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Alloantibody induced platelet responses in transplants: Potent mediators in small packages

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Abstract

The early histological studies of organ allografts noted platelets attached to vascular endothelium. Platelets adhere to vessels before any morphological evidence of endothelial injury. Subsequently, in vitro and in vivo experiments have demonstrated that alloantibodies can induce exocytosis of von Willebrand factor and P-selectin from endothelial cells and attachment of platelets within minutes. Platelets also adhere to and stimulate leukocytes. These interactions are increased by complement activation. After attachment platelets degranulate, releasing preformed mediators. Some chemokines stored together in platelet granules can form heteromers with synergistic functions. Heteromers containing platelet factor 4 (PF4; CXCL4) are specific to platelets and provide insights to unique platelet functions and opportunities for therapeutic intervention.

Keywords

Platelets; antibody; monocyte; macrophage; chemokines

Introduction

Platelets evolved as extremely responsive monitors of endothelial integrity. Within the laminar flow of blood vessels, larger leukocytes and erythrocytes have less contact with the vascular wall than platelets that remain adjacent to the endothelium because of their small size and discoid shape [1]. Platelets also outnumber leukocytes (150 to 450 million/ml vs 3 to 11 million/ml) and each platelet contains numerous granules with preformed mediators for rapid expression on the plasma membrane or release. These mediators include a wide range of immunologically active cytokines, chemokines, adhesion molecules and growth factors. In addition, upon activation platelets change shape increasing the surface area for expression of ligands and receptors. As a result, platelets are attuned to changes in vascular endothelial cells and can rapidly orchestrate responses. Alloantibodies and complement cause rapid responses in endothelial cells that are arresting to platelets, including the exocytosis of von Willebrand factor (vWf) and P-selectin from Weibel-Palade storage granules as well as exposure of subendothelial matrix components (Figure 1).

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There have been several recent reviews of platelets as mediators of inflammation and some have highlighted atherosclerosis and allograft rejection [2–7]. This review will concentrate on the inflammatory responses elicited by alloantibodies in organ transplants. Cells that are of particular relevance to both platelet function and antibody-mediated rejection, notably endothelial cells, neutrophils and monocytes, will be discussed in detail.

Evidence of Platelet Activation in Antibody-Mediated Rejection

Early histological studies of transplants undergoing severe rejection, particularly hyperacute rejection, described platelets amassed in capillaries [8]. Sharma and colleagues [9] published a detailed description of platelet accumulation in the first minutes of hyperacute rejection of renal allografts in dogs. In these studies, the recipient was sensitized by a series of skin grafts from the prospective kidney donor. The kinetics and location of platelet responses are instructive. Within one minute after circulation was restored to the transplanted kidney, platelet aggregates were observed in the arterioles before any endothelial injury was evident on electron microscopic preparations. In subsequent biopsies during the first 15 minutes after reperfusion, platelet aggregates accumulated in the peritubular capillaries. The platelets that were adjacent to the vascular endothelium were degranulated and some had pseudopods in contact with the endothelium or extending through the endothelial layer.

Hobbs and Cliff [10] performed time-lapse cinematic microscopy of intravascular events in thin slices of allogeneic renal tissue that were implanted into observation chambers in rabbit ears. After these implants became revascularized, an orthotopic kidney was transplanted from the same donor, and the two transplants were found to reject simultaneously. Numerous platelets were observed to adhere to vascular endothelial cells within 2 days after transplantation. Some platelets detached from the endothelial cells and were replaced by leukocytes. Many platelets became attached to these adherent leukocytes and some platelets bridged between the leukocytes and endothelial cells. These rabbits were not deliberately sensitized, but the implanted renal slices required several days to establish revascularization in the ear chambers before the orthotopic renal allograft was performed. Therefore, it is not known whether alloantibodies mediated the platelet responses in this study. However, using a digital imaging camera to capture movements of fluorescently labeled platelets, we demonstrated that alloantibodies initiate rapid tethering of platelets to capillaries of skin allografts followed by leukocyte infiltrates [11]. For these experiments skin from ears of B10.A donors was transplanted to athymic Balb/c recipients. After 7 days when circulation was reestablished to the skin allografts, but the vessels were still of donor origin, fluorescently labeled Balb/c platelets were administered intravascularly followed by monoclonal antibodies to donor MHC antigens. Within 30 minutes of alloantibody administration, platelet rolling and tethering was evident. In the absence of complement activation, platelet flow returned to normal by 24 hours, but with complement activating antibodies, platelets returned to normal flow more gradually over 4 days. Repeated administration of alloantibodies caused repeated decreases in platelet flow. Although platelet flow decreased, most platelets did not fully arrest on the endothelium, but continued to roll and then returned to the circulation. Plasma samples from the systemic circulation contained platelets with evidence of activation including upregulation of P-selectin and membrane bound C3d. Immunohistology on skin grafts after passive transfer of alloantibodies confirmed that platelets were adherent to endothelial cells and that neutrophils infiltrated into the allografts. Depletion of platelets prior to administration of antibody greatly decreased neutrophil infiltrates indicating that platelets augment antibody induced leukocyte infiltrates. These interactions are not limited to skin allografts. In ongoing experiments, we have found that passively transferred alloantibodies rapidly induce complement deposition, platelet aggregates and monocyte adherence to capillary endothelium of cardiac and renal allografts in $RAG^{-/-}$ recipients [Kuo, H-H et al in preparation].

Complement deficient animals have been instructive in resolving the effects of different paths of the complement cascade on platelet activation in transplants. Rats deficient in C6, one of the complement components in the terminal complement cascade, allowed the effects of the membrane attack complex (MAC; C5b-C9) to be dissected from the effects of the preceding complement components. The absence of C6 did not alter rapid alloantibody production or C3d deposition in response to MHC class I incompatible cardiac allografts, but it did decrease the release of vWf from Weibel-Palade storage granules of endothelial cells and accumulation of intravascular platelet aggregates [12]. In C6 deficient recipients, vWf was largely retained in Weibel-Palade bodies of arterial endothelial cells even in sites of severe lymphocytic infiltrates. C6 deficient recipients also retained more vWf in capillary and venous endothelium than control recipients. These findings are not constrained to cardiac allografts because C6 deficiency was also associated with decreased vWf release and platelet aggregates in lung allografts [13].

These studies with C6 deficient animals do not indicate that endothelial cell lysis is necessary because many in vitro studies have demonstrated that endothelial cells express complement regulatory proteins such as CD59 that can control small amounts of C5b-C8 [14]. Small amounts of the complete MAC can be cleared by exocytosis or endocytosis [15]. As opposed to lysis, endothelial cells are activated by sublytic quantities of C5b-C9 or even C5b-C8, which assemble into transmembrane channels. These channels allow calcium flux and also interact with Gi-proteins that activate anti-apoptotic and proliferative pathways [16]. The immediate response to sublytic C5b-C9 or C5b-C8 is exocytosis of vWf and P-selectin [17]. In addition to releasing preformed mediators, endothelial cells activated by isolated terminal complement components in vitro synthesize tissue factor [18]. Tissue factor can expand the platelet responses by leading to thrombin production.

Transplants to B cell deficient recipients offer further evidence of the precipitous effects of alloantibodies on platelets. Cardiac transplants in B cell deficient mice elicit a vigorous cellular infiltrate but limited release of vWf or aggregation of platelets. Passive transfer of allantibodies to these mice results in complement deposition, vWf release and extensive platelet aggregates within 1 to 2 days [19, 20].

Over the years, several groups have reported that radiolabeled platelets localize to human renal transplants in the early stages of rejection [21, 22]. None of these studies subdivided rejections on the basis of antibody and cell-mediated diagnostic criteria. It is likely that platelets would localize to both types of rejection. A recent immunohistological study of human renal transplants reported that over half of the biopsies with C4d deposits had platelet aggregates that stained for CD61 in peritubular capillaries. However, this finding was not limited to antibody-mediated rejection; about one third of the biopsies with cellular rejection had intravascular platelet aggregates [23].

Taken together these studies indicate that platelets respond rapidly to endothelial disturbances in allografts, most notably to exocytosis of vWf and P-selectin. Antibodies alone, but even more effectively together with complement activation, cause endothelial cells to initiate platelet responses. Moreover, platelets attach to leukocytes and increase leukocyte interactions with endothelial cells.

Platelets as a dynamic source of immune modulators

In spite of their small size and lack of nucleus, platelets have a dynamic range of responses of relevance to transplants. These anucleate cells form as buds on pseudopodia of megakaryocytes that extend into the blood vessels in the bone marrow [24]. Large numbers of platelets (about 10^{11} per day) are sheared off the megakaryocytes into the circulation of adult humans. During inflammatory responses, platelet production can be increased; a

response referred to as reactive thrombocytosis. Proteomic arrays have identified over 300 mediators that platelets can secrete [25, 26]. Most of these mediators are loaded into cytoplasmic granules during platelet formation. Platelets contain three morphological types of secretory granules with different contents: alpha-granules, dense granules and lysosomes. The 40 to 80 alpha-granules in each platelet contain the majority of immunological mediators: adhesion molecules, cytokines, chemokines, and growth factors. Additional mediators can be imbibed from plasma and others can be synthesized by splicing mRNA transcripts or produced through the cyclooxygenase pathway [7, 27]. In order to deliver these mediators to sites of endothelial disturbance, platelets have membrane receptors for adhesion molecules and subendothelial matrix constituents.

Antibody induced endothelial responses of consequence to platelets

Actual disruption of vascular endothelial integrity as occurs in hyperacute rejection exposes subendothelial matrix constituents that initiate thrombus formation. However, platelet adhesion occurs in allografts before there is evidence of endothelial injury on an electron microscopic level [9]. Exocytosis of Weibel-Palade storage granules is the most consistent immediate response of endothelial cells to alloantibodies that is associated with platelet adherence and aggregation [11, 13, 19, 20]. Although in vitro and in vivo experiments indicate that antibodies to MHC class I antigens can elicit release of vWf in the absence of complement activation [11, 28], complement does augment vWf release. More prolonged platelet adherence resulted in skin allografts to $RAG^{-/-}$ mice following passive transfer of complement activating than non complement activating antibodies [11], and less vWf release was found in cardiac allografts to C6 deficient than C6 sufficient recipients [12]. Experiments with platelets under flow conditions indicate that the interaction between vWF and platelet membrane glycoprotein Ib (GPIb) in the GP Ib-IX-V complex provides the strongest binding [29]. In addition to vWf, the GP Ib-IX-V complex binds to P-selectin. P-selectin is stored together with vWF in Weibel-Palade granules and is brought to the surface with vWf and may serve as an anchor for vWf [30]. Following these first interactions, GPIIb/IIIa undergoes a receptor conformational change on the platelet membrane that permits binding to ICAM-1.

The interaction of activated platelets with endothelial cells causes further activation of both cells. In vitro incubation of activated platelets that express CD40L with human endothelial cells causes the endothelial cells to upregulate E-selectin, VCAM-1 and ICAM-1 as well as IL-8 and MCP-1 within 4 hours [31]. In addition to receptor-ligand interactions, IL-1β synthesized by activated platelets can cause expression of ICAM-1 and secretion of IL-6 and MCP-1 [32, 33].

Human platelets also express FcγRIIA (CD32) and C1qR. The interaction of these with alloantibodies on endothelium has not been studied. However, soluble immune complexes from lupus patients can activate platelets through FcγRIIA to upregulate CD40L (CD154) and P-selectin [34].

Platelet interactions with complement

Platelets express receptors that bind the collagen-like tails of soluble C1q and this inhibits platelet interactions with collagen, but clusters of C1q as would be present on vessels in antibody-mediated rejection, can initiate platelet aggregation [35]. Although platelets only express receptors for C1q, other components of complement bind to platelets and alter their function.

Sims and Wiedmer [36] studied the effects of sublytic amounts of C5b-C9 on platelets. They found that lysis does not occur when less than 1,000 C5b-C9 complexes form on the platelet

membrane. Instead platelets are activated to degranulate and to shed microparticles. This scenario could occur when antibody activates complement on endothelial cells and platelets are in the vicinity.

The interaction of platelets with C3 and C3 split products is less straightforward. Platelets do not express receptors for C3 and yet C3 is frequently associated with activated platelets. Del Conde and colleagues [37] reported that activated platelets could initiate the complete complement cascade through to the formation of C5b-C9. In these experiments, C3b was found to bind to P-selectin on the surface of platelets. Hamad and colleagues [38] confirmed that C3 binds to activated platelets, but in their experiments activation of C3 by cleavage was not required and C3 was not bound to P-selectin. Of greater relevance to antibody-mediated rejection, C3 on platelets was demonstrated to promote binding to complement receptor 1 (CR1; CD35) which is expressed on lymphocytes as well as neutrophils and monocytes. Therefore, C3 on the membranes of activated platelets would be expected to enhance platelet-leukocyte interactions.

Another interaction between platelets and C3 was recently reported, in which C3b on bacteria bound to platelets through GPIb [39]. This interaction promoted localization of opsonized bacteria to a subpopulation of dendritic cells in the spleen. The interaction between platelet GPIb and C3b could also facilitate platelet attachment to complement deposits in allografts. Moreover, if microparticles are shed from transplants with C3b attached, platelets could transport them to dendritic cells in the spleen.

Platelet interactions with neutrophils and monocytes

Neutrophils, monocytes and macrophages are characteristically found in biopsies of transplants with acute antibody-mediated rejection [40, 41]. In transplants, platelets quickly engage leukocytes and increase leukocyte interactions with vascular endothelium [10]. Platelets interact with leukocytes through several mechanisms including release of chemokines, shedding of microparticles and direct cell contact. In models of arterial injury, expression of P-selectin by platelets is the primary mechanism of initiating monocyte recruitment and activation [42–45]. We have found that P-selectin expressing platelets are associated with monocytes in the vessels of cardiac and renal transplants within an hour of administering alloantibody to $RAG^{-/-}$ recipients (Figure 2). P-selectin on platelets binds to PSGL-1 on monocytes and signals through NFκB to cause secretion of MCP-1, TNFα, IL-8 and IL-1β [42, 43]. Of the many chemokines in platelet alpha-granules, RANTES (CCL5) is integral to monocyte recruitment [44, 45]. When compared to the two platelet specific chemokines, PF4 and neutrophil-activating protein-2 (NAP-2), RANTES was over twice as effective at binding to inflamed endothelium and arresting monocytes [46]. RANTES can be released directly by platelets or from microparticles shed from platelets, and then associates with the glycosaminoglycans on the surface on the endothelial cells. Platelet derived RANTES has a distinctive property of forming heteromers with PF4 in the alpha-granules [47, 48]. These heteromers have enhanced chemokine functions that are thought to be due to increased binding of RANTES to leukocytes. The combined stimulation through P-selectin and RANTES-PF4 heteromers polarizes monocytes towards a proinflammatory function [6]. In contrast, macrophages stimulated with recombinant PF4 alone express a transcriptome distinct from either inflammatory or alternatively activated macrophages [49]. PF4 also prevents monocytes from undergoing spontaneous apoptosis during 72 hours of culture and induces differentiation to macrophages as evidenced by morphological and functional changes including expression of the co-stimulatory molecule CD86 and secretion of TNFα [50]. After activation, macrophages have been noted to phagocytize platelets and this process further inhibits apoptosis and enhances secretion of TNFα, IL-6 and IL-23 [51, 52].

In time-lapse cinematic microscopic studies of renal implants, Hobbs and Cliff [10] observed platelets attach to leukocytes that were adherent to vessel walls: “Up to 10 platelets may be seen adhering to one leukocyte, producing a pincushion or porcupine effect.” Such platelet-leukocyte conjugates are found in the circulation in inflammatory diseases. In addition, platelet microparticles form conjugates with leukocytes. Formation of these conjugates depends on P-selectin binding to PSGL-1. Platelets stimulate upregulation of integrins (CD11/CD18) on monocytes and promote migration into tissues [53]. In recent studies, it has been suggested that platelet microparticles may be transported into tissues by “piggy-backing” on neutrophils or monocytes [54]. More recently, evidence has been presented that platelets themselves may migrate into tissues under the influence of stromal derived factor-1 [55, 56]. The fact that platelets are capable of extensive shape change and motility is well documented. It is estimated that actin constitutes about a fifth of the total protein in platelets [57]. Platelets are also known to express several chemokine receptors including CCR1, CCR3, CCR4, and CXCR4 [58]. The concept of platelet migration into tissues has significant implications for transplantation because it provides a mechanism for platelets to enhance interactions among macrophages, dendritic cells and T cells in the interstitium of transplanted organs.

Under in vitro conditions, more activated platelets are bound to monocytes than neutrophils [59]. This preferential binding may contribute to the observation that macrophages often are more prevalent than neutrophils in antibody-mediated rejection, [41, 60, 61]. Macrophages are also much more numerous than neutrophils in experimental cardiac and renal transplants following passive transfer of alloantibodies to $RAG^{-/-}$ recipients (Kuo, H-H et al, in preparation).

Platelet interactions with lymphocytes

Antibody-mediated rejection often has some mixture of cellular rejection. Similarly, platelet interactions are not limited to neutrophils and monocytes [5]. Platelets express both CD40 and CD40L, which are integral to interactions with lymphocytes. When T cells are activated through CD3 and CD28, their production of IFNγ is enhanced by platelets [62]. This interaction is dependent on platelets binding to T cells and is blocked by antibodies to P-selectin, CD40L and GPIIb/IIIa. Platelets and T-cells can engage in a feedback loop in which platelets secrete RANTES to recruit T cells, and then activated T cells stimulate platelets through CD40-CD40L interactions to secrete RANTES thereby recruiting more T cells to the site of interaction [63, 64]. The relevance of platelet derived CD40L has been demonstrated by Kirk and colleagues [65]. Platelets are the major source of soluble circulating CD40L, and the authors demonstrated that purified CD40L trimers can induce rejection of cardiac allografts in CD40L knockout mice.

Platelets may sustain T cell recruitment to allografts through additional mechanisms including the secretion of glutamate. Activated platelets secrete glutamate, which can feed back in an autocrine loop through their glutamate receptors to increase thromboxane production [66]. Treatment with glutamate receptor antagonists or platelet depleting antibodies increases survival of skin grafts on athymic mice that are reconstituted with limited numbers of T cells [67]. These treatments delay CD4 and CD8 T cell infiltration of the skin grafts, and decrease thromboxane, PF4 and IFNγ levels in the plasma.

Platelets can also supplement low levels of T cell help for antibody production in models of viral infection. In studies of antibody responses to adenovirus, Elzey and co-workers [68] demonstrated that passive transfer of CD40L expressing platelets could provide help in CD40L deficient mice for isotype switching to IgG antibody production. This may be

relevant to possible local alloantibody production in tertiary lymphoid structures that form in some clinical transplants [69, 70].

Inhibition of platelet interactions

Although no therapeutic interventions specifically targeted to platelets are administered to transplant recipients currently, aspirin has been demonstrated to have beneficial effects on renal allograft outcomes [71]. Knowledge of the major mechanisms of platelet interactions with endothelial cells and leukocytes may provide new approaches to modulate inflammation.

Monoclonal antibodies and soluble inhibitors for P-selectin and RANTES have been used to inhibit platelet-leukocyte interactions experimentally [48]. However, these inhibitors do not just limit platelet functions. The most promising approach is to focus on platelet specific mediators. In terms of transplantation, inhibition of the RANTES-PF4 heteromer would have many advantages. Koenen and colleagues [47] have developed small molecules designed to prevent RANTES-PF4 heteromer formation. Human and mouse peptides have been demonstrated to inhibit RANTES mediated adhesion of monocytes to endothelial cells in vitro. They have applied one such inhibitor (MKEY) to prevent acute lung injury in mice that was induced by 3 different stimuli: aerosolized LPS, sepsis or acid [48]. This strategy prevented neutrophil infiltration and edema in the lungs to a similar degree as blocking antibodies to either PF4 or RANTES, but it did not interfere with general neutrophil functions such as phagocytosis or killing of bacteria.

Summary

The increasing data linking platelets with vascular inflammatory responses has obvious implications for allograft rejection. The functional versatility of platelets makes it likely that platelets modify many aspects of both humoral and cellular rejection. The interactions of platelets with endothelial cells and monocytes are particularly relevant to the pathological findings in antibody-mediated rejection.

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Abbreviations

MHC	Major Histocompatibility Complex
vWf	von Willebrand factor
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
PF4; CXCL4	Platelet Factor 4
NAP-2	neutrophil-activating protein-2
C1qR	C1q receptor
FcγR	Fc gamma receptor

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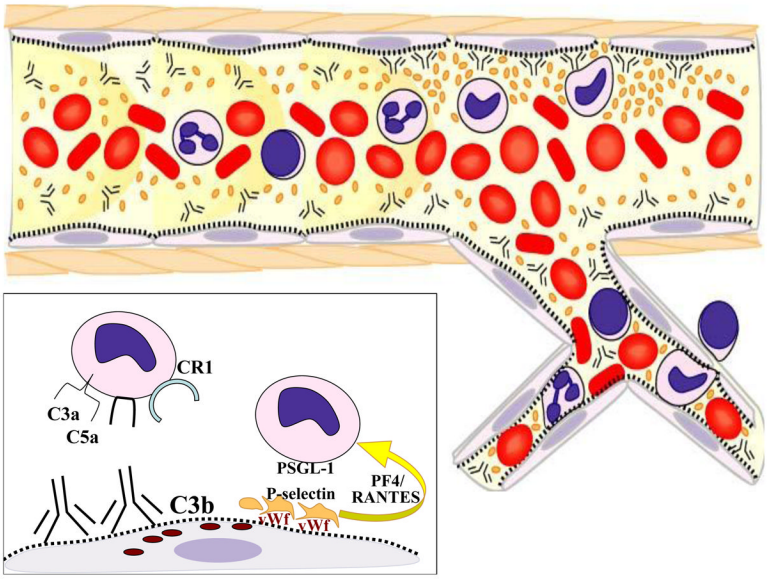

Figure 1.

The laminar flow of blood in arteries and arterioles causes platelets to be more concentrated adjacent to the vessel wall. As illustrated in the inset, alloantibodies cause exocytosis of von Willebrand factor (vWf) from storage granules in endothelial cells. Platelets bind to vWf and become activated. Activated platelets release chemokines from their alpha granules and express P-selectin on their membranes. Heteromers of PF4 and RANTES recruit monocytes, which are further activated by PSGL-1 receptors binding to P-selectin on platelets. Complement activation products provide additional chemoatraction (C3a and C5a) as well as ligands (C3b for CR1) to increase leukocyte localization to the endothelium.

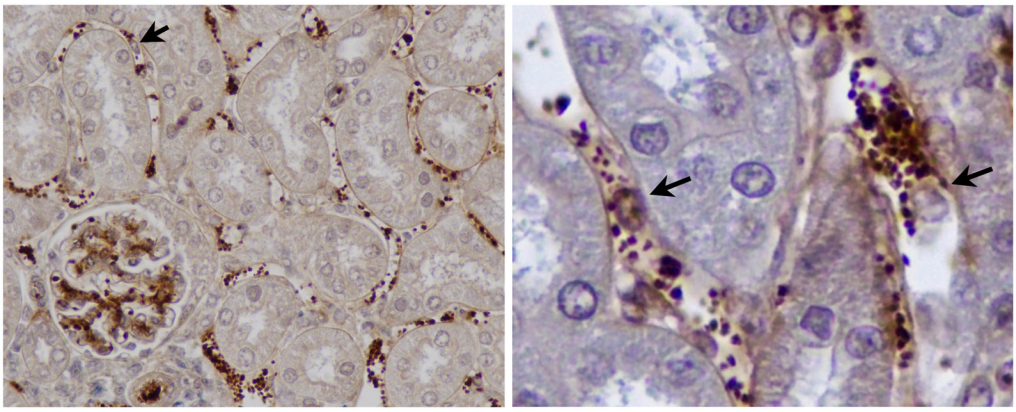

Figure 2.
Passive transfer of alloantibodies to C57BL/6 RAG$^{-/-}$ recipients of B10.A renal transplants causes release of vWf and extensive platelet accumulation in peritubular capillaries and glomeruli within 1 hour. Platelets (stained brown for vWf) interact extensively with endothelial cells and leukocytes (arrows). Leukocytes with adherent platelets are better appreciated on the enlarged image on right.