Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

HLA-G on peripheral blood CD4$^+$ T lymphocytes: a potential predictor for acute renal rejection

Nan Lu,[1,2] Yi Zhang,[3] Xiong Zou,[3] Xiaojing Yang,[3] Jun Tian,[4] Junhui Zhen,[5] Yuxia Zhou,[3] Shengmei Zhao[3] and Wenfeng Shi[4]

1 Institute of Diagnostics, School of Medicine, Shandong University, Jinan, P. R. China
2 Institute of Immunopharmacology and Immunotherapy, School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong
3 Department of Clinical Laboratory, Qilu Hospital, Shandong University, Jinan, P. R. China
4 Department of Renal Transplantation, Qilu Hospital, Shandong University, Jinan, P. R. China
5 Institute of Pathology, School of Medicine, Shandong University, Jinan, P. R. China

Keywords
acute graft rejection, CD4$^+$ T cells, HLA-G, renal transplantation.

Correspondence
Dr. Nan Lu, Institute of Diagnostics, School of Medicine, Shandong University, 44#, Wenhua Xi Road, Jinan, Shandong, 250012 P.R. China. Tel.: 086 531 88382051; fax: 086 531 88383782; e-mail: lunan129@126.com

Conflict of Interest
No conflict of interest needed to be declared.

Received: 19 October 2010
Revision requested: 2 January 2011
Accepted: 17 July 2011
Published online: 16 August 2011

doi:10.1111/j.1432-2277.2011.01314.x

Summary

HLA-G Expression in grafts and serum has been shown to improve graft acceptance. However, its expression on peripheral blood lymphocytes (PBLs) during acute rejection (AR) remains unknown. In this study, we serially monitored HLA-G expression on CD4$^+$ and CD8$^+$ PBLs of 66 recipients undergoing renal transplantation using flow cytometry at different time points before and after transplantation, as well as during AR episode. In stable recipients, HLA-G expression on CD4$^+$ PBLs declined during the first week after transplantation and increased continuously with immunosuppressive therapy. Then, expression declined gradually after 1 month and remained at a higher level compared with pretransplantation. When AR occurred, HLA-G expression decreased significantly compared with the stable level. In three recipients suffering from recurrent rejection, it remained at a low level despite impact immunosuppressive treatment. With mix lymphocyte assay, HLA-G$^+$ CD4$^+$ T cells showed inhibitory role on proliferation of peripheral blood mononuclear cell. HLA-G expression on CD8$^+$ PBLs was almost undetectable at different time points in the recipients and healthy controls. Our results suggest that HLA-G on CD4$^+$ PBLs might provide a potential marker for the early diagnosis of renal AR and for the immunosuppressive status of recipients.

Introduction

At present, great improvements in clinical technologies have made renal transplantation the most effective therapeutic measure for end-stage renal failure. However, acute rejection (AR) is still regarded as the major factor influencing short-term and long-term allograft survival [1]. Hence, one of the most important tasks for both scientists and clinicians is to accurately distinguish patients with a high risk of developing AR from other patients. There is still no universally accepted marker for renal rejection, although graft biopsies are the gold standard for rejection diagnosis. However, graft biopsies are invasive procedures with serious potential complications. Therefore, most patients are unwilling to accept these associated risks, which severely restricts the clinical applications of biopsies [2].

In the past two decades, the short-term outcomes of kidney transplant recipients have improved dramatically because of the availability of more potent immunosuppressive drugs to prevent AR [3]. However, immunosuppressive treatment is a double-edged sword [4]. The side effects of these immunosuppressants play a negative role in long-term morbidity and mortality of recipients. Several studies have observed an increased incidence of cancer [5,6] and serious infections [7] in renal transplant patients. So rational immunosuppressive therapy is regarded as a vital issue during post-transplantation [8]. Physicians generally find it difficult to maintain a balance

between increasing the dosage of immunosuppressants to prevent AR for high-risk patients and decreasing the drug doses to reduce or eliminate unnecessary side effects for the other patients.

Recently, HLA-G has been introduced in transplantation studies. HLA-G is a nonclassical HLA-I molecule, which, unlike classical HLA molecules, exhibits a more limited polymorphism and a restricted pattern of tissue expression [9]. HLA-G was originally observed on fetal cytotrophoblasts during early pregnancy and plays an essential role in maintaining maternal–fetal immune tolerance. In pathological conditions, HLA-G expressed on different tissues and cells, including peripheral blood mononuclear cell (PBMCs) [10]. Studies in the transplantation field have shown that the expression of HLA-G in serum [11,12] and allografts [13,14] is associated with allograft acceptance.

Our previous study with mice skin transplantation model showed time-dependent dynamic expression of Qa-2 (the murine homolog of HLA-G) mRNA on PBMCs during entire episodes of AR, which is closely related to AR occurrence and immunosuppressive treatment [15,16]. Recently, Racca *et al.* reported that HLA-G1 mRNA levels in PBMCs from patients with AR are below the median value of stable recipients [17]. To establish a noninvasive method for predicting AR, we investigated the expression of HLA-G on $CD4^+$ and $CD8^+$ PBLs using flow cytometry in patients undergoing renal transplantation.

Patients and methods

Subjects

The study comprised 66 patients (51 men and 15 women), with a median age of 41 years (range of 23–62 years old), who underwent renal transplantations from June 2007 to February 2009. All of the donor organs were from living relatives or an organ donation program. We obtained informed consent from each patient and the study protocol was approved by the Ethical Committee of Qilu Hospital of Shandong University. The study was performed in accordance with the ethical standards of the 2000 Declaration of Helsinki and the 2008 Declaration of Istanbul. The follow-up periods ranged from preoperation to more than 3 months postoperation. Recipients were categorized into three groups: Group Stable, Group Borderline Change (BL) and Group Rejection. Group Stable consisted of 32 patients (26 men and 6 women) with normal renal function and serum creatinine level within the normal range (<1.14 mg/dl) at the follow-up period after transplantation. Group BL comprised nine patients (six men and three women) who were diagnosed as borderline change at a scheduled biopsy examination performed at 4 weeks after operation. They either presented with an infiltration of mononuclear cells in <25% of the parenchyma (i0, i1) or foci of mild tubulitis, (t0, t1) was detected. All of these patients had normal renal function. Group Rejection comprised 25 patients (19 men and six women) who experienced at least one acute rejection episode after renal transplantation. The diagnosis of acute rejection episode was confirmed by renal biopsy according to the Banff 2003 system [18]. The demographic data for these patients are summarized in Table 1. The exclusion criteria and the amount of the excluded patients are shown in Table 2.

Immunosuppressive protocol

Patients were treated with a standard triple-therapy immunosuppressant protocol consisting of tacrolimus

Table 1. Patient characteristics and transplant courses.

Patient characteristics	Group Stable (n = 32)	Group Rejection (n = 25)	Group Borderline (n = 9)
Gender (male/female)	26/6	19/6	6/3
Age (year) mean ± SD	41.5 ± 9.64	42 ± 12.42	39.2 ± 6.61
PRA	negative	negative	negative
Anti-HLA antibody	<10%	<10%	<10%
Primary renal disease			
Chronic glomerulonephritis	13 (40.6%)	12 (48%)	5 (56%)
Hypertensive nephrosclerosis	9 (28.1%)	10 (40%)	2 (22%)
Diabetic nephropathy	6 (18.8%)	3 (12%)	2 (22%)
Autosomal dominant polycystic kidney disease	3 (9.4%)	0	0
Obstructive uropathy	1 (3.1%)	0	0
Donor			
Live/deceased	13/19	9/16	3/6
Age (year) mean ± SD	33.1 ± 4.82	30.6 ± 6.25	34.8 ± 10.47
Cold ischemia time (h)	14.8 ± 7.46	13.9 ± 8.21	11.9 ± 9.67
HLA mismatch A/B/DR	≤3	≤3	≤3

Table 2. Excluded patients.

Exclusion criteria	Patients (number)
<18 year old	3
Lost during the follow-up	17
Second or Third transplantation	2
HBV Carrier and chronic hepatitis B	11
Other virus infection (CMV, HSV)	3
CNI Drug poisoning	2
Antibody-mediated rejection (hyperacute rejection)	3
Total	41

HBV, hepatitis B virus; CMV, cytomegalovirus; HSV, herpes simplex virus; CNI, calcineurin inhibitor.

(FK506), mycophenolate mofetil (MMF), and prednisone (PRE). All patients received 50 mg of Thymoglobulin (Rabbit Anti-human Thymocyte Globulin, Imix-anga, France) and 1 g of methylprednisolone (Pfizer Pharmaceuticals Limited, New York, NY, USA) on the day of transplantation. After operation, all patients received 500 mg/day methylprednisolone and 50 mg/day Thymoglobulin for 2 days. At 3 days after transplantation, two doses of FK506 were administered at 0.1–0.15 mg/kg/day, and whole blood trough levels of FK506 were controlled according to post-transplantation time (10–20 ng/dl from 0 to 15 days, 7–15 ng/dl from 16 to 30 days, and 3–7 ng/dl thereafter). MMF was administered simultaneously with FK506 at a maintenance dose of 1–1.5 g/day that was divided into two doses. PRE was administered at 1 mg/kg/day at the beginning, reduced gradually, and finally maintained at 5–10 mg/day thereafter.

Rejection treatment consisted of three doses of methylprednisolone (500 mg/dose). If serum creatinine remained high, then Thymoglobulin (50 mg/day) was administered for 7 days.

Collection of peripheral blood

EDTA anti-coagulated peripheral blood samples (2 ml) were taken from each patient before the operation, on days 3 (D3) and 7 (D7), at weeks 2 (W2) and 3 (W3), months 1 (M1) and 2 (M2), and more than 3 months (M3) after transplantation.

All patients were instructed to return to hospital immediately if they experienced fever, lumbodynia, gross hematuria, oliguria, de novo or aggravated proteinuria, or if blood pressure, creatinine clearance or blood urea nitrogen was increased. For these patients, blood samples were collected immediately before the impact immunosuppressive treatment (D0) and on D3, D7, D14, and D21–60 after impact treatment.

Biopsy

Surveillance biopsies were scheduled at weeks 4, 8, 12, and 24 as part of the typical post-transplantation protocol in our transplant unit. In addition, patients underwent biopsies if AR was suspected.

Isolation of HLA-G$^+$ CD4$^+$ T cells

Peripheral blood mononuclear cell were isolated from peripheral blood by Ficoll density gradient centrifugation (Ficoll-Hypaque, 1.077 *g*/ml, Haoyang CO, China) at $400 \times g$ for 25 min, and then washed twice with PBS by centrifugation at $300 \times g$ for 10 min at room temperature.

Then, PBMC were first negatively isolated using the CD4$^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated with the biotin conjugated HLA-G-specific 87G antibody for 45 min. Subsequently, labeled cells were positively selected using anti-biotin microbeads (Miltenyi Biotec) to purified HLA-G$^+$ CD4$^+$ T cells from peripheral blood.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and Mixed lymphocyte culture (MLC)

Isolated PBMC were labeled with CFSE (Molecular Probes, Inc., Eugene, OR, USA) at a final concentration of 5 μM according to the manufacturer's instruction.

The CFSE-labeled PBMC from five recipients were treated as responder cells. The stimulator cells from five healthy donors were incubated with Mitomycin C (50 μg/ml) for 1 h at 37 °C and 5% CO_2 to inactivate their proliferation ability. Responder cells and stimulator cells (1×10^6) were mixed in medium containing 10% FCS and incubated at 37 °C and 5% CO_2 for 5 days.

To assess the inhibitory function of HLA-G$^+$ CD4$^+$ T cells, 2×10^5 purified HLA-G$^+$ CD4$^+$ T cells from the same donor of responder cells were added into MLC system with same amount of PBMC as control.

Flow Cytometry (FCM)

About 100 μl of whole blood from each patient was taken for FCM. Monoclonal antibodies against CD3 (PerCP-Cy5.5 and FITC, Clone: OKT3), CD4 (FITC, Clone: OKT4), or CD8 (PE-Cy5, Clone: RPA-T8) and HLA-G (PE, Clone: 87G) (eBioscience, San Diego, CA, USA) were added to each sample and incubated for 25 min at room temperature in the dark. Red blood cells were lysed with FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA) and then incubated for another 8–12 min at room temperature in the dark. After centrifugation for

5 min at 500 *g*, the supernatants were discarded. The cells were washed twice in cold sheath fluid (Becton Dickinson) and diluted with 100–200 μl of sheath fluid.

Flow cytometry was conducted using a FACS Calibur (Becton Dickinson). To analyze the data, total lymphocytes were separated from peripheral blood and identified as Gate 1, whereas the $CD3^+$ population was separated from Gate 1 and identified as Gate 2. To obtain the $CD3^+CD4^+$ and the $CD3^+CD8^+$ population, the cells obtained from Gate 2 were further subdivided by addition of Gate 3. Finally, the level of HLA-G on the $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes was measured.

The rate of positive cells (RPC) of HLA-G on peripheral blood $CD4^+$ and $CD8^+$ T-lymphocytes were calculated using Cellquest-pro software (Becton Dickinson). To ensure the quality of HLA-G antibody, JEG-3 choriocarcinoma cells (HLA-G positive cell line) were used as a positive control. In addition, FITC-mouse IgG2a, PE-mouse IgG2a, PerCP-Cy5.5-mouse IgG2a, and PE-Cy5-mouse IgG2b (eBioscience) were used as isotype-negative controls for compensating and gating when setting up the FCM. To control the intra-assay error and ensure the quality and accuracy of the cytometry, whole blood quality controls obtained from Jianzhong Wang (First Hospital, Peking University) were used.

The proliferation of responder cells in MLC were also measured using FCM. All live cells were gated, analyzed for CFSE level, and visualized in a histogram.

Statistical analysis

All data were analyzed using SPSS13.0 (SPSS Inc, Chicago, IL, USA) (version 13.0), MedCalc for Windows (version 9.3.8, MedCalc Software, Mariakerke, Belgium), and GraphPad Prism (version 5.0). The results are shown as mean values ± SD. ANOVA was used for serial measurements in a group or different groups and significant values were analyzed *post hoc* using the Student–Newman–Keuls test. Student's *t*-test analysis was used for analysis of differences between different groups. A P value of less than 0.05 was regarded as a significant difference and a P value of less than 0.01 was considered highly significant.

Results

The HLA-G expressions on peripheral blood $CD4^+$ T lymphocytes from Group Stable were plotted from before transplantation to more than 3 months after the operation (Fig. 1a). The RPC of HLA-G on $CD3^+CD4^+$ lymphocytes of pretransplantation was 2.27 ± 0.72%. In the first week of post-transplantation, the HLA-G was nearly undetectable on $CD3^+CD4^+$ lymphocytes and levels were considerably less than the pretransplantation level. After the first week, the HLA-G positive cells in the peripheral blood $CD4^+$ T lymphocytes increased continuously during the first month, reaching a peak 1 month of post-transplantation (10.65 ± 2.86%), before declining gradually to maintain a stable level (6.52 ± 2.54%) after 2 months.

Compared with Group Stable, the dynamic change of HLA-G in Group BL showed a similar tendency and increased as the immunosuppressive therapy (Fig. 1b). However, after 1–2 months post-transplantation, when borderline changes of rejection were detected, the increase of HLA-G expression was lower than that of Group Stable.

Among the 66 transplantation recipients, 25 of them experienced AR, which occurred from 15 days to 4.5 months after transplantation. All the patients with AR were confirmed by biopsy. The levels of HLA-G in these 25 patients were monitored when the acute rejection episode occurred and followed for more than 3 weeks (Fig. 2a). The first data point represents the levels of HLA-G during the previous routine check-up with no symptoms of AR. When AR occurred, HLA-G positive cells decreased significantly, even reaching an undetectable level. The patients were administrated methylprednisolone immediately. Three days later, the HLA-G levels up-regulated gradually until 2 weeks after the AR (4.16 ± 1.63%). Three weeks after AR, the HLA-G positive cells declined again (2.40 ± 0.82%). In Group Rejection, there were three patients suffering from recurrent rejection. The RPC of HLA-G on $CD3^+CD4^+$ lymphocytes remained at a low level (1.18 ± 0.56%), both in the relatively stable stage and during rejection episodes (Fig. 2b).

The dynamic change of HLA-G levels in the stable stages of Group Rejection, (except for three patients) showed a similar tendency with that of Group Stable and Group BL after transplantation.

The average levels of HLA-G expression on $CD4^+$ T lymphocytes in different groups, including healthy controls, patients before transplantation, in stable stage and during BL or AR were detected and analyzed. No statistically significant differences were found between healthy controls and pretransplant patients. When patients were stable with normal kidney function and no rejection symptoms, the levels of HLA-G expression were much higher than pretransplantation levels. In contrast, when patients were suffering from rejection or borderline changes, the expression of HLA-G was much lower than stable levels (Fig. 3).

To assess the inhibitory role of HLA-G^+ $CD4^+$ T cells, we determined the allogeneic proliferation of CFSE-labeled PBMC with addition of HLA-G^+ $CD4^+$ T cells to PBMC mixed lymphocyte culture. The result showed that addition of HLA-G^+ T cells led to a significant reduction

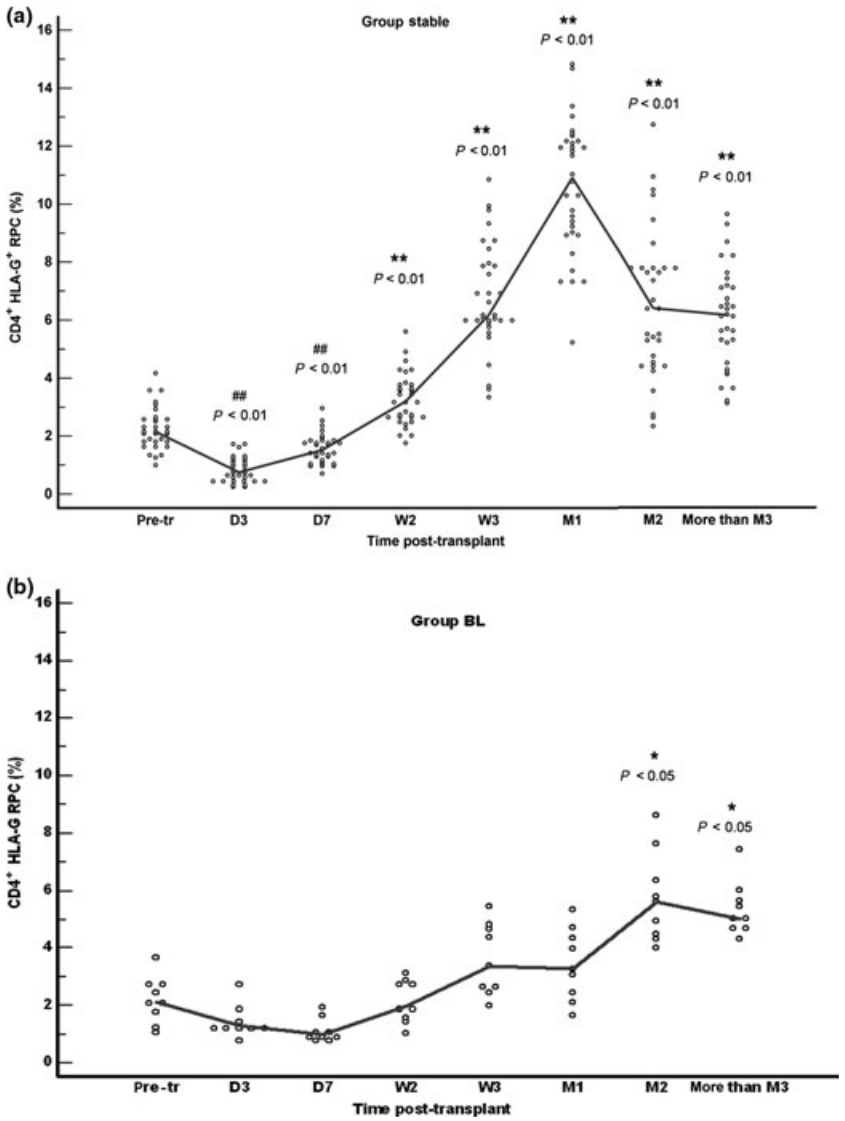

Figure 1 Dynamic HLA-G expression on $CD4^+$ PBLs in Group Stable (a) and Group BL(b). Peripheral blood samples were obtained before the operation (day 0), as well as at days 3 and 7, weeks 2 and 3, months 1 and 2, and more than 3 months after transplantation. All recipients in Group BL were detected borderline changes during a scheduled biopsy examination on month 1 after transplant. The value of each patient is plotted and the solid line represents the mean value of HLA-G expression at each time point. $*P < 0.05$ higher than the level at pretransplantation. $**P < 0.01$ significantly higher than the level at pretransplantation. $^{\#\#}P < 0.01$ significantly lower than the level at pretransplantation. PBL, peripheral blood lymphocytes; BL, borderline change.

of proliferation of PBMC from 79.92 ± 3.95% to 52.87 ± 5.08% (Fig. 4).

The HLA-G expression on peripheral blood $CD3^+CD8^+$ T lymphocytes of all patients was also monitored serially. The results indicated that the RPC of HLA-G cells in $CD3^+CD8^+$ lymphocytes remained at a rather low level (1.35 ± 0.43%), suggesting that there was no significant difference in Group Stable, Group BL or Group Rejection compared with the preoperation levels.

Discussion

Acute rejection of human renal allograft remains a serious clinical complication. It usually occurs within the first 3 months after transplantation [19]. In 2002, Galante *et al.* followed 1544 patients after renal transplantation and found that the 5-year survival rate of the nonacute rejection group was 82.2%, whereas the rate of the acute group was only 62.4% ($P < 0.001$) [20]. At the same time, AR is also a major risk factor for development of chronic rejection and reduction of allograft life [21]. Although graft histopathology has proven to be the gold standard for evaluating AR episodes [2], it is hardly utilized as a routine follow-up procedure because it is invasive and might cause kidney injury sometimes. Therefore, a less- or noninvasive technique for diagnosis of AR at earlier stages is an urgent need in the field of renal transplantation.

At the same time, despite the combination of powerful immunosuppressive agents that have led to significant improvements in renal allograft survival, individual differences and severe side effects resulting from insufficient or

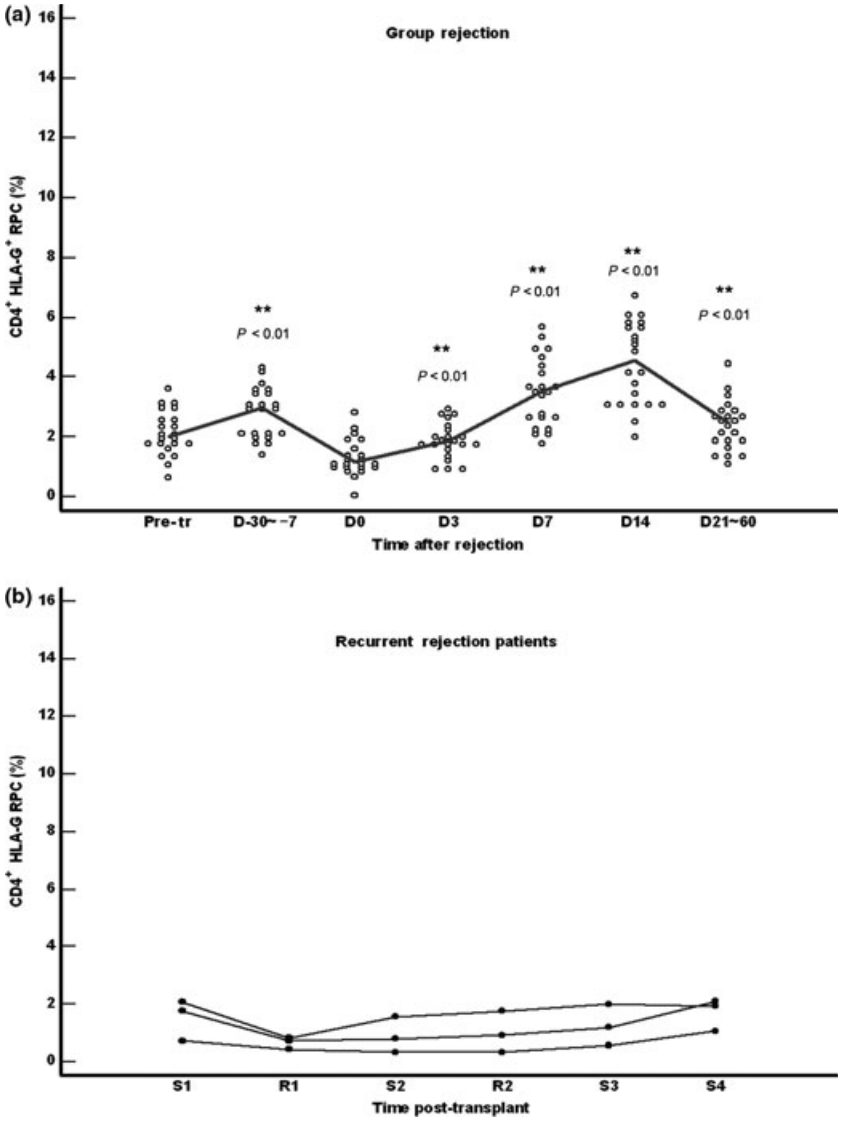

Figure 2 HLA-G expression on CD4+ PBLs during the acute rejection progress. (a) In Group Rejection, peripheral blood samples were taken six times during acute rejection. D-30–D-7: The last routinely checkout before AR occurred; D0: The laboratory or clinical dysfunction occurred on patients (the biopsy day); and others is different time point after AGR. $^{**}P < 0.01$ versus the level on AGR episode. The value of each patient is plotted and solid line represents the mean value of HLA-G expression on each time point in part A and B. (b) For three recipients suffering from recurrent rejection, six times of peripheral blood samples were taken. S1: The last routinely checkout before first AGR occurred; R1, R2: first and second rejection occurred; S2, S3, S4: relative stable stage between rejection. Each patient is represented by individual line. PBL, peripheral blood lymphocytes; AR, acute rejection; AGR, acute graft rejection.

excessive immunosuppression are still responsible for graft loss [8]. To date, there are no applicable methods to assess rational immunosuppressive drug doses for each patient.

Currently, renal function laboratory tests, such as the measurement of serum creatinine level, are the most widely used indicators of allograft function. However, they are not elevated until allograft damage has already occurred and the damage is usually irreversible. In addition, increased serum creatinine levels can also be caused by post-transplantation complications other than rejection [22].

In 2000, Lila *et al.* first reported that the occurrence of acute heart graft rejection in HLA-G-positive patients was lower than that of HLA-G-negative patients [23]. Since then, HLA-G has been observed in the grafts and serum of recipients who did not reject their allograft after heart [14], kidney [24], liver [25], or liver/kidney transplantations [26]. However, studies on the dynamic expression of HLA-G, particularly on PBLs, after transplantation and during AR episodes have not been conducted.

In this study, we monitored the HLA-G expression of PBLs in 66 kidney transplantation recipients. The levels of HLA-G on CD4+ PBLs decreased during the first week post-transplantation. At the early stages of post-transplantation, T lymphocytes were activated by HLA mismatch, although all recipients were treated with Thymoglobulin and large doses of methylprednisolone during the first 3 days.

From 2 weeks onwards, the amount of HLA-G positive cells increased gradually and was much higher than the levels of pretransplant, early stage of post-transplant and

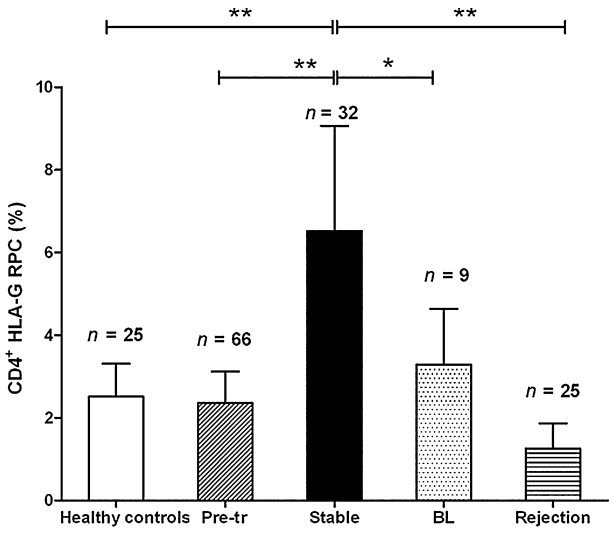

Figure 3 Analysis of HLA-G expression on $CD4^+$ T lymphocytes of healthy controls, pretransplantation patients, and recipients at stable stage, when BL was detected or when AR occurred. The RPC of HLA-G on $CD4^+$ T lymphocytes was measured using FCM. *($P < 0.05$) and **($P < 0.01$) vs. level of Group Stable. Dates are depicted as mean ± SD. BL, borderline change; AR, acute rejection; RPC, rate of positive cells.

during episodes of AR. Other studies have also reported that patients with graft expression of HLA-G and high levels of sHLA-G are associated with better graft acceptance [27,28]. The reasons for these results are not certain. By analyzing the clinical immunosuppressive protocol, we found that after 3 days of operation, all patients were treated with triple-therapy immunosuppressant protocol consisting of FK506, MMF, and PRE. Studies have reported that immunosuppressive agents, such as CsA, methylprednisolone, and MMF, could up-regulate Th-2 cytokines (such as IL-4, IL-10, and IL-12) [29]. Moreau *et al.* reported that these cytokines selectively induced HLA-G expression in human trophoblasts and monocytes [30]. Immunosuppressive agents might induce increased HLA-G expression on PBMCs by up-regulating the level of Th-2 cytokines.

During this process, HLA-G might participate in the protection of transplants against rejection and prolonging allograft survival by inhibiting the proliferation function of cells involved in graft rejection. Wiendl *et al.* have suggested that HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and found that CD4 HLA-G^+ T cells had suppressive effect on lymphocyte proliferation. Our study showed that addition of HLA-G^+ $CD4^+$ T cells could reduce mixed lymphocyte activated allogeneic proliferation of CFSE-labeled PBMC. Other studies have reported that HLA-G molecule inhibits the cytotoxic activity of $CD8^+$ T cells and NK cells [31], the proliferation of $CD4^+$ T cells [10], the cell cycle progression of alloreactive T cells [32] and the maturation of APCs [33]. And furthermore, some researchers have found that HLA-G efficiently induces regulatory T cells [34].

It is worthwhile to note that three patients suffering from recurrent rejection showed continuously low-levels of HLA-G expression despite a high-dose of immunosup-

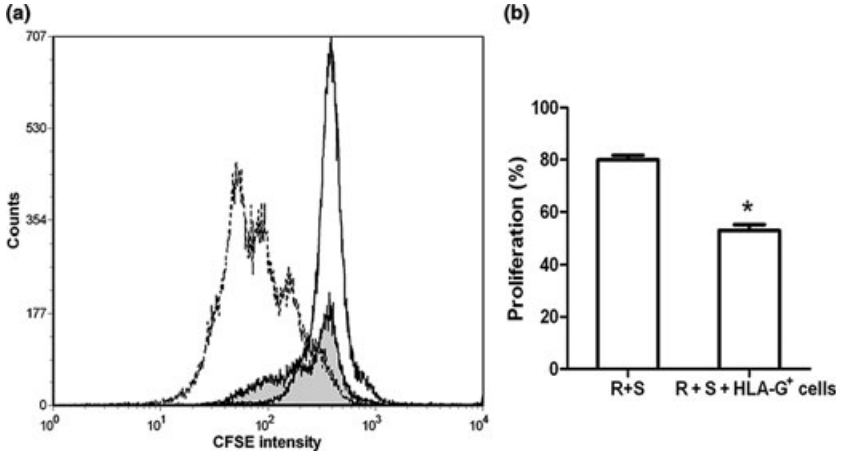

Figure 4 The role of HLA-G^+ $CD4^+$ T cells on PBMC proliferation to allogeneic stimulation. The CFSE-labeled PBMC from five recipients (responder cells) were co-cultured with PBMC from five health controls (stimulator cells). The proliferation of CFSE-labeled responder cells was measured with FCM. (a) Representative results with percentages of proliferation stated in overlay histogram. Solid line: CFSE-labeled recipient PBMC without stimulator cells. Dot line: CFSE-labeled recipient PBMC with stimulator cells. Shaded area: CFSE-labeled recipient PBMC were co-cultured with stimulator cells with the addition of 2×10^5 responder HLA-G^+ $CD4^+$ T cells. (b) Proliferation of PBMC from five recipients in mixed lymphocyte culture system. R+S: mean responder cells and stimulator cells in MLC. R+S+HLA-G^+: mean responder cells + stimulator cells with the addition of purified HLA-G^+ $CD4^+$ T cells. PMBC, peripheral blood mononuclear cell; CFSC, carboxyfluorescien diacetate succinimidyl ester; FCM, flow cytometry.

pressive agents. One possible explanation for this result is that individual patient has different susceptibility to immunosuppressive treatments. Patients in whom intensive immunosuppressive therapy failed to up-regulate their HLA-G expression may be at a high risk of recurrent severe rejection episodes.

The most important finding of our research is that the levels of HLA-G decreased significantly in all patients of Group Rejection when acute rejection episodes occurred compared with their last routine check-up before AR ($P < 0.01$) and follow-up after AR ($P < 0.01$). These results point to an inverse relationship between HLA-G^+ $CD4^+$ T lymphocytes and episodes of AR. The percentage of HLA-G^+ T lymphocytes might reflect the immune status of the host. Recently, Racca *et al.* also showed that HLA-G and HLA-G1 mRNA levels in PBMCs from patients with AR were lower than stable patients [17]. When AR occurred, host T lymphocytes and monocytes (the majority of PBMCs) were activated through direct and indirect recognition. However, the mechanisms underlying these changes need to be elucidated.

By analyzing the data of Group BL, we found that HLA-G^+ $CD4^+$ T lymphocytes decreased during borderline changes were found. During cell-mediated acute rejection process, activation of T lymphocytes is the initial stage and much more earlier than organ damage occurrence. Hence, the change in HLA-G expression on PBMCs may precede changes of kidney function markers. And the decrease of HLA-G may be valuable for early diagnosis of AR.

Both our previous study [15] and the study by Racca [17] have shown that HLA-G mRNA level are down regulated during AR. FCM is easier and less labor-intensive than PCR and is widely available in most clinical laboratories. Therefore, regular monitoring of HLA-G expression on peripheral blood $CD4^+$ T lymphocytes would be beneficial for the early diagnosis of AR.

In conclusion, the percentage of HLA-G^+ $CD4^+$ PBLs might reflect the immune status of the recipient and is related to AR episodes and immunosuppressive therapy. Therefore, we propose that the levels of HLA-G on $CD4^+$ PBLs may serve as a useful indicator in predicting AR episodes after renal transplantation. Further investigations with larger patient populations are required to confirm these findings. In addition, the exact mechanisms of HLA-G down-regulation on $CD4^+$ PBLs still need to be elucidated.

Authorship

NL, YZ and XZ: participated in research design, data analysis, and manuscript writing. NL, XY, YZ and YZ: performed the research. JT and WS: managed patients clinically and offered related clinical materials. NL, YZ, WS and SZ: participated in sample collection. JZ: analyzed pathological results of graft biopsy.

Funding sources

This work was supported by grants from Science & Technology Fund Planning Project of Shandong Province (2008GG30002017), Shandong University (200900114), Medical Science and Technology Development Foundation of The Health Department of Shandong Province (2009HW038) and Jinan Science and Technology Bureau (201004050).

References

1. Issa F, Schiopu A, Wood KJ. Role of T cells in graft rejection and transplantation tolerance. *Expert Rev Clin Immunol* 2010; **6**: 155.
2. Solez K, Colvin RB, Racusen LC, *et al.* Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant* 2008; **8**: 753.
3. Cooper JE, Wiseman AC. Novel immunosuppressive agents in kidney transplantation. *Clin Nephrol* 2010; **73**: 333.
4. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; **351**: 2715.
5. Zafar SY, Howell DN, Gockerman JP. Malignancy after solid organ transplantation: an overview. *Oncologist* 2008; **13**: 769.
6. Navarro MD, Lopez-Andreu M, Rodriguez-Benot A, Aguera ML, Del CD, Aljama P. Cancer incidence and survival in kidney transplant patients. *Transplant Proc* 2008; **40**: 2936.
7. Fishman JA. Introduction: infection in solid organ transplant recipients. *Am J Transplant* 2009; **9**(Suppl 4): S3.
8. Chadban S, Morris R, Hirsch HH, Bunnapradist S, Arns W, Budde K. Immunosuppression in renal transplantation: some aspects for the modern era. *Transplant Rev (Orlando)* 2008; **22**: 241.
9. Menier C, Rouas-Freiss N, Favier B, LeMaoult J, Moreau P, Carosella ED. Recent advances on the non-classical major histocompatibility complex class I HLA-G molecule. *Tissue Antigens* 2010; **75**: 201.
10. Feger U, Tolosa E, Huang YH, *et al.* HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation. *Blood* 2007; **110**: 568.
11. Wu J, Zhang W, Hernandez-Lopez P, *et al.* Isoforms of human leukocyte antigen-G and their inhibitory receptors in human kidney allograft acceptance. *Hum Immunol* 2009; **70**: 988.
12. Lila N, Amrein C, Guillemain R, Chevalier P, Fabiani JN, Carpentier A. Soluble human leukocyte antigen-G: a new strategy for monitoring acute and chronic rejections after

heart transplantation. *J Heart Lung Transplant* 2007; **26**: 421.
13. Brugiere O, Thabut G, Pretolani M, *et al.* Immunohistochemical study of HLA-G expression in lung transplant recipients. *Am J Transplant* 2009; **9**: 1427.
14. Sheshgiri R, Rouas-Freiss N, Rao V, *et al.* Myocardial HLA-G reliably indicates a low risk of acute cellular rejection in heart transplant recipients. *J Heart Lung Transplant* 2008; **27**: 522.
15. Lu N, Wan F, Yang X, *et al.* Qa-2 mRNA in peripheral blood mononuclear cells: a potential marker of acute allograft rejection. *Med Sci Monit.* 2009; **15**: R99.
16. Lu N, Wang C, Yang X, *et al.* Dynamic expression of Qa-2 during acute graft rejection. *Mol Med* 2011; **17**: 248.
17. Racca AL, Veaute CM, Bailat AS, *et al.* Expression of HLA-G and MICA mRNA in renal allograft. *Transpl Immunol* 2009; **21**: 10.
18. Racusen LC, Halloran PF, Solez K. Banff 2003 meeting report: new diagnostic insights and standards. *Am J Transplant* 2004; **4**: 1562.
19. Kamoun M. Cellular and molecular parameters in human renal allograft rejection. *Clin Biochem* 2001; **34**: 29.
20. Galante NZ, Tedesco HS, Machado PG, Pacheco-Silva A, Medina-Pestana JO. Acute rejection is a risk factor for long-term survival in a single-center analysis of 1544 renal transplants. *Transplant Proc* 2002; **34**: 508.
21. Gierej B, Gornicka B, Wasiutynski A. Role of C3d and C4d complement fragments in the diagnostics of acute allograft rejection after transplantations. *Ann Transplant* 2009; **14**: 61.
22. Lachenbruch PA, Rosenberg AS, Bonvini E, Cavaille-Coll MW, Colvin RB. Biomarkers and surrogate endpoints in renal transplantation: present status and considerations for clinical trial design. *Am J Transplant* 2004; **4**: 451.
23. Lila N, Carpentier A, Amrein C, Khalil-Daher I, Dausset J, Carosella ED. Implication of HLA-G molecule in heart-graft acceptance. *Lancet* 2000; **355**: 2138.
24. Crispim JC, Duarte RA, Soares CP, *et al.* Human leukocyte antigen-G expression after kidney transplantation is associated with a reduced incidence of rejection. *Transpl Immunol* 2008; **18**: 361.
25. Basturk B, Karakayali F, Emiroglu R, *et al.* Human leukocyte antigen-G, a new parameter in the follow-up of liver transplantation. *Transplant Proc* 2006; **38**: 571.
26. Creput C, Le Friec G, Bahri R, *et al.* Detection of HLA-G in serum and graft biopsy associated with fewer acute rejections following combined liver-kidney transplantation: possible implications for monitoring patients. *Hum Immunol* 2003; **64**: 1033.
27. Qiu J, Terasaki PI, Miller J, Mizutani K, Cai J, Carosella ED. Soluble HLA-G expression and renal graft acceptance. *Am J Transplant* 2006; **6**: 2152.
28. Apps R, Gardner L, Moffett A. A critical look at HLA-G. *Trends Immunol* 2008; **29**: 313.
29. Daniel V, Naujokat C, Sadeghi M, Wiesel M, Hergesell O, Opelz G. Association of circulating interleukin (IL)-12- and IL-10-producing dendritic cells with time posttransplant, dose of immunosuppression, and plasma cytokines in renal-transplant recipients. *Transplantation* 2005; **79**: 1498.
30. Moreau P, Adrian-Cabestre F, Menier C, *et al.* IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int Immunol* 1999; **11**: 803.
31. Riteau B, Rouas-Freiss N, Menier C, Paul P, Dausset J, Carosella ED. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. *J Immunol* 2001; **166**: 5018.
32. Bahri R, Hirsch F, Josse A, *et al.* Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. *J Immunol* 2006; **176**: 1331.
33. Liang S, Ristich V, Arase H, Dausset J, Carosella ED, Horuzsko A. Modulation of dendritic cell differentiation by HLA-G and ILT4 requires the IL-6–STAT3 signaling pathway. *Proc Natl Acad Sci USA* 2008; **105**: 8357.
34. Le Rond S, Azema C, Krawice-Radanne I, *et al.* Evidence to support the role of HLA-G5 in allograft acceptance through induction of immunosuppressive/regulatory T cells. *J Immunol* 2006; **176**: 3266.