In Vivo Platelet–Endothelial Cell Interactions in Response to Major Histocompatibility Complex Alloantibody

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Abstract—Platelets recruit leukocytes and mediate interactions between leukocytes and endothelial cells. Most studies examining this important platelet immune function have focused on the development of atherosclerosis, but similar mechanisms may contribute to acute and chronic vascular lesions in transplants. Platelets have been described as markers of transplant rejection, but little investigation has critically examined a role for platelets in transplant vasculopathy and, in particular, alloantibody-mediated transplant rejection. We now demonstrate using a skin transplant model that alloantibody indirectly induces platelet activation and rolling in vivo. Repeated IgG2a alloantibody injections result in sustained platelet–endothelial interactions and vascular pathology, including von Willebrand factor release, small platelet thrombi, and complement deposition. Maintenance of continued platelet–endothelial interactions are dependent on complement activation. Furthermore, we demonstrate that platelets recruit leukocytes to sites of alloantibody deposition and sustain leukocyte–endothelial cell interactions in vivo. Taken together, our model demonstrates an important role for platelets in alloantibody induced transplant rejection. (Circ Res. 2008;102:0-0.)

Key Words: platelet □ transplant □ alloantibody □ endothelial □ leukocyte

Platelets were included in the first pathological descriptions of hyperacute and acute transplant rejection.1,2 Subsequent to these reports, radioactively labeled platelets were used to demonstrate that platelet accumulation was an early indicator of transplant rejection.3,4 More recent studies have also described intravascular platelet aggregates in clinical and experimental models of antibody-mediated rejection.5,6 When immunohistology is used to detect platelet markers, the majority of biopsies from renal transplants contain platelet aggregates in peritubular capillaries.5 Mechanistic studies of platelets in mediating transplant rejection have been performed in xenotransplants but not in allografts.7,8

Many mechanisms may account for the accumulation of platelets in vessels of transplants. In hyperacute rejection, vascular integrity is disrupted and extracellular matrix components such as collagen are exposed, which interact with platelet receptors. Platelets also interact with an intact endothelial layer and promote inflammation. An activated endothelial cell layer expresses increased proinflammatory molecules including P-selectin and integrins such as intercellular adhesion molecule and vascular cell adhesion molecule (VCAM). Platelets have receptors for these endothelial activation markers and will transiently localize to the site of vascular inflammation (roll and tether) and eventually adhere. P-selectin also binds to exocytosed von Willebrand factor (vWF) and promotes platelet adhesion via GPIbα interactions.9,10

The interaction of platelets with vascular endothelium is particularly relevant to transplants. Adherent platelets release their granule contents, produce inflammatory mediators such as thromboxane and leukotrienes, and activate surface receptors such as GPIIb/IIIa that bind fibrin and vWF. Platelet secretion of inflammatory molecules includes the release of RANTES, interleukin-1β, β-thromboglobulin/CXCL7, macrophage inflammatory protein-1, stromal cell derived factor-1, and platelet factor (PF4), setting up a cycle of inflammation.

The observed association of platelet aggregates with antibody-mediated rejection suggests many mechanisms by which antibodies could initiate activation of platelets. Bivalent IgG antibodies have been shown to crosslink major histocompatibility complex (MHC) antigens and induce rapid exocytosis of Weibel–Palade storage granules, releasing contents such as vWF and P-selectin that can interact with both platelets and leukocytes.11 The deposited antibodies may also interact with platelets and leukocytes via Fc receptor interactions and initiate localization and activation of these cells. In addition, some subclasses of antibody bound to endothelial cell antigens can initiate activation of the complement sys-

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tem. Endothelial cells, leukocytes, and platelets have receptors for complement products that lead to accumulation and activation of inflammatory cells at the site of deposition.

To examine the role of platelets in alloantibody-mediated transplant rejection, we developed a skin graft model in which real-time interactions of platelets, endothelial cells, and leukocytes can be visualized. In our model, skin from a B10.A mouse is transplanted onto a Balb/c nude mouse. B10.A skin grafts elicit a vigorous T-cell response in immune competent Balb/c recipients that results in acute rejection of the skin graft.12,13 However, nude mice do not have T cells, allowing for a transplant that develops vascular connections in the absence of rejection and elicits no IgG antibodies. B10.A and Balb/c mice differ at major and minor histocompatibility antigens. Alloantibody to B10.A MHC, therefore, can be used to induce an immune response directed to the transplant endothelium. Using this model, we demonstrate in vivo that antibody to transplant endothelium induces platelet rolling and activation. Importantly, we also demonstrate that platelet activation in the transplant tissue bed mediates leukocyte localization.

Materials and Methods

Transplants

All procedures were performed under protocols approved by The Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee. Balb/c nude mice were anesthetized with ketamine and xylazine (80 and 13 mg/kg, respectively) and transplant beds prepared by removal of the epidermis and dermis. The thin skin from ears of H-2 incompatible B10.A mice (H-2Kk) were grafted into fitted beds on 10- to 12-week old Balb/c nude mice (H-2Kk). Mice were bandaged for 1 week to allow for graft healing and establishment of vascular connections.

In Vivo Studies

Platelets from control Balb/c mice were isolated, fluorescently labeled with calcein-acetoxymethyl ester dye and injected into a transplant recipient mouse, as we have described previously.14 Mice were anesthetized with ketamine and xylazine (80 and 13 mg/kg, respectively) and transplanted with 100 μl of circulating platelets from skin-transplanted mice and control mice (Figure 1C). Thirty minutes later, images were acquired in transplant recipient mouse skin. Platelets were then imaged in transplant blood vessels or in normal adjacent skin to establish baseline platelet velocity. Mice were then injected with 100 μg (<1% of total IgG) of an IgG2a monoclonal antibody specific for the MHC class I antigen (H-2Kk) expressed by B10.A mice. Thirty minutes later, images were acquired in transplanted tissue and adjacent normal tissue and platelet-rolling velocity again determined by measuring the distance that platelets travel between image frames (Figure 1A). The baseline platelet velocity is the same in transplanted and control skin (Figure 1B). Thirty minutes after antibody injection, the average platelet-rolling velocity in transplant tissue was reduced by ~5 times but was unchanged in normal control skin (Figure 1B). These data demonstrate that antibody to MHC class I antigen induces increased platelet–endothelial cell interactions in transplant tissue but has no effect in control recipient skin.

We next determined whether antibody-treated mice have circulating platelets with markers of activation. To do so, we isolated platelets from transplanted mice and control mice treated with nothing or with alloantibody, and platelets were incubated with fluorescent antibody to P-selectin. Flow cytometry demonstrated increased P-selectin expression on circulating platelets from skin-transplanted mice treated with donor-specific antibody (Figure 1C), but P-selectin was not increased on platelets when antibody was injected into nude mice that had not received a transplant (Figure 1D). Others have demonstrated complement deposition on activated platelets and that platelet P-selectin can initiate complement activation of inflammatory cells at the site of deposition.

Platelet Activation Studies

Prepared washed platelets were diluted at approximately 1:20 in cation-free Tyrode’s buffer and fluorescein isothiocyanate–conjugated antibody to P-selectin (BD Pharmingen) or C3d (Dako) was added. Platelet activation was determined by flow cytometry (FACS Calibur). Immobilized antibody was prepared by coating a 96-well plate with 4 mg/mL antibody overnight. Platelet activation was determined using an ELISA for PF4 (R&D Systems).

Immunohistochemistry

Harvested transplants were fixed in methanol–water–acetic acid (60%–30%–10%). Tissue was then embedded and sectioned, and immunohistochemistry was performed using protocols and procedures described previously with antibodies to vWF, myeloperoxidase (MPO),11 and C4d.15

MPO ELISA

On day 7, the central 0.5×0.5 cm portion of isolated skin graft was scraped and diced with a razor blade, resuspended in NP-40 lysis buffer, and homogenized. The protein concentration was determined, and sample concentration was normalized before the MPO ELISA (Cell Sciences).

Results

Antibody to MHC I Antigens on Skin Transplants Induces Platelet Activation In Vivo

To determine whether antibody that recognizes MHC I on transplant endothelial cells initiates platelet rolling and activation, we established a skin transplant model using the thin skin from ears of B10.A mice (H-2Kk) grafted onto Balb/c nude mice (H-2Kk). After allowing for graft healing and establishment of vascular connections, platelets from control Balb/c mice were isolated, fluorescently labeled, and injected into transplant recipient mice. Platelets were then imaged in transplant blood vessels or in normal adjacent skin to establish baseline platelet velocity. Mice were then injected with 100 μg (<1% of total IgG) of an IgG2a monoclonal antibody specific for the MHC class I antigen (H-2Kk) expressed by B10.A mice. Thirty minutes later, images were acquired in transplanted tissue and adjacent normal tissue and platelet-rolling velocity again determined by measuring the distance that platelets travel between image frames (Figure 1A). The baseline platelet velocity is the same in transplanted and control skin (Figure 1B). Thirty minutes after antibody injection, the average platelet-rolling velocity in transplant tissue was reduced by ~5 times but was unchanged in normal control skin (Figure 1B). These data demonstrate that antibody to MHC class I antigen induces increased platelet–endothelial cell interactions in transplant tissue but has no effect in control recipient skin.
We therefore measured C3d deposition on platelets by flow cytometry. Platelets from transplanted mice treated with alloantibody have increased C3d deposition (Figure 1E).

Antibody to Transplant MHC I Induce Acute Vascular Pathology

Skin grafts were harvested to perform immunohistochemistry for evidence of acute antibody-induced vascular pathology. Grafts were harvested 45 minutes following intravenous injections of antibody and fixed. In control untreated skin allografts, blood vessel lumens were open and vWF was confined to endothelial cells (Figure 2A, left, brown staining). In contrast, transplant blood vessels in mice treated with intravenous injections of alloantibody contained numerous small vessels that were occluded and small to medium vessels that contained aggregates of platelets that stained for vWF (Figure 2A, right, arrow). We also performed immunohistochemistry for C4d. Control untreated allografts had few blood vessels that stained with antibody to C4d (Figure 2B), whereas transplant recipients treated with antibody had numerous blood vessels with C4d deposition (Figure 2B). These data demonstrate that antibodies to MHC I antigens of transplants induce acute small vessel pathology including platelet thrombi and complement deposition in vessels.

Antibody to MHC I Antigens Does Not Directly Stimulate Platelets

To determine whether antibodies to the transplant directly activate platelets, or induce platelet activation secondary to vascular effects, platelets from control Balb/c mice were isolated and incubated with 50 μg/mL antibody to MHC class I (approximately equal to in vivo experiments). After incubation with antibody, platelets were also stimulated with thrombin (0.25 U/mL), and platelet activation was determined by P-selectin expression. Antibody did not induce platelet activation (Figure 3A, left) nor did it increase platelet activation in response to an agonist (Figure 3A, right). Furthermore, to determine whether immobilized alloantibody can stimulate platelets, a 96-well plate was coated with antibody or control. Washed Balb/c mouse platelets (5×10^5) were added to antibody-coated or control wells for 30
minutes, and as a positive control, wells were also stimulated with thrombin (0.25 U/mL). Platelet activation was determined by ELISA for PF4 release into the buffer. Platelets incubated in antibody-coated wells and those incubated in control wells had the same PF4 release, indicating that immobilized antibody to MHC class I does not directly stimulate platelet activation (Figure 3B, left). Platelets incubated in antibody-coated wells also did not have increased agonist-induced activation (Figure 3B, right). Taken together, these data demonstrate that the allograft antibody does not directly activate platelets; rather, platelets are indirectly stimulated by alloantibody in vivo.

Effects of Antibody to MHC I Antigens Are Acute and Short Acting

Antibodies can have a prolonged circulating time. Therefore, we next sought to determine whether the platelet-activating effect was persistent over a longer time frame following in vivo antibody administration. To demonstrate this, we imaged skin grafts and analyzed platelet-rolling velocity 30 minutes and 24 hours after antibody injection. As before, 30 minutes after injection of antibody to MHC class I antigens, there was a reduction in platelet-rolling velocity (Figure 4A). Twenty-four hours after antibody treatment, fluorescent platelets injected into the treated mice had a partial recovery of rolling velocity that was still significantly reduced compared with pretreatment (Figure 4A). By day 4, there was a total recovery of injected platelet-rolling velocity (Figure 4A). These data indicate that a single injection of antibody induced a short-lived vascular inflammation.

As further evidence of the short-lived effects of antibody to MHC I, 7 days after single antibody treatment, tissue was harvested and immunohistochemistry was performed. A single injection of antibody did not induce significant changes in staining for vWF or C4d (Figure 4B, vWF, and 4C, C4d deposition).

Repeated Injections of Antibody Worsens Vascular Pathology

Because a single injection of antibody did not alter the vascular pathology, we sought to determine whether multiple injections of antibody induces a sustained vascular inflammatory state. Mice were again injected with complement-activating IgG2a alloantibody specific for transplant tissue on days 0, 2, and 4, and fluorescently labeled platelets were imaged circulating in the transplant tissue before antibody and 30 minutes after antibody. As before, platelet-rolling velocity was greatly decreased immediately after antibody injection (Figure 5A). Two days and 4 days after antibody treatment, new labeled platelets were injected and imaged before and after antibody treatment. On day 2 after the first antibody injection, platelet-rolling velocity was significantly less than control but was partially restored to baseline (Figure 5A). Subsequent antibody injection suppressed rolling velocity to initial treatment velocities (Figure 5A). These data were replicated on day 4, with a partial return of velocity that was suppressed by antibody treatment (Figure 5A). In contrast,
reduces platelet velocity (Figure 5F). However, in contrast to non–complement-activating IgG1 antibody specific for allo-rejection.

Transplanted tissue treated with IgG2a anti-allograft antibody also had numerous infiltrating neutrophils compared with control-treated allografts, as demonstrated by MPO staining (Figure 5E). These data indicate that sustained exposure of a small vessel filled with vWF-stained platelet thrombi in mediating leukocyte interactions and trafficking to antibody-activated endothelium.

To examine the effect of platelets on vascular pathology, platelet-depleted and control mice were injected with antibody on days 0, 2, and 4, and grafts were harvested on day 7. Tissue was stained for MPO, and skin grafts were homogenized to quantify neutrophil infiltrates using a MPO ELISA. In support of the leukocyte-rolling studies, platelet-depleted mice had reduced numbers of infiltrating MPO-positive cells (Figure 6B) and a reduced tissue MPO concentration as compared with control mice (Figure 6C). Tissue was also stained for vWF and C4d, and platelet-depleted mouse vessels had fewer vWF plugs in vessel lumens and reduced C4d-positive staining, particularly in medium-sized arterioles (Figure 6D and 6E).

Platelets Mediate Transplant Endothelial Cell Inflammatory Molecule Expression

To identify important platelet-driven leukocyte–endothelial cell interactions, 1-μm fluorescent beads were coated with anti–P-selectin antibody, and beads were injected into control and platelet-depleted mice. Baseline images were acquired, and bead velocity was determined as an indicator of vessel P-selectin expression. Mice were then treated with alloantibody, and images were acquired again 30 minutes later.
Anti–P-selectin–coated beads have a decrease in velocity in transplant vasculature in control mice following alloantibody injection (Figure 7A), but there is no velocity change in transplant host tissue (data not shown). In contrast, platelet-depleted mice do not have a change in bead velocity (Figure 7A), indicating that platelets are a significant source of P-selectin in recruiting leukocytes. On day 2 postalloantibody, the anti–P-selectin–coated bead velocity has partial recovery to baseline in control mice (data not shown).

We also coated beads with anti-C3d antibody and performed a similar study. Thirty minutes after alloantibody treatment, anti–C3d–coated bead velocity is approximately equally decreased in control and platelet-depleted mice (Figure 7B) but unchanged in control host skin; data not shown). However, 24 hours later, the decrease in velocity is sustained in control mice but not in platelet-depleted mice (Figure 7B), demonstrating that platelet activation sustains complement deposition in transplant endothelium.

We next sought to determine whether platelets also promote an increase in integrin expression in transplant endothelium by measuring anti-VCAM–coated bead velocity on day 0 before alloantibody injection in control and platelet-depleted mice and then treating mice as previously with alloantibody on days 0, 2, and 4. Anti-VCAM bead velocity was determined again on day 5 and expressed as a change in velocity from baseline. The decrease in velocity is significantly less in platelet-depleted mice as compared with control mice (Figure 7C), indicating that platelets also promote the expression of firm adhesion molecules in transplant endothelium.

Taken together, these data demonstrate that platelets are significant effectors of vascular inflammation in antibody-mediated transplant rejection, in part, by having a role in localizing white blood cells to the inflamed vascular beds by promoting endothelial cell adhesion molecule expression.

**Discussion**

Transplant rejection is a complex process that involves many pathways and initiators, including alloantibody.23 As new...
Markers have been developed to detect antibody-mediated rejection, immune responses initiated by alloantibodies are becoming progressively appreciated. These include deposition of complement split products and the accumulation of neutrophils or macrophages in transplant microvasculature. Our data demonstrate that antibody to transplant MHC class I antigens induces platelet activation and rolling on transplant endothelium. Antibodies could induce an interaction between platelets and endothelial cells by several mechanisms. Mouse platelets express FcγRIIB; therefore, platelets could bind directly to IgG deposited on endothelial cells. However, in our model, the antibody effect appears to be indirect; soluble and immobilized alloantibody does not stimulate platelets. This does not rule out a secondary effect mediated by FcR on platelets in vivo.

There are 2 other potential acute mechanisms of alloantibody induction of platelet–endothelial interactions. In vitro studies have demonstrated that antibodies to MHC class I antigens cause exocytosis of Weibel–Palade bodies that contain mediators of platelet localization and activation, such as P-selectin and vWF. Our immunohistological studies confirm that increased vWF is associated with platelet aggregates in skin grafts of antibody-treated recipients. VWF is an important platelet activator and has a prominent role in mediating firm platelet adhesion. In addition, we demonstrated that following administration of alloantibody, circulating platelets express increased P-selectin. Platelet P-selectin not only binds to leukocyte PSGL-1 but also interacts with vWF.

Alloantibody may also activate complement. Platelets have C1q receptors that can localize platelets to the site of complement activation, and complement products can activate platelets. We demonstrated that complement was activated in the skin grafts and that circulating platelets express C3d after treatment with complement-activating antibodies.

We propose that in our model system, both mechanisms, alloantibody-induced endothelial degranulation and complement activation, participate together to increase and sustain platelet–endothelial cell interactions (Figure 8). Mice treated with complement-activating IgG2a antibody had an acute reduction in platelet-rolling velocity that was sustained 2 days later. Mice treated with non–complement-activating IgG1 antibody had a similar immediate decrease in platelet-rolling velocity, but 2 days later, platelet velocity was restored to baseline. These data indicate that alloantibody may induce acute endothelial cell degranulation and platelet adhesion but that complement activation by alloantibody is necessary for a sustained increase in platelet–endothelial interactions.

The importance of platelets to leukocyte activation and recruitment was also evident in our model. The data presented...
demonstrated that increased platelet endothelial interactions lead to increased and prolonged leukocyte interactions with the endothelial cell layer and worsened transplant vessel pathology. Several mechanisms may account for this important platelet-mediated outcome. Once the platelets tether, roll, and are activated, they release their granule contents locally and thus promote an inflammatory environment. There are numerous inflammatory molecules either released or retained on the surface of platelets that have effects on leukocytes, including P-selectin, PF4, β-thromboglobulin, and serotonin. In addition, platelets release complement components, and platelet P-selectin acts as a site of complement activation and localization.

Our data confirm that platelets are a major source of P-selectin and promote complement deposition. Immunohistochemistry on tissue from platelet-depleted mice helped to confirm the importance of platelets to leukocyte activation and recruitment. These mice had decreased C4d staining and, as confirmed by ELISA, fewer MPO-positive infiltrating cells (MPO is expressed by neutrophils in mice), clearly demonstrating a role for platelets in leukocyte recruitment and complement activation during antibody-mediated transplant rejection. Platelet-depleted mice also have less P-selectin expression in response to alloantibody. Furthermore, platelets promote the expression of firm adhesion molecules on endothelial cells, such as VCAM, that mediate leukocyte trafficking in response to alloantibody.

The experiments examining platelet-rolling velocity demonstrated that platelet velocity trends back toward baseline at 2 days (~50% recovery) and is not significantly different from baseline by 4 days post–alloantibody injection. On day 2 post–antibody treatment, the leukocyte-rolling velocity has less recovery, only ~25%. This may highlight a shortcoming of our model system that could account for the observed difference between cell types. For each imaging study, platelets from control mice are freshly isolated and fluorescently labeled, whereas the leukocyte-rolling studies represent endogenous white blood cells that have been persistently exposed to the inflamed endothelium. However, this difference in technique may not be of functional significance because activated platelets are rapidly cleared from the circulation.

The leukocyte-rolling velocity studies also highlight another interesting finding: leukocyte-rolling velocity in control and platelet-depleted mice was similar acutely following alloantibody injection but, 1 and 2 days later, returned to baseline only in platelet-depleted mice. This may be accounted for by many integrated mechanisms. Yamakuchi et al have demonstrated that alloantibody can induce endothelial cell activation and degranulation, initiating expression of molecules, such as endothelial P-selectin and vWF, that can localize and recruit platelets and leukocytes. Neutrophil tethering under physiological shear stress is dependent on platelet P-selectin–mediated interactions, and the presence of platelets strongly increases neutrophil adhesion. Platelet-depleted mice have reduced transplant vascular P-selectin expression and C3d deposition that is not sustained (Figure 7). We therefore hypothesize that alloantibody induces endothelial stimulation that increases leukocyte interactions acutely. However, without platelet-derived adhesion molecules and inflammatory mediators, the interactions are transient and do not sustain leukocyte interactions and trafficking.
Although our studies represent an acute setting, they are very relevant to the pathogenesis of transplant rejection and accelerated graft arteriosclerosis. The development of accelerated graft arteriosclerosis has been linked to acute events of ischemia reperfusion. Therefore, if the early inflammatory response can be limited, then perhaps graft survival can be improved. This study demonstrates that in the early stages of antibody responses to transplants, platelets are key mediators of leukocyte localization and rolling on the transplant endothelium. More aggressive platelet inhibitor therapy may, therefore, be beneficial in promoting transplant survival.

This study clearly demonstrates a role for platelets in antibody-mediated transplant rejection. Definitive mediators are yet to be determined; however, small vessel thrombi, increased complement activation, and leukocyte recruitment are all likely to contribute.

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**Disclosures**
None.

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Alloantibody has no effect on host control vasculature. Host skin adjacent to the transplant tissue was immunostained for vWF, C4d, and MPO. Tissue sections are from mice on day 7 after alloantibody treatment on days 0, 2, and 4.