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Severe bleeding tendency and impaired platelet function in a patient with CalDAG-GEFI deficiency

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Background: Platelets are essential in normal hemostasis and pathological thrombosis, and their function is tightly regulated in circulation. Study of patients with bleeding disorders of platelet function provides quite important clue to find a signaling pathway and molecules required for platelet activation.
Aims: To investigate the cause of bleeding problem in 15-year-old Japanese girl.
Methods: The proband has been suffering from repeated severe spontaneous nasal bleeding which required transfusions since she was 1 year old. The initial laboratory assessment indicated normal platelet count, normal PT/APTT, markedly prolonged bleeding time, and reduced platelet aggregation responses to ADP and collagen. Recently she started suffering from menorrhagia and she was referred to our hospital. Peripheral bloods obtained from the proband and her parents were analyzed by flow cytometry, Western blotting, and sequencing. Written informed consent was obtained from all subjects.
Results: Expression levels of her platelet surface glycoproteins were comparable to those of control. Although PMA-induced integrin αIIbβ3 activation was normal, αIIbβ3 activation induced by various agonists was impaired. Platelet granule release was also decreased. In addition, slower αIIbβ3 activation kinetics was observed by 'initial velocity assay'. These results, along with normal calcium mobilization and impaired Rap1 activation, suggest impaired activation process of αIIbβ3. Western blotting revealed the deficiency of CalDAG-GEFI, but normal talin and Kindlin-3 expression in her platelets. Sequencing results revealed compound heterozygous mutation, R360del and K309X, in CalDAG-GEFI. Introduction of expression vector for mutant CalDAG-GEFI into 293T cells confirmed that these mutations are responsible for the deficiency of CalDAG-GEFI.
Conclusion: We identified new mutations of CalDAG-GEFI causing severe bleeding problem. Our results indicate the essential role of CalDAG-GEFI in αIIbβ3 activation as well as hemostatic function of platelets.
Disclosure of Interest: None declared.

Platelets and cancer

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15(S)-HETE is a pro-angiogenic factor produced by platelets through COX-1

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Background: Thromboxane A_2 (TXA_2) is a potent pro-thrombotic hormone produced at high concentrations by platelets through the activity of cyclooxygenase-1 (COX-1). Aspirin, by inhibiting COX-1, reduces TXA_2 levels which explains its use for secondary prevention of vascular thrombotic events. Epidemiological and observational analyses also demonstrate that chronic use of low-dose aspirin is associated with a reduction in risk for certain types of cancer, consistent with the anti-angiogenic properties of this drug.
Aims: Here we have identified 15-hydroxyeicosatetraeinoic acid (15(S)-HETE) as a major COX-1 product of platelets and investigated its role in angiogenesis.
Methods: Blood from healthy volunteers was incubated with aspirin either intact or as platelet rich plasma (PRP) prior to stimulation with thrombin receptor-activating peptide-6 (TRAP-6) or vehicle. Plasmas were separated and the levels of TXB_2 (TXA_2 marker) and 15-HETE were determined by LC-MS/MS, and those of the 15(S)-HETE stereoisomer by EIA assay. The angiogenic properties of 15(S)-HETE were studied by assessing tube formation of HMEC-1 cells and sprout formation by rat aortas incubated with plasmas obtained from TRAP-6-stimulated platelets pre-treated with or without aspirin.
Results: TRAP-6 caused marked increases in the platelet productions of TXA_2 and 15-HETE both of which were strongly inhibited by aspirin. Treatment of platelets with aspirin also strongly reduced the pro-angiogenic effects of platelet plasmas in both the endothelial tube formation and rat aorta sprouting models. Angiogenic responses were fully restored by addition of exogenous 15(S)-HETE.
Conclusion: 15(S)-HETE is a major platelet COX-1 product which, in association with other growth factors, has strong pro-angiogenic effects. Thus, 15(S)-HETE represents a potential target for the development of novel anti-angiogenic therapeutics.
Disclosure of Interest: None declared.

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Platelets engineered to store interleukin-24 inhibited melanoma growth in mice

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Background: Activated platelets secrete agents (e.g., cytokines) that can promote solid tumor growth & cancer metastasis. Previous studies showed hematopoietic stem cells (HSC) can be genetically modified to induce platelets to express & secrete proteins to establish hemostasis in animal models of bleeding disorders. Interleukin-24 (IL-24) is a cytokine (normally produced by activated monocytes, macrophages & T–helper cells) with cytotoxic & anti-angiogenic activity preferentially towards cancer cells. Thus, we hypothesized it may be feasible use HSC gene transfer to target platelet synthesis, storage & secretion of IL-24 to inhibit tumor growth.
Aims: To investigate if HSC gene transfer targeting IL-24 synthesis, storage & secretion from platelets can inhibit melanoma in mice.
Methods: C57BlL/6 mice were transplanted with bone marrow transduced with a lentiviral construct encoding a megakaryocyte–specific *ITGA2B* gene promoter driving synthesis of IL-24 gene. Four weeks after transplant, IL-24 protein was characterized in platelets by flow cytometry. Murine melanoma cells (1×10^6) were implanted in mice at 5 weeks after transplant. Tumor size was measured in IL–24 & control groups using a digital caliper periodically for 4 weeks. Then mice were sacrificed to record tumor mass.
Results: Flow cytometry showed that HSC gene transfer of murine bone marrow led to synthesis & storage of IL-24 in platelets. At 30 days after implant of melanoma cells, platelet IL–24 mice displayed significantly smaller (≈50%) tumor size (550 ± 101 mm^3) & weight (599 ± 120 mg) compared to control mice with larger tumor size (1120 ± 114 mm^3) & weight (1391 ± 134 mg) $n \approx 5$ mice/group ($P < 0.05$) indicating that platelet IL–24 inhibited tumor growth *in vivo*.
Conclusion: HSC gene transfer can be utilized to induce synthesis & storage of anti-oncogenic agent IL–24 in platelets. Melanoma tumor challenge in mice with platelet IL–24 showed a significant decrease in

tumor growth suggesting that platelets may serve as a therapeutic vehicle to treat cancer.
Disclosure of Interest: None declared.

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CLEC-2 facilitates hematogenous tumor metastasis and *in vitro* tumor growth, but not *in vivo* tumor growth

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Background: We reported that a platelet activation receptor, C-type lectin like receptor-2 (CLEC-2) facilitates hematogenous metastasis by binding to podoplanin, a membrane protein of tumor cells in ISTH 2013. We also reported that hematogenous metastasis of podoplanin-positive B16F10 melanoma, but not tumor growth, was inhibited in CLEC-2 −/− bone marrow chimeric mice, although tumor cell proliferation *in vitro* was facilitated by platelets depending on CLEC-2.
Aims: The aim of this study is to investigate why tumor growth *in vivo* is not inhibited in the absence of CLEC-2, although *in vitro* proliferation is increased by platelets depending on CLEC-2.
Methods: CLEC-2-depleted mice were generated by injection of anti-CLEC-2 antibody, 2A2B10 every 7 days. 1×10^6 cells of B16F10 was inoculated into the dorsal skin of mice 4 days after 2A2B10 or control IgG injection. Tumor growth was evaluated by measuring the size of tumors. Intra-tumor angiogenesis and thrombosis were analyzed by immunohistochemistry. Intra-tumor functional vessels were determined by injection of FITC-dextran from the tail vein before sacrifice. Survival was analyzed by Kaplan–Meier method.
Results: Tumor growth was not inhibited in CLEC-2-depleted mice and the number of intra-tumor vessels did not differ between CLEC-2-depleted and control mice. However, the number of functional vessels in tumors significantly increased in CLEC-2-depleted mice. Intra-tumor thrombus formation was decreased in CLEC-2 depleted mice. Unexpectedly, CLEC-2-depleted mice exhibited prolonged survival.
Conclusion: We suggest that CLEC-2 depletion results in decrease in intra-tumor thrombus formation, which leads to increase in intact tumor vessels and efficient supply of oxygen and nutrition to tumor cells *in vivo*. As a result, the growth-stimulating effect of platelets via CLEC-2 observed *in vitro* is canceled. Less thrombotic tendency may be related to prolonged survival, although the cause for this finding is now under investigation.
Disclosure of Interest: None declared.

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Identification of a novel C-type lectin-like receptor 2 inhibitor that suppresses podoplanin-induced platelet aggregation and cancer metastasis

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Background: The binding of podoplanin (PDPN) to C-type lectin-like receptor 2 (CLEC-2) is crucial in platelet activation, lymphagenesis and cancer metastasis. Blockage of CLEC-2 signaling is a rationale scheme for development of anti-cancer metastasis and anti-thrombosis regimens.
Aims: We aim to identify synthetic compound to intervene CLEC-2 signaling. The mechanistic insight and the potential application of the compound were investigated.
Methods: A pool of synthetic compounds was examined for their effects on agonists-induced platelet aggregation. The compound that shows specific inhibition on PDPN-induced platelet aggregation were selected for characterizing the mechanistic insight using phospho-specific antibodies, protein kinase profiling, molecular modeling and surface plasmon resonance analyses. The anti-cancer metastasis efficacy of the compound was evaluated by tumor cell-induced platelet aggregation (TCIPA) assay and measuring tumor metastasis in a xenograft mouse model.
Results: A compound 2CP was identified to selectively inhibit PDPN- but not other agonists-induced platelet aggregation (IC50 = 12.1 μM). The activities of all CLEC-2 downstream signaling proteins examined, including the newly identified mediators Akt1/PDK1 and PKCμ, were all affected by 2CP. Nevertheless, 2CP did not inhibit the activities of 25 selected protein kinases that play a role in platelet signaling. 2CP thereby does not appear to directly act on the cytoplasmic signaling proteins. Instead, 2CP bound CLEC-2 (Kd = 33.2 μM) and competed for the same PDPN binding residues of R107, R118, R152 and R157 in CLEC-2. 2CP was further defined to possess anti-cancer metastatic activity by impeding PDPN-mediated TCIPA and augmented the therapeutic efficacy of cisplatin. Pulmonary tumor foci formation and the body weight loss associated with the increase in tumor burden were both attenuated by cotreatment of 2CP and cisplatin.
Conclusion: 2CP is the first defined CLEC-2 inhibitor with potential for developing anti-cancer metastasis regimens.
Disclosure of Interest: None declared.