Human Leukocyte Antigen-G Expression After Heart Transplantation Is Associated With a Reduced Incidence of Rejection

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Background—Human leukocyte antigen (HLA)-G, a nonclassic major histocompatibility complex class I molecule expressed in the extravillous cytotrophoblast at the feto-maternal interface, is known to protect the fetus from maternal cellular immunity. In a preliminary study, we showed that HLA-G is expressed in the hearts of some patients after heart transplantation.

Methods and Results—In the present study, a larger number of patients was investigated to confirm this finding and to look for possible correlations between HLA-G expression and the number and types of rejection. Expression of HLA-G in endomyocardial biopsy specimens was investigated by immunohistochemical analysis, and detection of the soluble HLA-G in the serum was performed by immunoprecipitation followed by Western blot analysis. HLA-G was detected in the biopsy specimens and serum of 9 of 51 patients (18%). The number of episodes of acute rejection was significantly lower in HLA-G–positive patients (1.2±1.1) as compared with HLA-G–negative patients (4.5±2.8) (P<0.001). No chronic rejection was observed in HLA-G–positive patients, whereas 15 HLA-G–negative patients had chronic rejection (P<0.032). A longitudinal study of these patients reveals that the status of HLA-G expression was maintained after 6 months both in serum and in biopsy specimens. During this period, HLA-G–positive patients did not have chronic rejection.

Conclusions—There is a significant correlation between rejection and HLA-G expression in the heart after transplantation. HLA-G expression and its effect in reducing the incidence and severity of rejection seem to be stable throughout the evolution. (Circulation. 2002;105:1949-1954.)

Key Words: transplantation ▪ genes ▪ rejection ▪ grafting
a larger number of patients followed for a longer period of time and to assess whether there was a correlation between the rejection phenomena and HLA-G expression.

A longitudinal study of HLA-G expression was performed 6 months after the first investigation to evaluate the persistence of HLA-G expression and the clinical evolution of these patients.

Methods

Patients

This study comprises 51 patients (42 men and 9 women) whose ages ranged from 20 to 73 years (mean, 55.5 years) and who received a heart transplant in our institution. Heart transplantation had been performed because of dilated cardiomyopathy in 28 patients (54.9%), coronary artery disease in 12 (23.5%), valvular disease in 6 (11.8%), and congenital heart disease and other reasons in 5 patients (9.8%). Donors included 42 men and 9 women with a mean age of 32.5 years. All recipients were screened before transplantation for HLA antibodies, and no patient was found to have HLA-specific antibodies. All recipients and donors were typed for HLA-A, HLA-B, and HLA-DR by means of routine serological methods.

Length of follow-up was 1 to 5 years for 13 patients, 6 to 10 years for 22 patients, and 11 to 15 years for 16 patients. All patients received induction therapy with rabbit anti-thymocyte immunoglobulin (2.5 mg/kg body weight per day for 3 days). Immunosuppressive treatment consisted of cyclosporine, prednisone, and azathioprine or mycophenolate. Prednisone was given on the first day after transplantation at 4 mg/kg body weight for 3 days. The dose of prednisone then was decreased gradually in increments of 0.2 mg/kg body weight per day. Thereafter, an attempt was made in all patients to reduce or stop prednisone according to the results of endomyocardial biopsy specimens, the body weight, and possible side effects.

Azathioprine was given on the first day after transplantation at doses of 3 mg/kg body weight per day. In 1997, azathioprine was replaced by mycophenolate at a rate of 2 to 3 g/d, according to leukocyte counts (target, 4000 to 6000/mm) and digestive tolerance.

Cyclosporine was given the third day after transplantation at 3 to 4 mg/d. The dose of cyclosporine was adapted according to its blood concentration measured by radioimmunoassay (range, 100 to 150 ng/mL the first year after transplantation), renal function, and the number of episodes of rejection. The episodes of acute rejection were treated, according to the grade and type of rejection, either by reinforced doses of prednisone at 15 mg/kg body weight per day (orally) or by using the prednisone with rabbit anti-thymocyte immunoglobulin.

The degree of rejection was classified according to the working formulation of the International Society for Heart and Lung Transplantation (ISHLT)²⁰: 0, no rejection; 1A, minimal graft rejection; 1B, mild to moderate rejection; 2A, moderate to severe graft rejection; and 4, very severe graft rejection.

Five to six endomyocardial specimens were taken from the right ventricle of each patient after transplantation; at least 4 of the biopsy specimens were used for the histopathological diagnosis of rejection, and 1 biopsy specimen was used for immunohistochemical analysis of HLA-G expression. The number of 1B and 3A acute rejections during the first postoperative year was assessed.

Chronic rejection and its characteristic graft vascular disease were detected by systematic angiography of the coronary arteries once per year in every patient. Patient characteristics and associated diseases were recorded: age, sex, race, donors (age, sex, and race), tumor, diabetes, arterial hypertension, serum creatinine level, and dyslipidemia.

Laboratory Investigation

Expression of the HLA-G molecule was analyzed by immunohistochemical analysis of endomyocardial biopsy specimens, and detection of its soluble forms in the serum was achieved by immunoprecipitation followed by Western blot analysis.

In addition to the biopsy specimens used for histopathological analysis and immunohistochemistry, control specimens were obtained from 4 human trophoblasts as positive control and healthy skin from nonpregnant women (after mammoplasty) as negative control of HLA-G expression.

Blood was collected from the patient at the time of the biopsy and centrifuged to obtain the serum. Serum of 8 healthy volunteer donors (4 men and 4 nonpregnant women) was used as control.

Monoclonal and polyclonal antibodies were used for the detection of HLA-G molecules in the biopsy specimens and serum of patients.

Monoclonal antibodies (mAbs) used were 87G IgG2a anti-HLA-G1 and -G5 (provided by D. Geraghty, Fred Hutchinson Cancer Research, Seattle, Wash), 4H84 IgG1 antidenatured HLA-G heavy chain (provided by M. McMaster, University of California, San Francisco), and W6/32 IgG2a anti-HLA class I heavy chains associated with β2m (Sigma, Milwaukee, Wis). An isotype-matched antibody (Sigma) was used as control.

A rabbit polyclonal antibody PAGS-6 generated against the C-terminal peptide of the HLA-G α-chain encoded by intron 4 sequences was used to specifically recognize the soluble forms HLA-G5 and HLA-G6.

Histology and Immunohistochemistry

For histology, 4-μm-thick sections were obtained from each paraffin block and stained with hematoxylin and eosin for diagnosis of rejection.

For immunohistochemical studies, 6-μm-thick sections of frozen tissues were fixed for 10 minutes in cold acetone, dehydrated, and permeabilized with saponin in PBS. Staining procedures were processed with the Dako Envision System (DAKO). Samples were incubated for 30 minutes in 50% human normal serum in PBS to eliminate nonspecific bindings. Samples were incubated with the following primary mAbs for 30 minutes: W6/32, 87G, 4H84 mAbs and control antibody and followed by incubation with a secondary conjugated goat anti-mouse/antibody coupled with peroxidase (DAKO) for 30 minutes. After incubation for 10 minutes with substrate, sections were counterstained with hematoxylin dye and mounted with antimounting medium (DAKO).

Immunoprecipitation and Western Blot Analysis

The class I positive M8 melanoma cell line kindly provided by F. Jotereau (INSERM U211, Nantes, France) was transfected with a full-length HLA-G5 cDNA subcloned in a vector pcDNA (Invitrogen, San Diego, Calif), as previously described. The M8-HLA-G5 transfected and M8-pcDNA (transfected with the vector alone) were used, respectively, as positive and negative controls for Western blot analysis. One milliliter of both soluble HLA-G5 transfectant superantigen and serum samples were incubated overnight at 4°C with PAGS-6 polyclonal antibody.

Protein A–sepharose beads were added and incubated for 1 hour at 4°C. The beads bearing the immune complexes were washed and incubated for 5 minutes at 95°C with 1% SDS sample buffer and 150 μM/L β-mercapto ethanol; 25-μL aliquots of total protein from the isolated samples were separated on 12% SDS-PAGE and transferred onto nitrocellulose membrane (Hybond-C, Amersham). After blocking in 5% nonfat milk in PBS–0.2% Tween-20 (Sigma), the membrane was incubated with the 4H84 mAb overnight at 4°C. After washing in PBS–0.2% Tween, the membrane was incubated for 30 minutes at room temperature with anti-mouse peroxidase conjugate reagent (Amersham). After washing, the staining reaction was carried out with the use of enhanced chemiluminescence Western blotting detection (Amersham), after which the membrane was exposed to Kodak film.

Statistical Analysis

Data are presented as mean ± SEM. The Student’s t test was used, and a value of P<0.05 was considered significant.
Results

Expression of HLA-G in Endomyocardial Biopsy Specimens

Expression of HLA-G antigens in sections of endomyocardial biopsy specimens after heart transplantation was investigated through immunohistochemical analysis with the use of HLA-G mAbs: 87G IgG2a specific for HLA-G1 and HLA-G5 and 4H84 IgG1, which recognizes the α1 domain (pan-HLA-G).

We detected HLA-G proteins in 9 of 51 patients. Seven patients’ specimens were positively stained by both 87G and 4H84 mAbs and two were positively stained by 4H84 mAb only. Trophoblasts used as positive control exhibited strong staining with 87G and 4H84 mAbs, whereas skin biopsy specimens taken from healthy nonpregnant women (after mammoplasty) were negative (Figure 1). Staining with W6/32 IgG2a was used to control the presence of intact HLA class I molecules in different tissues and was positive in all tested tissue biopsy specimens (data not shown).

Detection of Soluble HLA-G Proteins in the Serum of Heart-Transplanted Patients

We characterized the HLA-G-soluble antigens in the serum of 9 of 51 patients investigated by immunoprecipitation with PAG5-6, a rabbit polyclonal antibody specific for the soluble HLA-G5 and HLA-G6 proteins, followed by Western blot analysis with 4H84 mAb.

Two isoforms of HLA-G antigens, with the sizes of 37 and 27 kDa corresponding to the reported products of the alternatively spliced transcripts sHLA-G5 and HLA-G6, could be detected in the serum. In 7 patients, the soluble form HLA-G5 was detected, and in 2 patients, a band corresponding to the soluble form G6 was found (Figure 2).

In summary, HLA-G was detected both in the serum and myocardial biopsy specimens in 9 patients (18%). Among these patients, 7 were positive for 87G and 4H84 mAbs, and the soluble HLA-G5 was detected in their serum. The remaining 2 patients were positive only for 4H84 mAb, and the soluble HLA-G6 was detected in their serum. The
longitudinal study of these patients reveals that the status of HLA-G expression was maintained after 6 months both in the biopsy specimens and in the serum. HLA-G–positive patients remained positive, and HLA-G–negative patients remained negative.

Clinical Analysis
HLA-G–positive patients (18%) were 7 men and 2 women (all white), with a mean age of 58 ± 11.3 years (Table). The expression of HLA-G was detected during the following posttransplantation period: (1) 1 to 5 years in 2 patients, (2) 6 to 10 years in 5 patients, and (3) 11 to 15 years in 2 patients. Three HLA-G–positive patients (33.3%) had a pretransplantation diagnosis of coronary artery disease, and 6 had dilated cardiomyopathy (66.7%). None of the HLA-G–positive patients had a history of diabetes mellitus when compared with 6 (14.3%) HLA-G–negative patients. Five HLA-G–positive patients (55.6%) had a history of hypertension compared with 38 HLA-G–negative patients (90.5%) (P<0.008). Renal function, as reflected by mean serum creatinine, was 140 ± 45.12 in HLA-G–positive patients compared with 187.5 ± 131.02 in HLA-G–negative patients. No tumor was detected in HLA-G–positive patients, whereas tumors developed in 8 HLA-G–negative patients. No significant difference in age, sex, or race was observed between HLA-G–positive and HLA-G–negative patients.

Acute and Chronic Rejection
Clinical analysis of the patients showed that the number of episodes of acute rejection in the first year after transplantation in HLA-G–positive patients was significantly decreased, 1.2 ± 1.1 when compared with 4.5 ± 2.8 in HLA-G–negative patients (P<0.001). In addition, no chronic rejection could be detected in HLA-G–positive patients, whereas 15 HLA-G–negative patients had chronic rejection (P<0.032).

HLA Match in HLA-G–Positive and HLA-G–Negative Patients
In HLA-G–positive patients (n=9), 2 patients had 1 HLA-A and 1 HLA-B match, 2 patients had 1 HLA-A and 1 HLA-DR match, 2 patients had 1 HLA-B match, 2 patients had 1 HLA-DR match, and 1 patient had no HLA-A, HLA-B, or HLA-DR match.

In HLA-G–negative patients without chronic rejection (n=27), 6 patients had 1 HLA-A match, 2 patients had 1 HLA-B match, 2 patients had 1 HLA-DR match, 2 patients had 1 HLA-A, 1 HLA-B, and 1 HLA-DR match, and 15 patients had no HLA-A, HLA-B, or HLA-DR match.

In HLA-G–negative patients with chronic rejection (n=15), 5 patients had 1 HLA-A match, 1 patient had 1 HLA-A and 1 HLA-B match, 1 patient had 1 HLA-A and 1 HLA-DR match, 1 patient had 1 HLA-B match, and 7 patients had no HLA-A, HLA-B, or HLA-DR match.

Discussion
The development of tolerance requires that the immune system may utilize several strategies for neutralizing self-
reactive T cells. These strategies include deletion,21 energy,22 and immunoregulatory pathways.23

This study suggests that the expression of HLA-G after heart transplantation may allow an alternative strategy to downregulate the host immunoresponse and to limit graft rejection. In vitro, soluble HLA-G has been demonstrated to induce apoptosis of activated CD8+ T cells24 and to modulate NK25 and allo-CTL response,26 whereas membrane-bound HLA-G proteins have been shown to inhibit both NK and T cell–mediated cytosis.6,18 to suppress the proliferation of allospecific CD4+ T lymphocytes,27 and to induce Th2 cytokine profile.28 Interestingly, we recently demonstrated that soluble HLA-G protein secreted by allo-specific CD4+ T cells suppresses the allo-proliferative response.29

In the present study, HLA-G expression was found in 18% of the 51 patients who received a heart transplant 1 to 15 years before this study was undertaken.

In HLA-G–positive patients, the incidence of acute rejection was significantly decreased ($P<0.001$), and there was no case of chronic rejection ($P<0.032$) when compared with HLA-G–negative patients. Expression of HLA-G after a 6-month interval persisted both in the biopsy specimens and in the serum of HLA-G–positive patients, whereas the HLA-G–negative patients remained so. No significant difference between age, race, or sex could be detected between HLA-G–positive and HLA-G–negative patients. It is of note that the two women expressing HLA-G in this series were not pregnant after transplantation and that none of the HLA-G–positive patients had a tumor. Therefore, the expression of HLA-G was likely activated during the process of transplantation, not by pregnancy or tumor development.

In heart-transplanted patients, HLA-G expression may allow escape from recognition and destruction of the graft by alloreactive T cells. Furthermore, the soluble HLA-G forms HLA-G5 and HLA-G6 detected in the serum of some patients may play an additional role in inhibiting the cytotoxic activity of NK cells.25 The expression of HLA-G in this series was recognized at different times after transplantation from 1 to 15 years. The fact that a repeated investigation after 6 months showed a persistent expression of HLA-G may indicate that this expression can be activated during the first year after transplantation and maintained by factors yet to be identified. Experimentally, numerous factors have been shown to up-regulate HLA-G expression, such as IL-10.16 An IL-10–HLA-G autocrine effect may contribute to graft tolerance. However, we cannot exclude that HLA-G–positive patients may carry out specific HLA-G alleles associated with high HLA-G production.30

In addition, the degree of histoincompatibility between donor and recipient may play a role in stimulating HLA-G production. Today, heart transplantation has been established as a valuable therapeutic approach to end-stage heart failure. The search for HLA-G expression in transplant recipients could provide a new understanding of the factors favoring graft tolerance and new modulation of immunosuppressive therapy. For example, the use of soluble forms of HLA-G could contribute to immunosuppression and make it possible to reduce the amount of other immunosuppressive agents.

The present study supports the hypothesis that HLA-G expression in heart-transplanted patients is associated with a better graft tolerance. HLA-G expression seems to minimize acute rejection and to protect the graft from chronic rejection. It remains to be understood why some heart-transplanted patients express HLA-G, whereas others do not.

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**References**


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