflammatory cytokine response and improve PI graft survival and function in vitro and in vivo.

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