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Detection of Donor-Derived Microparticles in the Peripheral Blood of a Hand Transplant Recipient During Rejection

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Background. Microparticles (MPs) are released from the plasma membrane of activated or dying cells and bear surface molecules from those cells. We examined whether donor-derived MPs in the peripheral blood of the recipient could serve as a marker of tissue damage due to rejection of a transplanted hand. **Methods.** Platelet-free plasma from the recipient of the transplanted hand was analyzed for MPs bearing the donor-specific HLA molecule A*02 using flow cytometry. Rejection status of the transplanted hand was monitored by histopathology of skin punch biopsies. **Results.** Donor-specific MPs expressing HLA A*02 were quantifiable in the peripheral blood of the recipient. Levels of these MPs increased with worsening rejection of the transplanted hand. **Conclusions.** These findings demonstrate the ability to detect donor specific MPs through staining of graft cell-specific HLA and promote further investigation into the potential utility of flow cytometry for donor-derived MPs as a noninvasive tool to assess rejection in solid organ transplantation patients.

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A cute rejection remains a significant complication after solid organ transplantation despite advances in immunomodulatory therapies.[1,2] Diagnosing rejection typically involves invasive biopsy sampling for histopathological analysis after organ dysfunction. This has variable sensitivity depending on the transplanted organ and can place patients at risk for procedural complications. Development of noninvasive measuring of biomarkers for rejection have had limited success in the ability to differentiate between rejection and other causes of graft failure.[3]

Peripheral blood microparticles (MPs) have been proposed as noninvasive general biomarkers for acute rejection.[4-7] MPs are submicron vesicles produced during cell activation or apoptosis. Generated through budding from the plasma membrane of cells, they bear surface proteins and intracellular contents from their cells of origin,[8] and may act as transcellular effectors in inflammation and thrombosis.[9] The level of MP-mediated procoagulant activity (procoagulant MPs) appears to increase in patients experiencing transplanted islet cell dysfunction due to rejection, and subsequently decrease after immunosuppressive treatment.[7] Endothelium-derived MP (EMP) levels identified by flow cytometry as CD31-expressing MPs has been shown to be elevated in renal transplant recipients with histological evidence of rejection compared with those with normal appearing graft tissue.[6] In a prospective study of heart transplant recipients, increased levels of procoagulant MPs bearing E-selectin, fas, and tissue factor has been associated with allograft rejection.[5]

In all these studies, the MPs and their markers (procoagulant molecules, apoptotic factors, and endothelium specific proteins) are used as general indicators of the immune response mediating rejection and/or tissue damage, and are not donor organ-specific. Here, we propose the specific measurement of donor cell MP release, taking advantage of HLA mismatch between donor and recipient.[10] In this proof of concept pilot study, we test the feasibility of detecting MPs shed specifically from donor cells during acute rejection experienced by the recipient of a hand transplant, using flow cytometry with donor HLA molecule staining of MPs.

MATERIALS AND METHODS

Study Subject and Sampling

The study participant was a woman (HLA A*02 negative) who was the recipient of a right hand transplant from a male donor (HLA A*02 positive). She was studied after providing informed consent under a research protocol approved by the UCLA Institutional Review Board. Clinical details of this case were previously reported.[11]

Histopathological Evaluation

Histopathological evaluation was performed by taking punch biopsy specimens of graft skin from the transplanted hand. These samples were examined by the UCLA Pathology Department and assigned rejection scoring based on standard Banff Criteria.[12]

Peripheral Blood MP Quantitation

Platelet-free plasma (PFP) samples were prepared as previously described using serial centrifugations (15 minutes at 1500g followed by 2 minutes at 13 000g), and stored at –80 °C until use. MP labeling was performed with 30 μL of PFP using Alexa 488 labeled anti-HLA A*02 antibody (Serotec Inc, clone BB7.2) or an isotype matched control conjugate (IgG2b) at a final concentration of 10 μg/mL for 30 minutes at room temperature protected from light, and then diluted with 500 μL with double filtered (0.25 μm) phosphate-buffered saline without calcium or magnesium (Mediatech). Counting beads (TruCount, BD Biosciences) were added as concentration standards. A Beckman-Coulter FC500 flow cytometer was used.[13-15] To limit background debris, sheath fluid (IsoflowTM; Beckman-Coulter) was prefiltered. All parameters were plotted on logarithmic scales. The forward scatter data were collected using the wide angle position (W = 1-19°), and MP measurements were performed according to the previously described standardized protocol using fluorescent 0.5 and 0.9 μm diameter beads (Megamix, BioCytex) to define the MP size gate. Because there is no current consensus on the threshold setting for MPs analyzed by flow cytometry, the threshold level was set based on the number of nonspecific background events observed using double filtered (0.2 μm) phosphate-buffered saline. Samples were run in triplicate.

RESULTS

As summarized in Figure 1 and described previously,[11] the participant had serial histopathological evaluations post-hand transplantation demonstrating ongoing mild to moderate rejection with adjustments of her immunosuppressive drug regimen. Severe rejection (grade 3) was noted on posttransplant day 717, which persisted despite treatment with high dose steroids and antithymocyte globulin. As a result, she elected to undergo graft resection on day 771.

Because some level of donor HLA-bearing MP release would be expected in the recipient under normal physiologic conditions while graft tissue is present,[8] the significance of an isolated MP level to indicate the presence of rejection mediated donor organ damage must be compared with a baseline level taken in the absence of rejection. Therefore, we sought to establish a baseline donor HLA-bearing MP level taken when significant rejection was not detected in the subject (day 642) as a control value and track the changes in levels longitudinally as rejection intensified. Given that the donor but not recipient had tissue type HLA A*02, serial measurements of donor-derived MPs were performed by flow cytometry of PFP with staining for HLA A*02 as shown in Figure 2. Assays were performed on samples from days 642, 717, 723, 744, 751, and 771 posttransplant, spanning the 130 days before graft resection (Figure 3). On days 642, 717, and 723, the plasma level of donor HLA-bearing MPs remained relatively stable
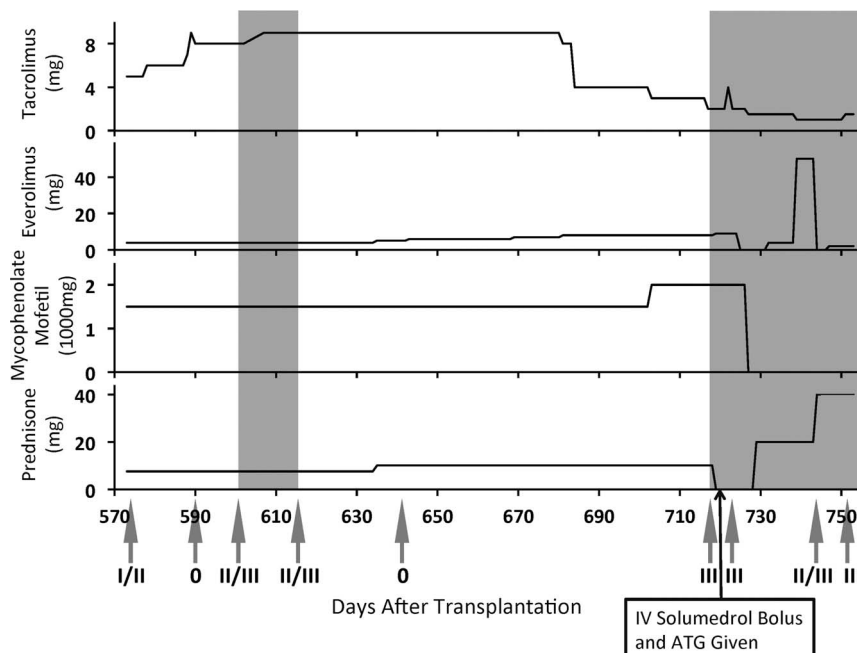

FIGURE 1. Rejection status and immunosuppressive treatment of the hand transplant recipient. Total daily prescribed doses of tacrolimus, everolimus, mycophenolate mofetil, and prednisone over time are indicated. Arrows indicate times of biopsies and Banff rejection scores. The shaded zones indicate clinical rejection noted on physical examination.
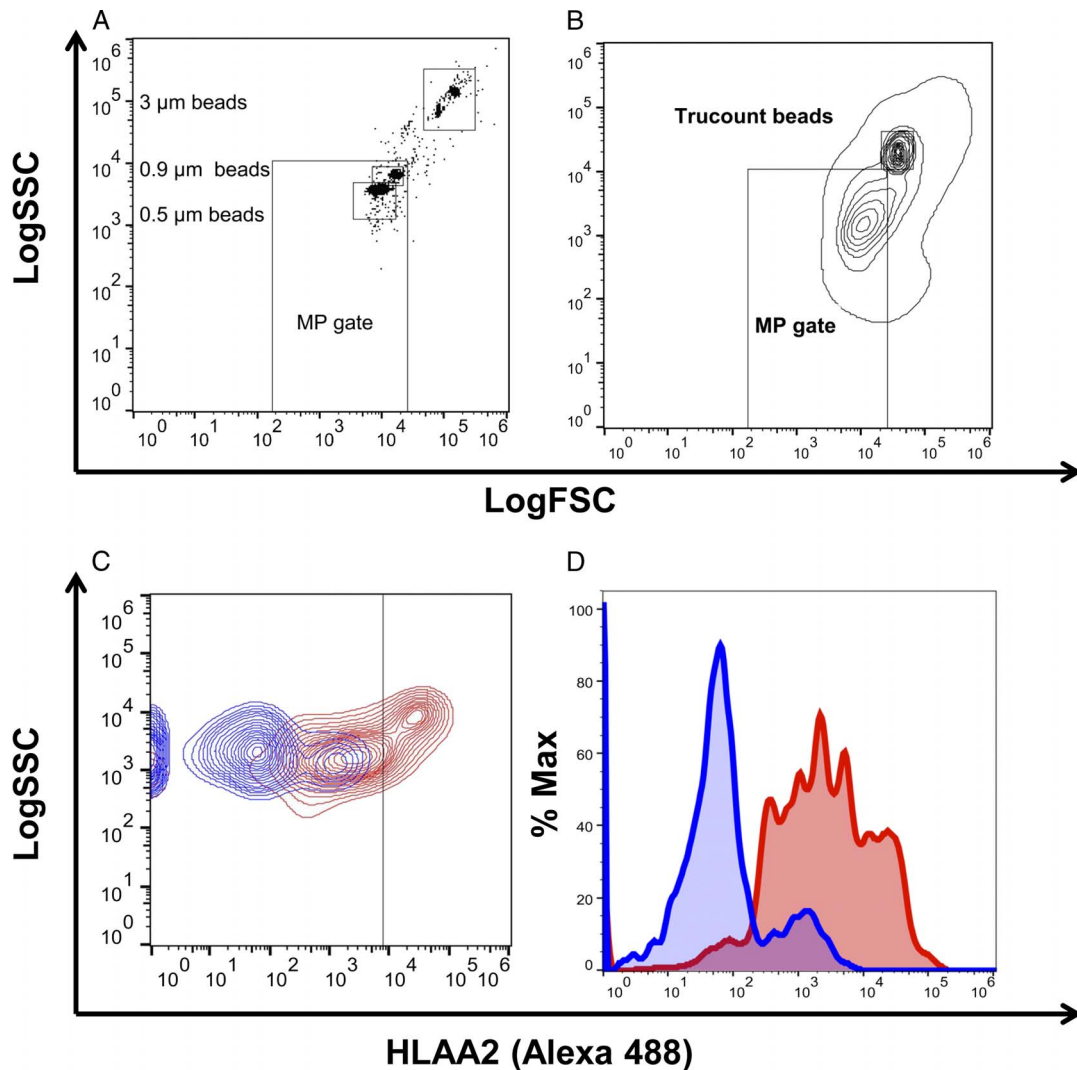
FIGURE 2. Methodology for detecting donor-specific HLA A*02-expressing MPs by flow cytometry. A, Forward scatter and side scatter gating for MPs was set based upon 0.5 and 0.9 μm diameter fluorescent bead standards. B, A representative analysis for total MPs using the above gating strategy is shown, including counting beads used as a concentration standard to estimate MP concentration. C and D, Representative staining for HLA A*02 is shown for the MPs defined above. The blue and red plots show staining with an isotype control or anti-HLA A*02 antibody, respectively.

(1.2-1.4/μL PFP). After the participant presented with grade 3 rejection on day 717, a steady rise in MPs was observed, peaking at 4.93/μL at the time of resection on day 771.

DISCUSSION

Although assessment of peripheral blood MPs has been investigated as a general indicator of tissue damage and/or immune activation in solid organ transplantation,[4-7] to our knowledge, this is the first study specifically quantifying donor HLA-bearing MPs as a potential marker for rejection. Differentiating between host-derived and graft-derived MPs by detecting donor cell-specific membrane proteins has previously been proposed, however this was suggested only for EMPs[16] in contrast to our approach to detect all MPs from donor tissues. Thus, this technique could provide an accurate assessment of overall graft tissue injury during rejection.

As the severity of rejection progressed in the study participant, there was a coincident rise in donor HLA-bearing MP
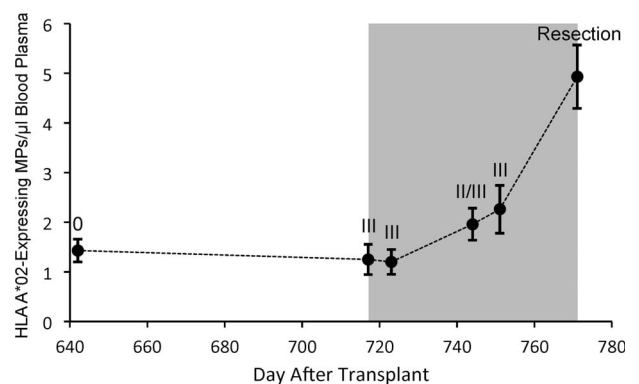
FIGURE 3. Levels of donor-derived MPs in the blood plasma of the hand transplant recipient over time. The concentrations of A*02-expressing MPs in the blood plasma of the subject are plotted over time. Simultaneous Banff rejection scores are indicated (0-3) above each point. Error bars indicate the standard deviation of triplicate measurements. The shaded zone corresponds to clinically apparent severe rejection of the transplanted hand.

levels. It is unclear whether the level of these MPs seen at baseline (without rejection) was due to normal cellular turnover in the transplanted hand versus ongoing low grade rejection demonstrated by our previous findings of persistent infiltrating oligoclonal CD8^{+} T cells in the graft.[11] Regardless, the MP concentration was relatively stable at a baseline on day 642 without rejection, and the first few days after initial clinical presentation of severe rejection, days 717 and 723. Unfortunately the next available sample was 3 weeks later on day 744, and thus it is unclear precisely how quickly MP levels rose after the onset of rejection.

Immunosuppression was increased at the onset of severe clinical rejection with the treatment of antithymocyte globulin and high-dose corticosteroids. The precise effects of different immunosuppressive agents on donor HLA-bearing MP levels remain to be determined because the biogenesis of MPs remains poorly understood. Formation of MPs may be driven by complex cellular processes, such as apoptosis. We have found that both annexin V+ and annexin V– MPs can be determined in plasma (unpublished data), suggesting that alternative cellular processes may be involved in release of MPs. Immunosuppressive agents may affect immune cells, such as lymphocytes and simultaneously attenuate inflammatory responses and rejection-related tissue destruction. We found that donor specific HLA2+ MP levels increased over time with immunosuppression and ongoing tissue rejection. Complex pleotropic effects of immunosuppression on both immune and endothelial cells (that also express HLA)[17,18] as well as tissue-related release of MPs in the setting of tissue rejection may regulate the overall levels of plasma HLA-bearing MPs. Thus, it would be important to also consider the effect of immunosuppression in future studies of donor HLA-bearing MPs.

Our data suggest that donor HLA-bearing MP levels have the potential serve as a useful noninvasive marker for cell death and tissue damage that occurs during transplanted organ rejection. Given the usual mismatch between recipient and donor HLA types and availability of anti-HLA antibodies, this could be generally applicable. However, it is unclear how sensitive this approach would be for rejection, because we did not observe elevated MPs in the first week after clinical presentation of our subject with severe rejection. It is possible that our "baseline" measurement (day 642) already reflected MP elevation from developing rejection. Alternatively, MP level elevations during rejection may be more rapid in more vascular transplanted organs, such as kidney or lung. Moreover, it may be possible to develop more sensitive assays for donor HLA-bearing MPs using dedicated hardware rather than a flow cytometer, for which MP detection is at the limits of its size resolution.

Because other processes affecting graft tissue can induce MP release, certain subpopulations of donor cell-derived MPs may be more specific for rejection. Specificity may therefore be enhanced by combining donor HLA staining with staining for other cell-specific markers to track levels of different MP subpopulations through specific gating and a multicolor approach. For example, endothelial-derived MPs can be detected through specific markers, such as CD144 and CD146, or T lymphocytes detected by staining with CD3.[19]

Because MPs may mediate the intercellular transfer of functional cellular components, such as cytoplasmic proteins, bioactive lipids, and nucleic acids,[8] MP production in graft

tissue may also reflect regulated events in addition to cell death. Additionally, tissue damage by mechanisms other than rejection, such as drug toxicity and infection, could also increase MPs. Sepsis has been shown to increase EMP and hepatic cell MPs after liver transplantation,[4] and EMP increases were also noted in transplant recipients with cardiovascular disease, renal failure,[20] and cytomegalovirus infection.[21]

In summary, this work is a pilot study of measuring donor-derived MPs in the peripheral blood of a hand transplant recipient, demonstrating that MPs are detectable and that levels shift during a clinical course spanning from clinical stability to graft rejection. MP levels were highest during severe clinical rejection, supporting the idea that MP monitoring can potentially serve as a specific biomarker for rejection of solid organ transplantation. More studies are necessary to determine if this approach could work as a sensitive and specific assay to obviate invasive tissue sampling for diagnosis of rejection and tissue damage.

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