Matrix Embedding Alters the Immune Response Against Endothelial Cells In Vitro and In Vivo

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Background—Endothelial cell (EC) dysfunction represents the first manifestation of atherosclerotic disease. Restoration of endothelium via seeding or transfection is hampered by local alterations in flow, inflammation, and metabolic activation. Perivascular EC implants are shielded from these forces and still control vascular repair. The host immune response to such implants, however, remains largely unknown. We investigated the effect of embedding of ECs within 3-dimensional matrices on host immune responses in vitro and in vivo.

Methods and Results—We compared expression of major histocompatibility complex (MHC), costimulatory, and adhesion molecules by free aortic ECs or ECs embedded in Gelfoam matrices by flow-cytometry. T-cell proliferation was assessed by \(^{3}H\) thymidine incorporation. Humoral immune response (ELISA and FACS analysis) and cellular (histopathology) infiltration were investigated after subcutaneous injection of free porcine aortic ECs (PAEs) or of a Gelfoam/EC block, or after concomitant injection of PAEs adjacent to Gelfoam in rats. Aortic ECs embedded in Gelfoam expressed lower levels of MHC class II, costimulatory, and adhesion molecules compared with free ECs \((P < 0.001)\), and induced 3-fold less proliferation of human CD4\(^+\) T-cells \((P < 0.0005)\). Implantation of a Gelfoam/EC block in rats nearly abrogated the immune response with 1.75- to 9.0-fold downregulation in tumor necrosis factor-\(\alpha\), interleukin-6, monocyte chemotactic protein-1, and PAE-specific immunoglobulin G \((P < 0.005)\) and 3.3- to 4.5-fold reduction in leukocytic tissue infiltration. Injecting PAEs adjacent to Gelfoam induced a significant response comparable to that of free implanted PAEs.

Conclusions—Embedding ECs within 3-dimensional matrices alters the host immune response by inhibiting expression of MHC class II, costimulatory, and adhesion molecules, offering the rationale to develop novel therapies for vascular diseases. (Circulation. 2005;112[suppl I]:I-89–I-95.)

Key Words: cells ◼ endothelium ◼ immune system

Several attempts have been undertaken to replace diseased endothelium with healthy, functional endothelial cells (ECs), such as EC seeding of vascular grafts or implantation of tissue-engineered vessels.\(^1\) The luminal surface is a harsh environment, however, and EC survival is poor after seeding or sodding.\(^2\) ECs survive far longer in the perivascular space away from flow and heightened metabolic activity.\(^3\) Interestingly, if embedded within 3-dimensional (3D) polymeric scaffoldings (Gelfoam), ECs can be grown to great density and confluence, and in this state retain their biochemical function and normal growth kinetics. Perivascular implants of 3D blocks with confluent ECs can continue to serve as powerful regulators of vascular disease.\(^3\)\(^-\)\(^5\) When placed in the perivascular space of injured arteries, these tissue-engineered EC constructs (TEECs) inhibit intimal hyperplasia by nearly 10-fold over control and unseeded implants and \(\geq\) 3-fold more than perivascular heparin release devices up to 3 months.\(^4\)\(^-\)\(^5\) Though autografts are tolerated better than allografts and xenografts, endothelial dysfunction is considered as a diffuse endothelial cell disease, and these cells are not ideal for reimplantation.\(^6\) Thus, nonautologous healthy ECs are phenotypically and biochemically far more desirable, but their implantation poses an immunological challenge. However, allogeneic and even xenogeneic ECs embedded in 3D Gelfoam-matrices evoked only a weak immune response compared with injected cells.\(^4\)

The purpose of this study was to characterize the extent of immune response to matrix-embedded ECs and to explain why and how this response is muted in comparison to direct injection of these same cells.

Methods

Porcine and Human Aortic ECs

Porcine aortic ECs (PAEs) were isolated from LargeWhite swine aortas by collagenase treatment. The aortas were washed extensively...
with cold phosphate buffered saline (PBS) supplemented with 200 U/mL Penicillin G and 200 μg/mL streptomycin. After removal of adventitial tissue and blood clots, sections of the aorta were clamped and filled with prewarmed collagenase II (0.75 mg/mL in PBS/PS: Worthington Biochemical) for 8 minutes. Detached cells were collected by flushing and plated in 35-mm polystyrene tissue culture plates (TCPS, Corning) containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum (HyClone), 100 U/mL Penicillin G, and 100 μg/mL streptomycin. Cells were split on confluence by incubation with 0.25% trypsin/0.04% EDTA (Life Technologies).

Human aortic ECs (HAEs) were obtained from Clonetics and grown in optimized endothelial growth medium-2 (Cambrex) supplemented with 5% fetal bovine serum. Medium (DMEM) supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum.

Endothelial growth medium-2 (Cambrex) supplemented with 5% fetal bovine serum.

Gelfoam sheets (Pharmacia & Upjohn) were cut into 2.5 × 1.0 × 0.3 cm³ blocks, hydrated in PBS for 30 minutes, and incubated with endothelial cells (0.8 × 10⁵ PAEs or 0.9 × 10⁵ HAEs) for 2 hours before transfer to 17 × 100-mm polypropylene tubes containing 3 mL complete DMEM or endothelial growth medium-2 medium.3–5 Under standard cell culture conditions for up to 2 weeks ECs invaded the blocks, lining the interstices of the 3D Gelfoam matrices. Homogeneous distribution of ECs within the Gelfoam was demonstrated by scanning electron microscopy and biosecretory function through assays for production of a standard panel of factors. Cell viability was determined by trypan blue exclusion and a LIVE/DEAD viability/cytotoxicity kit (Molecular Probes).

**EC Biosecretory Function**

Biosecretory function of ECs in Gelfoam and on polystyrene wells were compared. Total protein production was determined by Bicinchoninic Acid protein assay-kit (Pierce). Total glycosaminoglycan and heparan sulfate production were determined using a dimethyl-methylene blue assay before and after cell-conditioned medium treatment with chondroitinase ABC (0.1 U/sample, Seikagaku America) for 3 hour at 37°C to eliminate chondroitin and dermatan sulfate.7,8 Prostacyclin concentrations were determined by a 6-keto-prostaglandin F1α (clone A85–1), and anti-mouse IgM (clone C65–485), rat anti-mouse anti-human 4-1BB ligand (PE-labeled, clone C65–485), rat anti-mouse anti-human CD40 ligand (PE-labeled, clone MIH12) from R&D Systems, and rat anti-mouse IgG2a was from Southern Biotechnology. Mouse anti-human ox40-ligand was from MBL. PE-labeled antibodies included mouse anti-human Programmed Death Ligand 1 (PD-L1, clone MIH1), anti-human PD-L2 (clone MH1H8), and anti-human inducible costimulator ligand (clone MH1H12) from eBioscience.

**Confocal Microscopy**

Expression of CD31, HMC-II, CD58, intracellular adhesion molecule-1 (ICAM-1), and E-selectin were also analyzed by confocal microscopy. ECs were seeded on cover slips or embedded in Gelfoam matrices. After washing with PBS and fixation with 3% paraformaldehyde for 20 minutes (cover slips) or overnight (Gelfoam), ECs were blocked with rat serum (Bethyl Laboratories) for 30 minutes. Before staining with antibodies, Gelfoam matrices were cut into 2-mm thick slices. ECs were stained with the appropriate amount of antibodies for 1 (cover slips) or 2 hours (Gelfoam) and analyzed on a Zeiss LSM510 Laser scanning confocal microscope. Staining intensity was quantified with ImageJ software (National Institute of Health) and normalized against CD31 expression.

**Lymphocyte Proliferation Assay**

PAEs grown on polystyrene wells or embedded in Gelfoam were seeded in 96-well plates at 5 × 10⁴ cells/well and stimulated with 40 ng/mL porcine INF-γ for 48 hours, followed by mitomycin C treatment (50 μg/mL for 30 minutes; Sigma) to prevent background proliferation. Human CD4⁺ lymphocytes were purified by negative selection by guest on April 30, 2017 http://circ.ahajournals.org/ Downloaded from
selection with a CD4+ T-cell isolation kit II (Miltenyi Biotec) according to the manufacturer’s instructions and added at 2×10^6 cells/well. In some experiments, a murine antibody directed against HLA-DR,DQ,D [3H]-thymidine blocked activation via MHC class II molecules. We measured [3H]-thymidine incorporation on day 6 by 16 hour pulse (1 μCi/mL, Amersham). Thymidine uptake of mitomycin-treated PAEs, medium, or T-cells alone were used as negative controls.

**PAE-Induced Immune Reaction in Rats**

Fifty-four Sprague-Dawley rats (Taconic, Germantown, NY) received 5×10^5 PAE transplants in the subcutaneous dorsal space as Gelfoam-embedded cells, saline-suspended cell pellets, or pellets adjacent to empty Gelfoam. After dorsal incision, a small subcutaneous cavity was created with blunt dissection and Gelfoam matrices were carefully inserted into this space. In some animals, ECs were injected directly onto empty Gelfoam matrices. Empty control Gelfoam matrices were incubated in complete DMEM before implantation. Sera were collected serially from 0 to 56 days, aliquoted and stored at –70°C. Circulating rat immunoglobulins specific for the new implantation. Sera were collected serially from 0 to 56 days, aliquoted and stored at –70°C. Circulating rat immunoglobulins specific for the implanted PAEs were measured by flow cytometry. 2×10^5 PAEs were collected at various time points and fixed in 1% paraformaldehyde, and 104 cells were analyzed by flow cytometry.

We measured the presence of rat TNF-α, IL-6 (R&D Systems; detection limit 5 pg/mL), and rat IL-10 (R&D Systems; detection limit <5 pg/mL). Serum-concentrations were quantified by ELISA on days 0, 5, 12, and 28 after implantation. Measurements were performed at the same time by the same ELISA to avoid variations of assay conditions.

The cellular response to implantation was evaluated immunohistochemically in 6 rats from each group on day 28 after implantation. Five-micrometer paraffin sections were cut and antigen retrieval performed by microwave heating for 10 minutes in a 0.01 mol/L citrate buffer (pH 6.0). Leukocytes and T and B lymphocytes were identified by an avidin-biotin peroxidase complex method. The primary antibodies were mouse anti-rat CD45R0 to identify leukocytes (Research Diagnostics; 1:50 dilution), mouse anti-rat CD4 to identify CD4+ T-cells (Pharmingen; 1:10 dilution), and mouse anti-rat CD8 to identify CD8+ T-cells (Pharmingen; 1:50 dilution). Rat spleen was used as a positive control, and mouse IgG as negative control. Primary antibodies were applied for 1 hour at room temperature, and all sections were counterstained with Mayer’s hematoxylin solution (Sigma). Six nonoverlapping fields (×600) were examined by 2 blinded observers. The results for each group were averaged.

**Statistics**

Statistical analyses were performed with JMP software (SAS Institute Inc). Data were found to be normally distributed and expressed as mean±SD. Comparisons between 2 groups were analyzed by Student’s t test, and comparisons between >2 groups were analyzed by ANOVA followed by Bonferroni post hoc test. A value of P<0.05 was considered statistically significant.

**Results**

**Growth Kinetics and Biochemical Activity of Matrix-Embedded ECs**

ECs cultured within Gelfoam grew well within 3D matrices, with cell doublings approximately every 36 hour at a saturation density of 0.7×10^6 cells/cm^2 Gelfoam. Cell viability remained at 95% over the 2-week culture course. Matrix-embedded ECs retained their identity and normal postconfluent biosecretory ability. PAEs cultured in Gelfoam produced similar amounts of glycosaminoglycan (2.76±0.19 versus 2.77±0.13 μg/10^6 cells), heparan sulfate (1.53±0.02 versus 1.56±0.08 μg/10^6 cells), prostacyclin (72.3±3.8 versus 76.8±1.6 pg/10^6 cells), and transforming growth factor-β (844.3±123.4 versus 889.8±147.3 ng/10^6 cells) as cells grown in TCPS, and PAEs retained their ability to take up acetylated-low-density lipoprotein when cultured in Gelfoam.

**Embedding of ECs in Gelfoam Alters Expression of Surface Molecules**

Constitutive expression of CD58 was significantly reduced in PAEs embedded in Gelfoam compared with CD58 expression of PAE grown on TCPS (−60.4%, P<0.002; Figure 1). There was also a significant reduction in upregulation of MHC class II, costimulatory, and adhesion molecules on matrix-embedded PAEs compared with PAEs grown on TCPS under FACS-analysis (CD80: −64.9%, P<0.002; CD86: −65.4%, P<0.001; CD40: −53.8%, P<0.005).

**Figure 2.** Expression of CD58, ICAM-1, E-selectin, MHC-II, and CD31 were analyzed by confocal microscopy. Staining intensity was normalized against CD31 expression. Representative pictures of CD58 and MHC II expression on matrix-embedded PAEs and PAEs grown on TCPS.
ICAM-1: -68.7%, P<0.001; vascular cell adhesion molecule-1: -53.9%, P<0.005; E-selectin: -71.8%, P<0.0005; P-selectin: -79.9%, P<0.0002; MHC II: -78.3%, P=0.0002; Figure 1). There were no significant differences in surface expression of MHC class I and CD31 molecules. Confocal microscopy revealed reduced expression levels of CD58, ICAM-1, E-selectin, and MHC-II on matrix-embedded PAEs (whereas CD31 expression remained unchanged (P<0.02; Figure 2).

Similar results were obtained for HAEs. HAEs grown in a 3D matrix exhibited a significantly reduced expression profile of CD58 and showed a significant lack in upregulation of costimulatory and adhesion molecules (Figure 3). However, there were no significant differences in ICAM-1, E-selectin, MHC I, and CD31 expression levels between HAEs embedded in Gelfoam and HAEs grown on TCPS. Furthermore, there were no significant differences in constitutive expression of PD-L2 (100%, P=0.73) and in upregulation of PD-L1 (86%, P=0.09; Figure 3).

**Lymphocyte Proliferation Assay**
The proliferative responses of isolated human CD4+ T-cells to untreated and INF-γ treated PAEs (40 ng/mL, 48 hours) grown in TCPS or embedded in Gelfoam were assayed by thymidine incorporation. The strong CD4+ T-cell proliferation noted after exposure to PAEs pretreated with INF-γ was nearly eliminated when PAEs were matrix-embedded (17087.2±3749.75 versus 5367.8±1976.3 cpm, P<0.01; Figure 4). The presence of MHC II antibody blocked lymphocyte proliferation in response to INF-γ–treated PAEs by 65% to a level comparable to matrix-embedded PAEs (Figure 4). Mitomycin-treated PAEs (61±13 cpm) and isolated CD4+ T-cells alone (83±27 cpm) did not show a significant proliferation after 6-day culture.

**Effect of EC Embedding on Immune Response In Vivo**
We then explored the effect of embedding ECs in Gelfoam on immune responses in vivo. PAE embedding in Gelfoam significantly reduced formation of PAE-specific IgG (Figure 5A and 5B). Serum cytokines (MCP-1, IL-6, TNF-α) rose, peaking 5 days after implantation, in rats receiving free PAEs and injections of PAEs adjacent to Gelfoam. In contrast, cytokine levels did not increase above background in animals with matrix-embedded PAEs (Figure 5C).

Immunohistological studies revealed evidence of cellular infiltration into and around the implant/injection site at 28 days. After injection of free PAEs and injection of PAEs adjacent to Gelfoam, T-cells were abundant within the implant/injection side, whereas large numbers of CD45R0-positive leukocytes were also found at the periphery of the graft. In contrast, the tissue surrounding the implant and Gelfoam-PAE itself were infiltrated with 4.5-fold fewer leukocytes and CD4+ T-cells and 3.3-fold fewer CD8+ T-cells than the other cell implantation groups (Figure 6, Table).

**Discussion**
Vascular connectivity of xenogeneic organ transplants is associated with hyperacute and acute rejection. In contrast, xenogeneic cells or tissue implanted and not anastomosed to vessels remain at risk of a cellular immune response and may have size limitations but are largely unaffected by humoral immunity. This cellular reaction involves T-cell activation and graft infiltration, and is also seen in alloimplants.9

We have shown that matrix-embedding of cells like ECs, which are normally substrate-adherent, prolongs cell survival...
after implantation. Matrix-embedded ECs retain their proliferative, biosecretory, and biochemical activity. They also have significantly reduced expression of constitutive and cytokine-upregulated costimulatory and adhesion molecules compared with free ECs, and in particular a marked abrogation of MHC II regulated immune reactivity.

ECs activate T-cells through antigen-mediated processes, and T-cell activation can modify crucial EC function, including antigen presentation via activation by cytokines. ECs of transplanted organs play diverse and important roles in nonautologous immune responses. Antigen-presentation to CD4+ T-cells via MHC-II is essential for host immune recognition in the setting of nonvascularized xenogeneic implants. Cell-substrate anchoring had no effect on MHC-I expression but markedly muted the expected upregulation of MHC-II molecules. PAEs embedded in Gelfoam evoked only a modest proliferation of xenogeneic CD4+ T-cells in vitro, similar to the response seen with blockade of MHC-II binding in free PAEs.

Antigen-specific signals generated by T-cell receptors alone are insufficient for optimal T-cell activation. Full activation requires a second “positive” costimulatory signal provided during cognate interaction with antigen-presenting cells. Costimulation has therefore become a potential target by which to enable allograft and xenograft transplantation.

Matrix-embedded PAEs and HAEs exhibited significantly lower expression levels of costimulatory and adhesion molecules on activated ECs without affecting inhibitory co-molecules.

Matrix-embedded PAEs showed a lower stimulation of the initial event in the recruitment of leukocytes, which involve P-selectin and E-selectin, and of vascular cell adhesion molecule-1, which is closely associated with T-cell recruitment at sites of immune inflammation. The full panel of general and species-specific costimulatory molecules was downregulated by matrix embedding, including the first report of EC expression and suppression of 4-1BB-ligand. At the same time, expression and upregulation of PD-L1 and PD-L2, members of the B7-family that act as countervailing inhibitory molecules, remained intact after matrix embedding. These in vitro findings translated into a significantly muted immune reaction in rats after implantation of matrix-embedded PAEs. The immune tolerance for Gelfoam-embedded cells likely derives from a direct interaction between ECs and the Gelfoam matrix, rather than simple concomitant presence or contact of the 2 materials.

Whereas others have shown that implanted cells or proteins combined within tissue-engineered biomaterials can serve as a source of antigens, it appears from our data that the immune reaction to confluent ECs that remain matrix-
adherent is dramatically muted. Earlier results have demonstrated that Gelfoam is immunoneutral, and here we show that Gelfoam itself has no immune-protective effect, as injection of PAEs adjacent to Gelfoam evoked the same immune response as free injected ECs. The nature of ECs may therefore contribute to this unique form of immunomodulation. In particular, these cells have a sidedness, a basal surface that interacts with basement membrane and superior surface that interacts with flowing blood and cellular elements. Data from others and us suggest that EC function is anchorage- and density-dependent. Systemic diseases like hypertension, alterations in lipid and glucose metabolism, or exposure to toxins alter anchorage-dependent regulation, and the amplitude and nature of immune responses against the endothelium and phenotypic transformation of intact ECs from matrix-adherent to free might be critical to initiation of vascular disease. The importance of a 3D environment for activation of T-cells was just recently elucidated when Gunzer et al demonstrated that in the same 3D environment, naive T-cells respond with a spectrum of different interaction modes dependent on the type and activation state of the antigen-presenting cells.

Taken together, our results could have implications for the understanding of vascular and immune biology. Tissue engineering might serve as a tool to study the role of the immune response in vascular disease on the one hand and the role of matrix systems as enablers of EC implantation on the other. Our results are relevant for further definition of vascular disease and for the development of clinically applicable xenograft protocols.

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