Recipient mononuclear cell recognition and adhesion to graft endothelium after human cardiac transplantation. Lymphocyte recognition leads to monocyte adhesion.

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Research Article

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Recipient Mononuclear Cell Recognition and Adhesion to Graft Endothelium after Human Cardiac Transplantation
Lymphocyte Recognition Leads to Monocyte Adhesion

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Abstract

Transendothelial migration of mononuclear cells is crucial in the development of allograft rejection and transplant coronary disease. Adhesion of circulating cells to endothelium is the initial step in transendothelial migration. Human aortic endothelial cell cultures were established from aortic tissue harvested at the time of organ donation for cardiac transplantation which allowed specific recipient mononuclear cell–graft endothelial interactions to be studied. Confluent untreated endothelial cells were incubated with recipient mononuclear cells for 15 min to assess adhesion. Adhesion of recipient mononuclear cells to endothelium derived from their graft was threefold higher than adhesion to nonspecific endothelium (93 ± 20 vs. 30 ± 11 cells/high power field, $P < 0.005$). Graft-specific adhesion was inhibited by preincubation of the endothelium with antibodies to class I HLA (34 ± 16 cells/high power field, $P < 0.005$). Immunofluorescence performed after adhesion showed that $73\pm6\%$ of both specific and nonspecific adherent cells were monocytes. The use of purified lymphocyte and monocyte preparations showed that graft-specific lymphocytes induce unrelated monocytes to become adherent. These results suggest that lymphocytes are primed in vivo to recognize endothelium derived from their graft which leads to a rapid increase in lymphocyte and monocyte adhesion. Such allo-recognition may involve endothelial class I HLA molecules. (*J. Clin. Invest.* 1994. 94:2142–2147.) Key words: lymphocyte • monocyte • endothelium • heart transplantation • recognition

Introduction

Migration of monocytes and lymphocytes through arterial endothelium into the vascular wall and perivascular tissue is integral to the development of transplant arteriosclerosis and allograft rejection (1). The first step in transendothelial migration of mononuclear cells is adhesion to vascular endothelium using both specific and nonspecific adhesion molecules (2). Binding is followed by stable adhesion and migration into the tissue only after a second activation due to locally produced chemoattractants or adhesion to integrin activators (2). Events in the recognition and adhesion of lymphocytes to endothelium have been extensively studied (3). Lymphocyte adhesion to endothelium does not require a chemoattractant signal as crosslinking of the T cell receptor may lead to activation of lymphocyte function–associated antigen-1 (LFA-1),[1] the ligand for intercellular adhesion molecule-1 (ICAM-1) (4). Lymphocytes may be primed in vitro to recognize and bind specific endothelial cells after priming of recipient lymphocytes with donor splenocytes in a mixed lymphocyte culture for 12 d (5).

Monocyte adhesion to endothelial cells may differ significantly from lymphocytes, and this has been studied extensively in the context of lipid modification by arterial wall cells (6). Cells of the monocyte/macrophage lineage contribute significantly to the development of allograft rejection (7) and transplant coronary disease (8). Monocytes have not been shown to be capable of allo-recognition although they are active in antigen presentation. It is unclear if their adhesion to vascular endothelium is enhanced in the context of transplantation in the absence of endothelial activation. Adhesion to unstimulated endothelium would indicate a primary role for circulating mononuclear cells in the pathogenesis of transplant coronary disease.

The routine harvest and isolation of human aortic endothelial cells from heart transplant donors allows the subsequent investigation of interactions between mononuclear cells of the recipient and graft endothelium (9). This study sought to assess adhesion of mononuclear cells from human heart transplant recipients, primed only by in vivo exposure to the transplanted heart, to human aortic endothelial cells cultured from a recipient's graft, or to an unrelated graft. Immunofluorescence was used to document the adherent cell type(s). Antibody inhibition studies were performed in graft-specific interactions to elucidate

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1. *Abbreviations used in this paper:* HAEC, human aortic endothelial cells; HPF, high power field; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function–associated antigen-1.

the mechanisms responsible for the specificity of the adhesion of recipient cells to graft endothelium.

Methods

Endothelial cell isolation and culture. Human aortic endothelial cells (HAEC) were derived from aortic specimens retrieved at the time of organ harvest for cardiac transplantation as previously described (9). Briefly, each aortic ring was collected into M199 (Whittaker Bioproducts, Walkersville, MA) containing 20% heat-inactivated FBS (Hyclone Laboratories, Logan, UT), endothelial cell growth supplement (20 μg/ml, Becton Dickinson, Bedford, MA), and heparin (90 μg/ml). The endothelium was subjected to 0.1% collagenase digestion and cells were isolated. Primary and subsequent cultures were established in M199 containing 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.05% fungizone, 10% FBS, 10% pooled heat-inactivated human serum, 20 μg/ml endothelial cell growth supplement, and 90 μg/ml heparin (subsequently referred to as EC media). HAEC were plated into 75-cm² tissue culture flasks (Costar Corp., Cambridge, MA) that were pretreated with 0.1% gelatin (tissue culture grade, low endotoxin, Sigma Immunochemicals, St. Louis, MO). When confluent, the cells were passaged using trypsin digestion. Cells from passages 3–10 were used in these experiments.

Mononuclear cell preparation. Peripheral blood mononuclear cells (PBMC) were isolated from heart transplant recipients using Ficoll-Hypaque density gradient centrifugation. PBMC were not used if taken during an episode of cellular rejection and were taken from patients from 4 wk to 2 yr after transplantation. After centrifugation (800 g × 20 min at 22°C) the mononuclear cell band was washed once in phosphate-buffered saline (PBS) and resuspended in Dulbecco's modified Eagle's medium (DME low glucose; Whittaker Bioproducts) containing 5% calf bovine serum (Hyclone Laboratories, LPS < 0.025 ng/ml). Cell density was established using a hemocytometer. The proportion of monocytes and lymphocytes in the mononuclear cell isolates was determined by cytospin preparations stained with Wright's stain. Lymphocyte- and monocyte-rich subpopulations were obtained by differential adhesion, PBMC were plated into 60-mm Petri dishes and incubated at 37°C for 30 min before collecting nonadherent cells as lymphocyte-rich (86% CD3+ by immunofluorescence) and adherent cells as monocyte-rich (72% CD11c+ by immunofluorescence) subfractions. In the subpopulation experiments, lymphocytes and monocytes were reconstituted in either a 3:1 or 1:1 ratio at a final concentration of 10⁶/ml.

Endothelial adhesion assay. HAEC were cultured until confluent (72 h) in 8-well chamber slides (Nunc, Inc. Naperville, IL) or 48-well tissue culture plates (Costar Corp.) in EC media as defined above. Culture media were exchanged twice with DME (low glucose) containing 5% calf bovine serum, before the addition of PBMC. A 15-min adhesion assay was used as previously described (6). PBMC (100,000) at a concentration of 10⁶/ml in a final volume of 300 μl were added and incubated for 15 min at 37°C humidified with 5% CO₂. Nonadherent cells were rinsed off with two 0.5-ml DME washes and the wells were fixed in 1% glutaraldehyde. The number of adherent cells was then determined using inverted phase-contrast microscopy at a magnification of 250 and a 10-mm × 10-mm grid. The number of adherent cells was assessed by counting three uniformly covered central fields in each of three wells for each donor. Analysis of three central fields has been shown to give reproducible and consistent results (6). Group comparisons were performed using single and repeated measures ANOVA with significance defined at $P < 0.05$.

Antibody blocking of graft-specific mononuclear cell adhesion. In quadruplicate experiments endothelial cells were preincubated for 1 h at 37°C with a 1:100 dilution of a mouse monoclonal antibody to class I HLA (W6/32, IgG₁ mouse ascites, a kind gift from Dr. P. Terasaki, UCLA), class II HLA (H-4, Dr. P. Terasaki), ICAM-1 (CL39/4G8 [10], provided by Dr. C. W. Smith, Division of Pediatrics, Baylor College of Medicine, Houston, TX), or immunoglobulin isotype controls. Unbound antibody was removed by two washes with DME. In

three further experiments, 3 × 10⁵ donor-specific mononuclear cells were incubated at 4°C for 1 h with an antibody directed against CD18 (TS1/18 [11], kindly provided by Dr. C. W. Smith) or an immunoglobulin isotypic control, and then used in the adhesion assay.

Identification of adherent cells by immunofluorescence. Immunofluorescence was used to determine the cell populations in PBMC isolates and after adhesion assays to determine the adherent cell type. In these experiments, endothelial cell culture and adhesion assays were performed using 8-well tissue culture chamber slides (Nunc, Inc.). After mononuclear cells were allowed to adhere to endothelial monolayers, unfixed cells were incubated with a 1:100 dilution of CD3-fluorescein and CD11c-phycoerythrin monoclonal antibodies (Becton Dickinson Immunocytometry Systems, Mountain View, CA). In selected experiments, antibody to CD14-fluorescein and CD2-fluorescein (Becton Dickinson Immunocytometry Systems) were used. After a 30-min incubation on ice, the cells were gently rinsed with two volumes of DME, the well separators were removed, and slides were mounted using PBS/glycerol. The cells were counted using epifluorescence microscopy.

Results

The adhesion of cardiac transplant recipient mononuclear cells to unactivated endothelium derived from their specific graft was used in this series of experiments as a surrogate for adhesion of recipient mononuclear cells to endothelium of their allograft. Adhesion to unrelated endothelium after a 15-min incubation was used to investigate the specificity of adhesion. In a pilot experiment involving three recipient mononuclear-donor endothelium adhesion assays performed at the same time, there was a significant increase in adhesion of recipient mononuclear cells to endothelium specific for their graft compared with adhesion to endothelium from unrelated grafts (Fig. 1). A limitation in the number of PBMC isolated from recipient A did not allow simultaneous adhesion to endothelia B and C to be performed. In a series of 25 patient-graft combinations, adhesion of PBMC to endothelium derived from their allograft was 93±20 cells/high power field (HPF), which was threefold higher than the adhesion noted in 120 unrelated PBMC-endothelial adhesion combinations (30±11 cells/HPF, $P < 0.0001$, Fig. 2). Adhesion of PBMC to unstimulated endothelium was not dependent on the passage of endothelial cells used. Adhesion of heart transplant recipient PBMC to unrelated endothelium (30±11 cells/HPF) was not increased when compared with adhesion of PBMC isolated from normal individuals ($n = 13$, 26±12 cells/HPF). This suggests that increased adhesion is not due to activation of mononuclear cells in transplant recipients but that specific endothelial-dependent recognition and adhesion is occurring.

Since cardiac transplantation does not allow sufficient time for prospective HLA typing and matching, allograft-specific mononuclear cell adhesion may result from recognition of major histocompatibility complex determinants on graft endothelium. The role of HLA molecules in graft-specific adhesion was investigated using antibodies to class I HLA. Preincubation of the endothelium with antibodies to class I HLA molecules reduced graft-specific adhesion from 93±20 to 34±16 cells/HPF (Fig. 2, $P < 0.005$). Preincubation of the endothelium with irrelevant isotypic antibodies to class II HLA and a nonblocking ICAM-1 antibody in comparable concentrations did not affect mononuclear cell adhesion (Fig. 2). This was true even though ICAM-1 was present at high levels on the endothelial cell surface (12), suggesting that binding of any antibody to the endothelial cell surface did not block monocyte adhesion. Antibodies to class
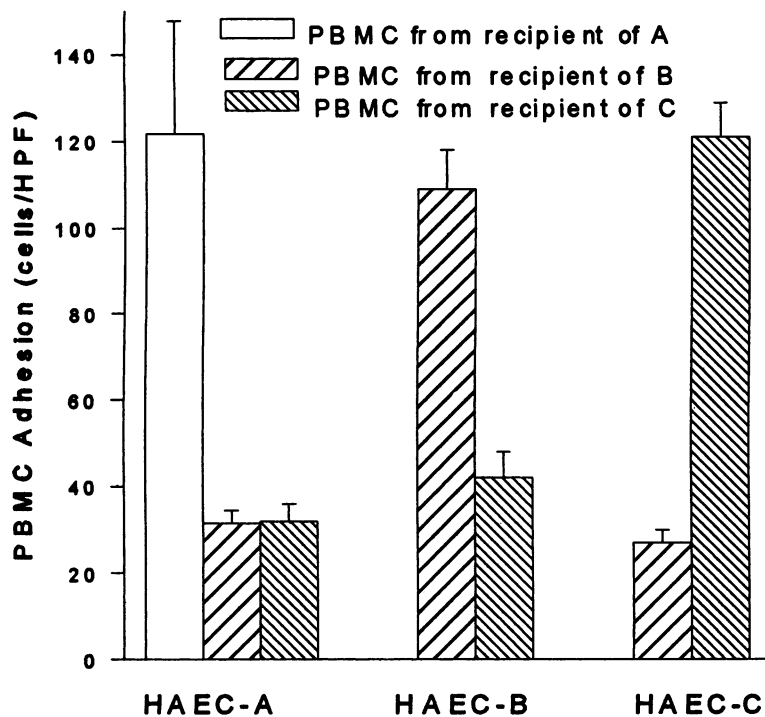
Figure 1. Adhesion of cardiac transplant recipient PBMC from patients A, B, and C to endothelia derived from allografts transplanted into A, B, and C. Adhesion to allograft-specific endothelia was markedly increased ($P < 0.005$) compared with adhesion to unrelated endothelium. The figure shows mean±SD.

II HLA and a nonblocking ICAM-1 antibody had no effect on nonspecific adhesion. Pretreatment of the PBMC with antibodies to CD18, the common subunit of both LFA-1 and Mac-1, was associated with a marked reduction in adhesion; graft-specific adhesion was reduced to 15±6.6 cells/HPF ($P < 0.005$) and nonspecific adhesion was reduced to 3.3±0.7 cells/HPF ($P < 0.005$).

Mononuclear cell preparations from cardiac transplant recipients were composed of 25±4% monocytes and 75±7% lymphocytes as determined by Wright's stain. This was similar to isolates from normal individuals (80±5% lymphocytes and

20±3% monocytes). Immunofluorescence was used to document the type of leukocytes involved in adhesion to endothelium after a 15-min incubation. In a representative study, analysis of cells bound to graft-specific endothelium showed that 78% of cells bound were CD11c positive (Fig. 3). In a separate series of experiments using CD14, 80% of the adherent cells were positive (data not shown), confirming that the majority of adherent cells were monocytes. Approximately 22% of cells adherent to the graft-specific endothelium were CD3 positive. A similar proportion of monocytes bound nonspecific endothelium (81%, Fig. 3, $P = $ NS). In a larger series ($n = 7$), 87±11%
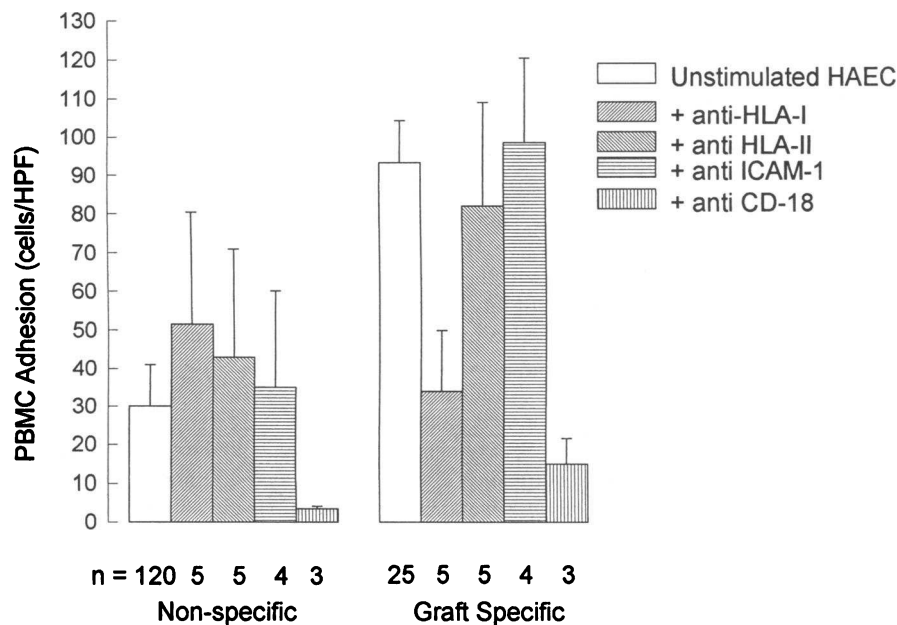

Figure 2. Mechanisms of allograft-specific PBMC adhesion. Allograft-specific adhesion of recipient PBMC is inhibited by preincubation of endothelial cells with an antibody to class I HLA and preincubation of the PBMC with antibodies to CD18. Antibody to endothelial class II HLA and a noncompetitive ICAM-1 antibody do not inhibit specific or nonspecific adhesion. The figure shows mean±SD.

Figure 3 (chart):

PBMC Adhesion (cells/HPF) — y-axis 0 to 70

Legend:
- CD11c(−) CD3(−)
- CD11c(+) CD3(−)
- CD11c(−) CD3(+)

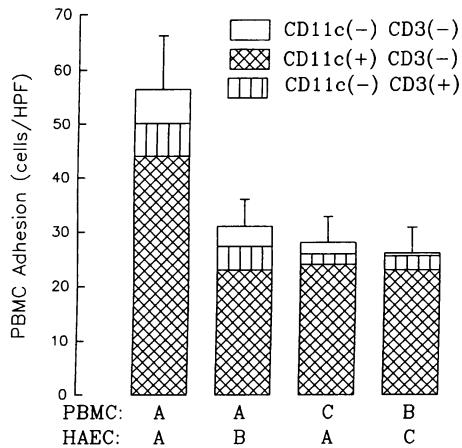| PBMC:	A	A	C	B
HAEC:	A	B	A	C

Figure 3. The proportion of recipient monocytes and lymphocytes adherent to monolayers of endothelium derived from their graft (A/A) or an unrelated graft (A/B) was determined by immunofluorescence. Adhesion of unrelated PBMC to graft-specific endothelium (C/A) and adhesion of unrelated PBMC to the specific third party endothelium (B/C) showed similar monocyte/lymphocyte ratios. This figure summarizes mean±SD for eight experiments.

Figure 4 (chart):

Adhesion (cells/HPF) — y-axis 0 to 140

Lymphocytes:	A	A	-	A	B	B	-	B	A
Monocytes:	A	-	A	B	-	A	B	B	B
HAEC:	A	A	A	A	A	A	A	A	C

Figure 4. Lymphocytes from a recipient which are graft specific (A) are able to induce adhesion of unrelated monocytes (B) to endothelium (A) equal to that seen with graft-specific monocytes (A). Graft-specific monocytes (A) do not confer any increase in adhesion alone or in the presence of nonspecific lymphocytes (B). Graft-specific lymphocytes (A) do not confer increased adhesion of unrelated monocytes (B) to a third party endothelium (C). This figure shows mean±SD for triplicates in a representative experiment performed for eight individual donor–recipient pairs.

of cells adherent to graft-specific endothelium were CD11c positive and 13±11% were CD3 positive. Nonspecific endothelia bound 78±13% monocytes and 22±12% lymphocytes. Thus, adhesion of both lymphocytes and monocytes to graft-derived endothelium was increased. More than 95% of all adherent cells on phase microscopy stained with one of the antibodies used. Less than 5% of adherent cells were CD2 positive.

To determine which cells were providing specificity for recognition of graft endothelium in recipient PBMCs, lymphocyte-rich and monocyte-rich subfractions were prepared, mixed in graft-specific and nonspecific combinations, and adhesion compared. The results of a representative experiment are shown in Fig. 4. Graft-specific lymphocytes stimulated the adhesion of unrelated monocytes equal to that seen when the combination of graft-specific lymphocytes or monocytes when used alone, suggesting a synergistic effect with both cell populations. A summary of eight experiments from eight individual recipients shows that graft-specific lymphocytes were able to stimulate the adhesion of nonspecific monocytes (85±44 cells/HPF) similar to that seen with graft-specific monocytes (90±52 cells/ HPF). Graft-specific monocytes in the presence of unrelated lymphocytes (46±23 cells/HPF) were no more adherent than the combination of nonspecific lymphocytes and nonspecific monocytes (48±23 cells/HPF). Immunofluorescence of adherent cells after the reconstitution experiments showed that 70–80% of adherent cells were monocytes. Adhesion assays were performed using lymphocyte/monocyte ratios of 3:1 and 1:1 and showed no difference in the number or phenotype of adherent cells.

Discussion

Adhesion of circulating mononuclear cells to endothelium is the first step in recruitment of a localized immune or inflammatory response in an organ or tissue (13). Stable adhesion of leukocytes to endothelium depends on transient binding with recognition followed by leukocyte activation and subsequent interaction
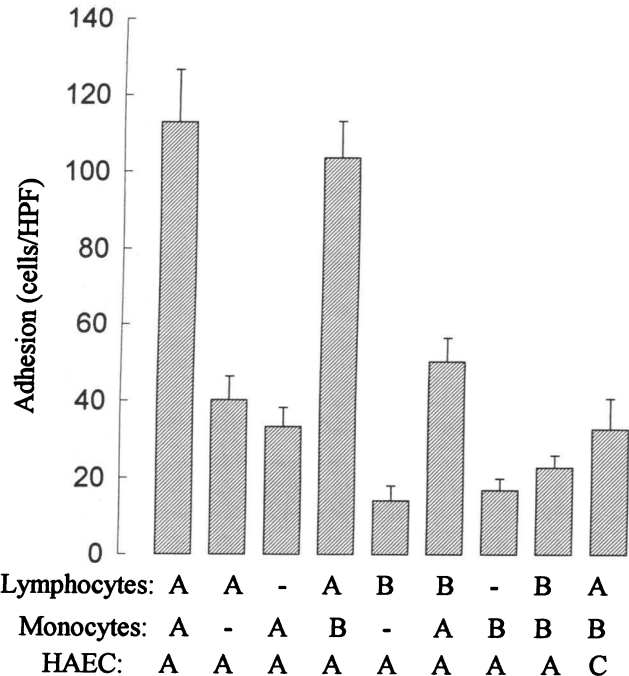
between adhesion molecules and their corresponding ligands (2). Inducible expression of a variety of adhesion molecules on the luminal surface of endothelial cells allows spatial and temporal selection of specific leukocyte subsets from the circulation (13, 14). Circulating lymphocyte sampling of endothelial major histocompatibility complex molecules has been suggested as a method of recruitment for specific T lymphocyte subsets (15). Although class I HLA molecules are constitutively expressed on endothelial cells, the expression of class I and II HLA molecules can be further induced by cytokines known to be induced during inflammation and rejection (14), although cytokine release may be secondary to mononuclear cell recognition and transendothelial migration (16). Specific recognition and adhesion of lymphocytes from heart transplant recipients to graft endothelium has been documented (5, 17), but only after in vitro priming of the lymphocyte population with a 12-d mixed lymphocyte-donor splenocyte culture followed by an 18-h incubation with the endothelium. Adhesion in these assays was shown to be class I HLA dependent.

In the series of experiments outlined in this paper, we sought to document the adhesion of cardiac transplant recipient mononuclear cells to human aortic endothelial cells derived from their allograft to detect in vivo priming. There was a threefold increase in adhesion of mononuclear cells to graft-specific endothelium to which the recipient had been exposed previously. This allograft-specific adhesion was not attributable to nonspecific mononuclear cell activation, as adhesion to third party endothelium was not increased. Recipient PBMC were not taken

at a time of clinical cellular rejection or active infection, and care was taken in the isolation of mononuclear cells to avoid steps which would lead to excess monocyte activation.

Immunofluorescence microscopy was used to document the cell type of adherent cells and showed that 87% of allograft-specific adhesion cells were CD11c(+) monocytes and only 13% were CD3(+), suggestive of lymphocytes. This is surprising in view of (a) the predominance of lymphocytes in the PBMC isolates, (b) the lack of allo-specificity of monocytes, and (c) the need, therefore, to invoke lymphocyte recognition and signaling followed by monocyte adhesion all occurring in a 15-min period.

Possible mechanisms involved in allograft-specific PBMC recognition of endothelium include class I HLA which is constitutively expressed (16), class II HLA which is inducibly expressed (10), and other less well defined endothelial antigens (19). These data showing that antibodies to class I HLA inhibit graft-specific adhesion suggest a role for endothelial histocompatibility complex molecule interactions with lymphocytes in producing graft-specific adhesion. Enhanced expression of both class I and II HLA molecules has been demonstrated in the context of acute cellular rejection (20), suggesting a role in transendothelial migration of mononuclear cells. Antibodies to class II HLA did not inhibit adhesion; the number of class II molecules constitutively expressed on endothelium was small and incubation with gamma interferon increased class II expression and PBMC adhesion, which could be inhibited by antibodies to class II HLA (data not shown). A potential mechanism linking endothelial HLA molecules to increased PBMC adhesion is the activation of LFA-1 demonstrated after crosslinking of the T cell receptor (4) by anti-CD3 which mimicks the combination of HLA and antigen (21), once again providing an alloantibody-equivalent substrate for signaling. Activation of LFA-1 by crosslinking of the T cell receptor occurs within minutes (4) and could explain the time course of adhesion noted in our studies. The inhibition of graft-specific adhesion by anti-CD18 suggests that LFA-1 or Mac-1 is involved in this process. While TS 1/18 inhibited both specific and nonspecific adhesion, the magnitude of inhibition was greater for specific adhesion. In fact, donor-specific adhesion in the presence of TS 1/18 was less than controls indicating a specific effect of TS 1/18 on this process. The antibody to ICAM-1 was not able to block adhesion in our assays; this antibody is directed at an epitope on ICAM-1 not involved in cellular adhesion (10) and does not rule out a role for ICAM-1, ICAM-2, or ICAM-3 in this phenomenon. The ability of graft-specific lymphocytes to enhance the adhesion of nonspecific monocytes equal to that of specific monocytes confirms that lymphocytes confer specificity in this interaction. It is not known if the lymphocyte-endothelial interaction induces an endothelial-based monocyte adhesion molecule, a monocyte adhesion ligand, or both. The rapidity of this interaction would make a soluble cytokine interaction between lymphocyte and monocyte unlikely although cell–cell signaling may occur. The endothelium of allografts does not appear to change to a phenotype which is specialized in lymphocyte trafficking (22).

The relationship of these findings to the development of transplant coronary disease and cellular or vascular rejection is limited by potential differences in HLA and adhesion molecule expression on aortic and coronary artery endothelium (23). Pathological involvement of the ascending aorta of transplanted hearts in a similar fashion to the coronary arteries suggests that similar mechanisms exist (24, 25). These findings need to be balanced by observations that the expression of HLA molecules and adhesion molecules differs in large and small arteries as determined by immunohistochemistry (20).

The observations made in this study suggest that PBMC are primed in vivo and are capable of allograft-specific recognition followed by adhesion to allogeneic endothelium in heart transplant recipients. Such priming is consistent with recent data demonstrating allograft-specific antibodies to vascular wall cells in all cardiac transplant recipients (Fyfe, A. I., manuscript submitted for publication). This graft-specific recognition appears to be on the basis of HLA class I molecules. Although monocytes represent the major adherent cell type in the graft-specific adhesion, this is secondary to lymphocyte recognition. Work is currently in progress to determine if donor-specific lymphocyte-mediated monocyte endothelial adhesion is caused by endothelial or mononuclear cell activation. Immune injury to the endothelium as a result of its role in the immune response is postulated in the development of cellular and vascular rejection and transplant coronary disease (1). This in vitro model which is based on human tissue integrates important components of cellular immune recognition, activation and adhesion after cardiac transplantation, and should be useful in gaining a further understanding of immune endothelial injury.

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