Novel Platelet and Vascular Roles for Immunoreceptor Signaling

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Abstract—The immunoreceptor signaling pathway has classically been defined by its role in mediating intracellular signals downstream of immune receptors on circulating cells, but recent studies have revealed 2 new and unexpected roles for this pathway in vascular biology. In platelets the immunoreceptor signaling pathway is coupled to 2 structurally distinct platelet collagen receptors, glycoprotein VI and integrin α2β1, and is required for the activation of platelets after exposure to vessel wall collagen during plaque rupture. During vascular development immunoreceptor signaling is required for proper formation of the lymphatic system, a role that has revealed the contribution of hematopoietic endothelial progenitors to that process. In conjunction with the identification of new biological roles in vascular cell types, new molecular mechanisms of activating this signaling pathway have been discovered, including activation by integrins and immunoreceptor tyrosine activation motifs (ITAMs) on receptors that do not function as part of the immune response. Here we discuss some of these recent findings and their implications for vascular biology and the treatment of human vascular diseases. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words:

Immune receptors are a family of multi-subunit receptors that play critical roles in the adaptive immune response. Classical immune receptors, such as the T cell receptor, B cell receptor, and Fc receptors, are composed of specialized subunits that participate in either ligand binding or intracellular signaling. Activation of intracellular signaling by multi-subunit immune receptors is triggered by tyrosine phosphorylation at a region known as the immunoreceptor tyrosine activation motif, or ITAM, on the signal transducing subunit.1 Once phosphorylated, tyrosine residues within the ITAM consensus sequence (YxxILxα6o Tyr) bind the tandem SH2 domains of either Syk or ZAP-70, 2 related nonreceptor tyrosine kinases, an interaction that leads to conformational release and activation of the kinase. Most signals downstream of immune receptors are mediated by these kinases, with ZAP-70 serving as the primary kinase in T cells and Syk as the primary kinase in other hematopoietic cell types, including platelets. Interestingly, immune receptors such as the T cell receptor contain up to 10 distinct ITAMs, whereas homologous receptors such as the platelet collagen receptor glycoprotein VI (GPVI) signal through a single ITAM unit. Presumably the number and structural context of these ITAMs provide a mechanism for modulation of signaling.2

The event that triggers signaling following the binding of immune receptors to ligand is ITAM phosphorylation by Src family tyrosine kinases, a large family of nonreceptor tyrosine kinases that transduce signals downstream of numerous cell surface receptors. While the molecular mechanism by which ligand binding drives ITAM phosphorylation is not well understood, clustering of receptors driven by binding to multivalent ligand appears necessary and may bring the ITAM-bearing subunits into contact with activated Src family kinases, including Syk and ZAP-70.3

Lipid rafts are cholesterol-rich regions of the cell membrane that are biochemically defined by resistance to detergent and are enriched in many of the signaling molecules utilized by immune receptors, including the Src family kinases and the transmembrane lipid raft adaptor molecular LAT (linker in activated T cells). The intracellular domain of LAT contains multiple tyrosines that when phosphorylated bind the SH2 domains of critical signaling effectors, including the adaptors GADS and GRB2 and the phospholipase PLCγ2.4 SLP-76 and its B cell homologue SLP-55 (also known as BLNK and BASH) are cytoplasmic adaptors that are also critical for immune receptor signaling. Recruitment of SLP-76 to the membrane by GADS-LAT interaction allows formation of the canonical immune receptor signaling complex. Essential roles for the major components of this signaling complex, including Syk, SLP-76, LAT, and PLCγ2, in the generation of adaptive immune responses have been demonstrated genetically by the generation of knockout mice with immune defects.5–8 Significantly, many of the components of this canonical immune signaling pathway are expressed in nonimmune hematopoietic cells such as platelets and osteoclasts, where the pathway lies downstream of receptors that are structurally and functionally homologous to...
immune receptors but participate in nonimmune biological roles, such as collagen signaling by the platelet GPVI collagen receptor.

The presence of alternative, noncanonical means of activating the immune receptor signaling pathway have emerged primarily from studies of Syk, the intracellular kinase responsible for activating this pathway outside of T cells. A link to integrin signaling was established by the observation that adhesion of platelets to fibrinogen through the αIIbβ3 integrin (also known as GPIIbIIIa) results in Syk phosphorylation and Syk co-immunoprecipitates with the cytoplasmic tails of β integrin subunits. Functionally, some integrin-mediated responses, such as the spreading of platelets on fibrinogen and the activation of neutrophils in response to integrin ligand, are deficient in cells that lack Syk, SLP-76, or PLCγ2. An important finding of such functional studies has been that the lipid raft adaptor LAT, critical for signaling downstream of immune-type receptors, is dispensable for integrin-mediated activation of this pathway. Thus a noncanonical mechanism of activating the immune receptor signaling pathway through integrins exists and appears to operate outside of lipid rafts (Figure).

As a result of the dual role played by many of the key components of this pathway in canonical and noncanonical signals, it is not known whether the loss of any particular cellular function observed in mice lacking Syk, SLP-76, or PLCγ2 is caused by loss of ITAM signaling, integrin signaling, or both. In the case of platelet responses to the extracellular matrix protein collagen, convergence of integrin and ITAM receptors at the level of intracellular signal transduction is accompanied by convergence at the level of extracellular ligand binding, as platelets express both an immune-type collagen receptor, GPVI, and a collagen-binding integrin, α2β1. Whether and to what extent integrin and immune receptor type activation of this signaling pathway operates in platelets and in other cell types is not yet known. A clear definition of the biological roles of noncanonical signaling through the immune receptor pathway remains an important future area for investigation.

Finally, in addition to activation by immune receptor ITAMs and by integrins, recent studies have demonstrated Syk binding and activation by ITAM-like sequences in proteins that are not homologous to immune receptors. A recent bioinformatics analysis identified no fewer than 48 proteins that met stringent criteria for an ITAM motif and up to 368 that met less stringent criteria. The biological role of these alternative ITAM-containing proteins in the activation of Syk and this pathway remains to be defined, but these novel ITAMs may explain the expression and function of Syk in cells outside the hematopoietic system.

**Immunoreceptor Signaling in Platelet Collagen Responses**

The possibility that the biological roles of canonical, ITAM-activated immunoreceptor signaling and those of noncanonical, integrin-activated immunoreceptor signaling may converge is highlighted by the utilization of this pathway in platelet collagen responses. Platelets are anuclear, small circulating cells in the blood that are activated during vessel wall injury and are required for hemostasis in the arterial system. In the absence of stimuli that trigger intracellular signals, resting platelets circulate in the blood in contact with both the endothelial lining of the vessel wall and blood proteins such as fibrinogen, but bind neither. In contrast, activated platelets participate in hemostasis and thrombosis by binding the injured vessel wall and each other (via dimeric fibrinogen molecules). A primary stimulus for platelet activation after vessel injury is the matrix protein collagen that is exposed after disruption of the vascular endothelium. Unlike platelet activation by thrombin, ADP and thromboxane, classic soluble activators of G protein-coupled signaling in platelets, the molecular basis of the platelet collagen response has only recently been understood. Syk and the ITAM-containing immunoreceptor signaling adaptor FcγR are noted to be required for platelet collagen responses even before the platelet receptor coupled to those signaling proteins was cloned. Biochemical studies revealed the collagen-binding subunit of this receptor to be GPVI, an Ig-domain containing receptor whose predicted amino acid sequence and genomic locus identify it as a homologue of immune receptors such as the Fc receptor for IgA. Genetic studies in mice have confirmed that loss of GPVI, SLP-76, or PLCγ2 results in the loss of collagen activation of platelets,
confirming the requirement for ITAM-activated, canonical immunoreceptor signaling for this platelet response.5,22,23

A second collagen receptor expressed on the surface of platelets is the integrin α2β1.24 Because α2β1, like other platelet integrins, is held in an inactive conformation and unable to bind ligand until released by inside-out signals,25,26 it has remained unclear whether or not this collagen-binding integrin participates in the generation of collagen signals.27 The integrin α2β1 is not required for wild-type platelet aggregation responses induced by fibrillar collagen,14,28 but is required when GPVI signaling is reduced either genetically or pharmacologically.14 α2β1 is also required for firm adhesion of platelets to immobilized fibrillar collagen under flow conditions that reproduce in vivo platelet-collagen interaction.29,30 These findings are consistent with either a secondary signaling role for α2β1 after GPVI activation of the integrin or an adhesive role for α2β1 that confers GPVI co-receptor function required for receptor activation by weak ligands or under flow conditions. These biological roles are not mutually exclusive. While at this time there are no definitive data that distinguish between these 2 possibilities, genetic studies in mice provide indirect support for direct α2β1-mediated signals. LAT-deficient platelets exhibit a severe loss of response to GPVI-specific ligands such as the snake venom convulxin or collagen-related peptides,31 but retain responses to collagen.14,29,32 Moreover, LAT-deficient platelet collagen responses require integrin α2β1. This requirement is not easily explained via GPVI co-receptor function because LAT-deficient platelets express normal levels of surface GPVI. Instead these studies may reflect the ability of integrins to activate the immunoreceptor signaling pathway in a lipid raft-independent, LAT-independent manner. Precise definition of whether and how integrins activate immunoreceptor signaling independently of ITAMs remains a significant open question for future investigation.

Immunoreceptor Signaling in Vascular Development: Revealing a Link Between Hematopoietic Cells and New Vessel Formation

An unsuspected link between immunoreceptor signaling and the vascular system emerged with the first report of mice lacking the Syk kinase.6,33 Syk-deficient mice appeared “hemorrhagic” and edematous in mid-gestation and died postnatally in association with the development of chyloous ascites. Similar phenotypes were observed in mice lacking SLP-7623 and PLCγ2.5 Further phenotypic evaluation of these animals revealed a surprising finding: the vascular abnormalities were the result of defects that arose during formation of the lymphatic vascular system and culminated in vascular connections between nascent lymphatics and pre-existing blood vessels.34 Mice lacking Syk, SLP-76, or PLCγ2 develop lymphatics at the same time and in the same manner as wild-type mice, but in a limited number of new lymphatic vessels connections are made to blood vessels, filling the developing lymphatic system with blood. Histologically the lymphatic vessels of mice lacking this pathway appear structurally normal but molecular analysis reveals the presence of endothelial cells carrying lymphatic molecular markers fused to blood endothelial cells lacking those markers.34 Examination of Syk, Slp-76, and PLCγ2 mRNA expression in developing embryos has confirmed expression in circulating blood cells but failed to reveal expression in either blood or lymphatic endothelial cells. These studies have established a new role for the immune receptor signaling pathway in vascular development, but the question of how the signaling pathway regulates vascular development was not understood until recently.

Studies designed to investigate this question have yielded unexpected insights into the relationship between blood and endothelial cells during vascular development. Consistent with the lack of detectable expression in embryonic endothelial cells, we have used a GATA1-GFPSlp-76 transgene that drives expression in blood but not endothelial cells to rescue the vascular phenotype of Slp-76--deficient mice (Mark L. Kahn, unpublished observations). The GATA1-GFPSlp-76 transgene drives expression of a GFPslp-76 fusion protein in a limited number of circulating cell types but not in endothelial cells, indicating that the endothelial requirement for immunoreceptor signaling is either indirect, ie, required in circulating cells to direct endothelial cell function, or is in endothelial cells that originate from hematopoietic cells. To distinguish between these possibilities we analyzed chimeric embryos in which deficient cells and wild-type cells were mixed to test cell autonomy. In this experiment, deficient cells conferred the vascular phenotype despite the presence of wild-type circulating cells, a result consistent with a cell autonomous defect in hematopoietic endothelial progenitors. Consistent with this mechanism, surviving Slp-76--deficient adult mice lack circulating endothelial precursors that express lymphatic molecular markers, a cell type that could link the hematopoietic cells to the lymphatic vascular system. A common origin has been established for blood and endothelial cells early in embryonic development, on the basis of close spatial and temporal association,35 genetic studies,6,37 and studies of cultured embryonic stem cells.38 Recent studies using adult animals have extended this relationship with evidence that mature hematopoietic stem cells can give rise to circulating cells that participate in adult vessel growth in response to angiogenic stimuli.39 Whether such cells contribute to normal vascular development after the establishment of the hematopoietic system has not been known, however, and the vascular defects in mice lacking immunoreceptor signaling provide new genetic evidence for such a role. How this signaling pathway is utilized in hematopoietic endothelial progenitor cells is not yet known, but further studies to fate-map hematopoietic cells that express Syk and Slp-76 during lymphatic vascular development and functional studies of circulating endothelial progenitor cells lacking this pathway should yield new insights into the how blood cells participate in vessel formation both during and after embryonic development.

Immunoreceptor Signaling in Vascular Biology: Clinical Implications

The recently identified roles of immunoreceptor signaling in platelets and in circulating endothelial progenitors (CEPs) suggest potential roles in the pathogenesis and treatment of
human vascular diseases. In the case of platelet collagen receptors, a clear path exists between the molecular understanding of how these cells respond to vessel wall injury and the development of novel agents to block that response during the first stages of myocardial infarction or stroke. In the case of immunoreceptor signaling in CEPs, it is possible that this pathway can be used to alter the number and/or identity of such endothelial precursors to drive or inhibit vascular growth, e.g., in the setting of ischemia or tumor angiogenesis. While clinical application of immunoreceptor signaling in progenitor/precursor cells remains entirely speculative at this time, more concrete clinical data exist in support of targeting platelet collagen receptors to treat human vascular diseases.

**Platelet Collagen Receptors as Potential Risk Factors and Therapeutic Targets**

Platelet collagen responses are believed to be a primary response of unactivated, circulating platelets to vessel wall injury, and platelet-activating signals generated by platelet collagen receptors may therefore play a determinative role in the thrombotic outcome of plaque rupture in the coronary and cerebral vasculature. Studies of human GPVI deficiency states and blockade of integrin α2β1 confirm that GPVI and α2β1 play similar roles in human and mouse platelets. This model has led to the hypothesis that an individual’s platelet collagen receptor activity may function as an independent risk factor for stroke and myocardial infarction and, conversely, that inhibition of that activity could provide protection against thrombus formation after plaque rupture. A straightforward mechanism by which individual platelet collagen responses may vary is receptor density. An early study of GPVI-mediated collagen signaling in a cell line revealed that GPVI affinity for collagen is relatively weak and that collagen responses were highly dependent on receptor density. Earlier studies of α2β1 polymorphisms in human subjects found polymorphisms associated with higher platelet receptor densities are also associated with an increased risk of myocardial infarction (MI) and stroke. In unactivated circulating platelets the integrin α2β1, like the αIbβ3 integrin, is held in an off state and unable to bind collagen. Thus, in the primary activation of platelets after plaque rupture and collagen exposure it appears that the GPVI receptor may play the largest role.

Several previous studies of healthy human subjects have demonstrated that platelet GPVI and α2β1 density are tightly regulated in healthy human volunteers, with only a 1.5- to 5.0-fold variation in both GPVI and α2β1 density. These studies also found only modest correlation between GPVI and α2β1 densities (r=0.35 to 0.46). We recently performed a study addressing the question of platelet collagen receptor density in 209 human subjects with a high prevalence of myocardial infarction. Through the use of a novel anti-glycoprotein VI (GPVI) monoclonal antibody and a previously characterized anti-integrin α2β1 antibody to directly measure the density of platelet collagen receptors by flow cytometry, we found a 16-fold variation in expression of GPVI and a 23-fold variation in expression of α2β1, with a strong correlation between GPVI and α2β1 density (r=0.68 P<0.0001). Thus, these findings differed significantly from those of previous studies in healthy human subjects. Interestingly, however, our findings are similar to another recent study of 367 patients with ischemic heart disease, which found a 16-fold variation in α2β1 levels and a 7-fold variation in GPVI levels, with a highly significant correlation between the 2 (r=0.702; P=0.001). In fact, this study showed a possible upregulation of both receptors in patients with an acute coronary syndrome. Thus, the degree of regulation of platelet collagen density between individuals, and in response to clinical status requires further study.

Studies of GPVI polymorphisms have been less clear. One study identified a GPVI coding region polymorphism (T683C) that correlates with lower GPVI receptor density and diminished platelet aggregation in response to collagen, suggesting that platelet GPVI polymorphisms associated with lower receptor densities confer less robust platelet responses, but a separate study of the same polymorphism found it associated with an increased risk of myocardial infarction. Thus whether and how GPVI polymorphisms can be used to predict receptor density and risk of atherothrombosis is unclear. To address this question we have recently performed a case-control study, comparing 77 patients with prior myocardial infarction to 77 control patients, matched on the basis of traditional risk factors for myocardial infarction. In this study, we did not find a strong association between heterogeneity at the GPVI 683C allele and GPVI receptor density, as measured using a monoclonal antibody and flow cytometry. However, a GPVI or α2β1 density within the lowest decile was associated with a significantly lower likelihood of prior myocardial infarction (odds ratio, 6.3; 95% confidence interval, 1.3 to 29.2; P=0.009). Thus while preliminary studies of human platelet collagen receptor density suggest a direct relationship to the risk of stroke and myocardial infarction, larger studies are necessary to confirm and quantify this risk and to determine if any particular polymorphisms can be used to predict receptor density.

In addition to predicting clinical risk, the inhibition of platelet collagen responses would appear to hold significant therapeutic promise. Present anti-platelet therapies have proven efficacy for treatment of both myocardial infarction and thrombotic complications secondary to percutaneous coronary intervention. Use of these agents is limited, however, by the fact that available agents either act as blanket inhibitors of platelet aggregation (e.g., the IIb/IIIa inhibitors) or function in a nonspecific manner to reduce autocrine platelet signaling (e.g., aspirin and ADP antagonists such as clopidogrel). Powerful IIb/IIIa inhibitors are difficult to use chronically because effective doses can result in unacceptable bleeding risk, while inhibitors of autocrine signaling through thromboxane generation or ADP are amenable to chronic use but are not powerful enough to antagonize strong thrombotic stimuli independently. An ideal anti-platelet therapy would target the causal event in atherothrombotic disease states but not incapacitate the platelet response to the point at which serious bleeding would prevent long term therapy. Targeting the initial event in platelet activation at sites of arterial vascular injury through the platelet collagen receptor GPVI might provide such a strategy. Loss of GPVI function results
in reduced arterial thrombosis in mice, although the extent of GPVI’s role in arterial thrombosis assays is dependent on the extent of vessel injury and may be strongly influenced by the generation of thrombin, a potent activator of platelets. GPVI deficiency in humans results in a mildly bleeding disorder, suggesting that anti-GPVI antibodies can reduce arterial thrombosis associated with myocardial infarction or stroke with a tolerable long-term bleeding risk. The fact that GPVI receptors on platelets are cleared in vivo for >2 weeks after a single injection of anti-GPVI antibodies in mice, the recent development of anti-GPVI antibodies capable of blocking collagen activation of GPVI as well as the recently solved crystal structure of the GPVI ectodomain suggest that multiple strategies could be used to develop novel anti-GPVI agents for clinical use.

Conclusions

Recent basic science and clinical studies have revealed diverse and unexpected vascular roles for an intracellular signaling pathway previously associated exclusively with immune responses. While based in the hematopoietic system, these roles include the regulation of hemostasis and thrombosis via collagen and integrin signals in platelets as well as the regulation of vascular development by circulating endothelial precursor cells. Remarkably, the biological diversity of this signaling pathway in the vascular system is matched by its molecular diversity, it can be activated by multi-subunit receptors that structurally resemble immune receptors, by integrins and perhaps by other classes of receptors that couple to novel ITAM-containing proteins as well. While the studies performed to date suggest broad and important vascular roles for this signaling pathway, the cellular and molecular basis for these roles remain mostly unknown and promise to be an exciting area of vascular biology in the future.

Disclosures

None.

References


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