Binding of Anti-HLA Class I Antibody to Endothelial Cells Produce an Inflammatory Cytokine Secretory Pattern

Eduardo Reyes-Vargas,¹ Igor Y. Pavlov,² Thomas B. Martins,² Jason J. Schwartz,³ Harry R. Hill,^{1,2} and Julio C. Delgado^{1,2*}

¹Department of Pathology, University of Utah, Salt Lake City, Utah

²ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah

³Department of Surgery, University of Utah, Salt Lake City, Utah

Current methods are inadequate for the diagnosis of early chronic allograft rejection. The goal of this study was to determine whether ligation of anti-HLA antibodies to endothelial cells is associated with a distinctive cytokine secretory pattern. Human iliac artery endothelial cells (HIAEC) cultured in vitro were incubated with w6/32, an anti-HLA class I mAb. Culture supernatants collected daily for up to 4 days were tested for secretion of 13 cytokines using a multiplexed fluorescent microsphere immunoassay. Culture of HIAEC with medium containing mAb

w6/32 supported the growth of HIAEC during the 4-day study period. Levels of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8, and TNF- α became significantly increased in supernatants of HIAEC incubated with the mAb w6/32. We conclude that ligation of anti-HLA class I antibodies to HLA class I antigens in endothelial cells initiates an acute inflammatory process and detecting an inflammatory cytokine secretory pattern might be useful to diagnose sub-clinical chronic allograft rejection. *J. Clin. Lab. Anal.* 23:157–160, 2009. © 2009 Wiley-Liss, Inc.

Key words: endothelial cells; HLA antibody; cytokines

INTRODUCTION

The use of current immunosuppressive regimens has led to important declines in acute cellular rejection and, therefore, chronic allograft rejection has become the dominant cause limiting the survival of allografts in solid organ transplant recipients. The suspicion of chronic allograft rejection relies primarily on the change of a physiological indicator (i.e., increase of serum creatinine in kidney transplantation), while confirmatory diagnosis requires invasive procedures to obtain graft biopsies for histological evaluation. Thus, current methods for diagnosis of chronic rejection are inadequate for detecting sub-clinical allograft damage that occurs before the establishment of irreversible graft injury.

Multiple factors can lead to the development of chronic allograft rejection including HLA alloantibodies, peri-transplant ischemia, calcineurin inhibitor toxicity, and infection by polyomavirus and cytomegalovirus, among others (reviewed in (1)). The occurrence of HLA alloantibodies post transplantation, however, appear to be the most important factor associated with chronic allograft rejection (reviewed in (2,3)). Ligation

of anti-HLA class I antibodies to endothelial cells in vitro induces cell proliferation and fibrosis, both seen in graft biopsies of patients with chronic rejection (2,4). Furthermore, data from several retrospective studies have shown strong associations between the occurrence of HLA alloantibodies and the incidence of chronic rejection in kidney (5–8), lung (9,10), and heart transplantation (11,12). These observations have led HLA clinical laboratorians to propose HLA alloantibody screening post transplantation as a measure to

Abbreviations: EGM, endothelial cell growth medium; HIAEC, human iliac artery endothelial cells.

Eduardo Reyes-Vargas and Igor Y. Pavlov contributed equally to this study.

*Correspondence to: Julio C. Delgado, University of Utah, 15 N Medical Drive E, Salt Lake City, UT 84112.

E-mail: julio.delgado@path.utah.edu

Grant sponsor: ARUP Institute for Clinical and Experimental Pathology.

Received 14 January 2009; Accepted 5 February 2009

DOI 10.1002/jcla.20310

Published online in Wiley InterScience (www.interscience.wiley.com).

diagnose early allograft rejection. The interpretation of the appearance of HLA alloantibodies post transplant, however, is difficult given the lack of temporal correlation between the occurrence HLA alloantibodies and the start of chronic rejection (3).

The development of clinical laboratory methods that allow detection of sub-clinical chronic allograft rejection would be an important step in improving the long-term survival of allografts. One possibility would be the discovery of surrogate markers of endothelial cell activation caused by HLA antibodies that can be examined in clinical specimens of transplanted patients. In this study, we employed an in vitro endothelial cell model to analyze the cytokine secretory pattern in response to activation with HLA antibodies. We find that ligation of an anti-HLA class I antibody to endothelial cells produces a distinctive increased inflammatory cytokine secretory pattern. Using this approach, we suggest that an assay detecting an inflammatory cytokine secretory pattern might be useful to diagnose sub-clinical events during chronic allograft rejection.

MATERIAL AND METHODS

Cells and Antibodies

Human iliac artery endothelial cells (HIAEC) from a single donor were obtained from Lonza (Walkersville, MD), and cultured at 37°C, 5% CO₂, in endothelial basal culture medium (Lonza) supplemented with 10 ng/ml human recombinant epidermal growth factor, 1.0 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, 3 mg/ml bovine brain extract, human fibroblast growth factor, human vascular endothelial growth factor, heparin, and 2% FBS, which will be referred in this study as complete endothelial growth medium (EGM). Experiments were performed on HIAEC monolayers that were between 80–90% confluent, and used between passages 2 and 3. The following antibodies were used: w6/32 (IgG2a), a mAb that binds a conformational epitope on HLA class I antigens (ATCC, Mannasas, VA); mouse IgG2a used as an isotype control (ATCC).

Cell Growth Assays

HIAEC were seeded into six-well plates at 96,000 cells/well and left to attach overnight in complete EGM. The next day (time 0) complete EGM was removed and anti-HLA class I mAb w6/32 (10 µg/ml) or control isotype were added to the cultures. HIAEC cell growth was assessed by absolute cell count determination using Flow-Count Fluorophores (Beckman Coulter, Miami, FL) in a FACSCanto flow cytometry (BD Biosciences, San Jose, CA). Briefly, HIAEC were detached with

0.125% trypsin/0.05% EDTA at 0, 24, 48, 72, and 96 hr. Cell pellets were washed twice with buffer containing PBS and 1% FBS. For absolute count determination, 100 µl of HIAEC suspensions were mixed with 100 µl of Flow-Count Fluorophores. Indirect HIAEC absolute count calculations were done using 1,000 fluorophore counts.

Cytokine Quantitation

Supernatants of in-vitro cultures of HIAEC stimulated with 10 µg/ml of mAbs and collected at different time points (0, 24, 48, 72, and 96 hr) were tested for the secretion of cytokines using a multiplexed fluorescent microsphere immunoassay developed at ARUP Laboratories, Salt Lake City, UT. The development and validation of the multiplexed cytokine panel used in this study has been described in detail (13). The panel comprises the following 13 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, INF-γ, TNF-α, IL-2r (CD25), and CD40 ligand.

Statistical Analysis

Data are presented as the mean ± standard deviation unless indicated otherwise. All data were evaluated with two-tailed unpaired Student's *t* test or compared by one-way analysis of variance. A *P* value of less than 0.05 was deemed significant for all tests.

RESULTS

To determine whether the treatment with anti-HLA class I antibodies has any effect on the growth of endothelial cells, HIAEC were cultured in the presence of w6/32, an antibody that binds a conformational epitope on HLA class I antigens. Incubation of HIAEC with medium containing mAb w6/32, in the absence of growth supplements, supported the growth of HIAEC during the 96 hr study period. The rate of growth of HIAEC upon stimulation with the w6/32 antibody was similar to the rate of growth of HIAEC cultured with complete EGM (Fig. 1). In contrast, the culture of HIAEC with medium containing the isotype control antibody IgG2a or the culture with medium in the absence of supplements did not support their growth (Fig. 1).

To determine whether ligation of HLA class I antibodies is associated with a particular pattern of cytokine secretion, we next measured the concentrations of several cytokines in tissue culture supernatants collected throughout the 4-day study period. We found that levels of several pro-inflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α became significantly increased in supernatants from HIAEC incubated with the mAb w6/32 over the duration of the culture

(Fig. 2). We also noted that concentration of other Th1 and Th2 cytokines, tested by the multiplexed assay used in this study, were found at very low levels and were not

significantly different after mAb w6/32 stimulation (data not shown).

DISCUSSION

In vitro culture of endothelial cells has been valuable for studying the potential role of HLA alloantibodies in the development of chronic allograft rejection. Ligation of HLA class I molecules with anti-HLA antibodies induces a cascade of intracellular events leading to endothelial cell proliferation (2,4,14). In this study we confirmed that the addition of anti-HLA class I antibody, in the absence of endothelial growth supplements, is sufficient to support the growth of HIAEC in vitro.

An important issue in solid organ transplantation is how to become aware of early chronic allograft rejection before irreversible graft injury takes place. The solution to this problem is the development of clinical laboratory indicators that are able to diagnose sub-clinical events during chronic allograft rejection and that allows transplant physicians to take the appropriate measures to prevent irreversible graft damage. The primary goal

Fig. 1. Stimulation of endothelial cell growth by anti-HLA class I antibody. HIAEC cells were cultured with 10 μ g/ml of mAbs to HLA class I antigens (w6/32), or murine IgG2a isotype control antibody, EGM, or complete EGM. Cells were recovered at 0, 24, 48, 72, and 96 hr. Cells were resuspended in PBS and 1% FBS. For absolute count determination, 100 μ l of HIAEC suspensions were mixed with 100 μ l of Flow-Count Fluorophores. HIAEC absolute count calculations were done using 1,000 fluorophore counts. The data are expressed as the mean count of two different experiments in triplicates.

Fig. 2. Induction of pro-inflammatory cytokine secretion by anti-HLA class I antibody in endothelial cells. HIAEC cells were cultured with 10 μ g/ml of mAbs to HLA class I antigens (w6/32), or murine IgG2a isotype control antibody. Culture supernatants were collected at 0, 24, 48, 72, and 96 hr. Levels of cytokines were measured using a multiplexed fluorescent microsphere immunoassay. The data are expressed as the mean $\pm$ standard deviations of two different experiments in triplicates. Significant increased levels ($P < 0.05$) of IL-1 β , IL-6, IL-8, and TNF- α were found in culture supernatants of HIAEC stimulated with the w6/32 antibody at all time points.

of this study was to determine whether ligation of anti-HLA class I antibody to endothelial cells is associated with a cytokine secretory pattern that could be used as a surrogate marker for endothelial cell activation by HLA antibodies. To answer this question, we used a multiplexed assay that allowed us to measure 13 cytokines at once in culture supernatants of HIAEC stimulated with the anti-HLA class I mAb w6/32 and the IgG2a mAb isotype used as a control. The results showed that ligation of the w6/32 antibody to HIAEC produced a significant increase in the secretion of the pro-inflammatory cytokines IL-1 β , IL-6, IL-8, and TNF- α .

The production of IL-1 β , IL-6, and TNF- α is rapidly induced in the course of acute inflammatory reactions associated with injury, trauma, stress, and infection (reviewed in (15–18)). IL-1 β , the prototypic pro-inflammatory cytokine, induces the secretion of a large number of cytokines including IL-6 and TNF- α , along with several chemokines and adhesion molecules (17). Many of the pro-inflammatory effect of IL-1 β can be explained on the basis of the effect of TNF- α on the vascular endothelium (16). The stimulation of endothelial cells by TNF- α promotes inflammation by inducing the expression of adhesion molecules for inflammatory leukocytes and by the release of several chemokines, including IL-8, which are responsible for the recruitment of neutrophils to the site of inflammation (15). Taken together, our results showing an increase of IL-1 β , IL-6, IL-8, and TNF- α in culture supernatant of HIAEC stimulated with the w6/32 mAb supports the idea that ligation of anti-HLA class I antibodies to HLA class I antigens in endothelial cells is associated with an initial inflammatory process.

Proliferation of smooth muscle cells and endothelial cells causing vascular vessel occlusion and fibrosis of transplanted organs are the most typical findings in biopsies of patients undergoing chronic allograft rejection. The results of this study provide additional support to the implications of HLA alloantibodies in the genesis of neo-vascularization and fibrosis in chronic allograft rejection. From our results showing an increased pro-inflammatory cytokine secretory pattern it can be concluded that ligation of HLA alloantibodies to HLA antigens expressed in the allograft endothelium is associated with increased secretion of endothelial growth factors and pro-inflammatory cytokines and chemokines, all of them acting in concert to produce neo-vascularization and fibrosis in the allograft.

In summary, in this study we find that ligation of an anti-HLA class I antibody to endothelial cells in vitro produces a distinctive inflammatory cytokine secretory pattern elevated from baseline. Future studies using a pro-inflammatory multiplexed cytokine panel to test prospective clinical specimens of patients post transplantation are

necessary to determine whether this characteristic cytokine secretory pattern can be used in clinical practice to detect sub-clinical chronic allograft rejection.

REFERENCES

1. Cornell LD, Smith RN, Colvin RB. Kidney transplantation: mechanisms of rejection and acceptance. *Annu Rev Pathol* 2008;3:189–220.
2. Harris PE, Bian H, Reed EF. Induction of high affinity fibroblast growth factor receptor expression and proliferation in human endothelial cells by anti-HLA antibodies: A possible mechanism for transplant atherosclerosis. *J Immunol* 1997;159:5697–5704.
3. Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: From association to causation. *Transplantation* 2008;86:377–383.
4. Bian H, Harris PE, Reed EF. Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation. *Int Immunol* 1998;10:1315–1323.
5. Mao Q, Terasaki PI, Cai J, et al. Extremely high association between appearance of HLA antibodies and failure of kidney grafts in a five-year longitudinal study. *Am J Transplant* 2007;7:864–871.
6. Mizutani K, Terasaki P, Rosen A, et al. Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure. *Am J Transplant* 2005;5:2265–2272.
7. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RW. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome. *Transplantation* 2003;75:1034–1040.
8. Lee PC, Terasaki PI, Takemoto SK, et al. All chronic rejection failures of kidney transplants were preceded by the development of HLA antibodies. *Transplantation* 2002;74:1192–1194.
9. Palmer SM, Davis RD, Hadjiliadis D, et al. Development of an antibody specific to major histocompatibility antigens detectable by flow cytometry after lung transplant is associated with bronchiolitis obliterans syndrome. *Transplantation* 2002;74:799–804.
10. Jaramillo A, Smith MA, Phelan D, et al. Development of ELISA-detected anti-HLA antibodies precedes the development of bronchiolitis obliterans syndrome and correlates with progressive decline in pulmonary function after lung transplantation. *Transplantation* 1999;67:1155–1161.
11. Stastny P, Lavingia B, Fixler DE, Yancy CW, Ring WS. Antibodies against donor human leukocyte antigens and the outcome of cardiac allografts in adults and children. *Transplantation* 2007;84:738–745.
12. Tambur AR, Bray RA, Takemoto SK, et al. Flow cytometric detection of HLA-specific antibodies as a predictor of heart allograft rejection. *Transplantation* 2000;70:1055–1059.
13. Martins TB, Pasi BM, Pickering JW, Jaskowski TD, Litwin CM, Hill HR. Determination of cytokine responses using a multiplexed fluorescent microsphere immunoassay. *Am J Clin Pathol* 2002;118:346–353.
14. Jaramillo A, Smith CR, Maruyama T, Zhang L, Patterson GA, Mohanakumar T. Anti-HLA class I antibody binding to airway epithelial cells induces production of fibrogenic growth factors and apoptotic cell death: A possible mechanism for bronchiolitis obliterans syndrome. *Hum Immunol* 2003;64:521–529.
15. Remick DG. Interleukin-8. *Crit Care Med* 2005;33:S466–S467.
16. Bradley JR. TNF-mediated inflammatory disease. *J Pathol* 2008;214:149–160.
17. Dinarello CA. Interleukin-1 β . *Crit Care Med* 2005;33:S460–S462.
18. Song M, Kellum JA. Interleukin-6. *Crit Care Med* 2005;33:S463–S465.