Progesterone Induces Human Leukocyte Antigen-G Expression in Vascular Endothelial and Smooth Muscle Cells

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Background—Human leukocyte antigen-G (HLA-G) expression in heart transplant patients has been negatively associated with acute cellular rejection and cardiac allograft vasculopathy. We assessed HLA-G expression in vascular human endothelial and smooth muscle cell cultures to determine if future therapeutic agents can be targeted toward inducing HLA-G expression to protect against allograft rejection and vasculopathy.

Methods and Results—Human coronary artery endothelial, aortic endothelial, and coronary artery smooth muscle cell cultures were exposed to cytokines (interferon-γ or interleukin-10), hypoxia/reoxygenation stress, immunosuppressive agents (cyclosporine, sirolimus, or tacrolimus), or progesterone. HLA-G was not expressed by untreated, normoxic cells. Furthermore, maximal doses of interferon-γ, interleukin-10, cyclosporine, sirolimus, or tacrolimus, as well as exposure to hypoxia/reoxygenation, failed to induce HLA-G expression. HLA-G, which has previously not been detected in adult vascular endothelial and smooth muscle cells, was detected by enzyme-linked immunosorbent assay and flow cytometry in human coronary artery endothelial, human coronary aortic endothelial, and human coronary artery smooth muscle cultures after incubation with progesterone in a dose-dependent manner (P<0.001) with no change in cellular proliferation ability or viability. This effect was partially blocked in the presence of mifepristone, a progesterone receptor antagonist (human coronary artery endothelial: 48.8±15.6%; human coronary aortic endothelial: 59.5±9.5%; human coronary artery smooth muscle: 59.8±9.8% of control; P<0.05). Progesterone-induced HLA-G expression was not protective against hypoxia/reoxygenation injury.

Conclusions—HLA-G is not expressed at baseline in vascular endothelial and smooth muscle cells but can be induced by exposure to progesterone. Although tightly regulated, induction of HLA-G expression in these cells may represent a promising and novel therapeutic strategy to protect against rejection and cardiac allograft vasculopathy after heart transplantation. (Circulation. 2008;118[Suppl 1]:S58–S64.)

Key Words: endothelium ■ HLA-G ■ progesterone ■ rejection ■ transplantation

H uman leukocyte antigen-G (HLA-G) is a nonclassical MHC-I protein expressed by extravillous cytotrophoblasts during early pregnancy and participates in maintaining maternal–fetal immune tolerance.1 The immunosuppressive role of HLA-G is apparent by its ability to inhibit CD8+ T cell toxicity,2 CD4+ T cell alloreactivity,3 natural killer cell-mediated cytolysis,4 and activate regulatory CD4+ T cells.5 The result of alternative splicing of primary mRNA transcripts, 4 membrane-bound, G1 to G4, and 3 soluble, G5 to G7, isoforms of HLA-G have been identified to date.6 Primarily expressed by trophoblasts, soluble and/or membrane-bound HLA-G isoforms have been detected in some patients after solid organ transplantation.7 Interestingly, HLA-G expression postheart transplant appears to be advantageous. Detection of HLA-G in endomyocardial biopsies of heart transplant recipients is negatively associated with both the number of moderate to severe acute rejection episodes and the presence of cardiac allograft vasculopathy (CAV).8 More recent studies suggest that high soluble HLA-G levels also appear to confer similar protection from graft rejection and vasculopathy.8,9 Although present in endomyocardial biopsies and sera of heart transplant recipients, it remains unknown whether vascular endothelial and smooth muscle cells are capable of HLA-G expression. Induction of HLA-G expression might represent a novel protective mechanism against acute rejection and CAV. Being one of the leading causes of late morbidity and mortality in patients undergoing heart trans-
plantation, CAV is a major complication characterized by a diffuse, concentric thickening of the arterial intima.\textsuperscript{10,11} Although a multitude of factors likely contribute to the occurrence of CAV, immunologic mechanisms play an important role because only the allograft, not native, vasculature is affected.\textsuperscript{10,11} Furthermore, the endothelium of the coronary arteries expresses MHC antigens, which are capable of inducing immune responses.\textsuperscript{12} T cells have also been detected in close proximity to the luminal endothelium of allograft vessels and may contribute to the progression of transplant vasculopathy.\textsuperscript{13} Consequently, vascular HLA-G expression might inhibit the immunologic processes that lead to CAV.

Although the precise pathways regulating HLA-G gene expression remain ill defined, several mechanisms have been proposed. Signaling molecules such as progesterone,\textsuperscript{14,15} interferon-\gamma,\textsuperscript{16} and interleukin-10,\textsuperscript{17} which are present at high levels during gestation, are capable of enhancing HLA-G expression. Interventions associated with transplantation such as hypoxic injury\textsuperscript{18} and immunosuppressive therapy\textsuperscript{9} might also regulate this expression. Additionally, genetic factors are known to modulate HLA-G gene expression, because HLA-G allelic differences are associated with differential HLA-G mRNA levels and isoform profiles.\textsuperscript{19} Our experiments were, hence, designed to assess HLA-G expression in vascular endothelial and smooth muscle cell cultures after treatment with various interventions shown to upregulate HLA-G in other cell types. Expression of HLA-G may represent an effective strategy to protect against acute rejection and CAV after heart transplantation.

**Methods**

**Cell Cultures**

Primary cultures of human coronary artery endothelial (HCAEC), aortic endothelial (HAEC), and coronary artery smooth muscle (HCASMC) cells (Lonza, Walkersville, Md) were grown to full confluence at 37°C and 5% CO\textsubscript{2} for all experiments. HCAEC and HCASMC were procured from the left and right coronary arteries and HAEC from the ascending and descending aortas of cadaveric donors. HCAEC and HAEC were cultured in EBM-2 medium supplemented with EGM-2 MV and EGM-2 bullet kits (Lonza), respectively, whereas HCASMC were grown in SmBM-2 medium supplemented with SmGM-2 (Lonza). All culture media were further treated with 100 U/mL streptomycin and 100 \(\mu\)g/mL penicillin.
Interventions for Inducing Human Leukocyte Antigen-G Expression

To determine if vascular endothelial and smooth muscle cells grown in culture could be induced into expressing HLA-G, they were treated with interventions that have been shown to modulate HLA-G levels in vitro and in vivo. Cells were subjected to cytokines: interferon-γ and interleukin-10 (Sigma, St Louis, Mo) at 0.1 to 100 ng/mL for 2 to 24 hours; hypoxia (pO2 = 21%) for 2 hours (H/R), which mimics the relevant ischemia and reperfusion time periods observed in clinical transplantation; immunosuppressive agents (LC Laboratories, Woburn, Mass): cyclosporine (100 to 1000 ng/mL), sirolimus, and tacrolimus (0.1 to 100 ng/mL) for 24 hours; and progesterone (100 to 10 000 ng/mL) for 24 hours with or without its receptor antagonist, mifepristone (Sigma). After the treatment period, cells were assessed for HLA-G expression by ELISA and flow cytometry.

Enzyme-Linked Immunosorbent Assays

Purified HLA-G protein standard containing all isoforms was obtained as previously described.14 For the ELISA, each well of a 96-well plate (Corning) was filled with 100 µL of the 0.2C8 mAb, which has substantially the same binding site on the HLA-G heavy chain as the 4H84 mAb, at a concentration of 10 µg/mL and kept at 4°C overnight. Each well was washed 3 times with a phosphate-buffered saline washing solution containing 0.05% Tween-20 and blocked with 5% milk for 4 hours at room temperature. Duplicate cell lysate samples, extracted with RIPA extraction buffer (Sigma), were added to a final volume of 100 µL in each well. After 1 hour at room temperature, the reactions were stopped by the addition of 50 µL of 1 mol/L HCl to each well and the plate was read at 450 nm on an automated ELISA plate reader (Molecular Devices, Sunnyvale, Calif). HLA-G levels were derived from a purified HLA-G protein standard curve. The lower detection limit of the ELISA was 10 ng/mL. Total protein concentrations in each cell lysate sample were determined by using a Bio-Rad protein kit (Bio-Rad Laboratories, Hercules, Calif). HLA-G concentrations in each cell lysate sample were then normalized using the total protein concentrations. The ELISA used was specific for all HLA-G isoforms. Measurement of HLA-G in cell lysates ensured that all membrane-bound and soluble isoforms could be detected. We did not measure levels of soluble and shed HLA-G in culture supernatants as they fell below the lower detection limit of the ELISA.

Flow Cytometry

To assess cell surface HLA-G expression, cultured cells were detached with Accutase cell detachment medium (eBioscience, San Diego, Calif) and incubated in PBSG/BSA (phosphate-buffered saline with 20 mMol glucose and 5% BSA) with the primary mouse antihuman HLA-G antibody, MEM-G/9 (Serotec, Oxford, UK) at 10 µg/mL for 45 minutes in the dark at 4°C. Cells serving as negative controls were incubated with isotype-matched mouse IgG (Serotec) at the same antibody concentration. After washing once with PBSG/BSA, cells were incubated in the dark for 30 minutes at 4°C in PBSG/BSA containing 2.5 µg/mL of secondary goat antimouse IgG conjugated to Alexa Fluor (Invitrogen). After 2 washing steps, cell surface HLA-G expression was detected by flow cytometry (Beckman Coulter Epics XL). Phenotypic characterization of endothelial cells (Figure 1A–B) was confirmed with a primary mouse antihuman CD31 antibody conjugated to phycoerythrin and compared with its isotype-matched mouse IgG conjugated to Alexa Fluor (Invitrogen). Before intracellular α-smooth muscle actin staining with cyanine 3-conjugated mouse antimouse actin (Sigma). Flow cytometry was performed by simultaneous fixation and permeabilization of cells with the Cytofix/Cytoperm kit (BD Biosciences, San Jose, Calif) before intracellular α-smooth muscle actin staining with cyanine 3-conjugated mouse antimouse actin (Sigma).

Viability Studies

The XTT (sodium 3’-[1-phenylaminocarbonyl]-3,4-tetrazolium]-bis[4-methoxy-6-nitro]-benzene sulfonic acid hydrate)-based Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, Ind) was used for the quantification of cell proliferation and viability. Cells were incubated at 37°C with 20% XTT labeling mixture in phenol red-free DMEM medium (Invitrogen) supplemented with 5% FBS for 4 hours. Cell viability was determined by the ability of metabolically active cells to convert the yellow XTT salt to an orange formazan dye. Sample absorbance was measured by an ELISA plate reader at 450 nm with a reference wavelength of 650 nm.

Statistical Analysis

Experiments were performed in duplicate at least 4 times. Data are represented as mean±SE. Comparisons between multiple groups...
were performed by 2-factor analysis of variance. For inhibition experiments involving comparisons between 2 groups, we applied unpaired $t$ tests. We considered $P<0.05$ to represent statistical significance.

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

**Results**

**Human Leukocyte Antigen-G Induction Experiments**
To determine HLA-G expression in HCAEC, HAEC, and HCASMC cultures, we subjected them to our interventions and assessed protein expression by ELISA and flow cytometry. Dose–response and time course experiments were performed in all cell types for all treatments. HCAEC, HAEC, and HCASMC cultures did not express HLA-G at baseline. Expression of HLA-G was not detected after treatment with cytokines (interferon-$\gamma$ and interleukin-10) and immunosuppressive agents (cyclosporine, sirolimus, and tacrolimus) at all doses and time points. We also did not detect HLA-G expression after exposure to different periods of H/R. However, HLA-G expression was induced in progesterone-treated HCAEC, HAEC, and HCASMC cultures.

**The Effect of Progesterone on Human Leukocyte Antigen-G Expression**
Because HLA-G expression was only induced after incubation with progesterone, cells were treated with varying progesterone doses for 24 hours. Total HLA-G protein expression increased in a dose-dependent manner ($P<0.001$) as detected by ELISA (Figure 2), whereas cell surface expression was confirmed by flow cytometry (Figure 3) in HCAEC, HAEC, and HCASMC cultures. Time course experiments showed maximal HLA-G expression within 12 hours in all cell cultures ($P<0.001$; Figure 4).

**Inhibition Experiments**
To determine if progesterone-induced HLA-G expression could be inhibited by the progesterone receptor antagonist,
mifepristone, cultured cells were incubated with progesterone (10,000 ng/mL) in the presence or absence of mifepristone (1000 ng/mL) for 24 hours. As measured by ELISA (Figure 5), HLA-G expression in HCAEC, HAEC, and HCASMC cultures was partially blocked by mifepristone ($P < 0.05$), indicating that the mechanism of progesterone-induced HLA-G expression was through receptor binding.

**Viability Studies**

Cells were assessed for proliferation ability and viability after 24-hour exposure to progesterone with or without mifepristone to ensure that differences in HLA-G expression were not the result of cellular injury. There were no significant differences in HCAEC, HAEC, or HCASMC viability after incubation with our interventions compared with vehicle, indicating that these cultures were not injured by progesterone or mifepristone (Table 1). To assess whether HLA-G expression might confer protection against H/R injury, cells were subjected to 12-hour hypoxia and 2-hour reperfusion after 24-hour progesterone treatment. Viability studies demonstrated that HCAEC, HAEC, and HCASMC cultures were not protected against H/R injury after progesterone-induced HLA-G expression (Table 2). There were no significant differences in proliferation ability and viability after H/R

**Figure 4.** Time course experiments assessing progesterone treatment (10,000 ng/mL) on HLA-G expression determined by ELISA. HCAEC (A), HAEC (B), and HCASMC cultures (C) showed maximal HLA-G expression within 6 to 12 hours of exposure ($P < 0.001$).

**Figure 5.** The effect of mifepristone, a progesterone receptor antagonist on progesterone-induced HLA-G expression in HCAEC, HAEC, and HCASMC cultures measured by ELISA. Compared with cells treated with 10,000 ng/mL progesterone alone (controls), cells incubated with 1000 ng/mL mifepristone in combination with 10,000 ng/mL progesterone had significantly reduced HLA-G expression. All cultures were treated for 24 hours. $P < 0.05$ compared with control.
stress in progesterone-treated cells compared with vehicle-treated cells, measured as percentages of vehicle-treated cells under normoxia.

### Discussion

Although soluble HLA-G has been detected in endothelial cells of chorionic vessels during embryonic development, this expression is lost in endothelial cells lining mature vessels. For the first time, our experiments illustrate in vitro HLA-G expression in adult vascular endothelial and smooth muscle cells. Interestingly, increasing HLA-G levels were detected after treatment with incremental progesterone doses in all cell types without any changes in cellular proliferation ability or viability. Yet, no expression was observed after exposure to other interventions of interest including cytokines, H/R stress, or immunosuppressive agents, all of which are present in the peri- or posttransplant milieu and have experimentally been demonstrated to induce or upregulate HLA-G expression.

Treatment of normal blood monocytes and macrophage cell lines with interferon-γ has been shown to raise HLA-G mRNA levels leading to increased intracellular and cell surface protein expression in vitro. This effect is not unexpected considering interferon-γ and its receptors are synthesized in first trimester human trophoblasts, which coincide with HLA-G expression. Additionally, interleukin-10 has been shown to induce HLA-G mRNA and protein expression in peripheral blood monocytes and trophoblasts. However, treatment with interferon-γ or interleukin-10 did not induce HLA-G expression in our studies, indicating that HLA-G expression appears to be tightly regulated and specific for cell type.

Ischemia/reperfusion injury and immunosuppressive medications may represent other possible mechanisms of HLA-G expression after heart transplantation. It has been shown that soluble and membrane-bound HLA-G mRNA expression is inversely related to oxygen concentration in primary cultures of extravillous cytotrophoblasts. Furthermore, hypoxia has been demonstrated to differentially affect HLA-G expression in various tumor cell lines by inducing HLA-G gene transcription in some HLA-G lines and decreasing constitutive expression in certain HLA-G lines, indicating that modulation of HLA-G expression by hypoxic treatment is dependent on cell type. Interestingly, the HLA-G promoter contains a heat shock element, which binds to heat shock factor 1, a transcriptional factor activated during conditions of environmental stress, providing more evidence that HLA-G expression might be stress-inducible. We therefore assessed the effect of H/R in our vascular and smooth muscle cell cultures, but did not detect HLA-G expression after injury. Although HLA-G upregulation might represent a protective response against hypoxic injury in tumor cells, progesterone-induced HLA-G expression did not protect against H/R injury in our cell cultures. We also measured HLA-G expression after treatment with cyclosporine, sirolimus, or tacrolimus, because recent in vivo studies have implicated immunosuppressive agents with increased serum HLA-G concentrations after heart transplantation. We were unable to detect HLA-G protein expression after these interventions, suggesting that HLA-G expression in response to hypoxic stress or immunosuppressive therapy might be specific for cell type.

Finally, we determined the effects of progesterone and its receptor antagonist, mifepristone, on HLA-G expression. Progesterone has been recently demonstrated to enhance HLA-G expression in the JEG-3 choriocarcinoma cell line and isolated first-trimester cytotrophoblasts through receptor activation followed by binding to a progesterone response element, which shares 60% homology to the wild-type mouse mammary tumor virus progesterone response element sequence. Furthermore, progesterone receptors are present in human heart as well as vascular endothelial and smooth muscle cells, indicating that these tissues are possible targets for progesterone-induced HLA-G expression. Intriguingly, our results show induction of this expression after treatment with progesterone and partial inhibition after coinubation with mifepristone in HCAEC, HAEC, and HCAEC.

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<tr>
<th>Table 1. Viability and Proliferation Ability of Treated HCAEC, HAEC, and HCASMC Cultures</th>
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<td>Progesterone/Mifepristone Dose (ng/mL)</td>
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<td>HCAEC 69.99±3.15</td>
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Data are represented as percentages of vehicle-treated cells under normoxic conditions.
HCASMC cultures, suggesting that the effect of progesterone was the result of progesterone receptor activation.

Induction of HLA-G expression in endothelial cells is of particular interest because they are primary targets of circulating T cells posttransplant due to expression of classical MHC-I and II antigens. After binding to classical MHC proteins, T cells secrete a host of cytokines, leading to recruitment of inflammatory cells and proliferation of smooth muscle cells, which can eventually progress into CAV. Endothelial HLA-G expression could represent a strategy to inhibit adjacent T cells and prevent the progression of CAV.

Allograft vasculopathy remains a major complication posthearth transplant. Hence, induction of HLA-G expression is of considerable clinical relevance due to its immunosuppressive properties and ability to protect against transplant vasculopathy. The beneficial effects of posttransplant HLA-G expression are evident throughout the solid organ transplant milieu. Yet, the mechanisms of posttransplant HLA-G expression remain vague. It is still unclear why HLA-G is detected in only certain patients. Although genetic factors may play a role, posttransplant interventions are also likely to be responsible for this phenomenon. It is important to elucidate the precise mechanisms that contribute to HLA-G expression after heart transplantation. Our experiments demonstrate that although HLA-G expression is tightly regulated in human tissue, vascular endothelial and smooth muscle cells express HLA-G in response to progesterone in vitro. Consequently, induction of this expression might represent a novel therapeutic strategy to protect against acute rejection episodes and CAV.

Disclosures

None.

References


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