Platelets promote osteosarcoma cell growth through activation of the platelet-derived growth factor receptor-Akt signaling axis

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Osteosarcoma is the most common primary malignant bone tumor of children and adolescents and is derived from primitive mesenchymal cells. This disease is highly aggressive, and distant metastasis develop in approximately 45% of patients despite treatment with a potent neoadjuvant, which consists of high doses of multiple chemotherapeutic agents. Approximately 20% of patients have metastatic sites in the lungs or bones at diagnosis and have a poor prognosis despite aggressive surgery and chemotherapy. Thus, more effective therapeutic approaches are required for treating these patients.

The interactions of tumor cells with platelets play a critical role in the progression of tumor malignancy. Tumor cell-induced platelet aggregation enhances the rate of tumor embolization in the microvasculature and protects tumor cells from immunological assault and blood-shear stress. Moreover, several factors such as transforming growth factor-β, vascular endothelial growth factor, and platelet-derived growth factor (PDGF) are stored in platelet granules and are released during platelet aggregation. Such platelet-derived factors promote the epithelial-mesenchymal transition, tumor vascular angiogenesis, and vascular permeability. Further, experimentally induced thrombocytopenia and antiplatelet agents decrease the rate of lung metastasis in mouse models, indicating the requirement for platelets in the formation of hematogenous metastasis.

Osteosarcoma cells possess the potential to induce platelet aggregation, and there is positive correlation between the expression level of platelet aggregation-inducing factors and the potential of osteosarcomas to metastasize to the lungs. However, the effect of osteosarcoma-platelet interactions on the proliferation of osteosarcoma cells is unknown. We report here that osteosarcoma-platelet interactions induce the release of platelet-derived growth factor (PDGF) from platelets, which promotes the proliferation of osteosarcomas. Co-culture of platelets with MG63 or HOS osteosarcoma cells, which could induce platelet aggregation, enhanced the proliferation of each cell line in vitro. Analysis of phospho-antibody arrays revealed that co-culture of MG63 cells with platelets induced the phosphorylation of platelet derived growth factor receptor (PDGFR) and Akt. The addition of supernatants of osteosarcoma-platelet reactants also increased the growth of MG63 and HOS cells as well as the level of phosphorylated-PDGFR and -Akt. Sunitinib or LY294002, but not erlotinib, significantly inhibited the platelet-induced proliferation of osteosarcoma cells, indicating that PDGF released from platelets plays an important role in the proliferation of osteosarcomas by activating the PDGFR and then Akt. Our results suggest that inhibitors that specifically target osteosarcoma-platelet interactions may eradicate osteosarcomas.

Materials and Methods
Plasmid construction. The open reading frame (ORF) of a human codon-optimized variant of wild-type Zoonthus sp. green fluorescence protein (ZsGreen) was subcloned from the pZsGreen-N1 vector (Takara Bio, Shiga, Japan) into the pQCXIN retroviral vector (Takara Bio), and the resulting construct was designated pQCXIN-ZsGreen. Retroviral infection was performed according to the manufacturer’s protocols.

Cell lines. The human osteosarcoma cell lines, MG63 and HOS, were purchased from the American Type Culture
Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (DMEM growth medium). MG63 and HOS cells that had stably transfected with ZsGreen gene (MG63/ZsGreen and HOS/ZsGreen, respectively) were cultured in DMEM growth medium containing 400 μg/mL of G418 (Life Technologies, Carlsbad, CA, USA).

**Platelet preparation and aggregation assay.** Whole blood was drawn by cardiac puncture from Jcl: ICR mice terminally anesthetized with chloroform and taken with 0.38% sodium citrate solution or 10 units/mL of heparin. The blood was centrifuged at 150 g for 8 min to obtain platelet-rich plasma (PRP) from the supernatant. Washed platelets were prepared from pellets of PRP by centrifugation at 500 g for 10 min following washing with modified Tyrode’s buffer (137 mM NaCl, 11.9 mM NaHCO3, 0.4 mM Na2HPO4, 2.7 mM KCl, 1.1 mM MgCl2, and 5.6 mM glucose). Washed platelets were resuspended in modified Tyrode’s buffer containing 1% murine PPP and 200 μM CaCl2 and then incubated with phosphate-buffered saline (PBS) or osteosarcoma cells (2.5 × 104 cells/mL) for 30 min at 37°C. After centrifuging twice at 10,000 g for 10 min, the supernatants of the reaction mixtures were designated PBS-platelet reactant and osteosarcoma-platelet reactant, respectively.

**Animal Care and Use Committee.** Animals. Jcl:ICR mice were purchased from Clea Japan (Tokyo, Japan). The animal procedures followed protocols approved by the Japanese Foundation for Cancer Research Animal Care and Use Committee.

**Statistical analysis.** The Student’s t-test was performed to determine the statistical significance of the results of the proliferation assays. Significant P-values are defined as **P < 0.01, *P < 0.05. NS indicates a value that is not significant. All statistical tests were two-sided.

**Results**

Osteosarcoma-platelet interaction promotes platelet aggregation and osteosarcoma cell growth. Osteosarcomas form pulmonary metastasis by inducing platelet aggregation.(16,17) To assess the role of osteosarcoma-platelet interactions in determining the malignant phenotype of osteosarcomas, we first measured the abilities of the human osteosarcoma cell lines MG63 and HOS to induce platelet aggregation. We found that each osteosarcoma cell line induced platelet aggregation to an extent that is consistent with published studies (Fig. 1a). We next examined the influence of platelets on the growth of the osteosarcoma cell lines. Because of the high concentration of adenosine triphosphate (ATP) in platelets, we were unable to determine the growth of osteosarcoma cells using proliferation assays that measure ATP. Therefore, we generated stable transfectants of MG63 and HOS cells that expressed ZsGreen (MG63/ZsGreen and HOS/ZsGreen, respectively) and measured ZsGreen fluorescence to determine the number of viable cells. Although the growth rates of MG63/ZsGreen and HOS/ZsGreen cells were slow in the presence of 0.5% FBS, the growth rate of each cell line was significantly enhanced in proportion to the number of washed platelets added to the cultured cells (Fig. 1b). We found that the addition of the supernatant of an osteosarcoma-platelet reactant, but not that of the PBS-platelet reactant, significantly enhanced the growth of MG63/ZsGreen and HOS/ZsGreen cells (Fig. 1c). These results indicate that the proliferation of osteosarcoma cells is increased in the presence of platelets as well as by supernatants of osteosarcoma-platelet reactant.

**Activation of the PDGFR-Akt axis in platelet-induced osteosarcoma proliferation.** Platelets contain many growth factors and cytokines, including transforming growth factor-β, vascular endothelial growth factors, and PDGFs(6,7) which are stored in platelet granules and released on platelet aggregation. Such platelet-derived factors promote the epithelial-mesenchymal transition, tumor vascular angiogenesis, and tumor growth.(8)
To identify the mechanism that mediated the effects of platelets and supernatants of osteosarcoma-platelet reactants on osteosarcoma cell proliferation, we used arrays comprising a panel of antibodies specific for cytokines, growth factor receptors, or downstream signaling components. Incubation of the array with cell lysates prepared from reactants of MG63 cells and platelets increased the intensity of the spots corresponding to the positions of antibodies against phosphorylated PDGFRα, phosphorylated PDGFRβ, phosphorylated epidermal growth factor receptor (EGFR), and phosphorylated Akt1⁄2⁄3 antibodies (Figs. 2a–d). To exclude the possibility that the addition of platelets altered the respective protein expression levels in MG63 cells, we performed western blot analysis. Consistent with the array data, we confirmed the increase in phospho-PDGFRβ in MG63 cells that were co-cultured with platelets (Fig. 2e). Because PDGFRβ was not detected in the platelet lysate, indicating that co-culture induced PDGFRβ phosphorylation in MG63 cells. An increase in the level of phospho-Akt induced by co-culture was also detected using western blotting (Fig. 2e). Because the elecrophoretic mobility of mouse Akt in platelets appeared higher compared with human Akt in MG63 cells, the intensity of the phospho-Akt signal may represent phosphorylation of human but not mouse Akt. We obtained similar results using another HOS cell line (Fig. 2f). These data suggest that osteosarcoma-platelet interactions activate the PDGFR-Akt signaling pathway.

PDGFs released on the osteosarcoma cell-induced platelet aggregation contribute to the activation of the PDGFR-Akt signaling axis. Activation of PDGFRα and PDGFRβ is mediated by PDGFs, and only PDGF-BB can activate both PDGFRs. To determine whether PDGF-BB was released during the osteosarcoma cell-mediated platelet aggregation, we measured the amount of PDGF-BB in the supernatants of the osteosarcoma-platelet reactants using an enzyme linked immunosorbent assay (ELISA). The level of PDGF-BB was increased in supernatants of osteosarcoma-platelet aggregates compared with platelets alone (Fig. 3a). To assess the contribution of PDGFs to the activation of PDGFR-Akt axis, we treated MG63⁄ZsGreen and HOS⁄ZsGreen cells with the supernatant of the osteosarcoma-platelet reactant in the absence or presence of PDGFRs inhibitor, sunitinib. We found that the levels of phospho-PDGFRβ and phospho-Akt increased in osteosarcoma cell lines in the absence of sunitinib.
absence of, but not in the presence of sunitinib (Figs 3b,c). These results indicate that PDGFs released from activated platelets by the initiation of osteosarcoma-platelet interactions activated the PDGFR-Akt signaling axis.

Activation of the PDGFR-Akt signaling axis contributes to the platelet-dependent proliferation of osteosarcoma cell lines. To assess the role of the activation of the PDGFR-Akt axis in the growth of MG63 cells, we determined the effects of sunitinib, LY294002, or erlotinib, which inhibit the activity of the PDGFRs, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), or EGFR, respectively. Sunitinib and LY294002, but not erlotinib, inhibited the growth of MG63 and HOS cells when they were co-cultured with platelets (Figs 4a,b). These results indicate that activation of the PDGFR-Akt axis contributes to the platelet-dependent proliferation of osteosarcoma cell lines.
contributes to the platelet-dependent proliferation of osteosarcoma cells.

Discussion

Osteosarcoma is highly aggressive with distant metastasis, and approximately 20% of patients have metastases in lung or bone at diagnosis. Although the frequency of 5-year disease-free survival of patients with nonmetastatic osteosarcoma is approximately 70%, the average survival rate after recurrence in distant organs is only 1 year. Therefore, metastasis is the most common cause of death of patients with osteosarcomas as well as other cancers. There is a positive correlation between the expression level of platelet aggregation-inducing factors and the potential of osteosarcomas to metastasize to the lung. Mehta et al. suggest that stimulation of platelets by osteosarcoma cells correlates with their potential to metastasize to the lung.

The platelet receptor glycoprotein Ibα, sialyl Lewisα/sialyl Lewisβ, integrins, thrombospondin-1 (TSP-1), and Aggrus/podoplanin has been reported to induce platelet aggregation. Among them, TSP-1 and Aggrus are key molecules that mediate osteosarcoma-induced platelet aggregation. TSP-1 and Aggrus are expressed on cell surface, indicating that they may serve as targets of therapeutic antibodies against osteosarcoma. In fact, we confirmed Aggrus expression in the used two human osteosarcoma cell lines (Fig. S1a) and the addition of our established neutralizing anti-Aggrus antibody (MS-1) suppressed the MG63-dependent platelet aggregation and the PDGF release from platelets at a significant level (Fig. S1b,c). These results suggest that Aggrus expression on osteosarcomas contributes to the interaction with platelets and that Aggrus could be a therapeutic target of osteosarcoma. We also observed the MG63-induced platelet aggregation was suppressed by the addition of antibody against von Willebrand factor, which is one of the platelet aggregation was suppressed by the addition of anti-Aggrus antibody (MS-1). We also observed the MG63-induced platelet aggregation and the PDGF release from platelets in the presence of DMSO, the epidermal growth factor receptor (EGFR) inhibitor erlotinib (1 μM), the PDGFR inhibitor sunitinib (1 μM), or the PI3K inhibitor LY294002 (20 μM). After a 48-h incubation, cells were lysed and the fluorescence of ZsGreen was measured to determine relative cell growth. The error bars indicate the mean ± SD of triplicate experiments. *P < 0.01 and **P < 0.005 (Student's t-test). N.S, not significant.

Platelet derived growth factor (PDGF) and the PDGFR are implicated in the pathogenesis of sarcomas such as Ewing sarcoma, chondrosarcoma, rhabdomyosarcoma, intimal sarcoma, and osteosarcoma. Immunohistochemical analysis revealed that PDGFRα and PDGFRβ are frequently expressed in osteosarcomas (79.6% and 86%, respectively, n = 54), and the prognosis of patients with co-expression of PDGFRα and PDGFR-AA is significantly poorer. In the present study, osteosarcoma-platelet interactions promoted the proliferation of osteosarcoma cell lines through the activation of the PDGFR-Akt signaling axis (Fig. 2). However, sunitinib treatment partially suppressed the platelet-dependent proliferation of osteosarcoma cells (Fig. 4). Moreover, the inhibitory effects of a PI3K inhibitor LY294002, which functions downstream in the signaling pathways of certain receptor tyrosine kinases, were increased compared with sunitinib (Fig. 4). These results suggest the participation of other signaling pathways in the platelet-dependent proliferation of osteosarcomas. For example, insulin-like growth factor-1 (IGF-1), which is released from the α-granules of activated platelets, increases the growth of MG63 cells. Although we did not detect other phosphorylated proteins, including the IGF-1 receptor, using antibody arrays, other proliferative signals may contribute to the platelet-dependent proliferation of osteosarcomas. Therefore, specific inhibitors that block osteosarcoma-platelet interactions may be useful for the suppression of platelet-dependent proliferation of osteosarcoma.

The use of chemotherapeutic agents is essential for the treatment of osteosarcoma patients; however, the efficacy of the current treatment regimen including adriamycin, which was originally developed in the mid-1980s, is limited. Apoptosis induced by adriamycin was attenuated by co-culture with platelets in osteosarcomas (Fig. S3). Moreover, their invasiveness was promoted by co-culture with platelets (Fig. S4). Because prior administration of neutralizing anti-Aggrus antibodies has been reported to prevent hematogenous metastasis of Aggrus-positive tumor cells in mouse models and to attenuate PDGF release from platelets (Fig. S1c), the combination therapy of anti-Aggrus antibodies with standard chemotherapeutic agents may be effective for inhibiting the proliferation of osteosarcomas and for preventing metastasis.

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Disclosure Statement

The authors have no conflict of interest.
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Supporting Information

Fig. S1. Involvement of Aggrus/podoplanin in platelet aggregation and PDGF release during osteosarcoma cell-induced platelet aggregation.

Fig. S2. Attenuation of MG63-dependent platelet aggregation by an anti-von Willebrand factor (vWF) antibody.

Fig. S3. Co-culture with platelets contributes to the resistance to apoptosis induced by adriamycin in osteosarcoma cells.

Fig. S4. Platelets promote invasiveness of osteosarcomas.