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Essential *in vivo* roles of the platelet activation receptor CLEC-2 in tumour metastasis, lymphangiogenesis and thrombus formation

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We have recently identified C-type lectin-like receptor 2 (CLEC-2) as a receptor for the platelet activating snake venom rhodocytin. CLEC-2 elicits powerful platelet activation signals in conjunction with single YxxL motif in its cytoplasmic tail, Src, Syk kinases, and phospholipase Cγ2. An endogenous ligand of CLEC-2 has been identified as podoplanin, which is a membrane protein of tumour cells and facilitates tumour metastasis by inducing platelet activation. Studies of CLEC-2-deficient mice have revealed several physiological roles of CLEC-2. Podoplanin is also expressed in lymphatic endothelial cells. In the developmental stages, when the primary lymph sac is derived from the cardinal vein, podoplanin activates platelets in lymphatic endothelial cells, which facilitates blood/lymphatic vessel separation. Moreover, CLEC-2 is involved in thrombus stabilization under flow conditions in part through homophilic interactions. The absence of CLEC-2 does not significantly increase bleeding tendency, implying that CLEC-2 may be a good target protein for anti-platelet drugs in addition to anti-metastatic drugs.

Keywords: CLEC-2/lymphangiogenesis/platelets/podoplanin/tumour metastasis.

Abbreviations: CLEC-2, C-type lectin-like receptor 2; FcR, Fc receptor; Fiaf, Fasting-induced adipose factor; GP, glycoprotein; ITAM, immunoreceptor tyrosine-based activation motif; LAT, Linker of Activated T cell; PLCγ2, phospholipase Cγ2; TNF-α, tumor necrosis factor.

CLEC-2 was identified as a receptor for a platelet activating snake venom, rhodocytin

Snake venom contains a vast number of toxins that target proteins in coagulation factors or platelets. Identification of these proteins has made an enormous contribution to understanding of thrombosis and haemostasis. The snake venom rhodocytin (also called aggretin) was purified from *Calloselasma rhodostoma* venom (*1*, *2*). Rhodocytin stimulates platelet aggregation depending on the Src family kinase, but independently of the collagen receptor glycoprotein VI (GPVI)/FcRγ-chain complex, which is well known to activate platelets depending on the Src family kinase (*3*–*5*). Initial studies indicated that rhodocytin induced platelet aggregation by binding to integrin α2β1 (*1*, *4*, *5*) and GPIb/IX/V (*5*). However, later studies reported that rhodocytin stimulates aggregation of platelets deficient in α2β1 or the extracellular domain of GPIb/IX/V (*6*), indicating that there was another activation receptor for rhodocytin, although the toxin may bind to α2β1 or GPIb/IX/V. Ultimately, we identified C-type lectin-like receptor 2 (CLEC-2) as the rhodocytin receptor using rhodocytin affinity chromatography (*7*).

When CLEC-2 was initially identified through a bioinformatic screen for C-type lectin-like receptors, reverse transcriptase-PCR and northern blot analysis indicated that CLEC-2 mRNA was expressed in the liver and in several haematopoietic cells, including monocytes, dendritic cells, NK cells and granulocytes (*8*). However, platelets and megakaryocytes were not investigated for expression. Later, CLEC-2 protein expression was systematically analysed, revealing that the CLEC-2 protein is also expressed in platelets, megakaryocytic cell lines, liver sinusoidal endothelial cells (*9*), and liver Kupffer cells (*10*) in humans. In mice, it has been reported that CLEC-2 is also expressed in neutrophils (*11*) and macrophages (*12*), where it mediates phagocytosis and increased expression of proinflammatory cytokines, including tumour necrosis factor α (TNF-α), and in Kupffer cells (*10*). CLEC-2 is highly and relatively specifically expressed in platelets and megakaryocytes, but is also present in other types of cells at low levels, particularly in mice.

CLEC-2 was first cloned through a computational screen for C-type lectin-like receptors (*8*). However, neither its function nor its ligand(s) were known for several years until we revealed that CLEC-2 is a signalling receptor expressed on the surface of platelets that induces platelet aggregation (*7*).

Mechanism of platelet activation by CLEC-2

CLEC-2 has a single YxxL motif in its cytoplasmic tail called hemi-immunoreceptor tyrosine-based activation motif (ITAM) hereafter. ITAM is a signalling motif, consisting of tandem YxxL motifs (YxxL-(X)10-12-YxxL), and found in immune receptors such as the T-cell receptor and the platelet collagen receptor GPVI/FcRγ-chain complex. The signalling pathway of the GPVI/FcRγ-chain has been intensively investigated. Cross-linking of GPVI leads to tyrosine phosphorylation of ITAM in the cytoplasmic domain of the

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FcRγ-chain, which is constitutively associated with GPVI, by the Src family kinases Fyn and Lyn. This leads to binding of the tandem SH2 domain of the tyrosine kinase Syk to the phosphorylated ITAM. Subsequent activation of Syk initiates downstream signalling events that culminate in tyrosine phosphorylation of Linker of Activated T cell (LAT), SLP-76 and Vav1/3 and activation of effecter enzymes including Btk, PI3-kinase, Rac/Cdc42 and phospholipaseCγ2 (PLCγ2) [reviewed in (*13*)] (Fig. 1A).

We have demonstrated that rhodocytin stimulates phosphorylation of the single YxxL motif, after which the tandem SH2 domains of Syk bind to the phosphorylated YxxL, and that the hemi-ITAM is necessary for CLEC-2 signal transduction (*7*, *14*). The groups of Watson and O'Callaghan proposed that CLEC-2 is present as a dimer in resting platelets, and that the tandem SH2 domains of Syk bind to the phosphorylated YxxLs of two CLEC-2 molecules with a stoichiometry of 2:1 (*15*, *16*). The ITAM of the GPVI/FcRγ-chain is tyrosine phosphorylated by the Src family kinases Fyn and Lyn, which are constitutively associated with the cytoplasmic tail of GPVI (*17*, *18*). This is followed by binding and subsequent activation of Syk and phosphorylation of downstream signalling molecules. In the case of CLEC-2, however, hemi-ITAM is mainly phosphorylated by Syk itself. R406, a specific Syk inhibitor, inhibits CLEC-2 hemi-ITAM phosphorylation induced by rhodocytin, but not GPVI ITAM phosphorylation induced by collagen in human platelets (*19*). A specific Src family kinase inhibitor, PP2, also inhibits CLEC-2 tyrosine phosphorylation (*7*), suggesting that CLEC-2 is phosphorylated by Syk and Src family kinases in human platelets. This is a new paradigm in signalling by YxxL-containing receptors that is different from the ITAM receptors, which are phosphorylated solely by the Src family kinases, followed by binding and subsequent activation of Syk.

The signalling pathway of CLEC-2 downstream of Syk is nearly the same as that of GPVI: tyrosine phosphorylation of LAT, SLP-76 and Vav1/3 and activation of effecter enzymes including Btk and PLCγ2 (*7*, *14*) (Fig. 1B). Murine platelets deficient in Syk or PLCγ2 failed to respond even to the maximum concentration of rhodocytin, suggesting that Syk and PLCγ2 are crucial for CLEC-2-mediated signal transduction. On the other hand, those deficient in the adaptor proteins LAT or SLP-76, or the guanine nucleotide exchange factor Vav1/3, did not respond to low concentrations but responded to high concentrations of rhodocytin, suggesting that these molecules are necessary, but compensatory, for CLEC-2 signalling (*7*). Adapter proteins play a critical role in signalling by YxxL motif-containing receptors by forming a protein scaffold that recruits PLCγ2 to the membrane.

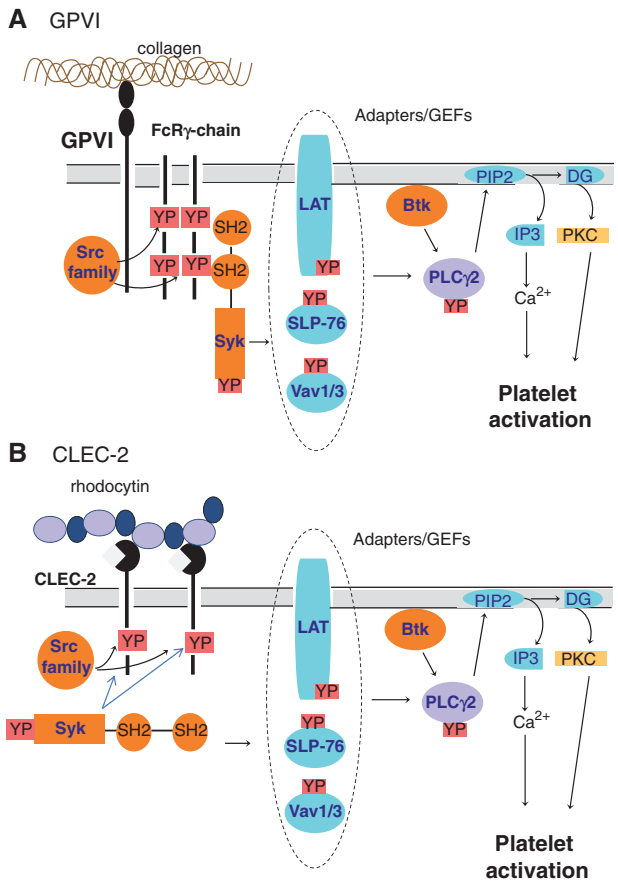

Fig. 1 Signal transduction pathway mediated through (A) GPVI/FcRγ-chain and (B) CLEC-2. YP indicates phosphorylated tyrosine.

Patho/physiological roles of CLEC-2

Tumour metastasis

It has long been recognized that platelets are involved in cancer metastasis and/or progression [reviewed in (*20*)]. Several kinds of tumour cells aggregate platelets, facilitating tumour growth and metastasis (*21*–*23*). Platelet aggregates surrounding tumour cells protect them from shear stress and NK cells (*24*) and facilitate formation of tumour cell nests in the blood stream. Growth factors released from activated platelets stimulate angiogenesis or tumour growth. Podoplanin is a type-I transmembrane sialomucin-like glycoprotein expressed on several kinds of tumour cells, including squamous cell carcinomas (*25*, *26*), seminomas (*27*) and brain tumours (*28*–*30*), and induces platelet aggregation [reviewed in (*31*)]. Recent investigations have supported the hypothesis that podoplanin expression may be associated with tumour metastasis or malignant progression (*28*, *32*). Therefore, identification of a podoplanin receptor in platelets has been urgently awaited, as it may be a good target protein for anti-metastatic drugs. We observed that the profile of podoplanin-induced platelet aggregation is quite similar to that of rhodocytin-induced platelet aggregation and identified CLEC-2 as a receptor for podoplanin in collaboration with Kato (*33*). These results were also confirmed by Christou *et al.* (*34*), who demonstrated that podoplanin binds to CLEC-2 in a kidney cell line. In an experimental metastasis model in mice, an anti-podoplanin blocking antibody significantly inhibited the number of metastatic lung nodules consisting of tumour cells expressing podoplanin, implying that

CLEC-2/podoplanin may be a promising target protein for anti-metastatic drugs (*35*).

Lymphatic/blood vessel separation

Podoplanin is also expressed in various normal tissues, including lymphatic endothelial cells, type I alveolar cells, and kidney podocytes [reviewed in (*31*)]. Podoplanin is expressed in lymphatic endothelial cells but not in vascular endothelial cells; hence it is used as a marker for lymphatic endothelial cells. However, under physiological conditions, CLEC-2 in platelets cannot interact with podoplanin in lymphatic endothelial cells. During organ development, the cluster of endothelial cells in the cardinal vein sprouts to form the primary lymphatic sacs from which a portion of the peripheral lymphatic vasculature is generated by further centrifugal growth [reviewed in (*36*)]. Recently, several lines of evidence have suggested that the interaction between CLEC-2 in platelets and podoplanin in lymphatic endothelial cells is necessary for lymph/blood vessel separation at the embryonic stage Fig. 2. Mice deficient in the signalling molecules Syk and SLP-76 have blood/lymphatic misconnections (*37*). These signalling molecules are necessary for CLEC-2-mediated platelet activation (*7*), but they are not expressed in vascular/lymphatic endothelial cells. Podoplanin-deficient mice have defects in lymphatic vessel patterning, suggesting that podoplanin is integrally involved in lymphatic vessel formation (*38*, *39*). Prox1-conditional knockout also showed the abnormal lymphatic/blood vessel separation with reduced expression of podoplanin (*40*). Moreover, endothelial cell *O*-glycan deficiency results in blood/lymphatic misconnections (*41*) and it has been demonstrated that sialic acid on the *O*-glycans of podoplanin is essential for binding to CLEC-2 (*33*). Carramolino *et al*. (*42*) provided evidence for the necessary role of platelets in blood/lymphatic vessel separation based on a study of Meis1-deficient mice, which completely lack megakaryocyte/platelets and fail to separate blood and lymphatic vasculature. All of these findings suggest that the CLEC-2/podoplanin interaction plays a crucial role in blood/lymphatic vessel separation.

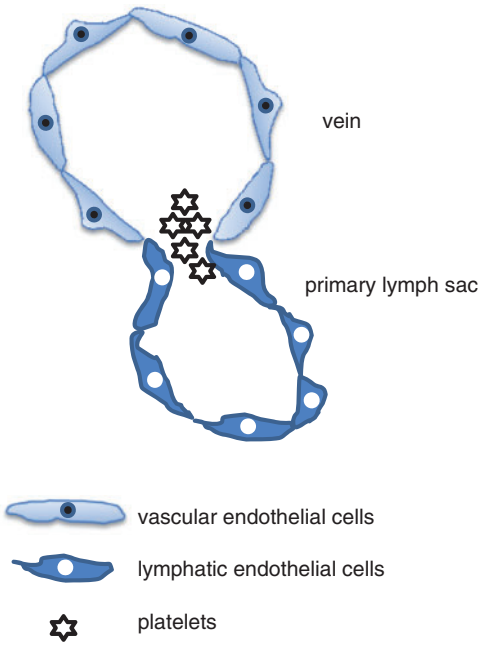

Fig. 2 Platelets help to separate the blood and lymphatic vascular systems.

To verify this hypothesis, CLEC-2-deficient mice were needed. Quite recently, Bertozzi *et al*. and we independently generated CLEC-2-deficient mice, which revealed that this hypothesis is correct. Both groups reported that these mice exhibit mortality at the embryonic/neonatal stages associated with disorganized and blood-filled lymphatic vessels and severe oedema due to abnormal blood/lymphatic vessel separation (*43*, *44*). Platelet factor 4-Cre-mediated deletion of SLP-76 (*43*) or CLEC-2 (Suzuki-Inoue *et al*., unpublished observation) is sufficient to confer lymphatic vascular defects, identifying platelets as the cell type required to regulate lymphatic vascular development. A follow-up research question has been determining the mechanism by which CLEC-2 in platelets regulates blood/lymphatic vessel separation. The finding that mice deficient in SLP-76 or Syk show a non-separation phenotype clearly indicates that platelet activation is required for the separation. Platelet activation results in granule release and platelet aggregation. Platelet granules contain large quantities of angiogenic factors, growth factors and extracellular matrix, implying that these factors may contribute to blood/lymphatic separation. Alternatively, platelet aggregates build up at the separation zone of lymph sacs and cardinal veins, which may physically assist separation. Uhrin *et al*. and Bertozzi *et al*. reported that platelet aggregates build up at the separation zone of podoplanin-positive lymph sacs and cardinal veins in wild-type embryos, but not in podoplanin-deficient or SLP-76-deficient embryos (*39*, *43*). Mice deficient in integrin αIIbβ3, which is necessary for platelet aggregation but not for granule release, do not show the non-separation phenotype (*43*). This finding supports the role of granule release. However, it has been reported that the non-separation phenotype is induced by inactivation of the *kindlin-3* gene required for platelet aggregation but not for granule release, suggesting that formation of platelet aggregates is more important for separation than granule release (*39*, *45*). Further study is required to definitively answer this question.

Mechanisms regulating blood/lymphatic separation, which appear to be unrelated to platelets, have also been suggested. Fasting-induced adipose factor (Fiaf) appears to regulate postnatal lymphatic partitioning from the blood vasculature in the small intestine. Mice deficient in Fiaf develop normally until birth, but have blood-filled lymphatic vessels in the small intestine after birth (*46*). Spred-1 and Spred-2 reportedly inhibit phosphorylation of MAP kinase following VEGF-C/VEGFR-3 signalling. Double-knockout mice that lack Spred-1 and Spred-2 showed embryonic lethality with dilated, blood-filled lymphatic vessels (*47*). The CLEC-2/podoplanin interaction may not be only one mechanism for regulating blood/lymphatic vessel separation.

Thrombus formation

It has recently been reported that anti-CLEC-2 antibody treatment of mice leads to loss of CLEC-2 in circulating platelets for several days. These CLEC-2-deficient platelets displayed normal adhesion under flow conditions, but subsequent thrombus formation was severely impaired *in vitro* and *in vivo* (*48*). In addition, tail-bleeding time was significantly prolonged in the antibody-induced CLEC-2-deficient mice, clearly revealing an essential role of CLEC-2 in haemostasis and thrombosis. This method involving antibody-induced removal of antigens from platelets is a valid approach, but certain questions remain. For example, CLEC-2 is also expressed on peripheral blood neutrophils in mice and mediates the production of TNF-α (*11*), which reportedly inhibits thrombus formation (*49*).

We have recently reported the phenotype resulting from genetically induced loss of CLEC-2 in mice. Because CLEC-2-deficient mice exhibit mortality at the embryonic/neonatal stages, we generated irradiated chimeric animals rescued by transplantation of a CLEC-$2^{-/-}$ fetal liver to investigate the role of CLEC-2 in thrombosis and haemostasis (CLEC-2 chimera) (*44*). In this study, CLEC-2-deficient platelets did not respond to rhodocytin as expected, but showed normal platelet aggregation in response to the classical platelet agonists collagen, thrombin and ADP, and normal platelet adhesion and spreading on surfaces coated with collagen, laminin, von Willebrand factor and fibrinogen. However, thrombus formation on the collagen-coated surfaces under flow conditions and laser-induced *in vivo* thrombus formation were inhibited in the CLEC-2 chimeras (*44*). These findings suggest that CLEC-2 is involved in thrombus stabilization *in vitro* and *in vivo*. We investigated the mechanism by which CLEC-2 stabilizes thrombus formation and proposed that CLEC-2 homophilically interacts with itself in a manner dependent on platelet activation (*44*). We observed that platelet adhesion to surfaces coated with recombinant CLEC-2 was significantly inhibited in CLEC-2-deficient mice, but CLEC-2-deficient platelets still bound to and spread on the surfaces (*44*). Therefore, we also proposed that there is another CLEC-2 ligand on the surface of platelets.

The phenotype of the CLEC-2 chimera is quite similar to that produced by antibody-induced removal of CLEC-2, further confirming the role of CLEC-2 in thrombosis. However, tail bleeding was different; the antibody-induced knockout, but not the CLEC-2 chimera, showed a significant increase in tail bleeding (*44*, *48*). Later, Hughes *et al*. independently generated CLEC-2 chimeras and reported that tail bleeding of the mice was not significantly increased (*50*), which is consistent with our findings. However, they concluded that CLEC-2 is not required for platelet aggregation at arteriolae based on their findings that *in vitro* thrombus formation under flow conditions on collagen-coated surfaces was not inhibited. The reason for the discrepancy between the two reports remains unclear, but it may be due to differences in genetic background (*44*, *50*) or subtle technical differences. Tang *et al*. (*10*) also reported on CLEC-2-deficient mice as a part of a project to generate a mouse knockout library for secreted and transmembrane proteins. Consistent with three other reports, lymph/blood misconnection and reduced viability were observed in their CLEC-2-deficient mice. Tail bleeding time was also compared between mice injected with recombinant CLEC-2-Fc and those injected with an isotype control. A soluble form of CLEC-2 led to a modest, non-significant ($P = 0.13$) increase in tail bleeding time when adult mice were infected (*10*), consistent with reports by Suzuki-Inoue *et al*. (*44*) and Hughes *et al*. (*50*).

While there is still controversy over the role of CLEC-2 in thrombosis and haemostasis, if CLEC-2 deficiency attenuates thrombus formation with only mild increase in bleeding tendency, CLEC-2 could be an ideal novel target protein for an anti-platelet drug that impairs pathological thrombus formation but not physiological haemostasis.

Concluding remarks

Investigation of the snake venom rhodocytin/aggretin has resulted in the discovery of the novel platelet activation receptor CLEC-2 and important new findings. Platelets regulate tumour metastasis and lymphangiogenesis through interaction between CLEC-2 and its endogenous ligand podoplanin. These results have stimulated new research areas linking platelet biology with oncology and embryology. Regarding clinical aspects of research on CLEC-2, research on the lectin receptor may produce therapeutic and preventive strategies for cancer and arterial thrombosis, which are leading causes of mortality in developed countries.

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Conflict of interest

None declared.

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