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Detection of HLA-G in Serum and Graft Biopsy Associated With Fewer Acute Rejections Following Combined Liver–Kidney Transplantation: Possible Implications for Monitoring Patients

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ABSTRACT: Human leukocyte antigen G (HLA-G) is a regulatory molecule that is expressed in the cytotrophoblast during implantation and is thought to allow the tolerance and the development of the semiallogeneic embryo. *In vitro*, HLA-G inhibits natural killer (NK) cell and CD8 T-cell cytotoxicity. HLA-G also decreases CD4 T-cell expansion. This suggests that it participates in the acceptance of allogeneic organ transplants in humans. We here describe the detection of high concentration of HLA-G in serum from liver-kidney transplant patients, but not in kidney transplant patients. This finding is supported by the ectopic expression of HLA-G in graft biopsies. Finally, its association with a low number of acute transplant rejections, especially in liver-kidney transplant patients led us to propose that HLA-G may serve to monitor transplant patients who are likely to accept their allograft and, thus, may benefit of a reduced immunosuppressive treatment. *Human Immunology 64, 1033–1038 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: liver-kidney transplantation; HLA-G; acute rejection

ABBREVIATIONS

DC	dendritic cells
ELISA	enzyme linked immunosorbent assay
LKT	liver-kidney transplantation
LT	liver transplantation
KT	kidney transplantation
mAb	monoclonal antibody

INTRODUCTION

Combined transplantations that include the liver and another organ favor allograft acceptance in animal models. In humans, the number of combined transplantations involving the liver has been increasing over the last 15 years due to cumulative organ dysfunction in some patients, physicians becoming more confident, and because the management of transplant patients has improved. The most frequent association is liver-kidney transplantation (LKT). This is mainly carried out in patients with glomerulonephritis associated with chronic alcoholic hepatitis, as a consequence of viral hepatitis [1]. The second most common situation is in patients initially transplanted for kidney or liver failure, who subsequently develop liver or kidney failure, respectively. Secondary liver failure is frequently caused by chronic viral hepatitis acquired during blood transfusion and/or hemodialysis, whereas secondary kidney failure is mainly due to the toxicity of anticalcineurin treatment. Some of these pa-

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tients develop chronic graft dysfunction and have to be re-transplanted or treated by dialysis. The initial rate of mortality following LKT is high, mainly due to infectious complications [2, 3]. Improvements in early post-transplantation care and patient selection have reduced the number of early deaths. The death rate in our unit has dropped below 4% during the last 3 years. Studies on small groups of patients have suggested that the incidence of acute renal rejection is lower following LKT than following renal transplantation alone [4, 5]. In contrast, Katznelson *et al.* [6] reported that the frequency of acute renal rejection was similar in LKT patients and in those transplanted with the contralateral kidney. However, they did not take the immunologic status of the recipient into account, even though this dramatically affects the graft outcome. Using a matched group of patients who had received only a single renal transplantation, we demonstrated that the frequency of renal acute rejection was lower following LKT even for re-transplanted patients [7]. These data suggested that the associated liver may facilitate renal allograft acceptance.

Several mechanisms may be involved in tolerance of LKT. These include expansion of inhibitory T cells induced by microchimerism, due to the presence of donated presenting cells within the liver graft [8], and Th2 immune polarization induced by hematopoietic precursors present in the liver graft [9]. However, we failed to observe expansion of inhibitory T cells in the peripheral blood of LKT patients. The reduction in acute rejection may also be due to production and secretion into the systemic circulation of soluble class I antigens by the liver allograft, because these antigens may neutralize alloantibodies and/or cytotoxic T lymphocytes, or may be due to the secretion of immunomodulatory factors by the liver graft. Human leukocyte antigen G (HLA-G) is of particular interest because it has immunoregulatory properties.

Human leukocyte antigen-G is a nonclassical major histocompatibility complex (MHC) class I molecule with low polymorphism and a restricted tissue distribution. HLA-G is only expressed in physiologic conditions in medullary thymic epithelial cells and in extra embryonic tissues. It can be expressed on mononuclear cells following activation by interferon or treatment with steroids. Four membrane-bound (HLA-G1 to -G4) and three soluble HLA-G isoforms (HLA-G5 to -G7) have been described. All of these isoforms are derived from the alternative splicing of the single primary transcription [10–12]. HLA-G interacts with inhibitory receptors such as ILT-2 and ILT-4. These receptors are mostly expressed on natural killer (NK) cells, but are also present on CD4 cells, CD8 cells, and antigen-presenting cells, suggesting that HLA-G can regulate the functions of these immune cells. Unlike during pregnancy, NK cells play a minor role in the allogeneic response of organ transplantation, whereas CD4 and CD8 are the most important cells. *In vitro*, HLA-G modulates the functions of several immune effectors: it acts on NK cells by inhibiting their cytotoxicity [13–16] and their transendothelial migration properties [17]. It inhibits antigen-specific $CD8^+$ T-cell cytolytic function [18] and induces apoptosis of phytohemagglutinin-activated $CD8^+$ T lymphocytes [19]. In addition, HLA-G interacts with CD4 T cells and dendritic cells (DC) that are involved in the initiation of the CD4 cell activation cascade, which is the key step of the alloimmune response. HLA-G suppresses $CD4^+$ T-cell proliferation in response to allogeneic stimulation [20–22] and promotes Th2 type responses [23]. HLA-G also inhibits DC maturation [24, 25], thus increasing allogeneic skin graft survival. These data suggested that HLA-G is involved in the inhibition of the alloreactive immune response, thus allowing the graft to be accepted.

MATERIALS AND METHODS

Patients, Samples, and Immunohistochemistry

Serum samples were obtained from 30 health individuals as a control group (C), 31 kidney transplant patients (KT), 21 liver transplant patients (LT), and 29 liver-kidney transplant patients (LKT). The KT and LT patients were matched to the LKT patients according to age, sex, liver or kidney disease etiology, and date of transplantation implying similar immunosuppressive treatment. Liver- and kidney-graft biopsies were obtained from LKT patients. Tissues were routinely fixed in 4% formalin and embedded in paraffin. The expression of HLA-G was evaluated in paraffin-embedded tissues using the EnVision antimouse peroxidase system and AEC as the substrate (Dako, Hamburg, Germany). Approval for this study was obtained from the local ethics committee.

Antibodies

The following monoclonal antibodies (mAbs) were used: W6/32, a mouse IgG2a anti-HLA class I heavy chain associated with β_2-microglobulin (β_2-m; Sigma Chemical, St. Louis, MO, USA); MEM-G/9, a mouse IgG1 recognizing HLA-G5 and shed HLA-G1 heavy chain associated with β_2-m (Exbio, Prague, Czech Republic); MEM-G/02, a mouse IgG1 recognizing free HLA-G heavy chain (kindly provided by V. Horejsi, Academy of Sciences, Prague, Czech Republic); and 4H84, a mouse IgG1 anti-HLA-G free heavy chain (kindly provided by S. Fisher and M. Mc Master, University of California, San Francisco, CA).

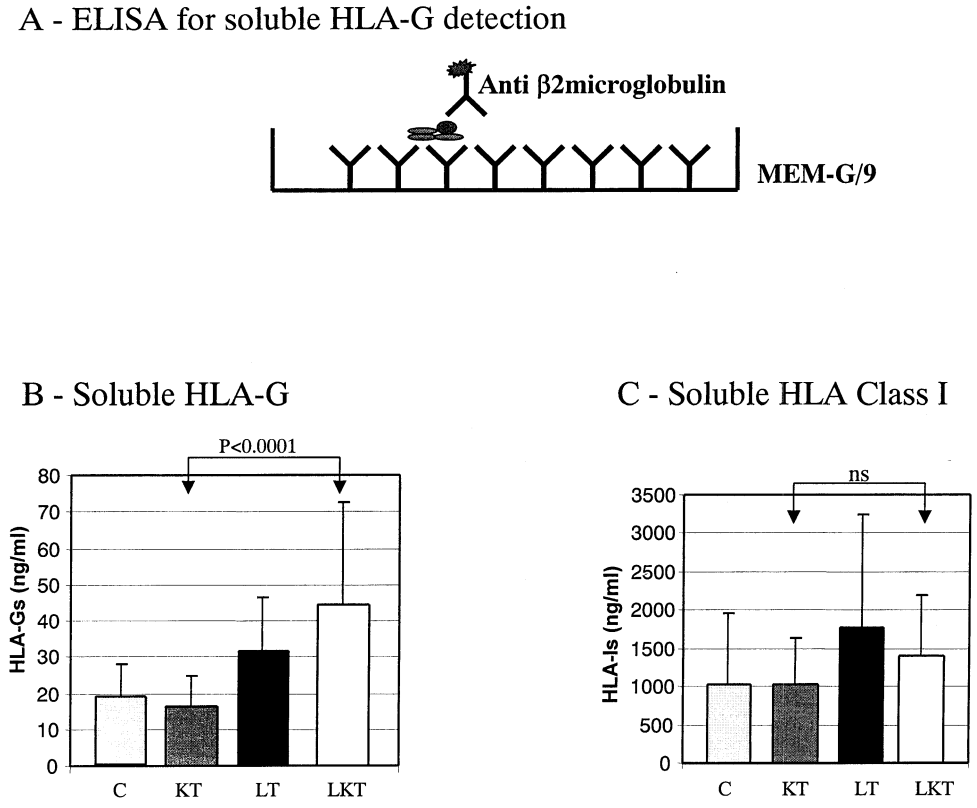

FIGURE 1 Detection of soluble human leukocyte antigen G (HLA-G) with enzyme-linked immunoabsorbent assay in sera of healthy individuals as a control group (C), kidney (KT), liver (LT), or combined liver-kidney (LKT) transplant patients. Sera from 30 C, 31 KT, 21 LT, or 29 LKT have been tested for soluble HLA-G using ELISA. Microtiters plates were coated with the MEM-G/9 monclonal antibodies (mAb) recognizing native HLA-G5 and shed HLA-G1 molecules, as a capture antibody for the detection of HLA-G (A) or with the W6/32 mAb recognizing a monomorphic determinant of β_2-microglobulin associated HLA class I heavy chains, as a capture antibody for the detection of soluble HLA class I molecules. (B) Concentrations of soluble HLA-G. (C) Concentrations of soluble HLA class I.

Enzyme-Linked Immunosorbent Assay

Microtiters plates were coated with the MEM-G/9 mAb (10 μg/ml) or with the W6/32 mAb (8 μg/ml), as a capture antibody for the detection of soluble HLA-G and soluble HLA class I molecules, respectively. After washes, pure serum for detection of soluble HLA-G or diluted at 1/50 for detection of soluble HLA class I were added to each well (100 μl) in triplicate and incubated for 1 hour. Anti-β_2-microglobulin HRP (Dako) was then added and plates were incubated for 1 hour. The chromogenic substrate (OPD [orthophenylenediamine dihydrochloride]; Dako) was added for 30 minutes in the dark and optical densities were measured at 490 nm. Standard curves were performed using serial dilutions of either calibrated supernatant of cells transfected with HLA-G5, or calibrated soluble HLA class I (Sangstat). Thus, the concentrations of soluble HLA-G and soluble HLA class I were determined from the value of optical density according to the standard curves. Detection limit of the enzyme-linked immunosorbent assay (ELISA) was 5 ng/ml.

Statistics

Statistical analysis was done with Statview SE software (Macintosh, version 5.0, Cupertino, CA, USA). Data were expressed as number of patients. The patients in whom HLA-G was expressed in biliary epithelial cells (BEC) were compared with those who did not by the nonparametric Chi-square test.

RESULTS AND DISCUSSION

We first determined the concentration of soluble class I molecules in serum samples from LKT patients. We have tested serum samples from 30 health individuals as a control group (C), 31 kidney transplant patients (KT), 21 liver transplant patients (LT), and 29 liver-kidney transplant patients (LKT). The concentration of soluble class I molecules was similar in all four groups (Figure 1). HLA-G was present in blood samples from transplant patients. The concentration of soluble HLA-G was similar in the control and KT groups, but was significantly elevated in the LKT group ($p < 0.0001$). Three KT and 22 LKT patients had a concentration of over 25 ng/ml of soluble HLA-G. This suggests that HLA-G could favor tolerance of kidney and liver grafts in LKT patients. This is in agreement of the results of Lila *et al.* [26, 27], who reported a significant correlation between the presence of soluble HLA-G in the blood of heart transplanted patients and a lower incidence of acute and chronic rejection episodes. These results suggest that HLA-G is a regulatory molecule that may act during human allo-

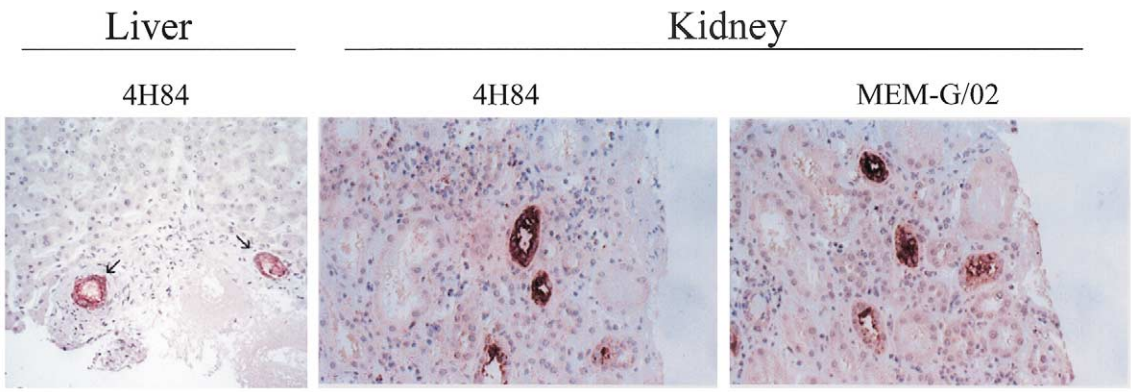

FIGURE 2 Expression of human leukocyte antigen G (HLA-G) in allograft liver and kidney biopsies. HLA-G was detected on paraffin-embedded section of allograft liver or kidney biopsies. HLA-G was detected in biliary epithelial cells of the liver allograft (arrows). Serial sections of one kidney allograft biopsy were incubated with the 4H84 monoclonal antibodies (mAb); a mouse IgG1, specific for the HLA-G-α1 domain common to all HLA-G isoforms; or with the MEMG/02 mAb, a mouse IgG1 that also recognizes all the HLA-G isoforms as a free heavy chain.

transplantation. However, HLA-G was rarely expressed in KT patients. More blood samples have to be analyzed to clarify this point.

Both the membrane-bound form (HLA-G1) and the soluble isoform (HLA-G5) display immunotolerant properties [28, 29]. For example, HLA-G1 protects targets from both NK cell mediated and cytotoxic T-lymphocyte mediated lysis, and only a few HLA-G1 positive cells are required to inhibit target destruction significantly [30]. Soluble HLA-G induces the apoptosis of $CD8^+$ T and NK cells by binding to CD8, and via a Fas/FasL-dependent mechanism in a model of PHA-stimulated lymphocytes [31]. Soluble HLA-G inhibits the allogeneic expansion of CD4 lymphocytes. For these reasons HLA-G could have a direct effect in LKT patients, through the expression of the membrane associated HLA-G1 isoform, on the membranes of hepatocytes and tubular cells of the kidney. In this study the soluble fraction detected in the serum of patients would correspond to the shedding of the molecule that was massively expressed on the cell surface. Alternatively, some cells may produce the soluble HLA-G5 molecule that can inhibit different T cell or DC functions.

In human allotransplantation, ectopic HLA-G expression has been demonstrated in graft biopsies and serum from heart or kidney/liver transplant patients with better graft acceptance [2, 26, 27]. No HLA-G has been detected in normal kidney and liver samples [2]. However, in LKT patients, we found that HLA-G was expressed by some tubular cells and not by glomerulus in the kidney graft biopsies. Two distinct monoclonal antibodies, namely 4H84 and MEM-G/02 were used [32]. Both antibodies recognize the free HLA-G heavy chain and they both gave similar pattern of staining in the kidney graft biopsy, as illustrated in Figure 2. HLA-G was also expressed by some BEC, but not by hepatocytes in the liver graft biopsies (Figure 2). It is of note that, for each patient tested, serial sections were obtained and tested systematically for classical HLA class I expression using the HC10 mAb, which recognizes HLA-B, -C free heavy chain, and for HLA-G expression using the 4H84 mAb. HC10 staining was always positive in all patients tested, exhibiting a high level of classical HLA class I molecule expression in both kidney and liver graft biopsies. In contrast, the 4H84 staining was positive in some patients and only in particular cells, such as BEC and tubular epithelial cells in liver and kidney allograft biopsies, respectively [2]. These observations led us to conclude that in our experiments the 4H84 antibody did not crossreact with autologous classical HLA class I molecules, but detected only HLA-G.

We obtained only few biopsies of renal allograft because of the absence of acute renal dysfunction, no biopsy was performed. In contrast, systematic biopsies of the liver were available, allowing us to perform correlation between the expression of HLA-G in BEC and episodes of acute rejection. We found that the expression of HLA-G in BEC correlated with the absence of liver rejection (Table 1). These data suggest a non-*in situ* inhibitory effect, through the expression of the membrane-bound form instead of through the secretion of the soluble isoform, by epithelial cells. Another hypothesis is that HLA-G may also be produced by hematopoietic cells because soluble HLA-G can be produced by monocytes and by CD4 and CD8 T lymphocytes, both *in vitro* and in mixed lymphocyte reactions (MLR) [33]. However the cells and mechanisms involved in this *de novo* expression of HLA-G remain unknown. Such HLA-G-positive T cells may act as inhibitory regulators of immune responses, thus limiting allograft rejection in hu-

TABLE 1 Association of HLA-G expression in BEC of liver allograft and episode of acute rejection in liver-Kidney transplant patients

	HLA-G positive BEC	HLA-G negative BEC	*p* Value*
Number of patients(%)	9 (22.5%)	31 (77.5%)	
Acute rejection			
Liver	0	11	< 0.05
Kidney	0	3	NS

* Nonparametric Chi-square test.

Abbreviations: BEC = biliary epithelial cells; HLA = human leukocyte antigen; NS = not significant.

mans following transplantation. These observations are supported by the fact that we found two LKT patients with peripheral blood $CD4^+$ and $CD8^+$ T cells expressing HLA-G *in vivo*. More data are required to identify factors that favor the expression of HLA-G on epithelial cells, monocytes, and T cells. For example, it would be important to study the HLA matching between the donor and the recipient, and the association of immunosuppressive drugs.

The discovery that HLA-G is associated with better allograft acceptance opens up new possibilities for monitoring transplant patients. Determination of soluble HLA-G levels in the serum of patients would make it possible to modulate the amount of immunosuppressive drugs given to patients. This is of particular interest for heart transplant patients or LKT patients, but a systematic analysis of the concentration of HLA-G is required in transplant patients.

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