Cell–Matrix Contact Prevents Recognition and Damage of Endothelial Cells in States of Heightened Immunity

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Background—Autoimmunity may exacerbate vascular disease, particularly in the form of anti-endothelial cell (EC) antibodies. The increased morbidity of cardiovascular diseases in concert with diabetes mellitus, hypertension, and other systemic illnesses may reflect the increase presence and potency of these antibodies. Matrix-embedded ECs act as powerful regulators of vascular repair accompanied by significant reduction in expected systemic and local inflammation. We compared the immune response against free and matrix-embedded ECs in naïve mice and mice with heightened EC immune reactivity.

Methods and Results—Mice were presensitized to EC with repeated (days 0, 21, 35) subcutaneous injections of saline-suspended porcine EC (PAE) (5×10^5 cells). Controls received saline injections. On day 42, mice received 5×10^5 matrix-embedded or free PAEs. Circulating PAE-specific antibodies and effector T-cells were analyzed via flow cytometry, and xenoreactive lymphocytes via ELISPOT, 90 days after implantation. PAE-specific antibody-titers, frequency of $CD4^+$-effector cells, and xenoreactive splenocytes were 2- to 4-fold lower ($P<0.0001$) when naïve mice were injected with matrix-embedded instead of saline-suspended PAEs. Though basal levels of circulating antibodies were significantly elevated after serial PAE injections (2210 ± 341 mean fluorescence intensity, day 42) and almost doubled again 90 days after injection of a fourth set of free PAEs, antibody levels declined by half in recipients of matrix-embedded PAEs at day 42 ($P<0.0001$). Levels of $CD4^+$-effector cells and xenoreactive splenocytes showed similar results.

Conclusions—Implantation of free PAE elicits a significant immune response in naïve mice and even more pronounced in mice with predeveloped anti-endothelial immunity. Matrix-embedding protects xenogeneic ECs against immune reaction in naïve mice and to a similar extent in mice with heightened immune reactivity. Matrix-embedded EC might offer a promising approach for treatment of advanced cardiovascular disease. (***Circulation***. 2006;114[suppl I]:I-233–I-238.)

Key Words: antibodies ■ endothelial cell ■ extracellular matrix ■ immune system

It is well-established that endothelial cell (EC) activation and damage elicited by different stimuli contribute to initiation and perpetuation of atherosclerotic disease. Activation and damage of ECs involves a stereotypical series of processes that create local inflammation inducing further changes in EC function and morphology. The 5 core changes of EC activation are loss of vascular integrity, altered expression of adhesion molecules, change in phenotype from antithrombotic to prothrombotic, changes in cytokine production, and upregulation of human leukocyte antigen (HLA) molecules.[1] Atherosclerotic disease in general[2] as well as a variety of cardiovascular risk factors[3–5] and autoimmune diseases[6] are associated with circulating anti-EC antibodies that directly damage and activate EC.[6,7] Yet the specific underlying pathophysiologic modalities allowing for phenotypic and genotypic changes of ECs are not fully delineated. Recent research on fibroblasts and smooth muscle cells has focused on a role of cell–matrix interactions in the activation and damage of cells and the induction of cellular immunogenicity.[8–10] There is a growing appreciation that effects in 2-dimensional culture do not necessarily translate into 3-dimensional systems.[10,11] In this regard we demonstrated muted immunogenicity and reduced activation of human and porcine ECs when embedded within a 3-dimensional collagen based matrix.[12]

This in-depth analysis of host immune reactions in immunocompetent mice investigated whether continuous cell–matrix contact would shield ECs from recognition and subsequent damage in vivo and if such immunoprotection would extent toward states of heightened anti-endothelial immunity.

Methods

Embedding and Implantation of Porcine Aortic Endothelial Cells

Porcine aortic ECs (PAEs) isolated from Large White swine aortae were either seeded on Gelfoam blocks ($2.5 \times 1.0 \times 0.3$ cm³; Pfizer,

New York, NY) as previously described[12] or grown to confluence on polystyrene plates. B6-mice received injections in the subcutaneous dorsal space on days 0, 21, and 35 of saline-suspended PAEs (5×10^5 cells, n=24, presensitized mice) or saline (n=24, naïve mice). On day 42, 12 mice from each group received 5×10^5 matrix-embedded or free PAE. All animal procedures were reviewed and approved by the local ethics committee on animal care and were conducted in accordance with the principles expressed in the Helsinki Declaration. Host immune reactions and lytic damage of ECs were studied for the next 90 days. Sera were collected serially from days 42 to 132, aliquoted, and stored at $-70°C$. Six mice of each group were euthanized on day 70, and the remaining were euthanized on day 132 for splenocyte isolation.

PAE-Specific Immunoglobulins

Serum levels of immunoglobulins specific for the implanted PAEs were measured by flow cytometry; 2×10^5 PAEs, same strain as the implanted cells, were detached from cell culture plates with 0.25% trypsin/0.04% EDTA, pelleted, washed, and resuspended in FACS buffer (phosphate-buffered saline [PBS], 1% fetal bovine serum [FBS], 0.1% sodium azide; Sigma Chemicals). These cells were then incubated with serum from recipient mice for 60 minutes at 4°C (diluted 1:10 in FACS buffer). After washing twice with FACS buffer, cells were incubated with FITC-conjugated rat anti-mouse IgG$_{2a}$ (clone H106.771; Southern Biotechnology, Birmingham, Ala), IgG$_1$ (clone A85–1), IgM (clone R6–60.2), or isotype controls (BD PharMingen, San Jose, Calif). After 30 minutes of incubation at 4°C, samples were washed twice with cold FACS buffer, fixed in 1% paraformaldehyde, and 10^4 cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson, Calif).

Splenocyte Isolation

Spleens were isolated aseptically in a laminar flow hood. Organs were cut in several pieces and clumps were further dispersed by drawing and expelling the suspension several times through a sterile syringe through a 19-G needle. Suspensions were filtered through a 200-μm mesh nylon screen. Cells were washed twice with RPMI (containing 2 mmol/L L-glutamine, 0.1 mol/L HEPES, 200 U/mL penicillin G, 200 μg/mL streptomycin, and 5% heat-inactivated calf serum) and immediately used. Isolated splenocytes in this state were evaluated in the following in vitro assays.

Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay

Immunospot plates (Millipore, Mass) were coated with 5 μg/mL of anti-mouse interferon (IFN)-γ, IL-2, IL-4, or IL-10 monoclonal antibodies (all BD Pharmingen) in sterile PBS overnight. Plates were then blocked for 2 hours with complete RPMI medium without phenol red, containing 10% heat-inactivated calf serum. Splenocytes and the same strain of PAEs used for implantation (both 0.5×10^6 in 100 μL complete RPMI medium) were then placed in each well and cultured for 48 hours at 37°C/5% CO$_2$. After washing with deionized water followed by PBS containing 0.05% Tween (PBST), 2 μg/mL of biotinylated anti-mouse IFN-γ, IL-2, IL-4, or IL-10 monoclonal antibodies (all BD Pharmingen) were added and incubated overnight. After washing 3 times with PBST, wells were incubated with horseradish peroxidase-conjugated streptavidin for 1 hour. After washing 4 times with PBST followed by PBS, plates were developed using 3-amino-9-ethyl-carbazole (all BD Pharmingen). The resulting spots were counted on a computer-assisted ELISPOT image analyzer (Cellular Technology). The number of spots in negative control wells (medium, splenocytes, or PAEs alone) were subtracted from xeno-responses to account for background during data analysis. Empty Gelfoam matrices had no effect on cytokine release by splenocytes.

Characterization of Host Effector Cells

2×10^6/mL splenocytes were stained with anti-CD4 FITC (clone L3T4), anti-CD8 FITC (clone Ly-2), anti-CD44 R-PE (clone Ly-24), and anti-CD62L allophycocyanin (clone Ly-22), and isotype controls (all BD PharMingen). CD4$^+$ and CD8$^+$ effector cells expressing CD44^{high} and CD62L^{low} were enumerated on a FACScalibur instrument, as previously described.[13]

Calcein-AM Release Assay

2×10^4 PAE/well (same strain as implanted cells) were incubated with 15 μmol/L calcein-AM (Invitrogen) for 40 minutes at 37°C with occasional agitation. After 2 washes with complete medium, splenocytes were added to the PAEs at final concentrations of 50:1, 25:1, 10:1, and 1:1. Spontaneous and maximum calcein release were examined as controls in 6 replicate wells that contained only PAEs or PAEs treated with 2% Triton X-100 for the last 20 minutes. After 3 hours of incubation at 37°C/5% CO$_2$ samples were measured using a Fluoroskan Ascent FL dual-scanning microplate luminofluorimeter (Thermo Electron Corporation). Data were expressed as arbitrary fluorescent units (AFU). Specific lysis was calculated according to the formula [(test release$-$spontaneous release)/(maximum release$-$spontaneous release)]$\times 100$.[14]

Statistical Analysis

All statistical analyses were performed with JMP software (2002; SAS Institute Inc). Data were validated as normally distributed and expressed as mean$\pm$SD. Comparisons between 2 groups were analyzed by Student t test, and comparisons between >2 groups were analyzed by ANOVA. A Spearman correlation determined relations between effector and cytokine-producing T-cells. $P<0.05$ was considered statistically significant.

Statement of Responsibility

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

EC Injections Induce Antibody Formation in Mice

In naïve B6 mice, 3 serial subcutaneous injections of xenogenic PAEs raised circulating anti-EC antibodies well above that seen with saline injections; 42 days after first injection of PAE IgG$_1$ antibody titers were 42-fold higher (2210 ± 341 versus 53 ± 12 mean fluorescence intensity [MFI]; $P<0.0001$) and IgM 2.8-fold higher (136 ± 39 versus 49 ± 14 MFI; $P<0.02$). There were no PAE-specific IgG$_{2a}$ antibodies detectable in serum of either mouse groups (data not shown).

Matrix-Embedding ECs Prevents Humoral Immune Reactivity

As previously demonstrated,[12] implantation of matrix-embedded xenogeneic ECs, in marked contrast to implantation of free cells, failed to induce a significant humoral immune response in naïve mice. In fact, IgG$_1$ antibody levels were 3.5-fold lower (210 ± 102 versus 735 ± 327 MFI; $P<0.001$) and IgM antibodies 5-fold lower (60 ± 11 versus 299 ± 51 MFI; $P<0.001$; Figure 1A and 1B) than after implantation of free cells. Injection of free PAE in presensitized mice resulted in an elevated humoral immune response with a pronounced increase in IgG$_1$ antibody levels (3795 ± 448 MFI; $P<0.0002$ versus naïve mice) and slight increase in PAE-specific IgM (164 ± 28 MFI). In marked contrast, implantation of matrix-embedded PAEs in presensitized mice did not increase PAE-specific antibodies. Moreover, antibody levels specific for the injected PAEs slowly decreased with time (IgG$_1$: 1578 ± 334 MFI; $P<0.0005$ versus free PAE; IgM: 69 ± 5 MFI; $P<0.01$ versus free PAE; Figure
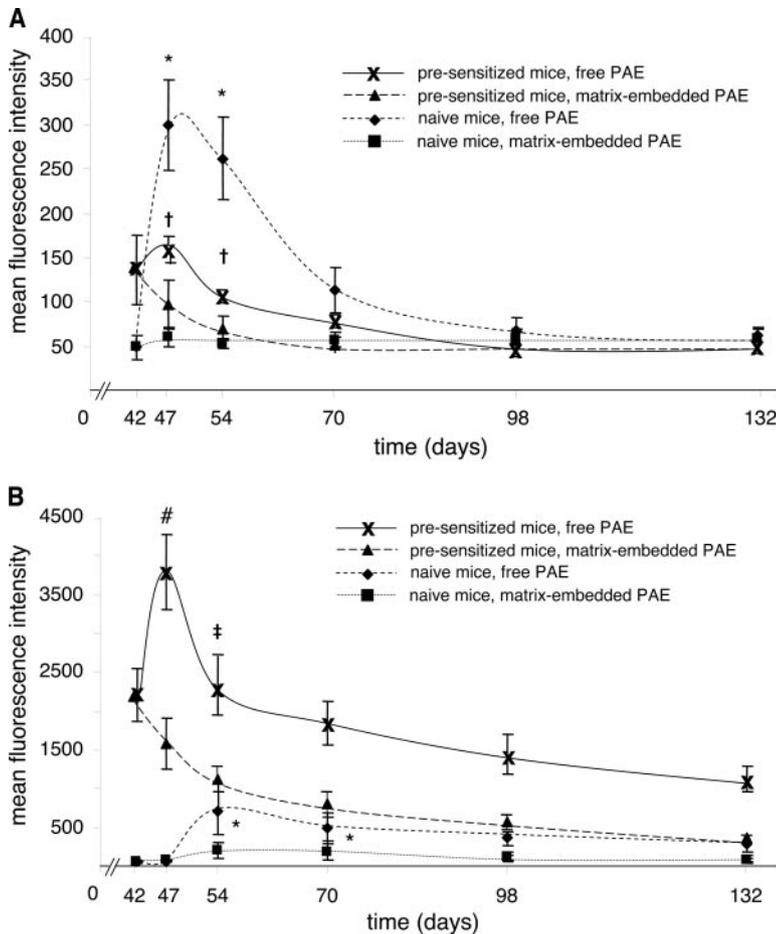
Figure 1. Circulating PAE-specific IgG$_1$ (A) and IgM (B) in naïve and presensitized mice after subcutaneous implantation of nonembedded or matrix-embedded PAEs were determined via flow cytometry. Graphic depiction of results from all mice (n=12/group to day 70, n=6/group day 71 to 132 after implantation) demonstrates significant differences between matrix-embedded and free PAE implantation. Antibody levels after implantation of matrix-embedded PAEs are slowly diminishing. Data are expressed as mean values±SD. *$P<0.001$, matrix-embedded vs free in naïve mice. †$P<0.01$, matrix-embedded vs free in presensitized mice. ‡$P<0.0002$, free PAE naïve vs presensitized mice. #$P<0.0005$, matrix-embedded vs free in presensitized mice.

1A and 1B). There was no increase in PAE-specific IgG$_{2a}$ antibodies in the 4 treatment groups (data not shown) supporting previous reports of a dominating T-helper cell 2 (Th2) response in xenografting.[15,16]

Matrix-Embedded ECs Are Poor Inducers of Cellular Immunity

Serial injections of free ECs induced xenogeneic Th2 cytokine (IL-4, IL-10) producing splenocytes in naïve and presensitized mice, with the latter 23% to 33% greater than the former (IL-4: 907±59 versus 680±129; $P<0.02$; IL-10: 1096±94 versus 888±151 number of spots; $P<0.02$; Figure 2A and 2B). Matrix-embedded PAEs elicited a far lower response in both mice subsets ($P<0.001$) with only a slight increase in IL-4 in presensitized hosts (322±75 versus 199±99 number of spots; $P<0.05$; $P<0.0005$ versus free PAE; Figure 2A) but not in IL-10 producing xenoreactive splenocytes (403±142 versus 451±135 number of spots; $P=0.27$; $P<0.001$ versus free PAEs; Figure 2B). The frequency of T-helper cell 1 (Th1) cytokine (IFN-γ and IL-2) producing splenocytes did not differ significantly between the 4 treatment groups, again supporting a predominant Th2 role in xenoreactivity (data not shown).

The increase in cytokine-producing splenocytes in mice receiving nonembedded PAEs was paralleled by an increase of CD4$^+$ and CD8$^+$ effector T cells over time (CD4$^+$: 44±2 naïve mice, 54±4% presensitized mice, $P<0.05$; CD8$^+$:

20±2; 21±2%; Figure 3A and 3B). Accordingly, differentiation of T cells into CD44^{high}/CD62L^{low} T-cells was significantly muted in naïve and presensitized mice exposed to matrix-embedded PAEs (CD4$^+$: 22±2 naïve mice, 21±3% presensitized mice; $P<0.01$ versus free PAEs; CD8$^+$: 12±2; 14±3%; $P<0.02$ versus free PAEs; Figure 3A and 3B). CD4$^+$ outnumbered CD8$^+$ effector T cells 1.7- to 2.6-fold in all treatment groups. A strong correlation was noted between the frequency of Th2-cytokine producing splenocytes and extent of T-cell differentiation cells into CD4$^+$CD44^{high}/CD62L^{low} (IL-4: $r=0.81$; $P<0.0001$; IL-10 $r=0.88$; $P<0.0001$; Figure 4) and CD8$^+$CD44^{high}/CD62L^{low} effector cells (IL-4: $r=0.79$; $P<0.0001$; IL-10 $r=0.86$; $P<0.0001$) across all treatment groups on day 132.

Matrix-Embedded ECs Are Shielded From Lytic Damage

The ability of host lymphocytes to damage xenogeneic ECs was characterized on days 70 and 132. Calcein release reached plateau at PAE effector:splenocyte ratios of 25:1. For this ratio, EC damage was 1.6-fold higher in naïve mice and 1.7-fold higher in presensitized mice when receiving nonembedded in place of matrix-embedded PAEs on day 70 ($P<0.001$). These ratios increased to 1.9 and 2.3, respectively, after 132 days ($P<0.0005$; Figure 5). The extent of endothelial damage in presensitized mice receiving matrix-embedded PAEs was significant lower when compared with
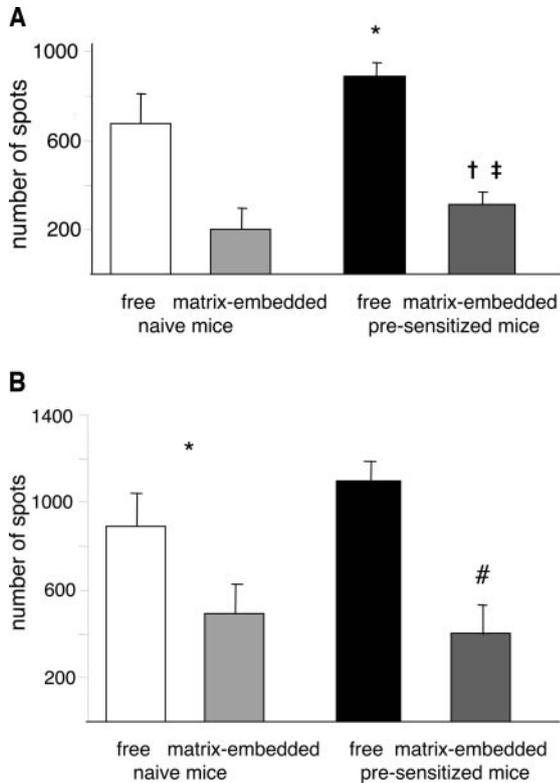
Figure 2. Frequency of xenoreactive cytokine-producing cells in recipients after implantation of free PAEs or matrix-embedded PAEs in naïve and presensitized mice. Data are expressed as mean±SD. Naïve and presensitized recipients of free PAEs exhibited significant increased frequencies of IL-4 (A) and IL-10 (B) producing splenocytes compared with recipients of matrix-embedded PAEs. *$P<0.02$, free PAEs naïve vs presensitized mice. †$P<0.05$, matrix-embedded PAEs naïve vs presensitized mice. ‡$P<0.0005$, matrix-embedded vs free in presensitized mice. #$P<0.001$, matrix-embedded vs free in presensitized mice.

Figure 3. Significantly increased CD4$^+$ (A) and CD8$^+$ (B) effector cells in mice receiving free PAEs. Splenocytes recovered from mice were analyzed by flow cytometry using CD62L and CD44 as markers for effector T-cells. No difference between naïve and presensitized mice when ECs are matrix-embedded. Data are expressed as mean values±SD. *$P<0.01$, matrix-embedded vs free. †$P<0.02$, matrix-embedded vs free. ‡$P<0.05$, naïve vs presensitized mice

naïve mice receiving free PAEs (20.9±2.3 versus 37.1±3.4% AFU; $P<0.001$; Figure 5) and did not increase with time in these animals.

Discussion

Transplantation of allogeneic cells is often accompanied by an immune response. The question that arises is whether this is a constitutive and immutable property of foreign cells or one that can be regulated. Cell transfusions are well-accepted unless surface donor antigens are mismatched with host. Cells from solid organs are not meant to circulate, and an autoimmune reaction can be seen when cells that are normally substrate adherent are freed and exposed to flow.[3–5] We examined whether the immunogenicity of cells that are normally anchored to basement membranes will be altered if implanted in a matrix-embedded rather than injected in a free, dislodged state. Our experiments were extended to include the influence of heightened anti-endothelial immunity that is a common clinical feature in a variety of autoimmune and endocrinologic diseases.

Serial injections of free PAEs raised circulating anti-PAE antibodies (Figure 1), elevating immunosensitivity, and the response to subsequent PAE injections was even greater than seen on first exposure. In contrast, when these same cells
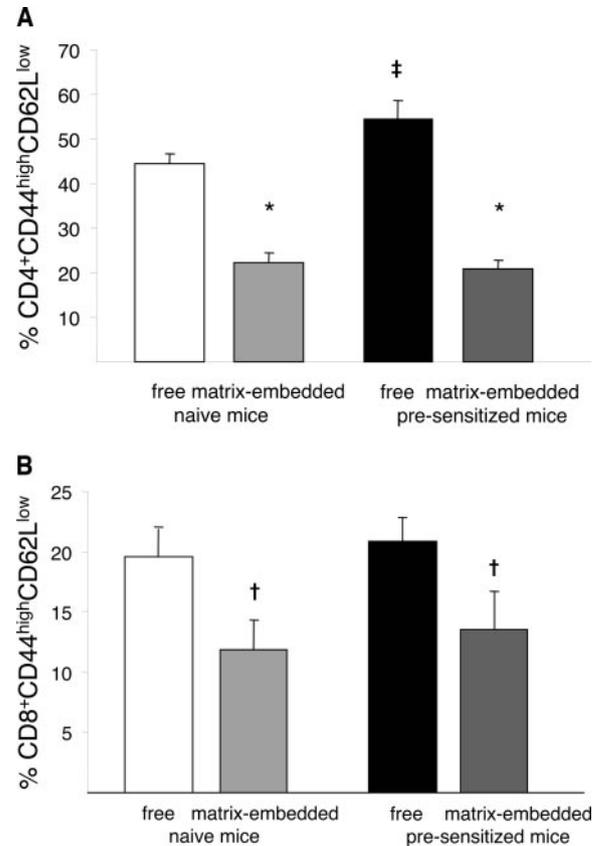
were implanted matrix-embedded, the immune response that followed what should have been presensitization decreased significantly over time. This is in line with previous observations that absence, withdrawal, or removal of antigen leads to a decline in circulating antigen-specific antibody levels. The initial response to EC injections is IgM-mediated, lower than the subsequent IgG response (Figure 1), and muted when preceded by serial injections (Figure 1B). The IgM response is far more robust in naïve than in presensitized animals and takes far longer to abate after free PAE injections than after implantation of matrix-embedded ECs. Presensitization of mice with suspensions of PAE resembles the IgG$_1$-driven anti-endothelial immunity seen in diabetes mellitus, hypertension, and autoimmune diseases.[3–7] The cellular immune response to free and matrix-embedded cells followed the pattern of humoral immunity. Repeated exposure to antigens results in increased formation of memory and subsequently in a more vigorous immune reaction by effector T cells.[17] Hence, the induction of xenoreactive IL-4 and IL-10 producing splenocytes (Figure 2) and effector T-cells (Figure 3) were elevated over time and only visible after implantation of free ECs in naïve and presensitized mice. In all mice, cytokine levels correlated linearly and precisely with effector
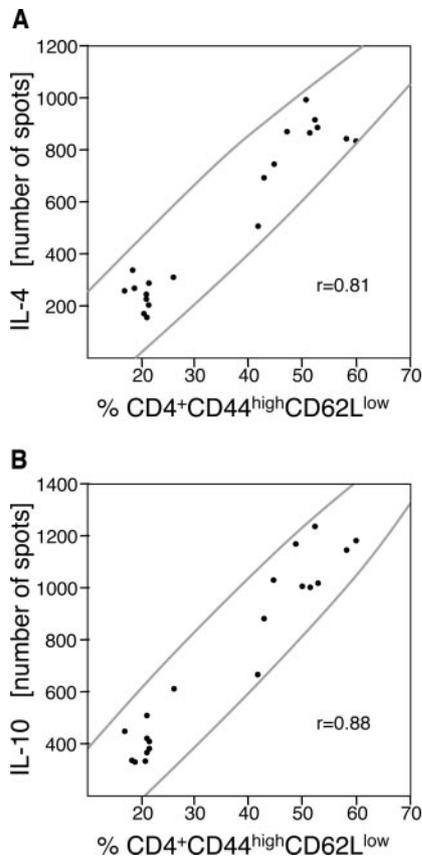
Figure 4. Spearman correlation of frequencies of Th2 cytokine-producing splenocytes and extent of T-cell differentiation into effector cells. Area of the density ellipse represents the 95% confidence interval.

T-cell induction (Figure 4), further supporting the notion of a Th2-driven cellular response in xenoreactivity[15,16] and accentuating the immunosilencing aspects of matrix-embedded ECs to activate adaptive immune mechanisms. This is in line with earlier reports demonstrating different behavior of cells in solution or attached to three- and two-dimensional surfaces.[9–12] The host damage to implanted ECs correlated with the extent of the immune response elicited. Implanted cells were most profoundly affected after presensitization and with free PAEs. The decreased induction of humoral and cellular immune responses in naïve mice and matrix embedding resulted in a lesser degree of damage by host immune cells (Figure 5).
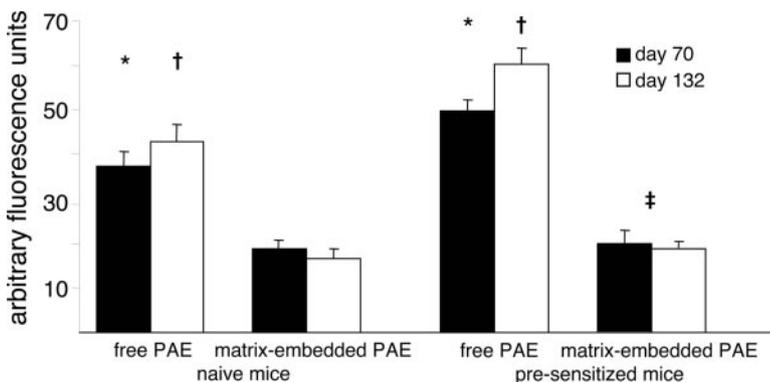
Our results provide novel insights into the activation of and damage to ECs, suggesting a pivotal role for cell–matrix contact. The honeycomb-like structure of our Gelfoam matrices, which are characterized by favorable biocompatibility,[12,18] allows ECs to attach and conform to the geometric configuration of the sponge, lining the internal surfaces of its matrix in a fashion simulating the appearance of confluent endothelium in quiescent vessels.[19] Thus, embedding ECs in Gelfoam resembles the physiological 3-dimensional state of intact endothelium. We now demonstrate that matrix embedding not only protects ECs from host immune reactions but also changes host perception of EC immunogenicity. Thus, the phenotypic transformation of ECs dislocated from a matrix-adherent to a free state might be critical to initiation and perpetuation of vascular disease. Our data suggest that EC detachment precedes expression of adhesion, costimulatory, and major histocompatibility complex molecules[12] that is then followed by attraction of immune cells, perpetuating endothelial activation and damage. In this regard, the immunobiological and immunoreactive qualities of ECs correlate with morphology and function. ECs from different vascular beds and divergent basement membrane connectivity demonstrate marked differences in constitutive and inducible expression of adhesion, costimulatory, and major histocompatibility complex molecules.[20] Further, there is growing appreciation that deposition of transitional extracellular matrix proteins such as fibronectin and fibrinogen into the subendothelial matrix as well as detachment of ECs from the basement membrane affects intra-endothelial signaling.[21,22]

Control of cell phenotype, immunogenicity, and function might be used to tailor the properties of tissue engineered constructs developed in vitro for regenerative purposes, particularly because cell-based therapies are limited by profound host immune reactions. Such potential is especially important for treatment of atherosclerotic disease because presence of activated immune cells and inflammation are key pathophysiologic components.[23] Heightened anti-endothelial immunity has been identified as a pivotal rate-limiting effect for EC-based therapies.[24,25] A more detailed understanding of how EC phenotypic shifts occur in vascular pathology, eg, via dearrangement of cell–matrix contact, would be of great benefit in developing appropriately targeted therapies.

Taken together, the results presented here demonstrate that the extracellular matrix can modulate immunogenicity of ECs. ECs embedded within a 3-dimensional matrix elicited

Figure 5. Endothelial damage via lysis is significantly reduced in naïve and presensitized mice receiving matrix-embedded compared with free PAEs. 2×10^4 PAEs were labeled with calcein and incubated with 5×10^5 splenocytes isolated after 70 and 132 days, respectively. *$P<0.001$, free vs matrix-embedded day 70. †$P<0.0005$, free vs matrix-embedded day 132. ‡$P<0.001$, presensitized matrix-embedded vs naïve free.

far less activation of host immune mechanisms and were subject to far lower attack and damage from host immune cells. Findings in naïve mice were amplified in hosts with heightened anti-endothelial immunity. This knowledge might be of importance in the field of vascular tissue engineering, because further immune protection of implantable cells might provide a basis for the rational design of extracellular matrices that exert control over cell function and immunogenicity, leading to improved tissue surrogates.

Sources of Funding

Disclosures

None.

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