

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

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Key words

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The interactions of tumor cells with platelets contribute to the progression of tumor malignancy, and the expression levels of platelet aggregation-inducing factors positively correlate with the metastatic potential of osteosarcoma cells. However, it is unclear how tumor-platelet interaction contributes to the proliferation of osteosarcomas. 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.

Osteosarcoma is the most common primary malignant bone tumor of children and adolescents and is derived from primitive mesenchymal cells.⁽¹⁾ 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.⁽²⁾ Approximately 20% of patients have metastatic sites in the lungs or bones at diagnosis⁽³⁾ and have a poor prognosis despite aggressive surgery and chemotherapy.⁽⁴⁾ 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.⁽⁵⁾ 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.^(6,7) Such platelet-derived factors promote the epithelial-mesenchymal transition, tumor vascular angiogenesis, and vascular permeability.^(8,9) 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.^(10–12)

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.⁽¹³⁾ 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 PDGF from platelets and enhance the proliferation of osteosarcomas. Co-culture of osteosarcoma cells with platelets promoted the proliferation of osteosarcoma cells. Analysis of phospho-antibody arrays revealed that the osteosarcoma-platelet interaction increased the phosphorylation of PDGFR and Akt. Using kinase inhibitors, phosphorylation of PDGFR and Akt were shown to be important for the platelet-dependent proliferation of osteosarcoma cells. These results suggest that osteosarcoma-platelet interactions initiate platelet aggregation, release PDGF from activated platelets, and activate the PDGFR-Akt signaling pathway to increase the growth of osteosarcoma cells.

Materials and Methods

Plasmid construction. The open reading frame (ORF) of a human codon-optimized variant of wild-type *Zoanthus* 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).

Immunoblot analysis. Sample preparation was performed as described previously.⁽¹⁴⁾ Briefly, cells were lysed in TENS buffer (50 mM Tris-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 1 mM Na₃VO₄, 1% NP-40, 0.1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride), and electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were transferred to a membrane and immunoblotted with an anti-Akt (pan) monoclonal antibody (mAb) (clone C67E7, Cell Signaling Technology, Danvers, MA, USA), anti-phospho-Akt (Ser473) mAb (clone D9E, Cell Signaling Technology), anti-PDGFRβ polyclonal antibody (P-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-PDGFRβ mAb (clone 42F9, Cell Signaling Technology), and anti-α-tubulin mAb (clone YL1/2, AbD Serotec, Kidlington, UK). The LAS-3000 mini system (Fujifilm, Tokyo, Japan) was used for visualization and quantification of signals.

Human phospho-RTK and human phospho-kinase arrays. Phosphorylation of signaling molecules was estimated using the Human Phospho-RTK Array Kit (ARY001B, R&D Systems, Minneapolis, MN, USA) and Human Phospho-Kinase Array Kit (ARY003B, R&D Systems) according to the manufacturer's protocols. Briefly, MG63 cells were co-cultured with buffer or platelets for 2 h. Three hundred micrograms of total cell lysates were incubated with each array. Proteins were detected using horse radish peroxidase (HRP)-conjugated mouse anti-phosphotyrosine antibody or streptavidin-HRP. Data were acquired using the LAS-3000 mini system. Image quantification was performed using Multi Gauge ver.3.0 software (Fujifilm). The signal intensities of duplicate spots were quantified.

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 1–2% murine platelet-poor plasma (PPP), and 200 or 250 µM CaCl₂ (each concentration used are shown in figure legends) was added to the platelet suspensions before starting the experiments. Platelet suspensions (200 µL) in the reaction tubes were stirred at 37°C and preincubated for 2 min before the addition of osteosarcoma cells. The platelet aggregation assay was performed using a platelet aggregometer (MCM HEMA TRACER 313M; SSR Engineering, Kanagawa, Japan) as previously described.⁽¹⁵⁾

Cell viability assay. MG63/*ZsGreen* and HOS/*ZsGreen* cells were suspended in DMEM medium containing 0.5% FBS (0.5 × 10⁴ and 2.0 × 10⁴ cells/mL, respectively) and seeded 0.1 mL in a 96-well plate. After overnight incubation, cells were co-cultured with washed platelets resuspended in modified Tyrode's buffer containing 200 µM CaCl₂. At the appropriate times, supernatants were removed, and TENS buffer was added to the cultured cells. The fluorescence of *ZsGreen*

in cell lysates was measured using a TriStar LB941 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). Buffer alone indicates the treatment of the cells with modified Tyrode's buffer containing 200 µM CaCl₂. In some experiments, the supernatant harvested from osteosarcoma-platelet reactants was added to the cultured osteosarcoma cells instead of platelets.

Preparation of supernatants of osteosarcoma cell-platelet reactants. Washed mouse platelets were prepared using 0.38% sodium citrate as described in the platelet preparation. Platelets (2.0 × 10⁸ platelets/mL) 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 × 10⁵ 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.

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⁾

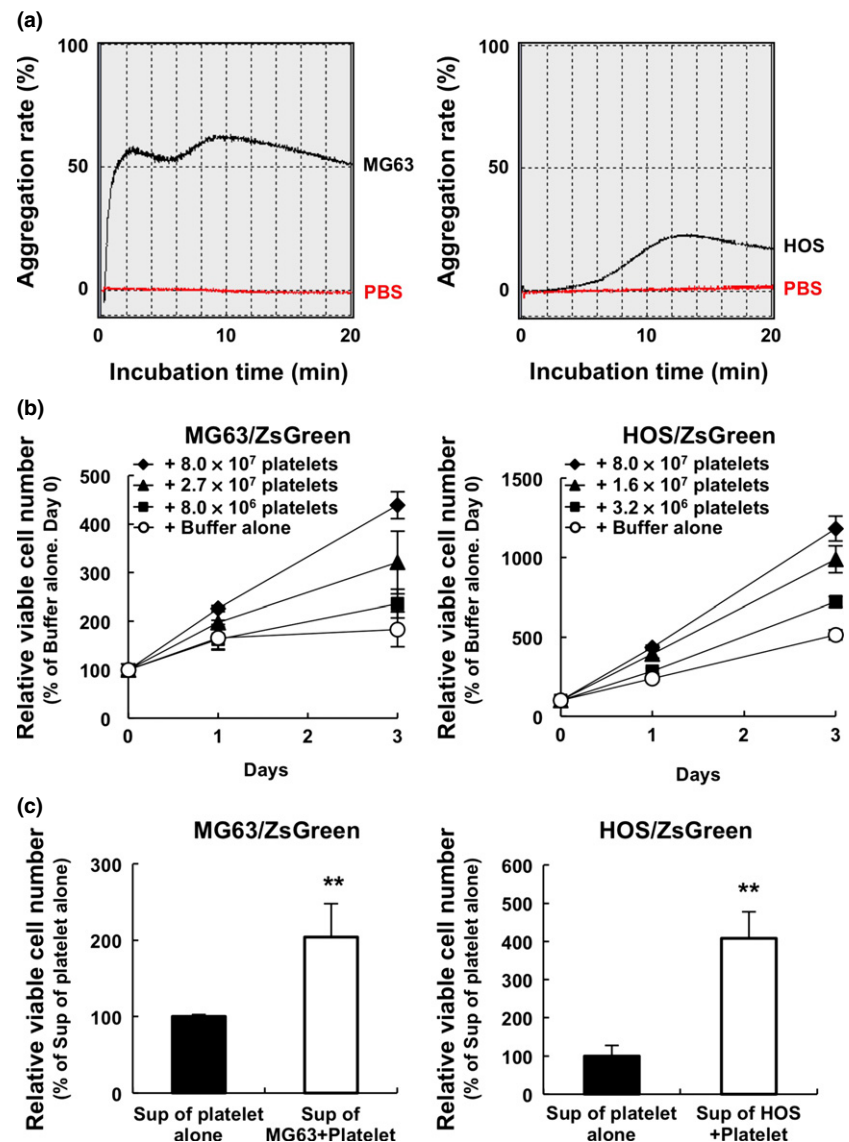


Fig. 1. Co-culture with platelets promotes platelet aggregation and the proliferation of osteosarcoma cells. (a) Platelets (4.0×10^7) prepared with heparin from the whole blood of Jcl:ICR mice were resuspended in modified Tyrode's buffer containing 2% PPP and $250 \mu\text{M}$ CaCl_2 , followed by incubation with PBS (red lines) or osteosarcoma MG63 (left panel, black line) and HOS (right panel, black line) cells (1.0×10^8 cells). The transmission of light by the samples was measured using an aggregometer to determine the aggregation rate. (b) MG63 and HOS cells stably transfected with ZsGreen gene, MG63/ZsGreen (left panel) and HOS/ZsGreen (right panel), respectively, were co-cultured with the indicated number of washed mouse platelets in medium containing 0.5% FBS. TENS buffer was added to each well at the times indicated, and the fluorescence of ZsGreen was used to determine the number of viable osteosarcoma cells. The error bars indicate the mean \pm standard deviation (SD) of triplicate experiments. (c) Washed mouse platelets (2.0×10^7 platelets) were resuspended in modified Tyrode's buffer containing 1% PPP and $200 \mu\text{M}$ CaCl_2 followed by incubation with PBS or osteosarcoma cells (2.5×10^4 cells) for 30 min. After centrifugation twice at 10 000 g for 10 min, the supernatant of osteosarcoma-platelet reactants or the PBS-platelet reactant was added to the cultures of MG63/ZsGreen (left panel) and HOS/ZsGreen (right panel) cells. After 24 h, TENS buffer was added to each well and the fluorescence of ZsGreen was used to determine the relative number of viable cells. The error bars indicate the mean \pm SD of triplicate experiments. ****** $P < 0.01$ by the Student *t*-test.

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 PDGFR α , phosphorylated PDGFR β , 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-PDGFR β in MG63 cells that were co-cultured with platelets (Fig. 2e). Because PDGFR β was not detected in the platelet lysate, indicating that co-culture induced PDGFR β 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 electrophoretic 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 PDGFR α and PDGFR β 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-PDGFR β and phospho-Akt increased in osteosarcoma cell lines in the

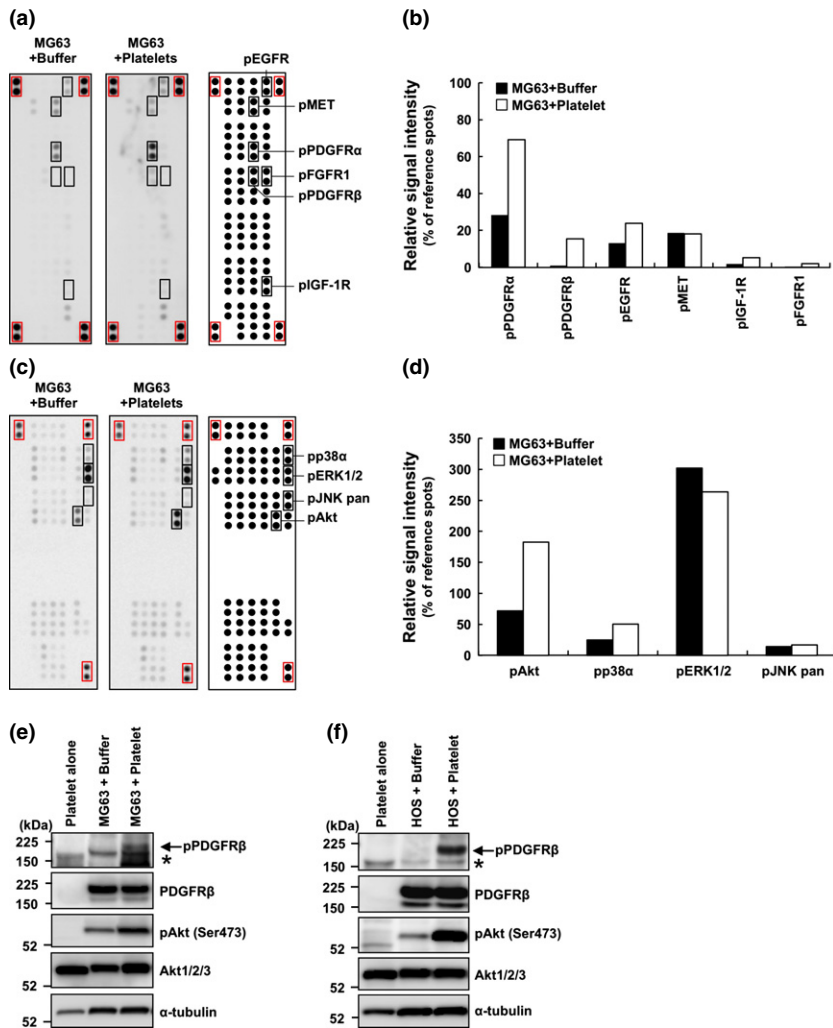


Fig. 2. Activation of the platelet derived growth factor receptor (PDGFR)-Akt signaling axis in MG63 cells co-cultured with platelets. (a, b) Platelets prepared with heparin from the whole blood of Jcl:ICR mice were resuspended in modified Tyrode's buffer containing 200 μ M CaCl₂. MG63/ZsGreen cells were co-cultured with buffer alone (MG63 + Buffer) or platelets (MG63 + Platelet) for 2 h. The preparation of cell lysates and incubation with the human phospho-RTK array were performed according to the manufacturer's protocol. Representative images of the probed arrays are shown (a). The signal intensity of each spot was determined using a LAS-3000 mini and quantified using Multi Gauge ver.3.0 software. The signal intensities of eight reference spots (within red squares) in each membrane were measured and defined as 100%. The relative intensities of duplicate spots are shown (b). (c, d) Analysis of the human phospho-kinase array using lysates prepared from MG63 cells co-cultured with platelets in modified Tyrode's buffer containing 200 μ M CaCl₂. MG63/ZsGreen cells were cultured with buffer alone (MG63 + Buffer) or with platelets (MG63 + Platelet) for 2 h. The preparation of cell lysates and incubation with the human phospho-kinase array were performed according to the manufacturer's protocol. Representative images of reacted membranes are shown (c). The signal intensity of each spot was measured using a LAS-3000 mini and quantified using Multi Gauge ver.3.0 software. The signal intensities of six reference spots (red squares) in each membrane were defined as 100%. The relative intensities of duplicate spots are shown (d). (e, f) Platelets prepared with 0.38% sodium citrate were resuspended Tyrode's buffer containing 200 μ M CaCl₂. MG63/ZsGreen (e) or HOS/ZsGreen (f) cells were incubated with buffer alone or platelets ($2.0 \times 10^7/24$ -well) for 2 h. Platelets alone, osteosarcoma cells alone (+Buffer) or co-cultures of osteosarcoma cells with platelets (+Platelet) were lysed and immunoblotted using the antibodies to phospho-PDGFR β , PDGFR β , phospho-Akt (S473), Akt, or α -tubulin.

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

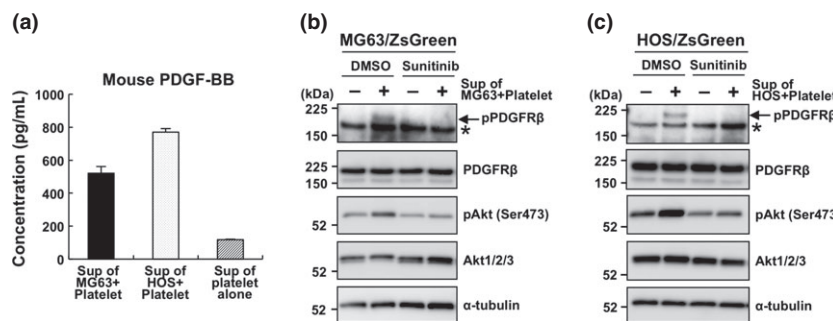


Fig. 3. Platelet-derived growth factors (PDGFs) released on the osteosarcoma-induced platelet aggregation contribute to the activation of the PDGFR-Akt signaling axis. (a–c) Washed mouse platelets (2.0×10^7 platelets) were resuspended in modified Tyrode's buffer containing 1% PPP and 200 μ M CaCl₂ followed by incubation with PBS or with the indicated osteosarcoma cells (2.5×10^4 cells) for 30 min. After centrifuging twice at 10 000 g for 10 min, the concentration of PDGF-BB in the supernatants was measured using an enzyme linked immunosorbent assay (ELISA) (a). The cultured MG63/ZsGreen (b) and HOS/ZsGreen (c) cells were treated with (+) or without (–) the supernatant of osteosarcoma-platelet aggregates in the presence of DMSO or 1 μ M sunitinib. After a 30-min incubation, cells were lysed with TENS buffer and immunoblotted with the indicated antibodies.

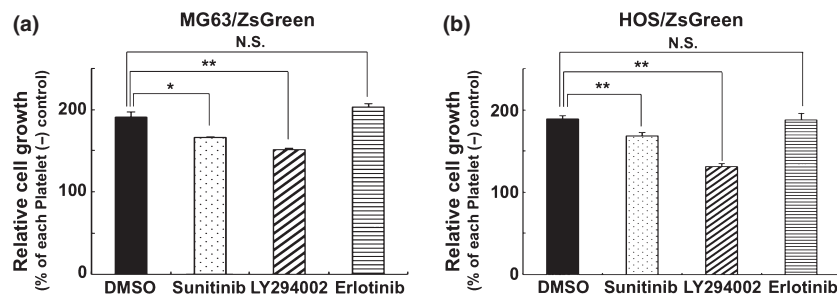


Fig. 4. Involvement of the platelet-derived growth factor receptor (PDGFR) -Akt signaling axis in the platelet-induced growth of osteosarcoma cells. (a, b) MG63/ZsGreen (a) or HOS/ZsGreen (b) cells in 24-well were co-cultured with platelets (2×10^7 in Tyrode's buffer containing $200 \mu\text{M}$ CaCl_2) in the presence of DMSO, the epidermal growth factor receptor (EGFR) inhibitor erlotinib ($1 \mu\text{M}$), the PDGFR inhibitor sunitinib ($1 \mu\text{M}$), or the PI3K inhibitor LY294002 ($20 \mu\text{M}$). 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 \pm SD of triplicate experiments. ** $P < 0.01$ and * $P < 0.05$ (Student's *t*-test). NS, not significant.

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.⁽¹⁸⁾ Although the frequency of 5-year disease-free survival of patients with nonmetastatic osteosarcoma is approximately 70%,⁽¹⁹⁾ the average survival rate after recurrence in distant organs is only 1 year.⁽²⁰⁾ 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.⁽¹³⁾ Mehta *et al.*⁽²¹⁾ suggest that stimulation of platelets by osteosarcoma cells correlates with their potential to metastasize to the lung.

The platelet receptor glycoprotein Ib α , sialyl Lewis^x/sialyl Lewis^a, integrins, thrombospondin-1 (TSP-1), and Aggrus/podoplanin has been reported to induce platelet aggregation.^(16,22–26) Among them, TSP-1 and Aggrus are key molecules that mediate osteosarcoma-induced platelet aggregation.^(17,18) 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)⁽¹⁴⁾ 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-mediating molecules and known to be overexpressed in metastatic osteosarcoma (Fig. S2).⁽¹³⁾ Thus, von Willebrand factor also plays some roles in osteosarcoma-mediated platelet aggregation.

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.^(27–30) Immunohistochemical analysis revealed that PDGFR α and PDGFR β are frequently expressed in osteosarcomas (79.6% and 86%, respectively, $n = 54$), and the prognosis of patients with co-expression of PDGFR α and PDGF-AA is significantly poorer.⁽¹⁹⁾ 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 osteosarcoma cells. 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.⁽³¹⁾ 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.⁽¹⁹⁾ 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^(8,14,32) 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

Additional supporting information may be found in the online version of this article:

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.