Role of Platelet Membrane Glycoproteins Ib/IX and IIb/IIIa, and of Platelet α-Granule Proteins in Platelet Aggregation Induced by Human Osteosarcoma Cells

Philippe Clezardin, Jeanne Drouin, Marie-Christine Morel-Kopp, Michel Hanss, Beate Kehrel, Claire-Marie Serre, Cécile Kaplan, and Pierre D. Delmas

INSERM Research Unit 234, Pavillon F [P. C., C-M. S., P. D. D.] and Pavillon E [M. H.], Hôpital Edouard Herriot, Place d’Arsonval, 69437 Lyon, France; Division of Hematology, Ottawa General Hospital, Ottawa, Ontario, Canada [J. D.]; Service Immunologie Léuco-Plaquettaires, Institut National de Transfusion Sanguine, Rue Alexandre-Cabanel, 75739 Paris, France [C. K., M-C. M-K.]; and Experimentelle Hämostaseforschung, Innere Medizin A, Universitätskliniken Münster, Germany [B. K.].

ABSTRACT

We have previously shown that the platelet-aggregating activity of human MG-63 and HOS osteosarcoma cells depends at least in part upon tumor cell surface-associated thrombospondin, and suggested that platelet-osteosarcoma cell interactions could occur through interactions with specific platelet membrane receptors. In this study, the platelet-aggregating activity of MG-63 and HOS cells was studied by using a variety of platelet disorders. Both osteosarcoma cell lines induced a biphasic platelet aggregation response when added to normal platelet-rich plasma, while the second phase of aggregation was absent when added to gray platelets (deficiency in α-granule proteins) and to aspirin-treated platelets. Platelets from two unrelated patients with type I Glanzmann’s thrombasthenia (deficiency in glycoprotein (GP) GPIb/IIa) did not aggregate at all with osteosarcoma cells. Using giant platelets from three patients with Bernard-Soulier syndrome (deficiency in GP Ib/IX), the aggregation response induced by MG-63 and HOS cells was metaphase and reversible when compared to normal-sized platelets and to giant platelets from a patient with May-Hegglin anomaly (no membrane GP defect). Because GPIb serves as a receptor for von Willebrand factor during hemostasis, aggregation experiments were also conducted with the platelet-rich plasma of two patients with a low plasma von Willebrand factor concentration (type I von Willebrand’s disease) before and after the infusion of deamino-arginine vasopressin. MG-63 and HOS cells induced biphasic platelet aggregation both before and after deamino-arginine vasopressin treatment, while the ristocetin-dependent binding of von Willebrand factor to platelets only occurred after deamino-arginine vasopressin treatment. Preincubation of normal platelet-rich plasma with monoclonal antibody SZ-2 directed against the von Willebrand binding domain of GPIb did not inhibit the platelet-aggregating activity of osteosarcoma cells, whereas anti-GPIb antibody SZ-2 did inhibit ristocetin-induced platelet agglutination. In addition, anti-GPIX antibodies did not affect platelet-osteosarcoma cell interactions. In conclusion, our data demonstrate that the first phase of the platelet-aggregating activity of human osteosarcoma cells is initiated by the interaction of these tumor cells with platelet membrane GPIb/IIa, whereas the second phase, even if plasma von Willebrand factor is deficient, involves platelet membrane GPIb and the participation of platelet α-granule proteins in membrane-mediated events, making aggregation irreversible.

INTRODUCTION

Metastasis is a complex phenomenon that involves tumor cell dissemination, and tumor cell interactions with host cells (platelets, endothelial cells) and subendothelial matrix (For review see Ref. 1). The survival of circulating metastatic cells and the attachment of tumor cells to endothelial cells and subendothelial matrix largely depend on the interaction of tumor cells with platelets (1-3). Such an interaction of tumor cells with platelets involves a series of sequential events (1, 4). Initially, a few platelets adhere to tumor cells which activate them through expression of prothrombogenic activities, depending on the origin of the tumor cells. This results in the recruitment of additional platelets, and formation of focal aggregates of activated, degranulated platelets attached to the tumor cell surface. Subsequently, tumor cells respond to activated platelets with the appearance of cellular processes that interdigitate with platelet aggregates, suggesting tumor cell cytoskeletal alterations. Several cell surface adhesive proteins have been implicated in linking platelets to tumor cells. Among these adhesive proteins are fibronectin (5), von Willebrand factor (5), fibrinogen (6), and thrombospondin (7, 8). During hemostasis, von Willebrand factor binds to platelet membrane glycoprotein complex GPIb/IX, and the membrane glycoprotein complex GPIb/IIa serves as a platelet receptor for fibrinogen, fibrinectin, von Willebrand factor, and thrombospondin (for review see Ref. 9). Adhesive proteins are also secreted from α-granules upon platelet activation and help to stabilize platelet aggregates (10). The functions of these major platelet glycoproteins have been elucidated by the study of platelets from patients with Bernard-Soulier syndrome, Glanzmann’s thrombasthenia, and gray platelet syndrome which lack GPIb/IX, GPIb/IIa and α-granule proteins, respectively (9, 11). Immunoinhibition has been widely used in evaluating the effects of antibodies against platelet GPIb/IIa on the platelet-aggregating activity of tumor cells (5, 12-15). Pretreatment of platelets with anti-GPIb/IIa monoclonal antibodies inhibit both the binding of mouse CT26 and human HCT8 colon carcinoma cells to platelets (5), and the platelet-aggregating activity of different tumor cell lines, including human HMV-I melanoma cells and M7609 colon carcinoma cells (12-15). By contrast, the significance of the results obtained with anti-GP Ib monoclonal antibodies remains unclear. Anti-GPIb monoclonal antibodies have no effect on the binding of mouse CT26 and human HCT8 colon carcinoma cells to platelets (5) and on the platelet-aggregating activity of M3Dau melanoma cells (14), while they inhibit the platelet-aggregating activity of human HMV-I melanoma cells and M7609 colon carcinoma cells (15). We have previously shown that the platelet-aggregating activity of human osteosarcoma cells depend at least in part upon tumor cell surface-associated thrombospondin, and suggested that platelet-osteosarcoma cell interactions could occur through interactions with specific platelet membrane receptors (8). However, it is not known whether platelet receptors such as GPIb/IX or GPIb/IIIa are involved during platelet aggregation induced by osteosarcoma cells, or if platelet-released α-granule proteins play a role in this context. The present study investigates the role of platelet membrane glycoproteins GPIb/IX and GPIb/IIa, and of α-granule proteins during platelet aggregation induced by human osteosarcoma cells, using...
were obtained from the American Type Culture Collection. Osteosarcoma cells bodies in polymorphonuclear leukocytes. Two patients (P. S. and W. M.) with investigated. Historical review revealed life-long easy bruising; hemorrhages bleeding problems. One patient (R. D.) with May-Hegglin anomaly was in-MATERIALS AND METHODS mission at 100%. The experiment was repeated twice with similar results. The addition of platelet syndrome, and a panel of monoclonal antibodies directed concentrations (0.6 to 2 x 10^6 cells/ml). Platelet count in platelet-rich plasma was platelet-rich plasma from patients with Glanzmann's thrombasthenia, human MG-63 osteosarcoma cells were added to platelet-rich plasma at various cell 0.2% (mass/volume) bovine serum albumin. Cell suspensions had viabilities of found to have thrombocytopenia (46 x 10^9/liter), giant platelets, and D6hle platelet syndrome, and a panel of monoclonal antibodies directed against GPIb and GPIX.

**PLATELET GPs IN OSTEOSARCOMA CELL-INDUCED PLATELET AGGREGATION**

**Fig. 1. Effect of osteosarcoma cell concentration on platelet aggregation.** EDTA-treated human MG-63 osteosarcoma cells were added to platelet-rich plasma at various cell concentrations (0.6 to 2 x 10^6 cells/ml). Platelet count in platelet-rich plasma was adjusted to 2 x 10^8 platelets/ml. Platelet-poor plasma was used to calibrate light transmission at 100%. The experiment was repeated twice with similar results. The addition of MG-63 cells is indicated by the short arrow.

platelet-rich plasma from patients with Glanzmann’s thrombasthenia, Bernard-Soulier syndrome, type I von Willebrand’s disease, or gray platelet syndrome, and a panel of monoclonal antibodies directed against GPIb and GPIX.

**MATERIALS AND METHODS**

**Patients.** Two unrelated patients with type I Glanzmann’s thrombasthenia (T. I.) and (SCH. U.) (14, 16), and one patient with gray platelet syndrome (K. A-M.) (17) have been described previously. Three patients with Bernard-Soulier syndrome (O. N.), (B. J-P.), and (B. G.) were studied; they had experienced severe epistaxes, hemorrhages following dental surgery, and menorrhagia. They all had a prolonged bleeding time, a platelet count of 30 to 60 x 10^9/liter, giant platelets, and absence of ristocetin-induced aggregation. Flow cytometry studies revealed a marked decrease of platelet GPIb by using monoclonal antibody SZ2. One obligatory heterozygote (B. L.), the mother of B. G. and B. J-P. was also studied; she had normal platelet function and no bleeding problems. One patient (R. D.) with May-Hegglin anomaly was investigated. Historical review revealed life-long easy bruising; hemorrhages occurred at her first delivery, after mammoplasty and dental surgery. She was found to have thrombocytopenia (46 x 10^9/liter), giant platelets, and Döhle bodies in polymorphonuclear leukocytes. Two patients (P. S. and W. M.) with type I von Willebrand’s disease were studied; they both had mild bleeding, a 50 to 70% decrease of the total amount of circulating von Willebrand factor, and absence of ristocetin-induced platelet aggregation.

**Cell Cultures.** Human osteosarcoma cell lines designated MG-63 and HOS were obtained from the American Type Culture Collection. Osteosarcoma cells to be used in aggregation studies were harvested from culture dishes with Ca^{2+}, Mg^{2+}-free Hanks' solution containing 10 mm EDTA. After incubation, EDTA-treated cells were diluted with a 5-fold excess of Hanks’ solution. Cell suspensions were then centrifuged at 160 x g for 4 min, the supernatants were removed, and the cell pellets were resuspended in Hanks’ solution containing 0.2% (mass/volume) bovine serum albumin. Cell suspensions had viabilities of approximately 80 to 90% by trypan blue exclusion. Antibodies. Mouse monoclonal antibodies SZ1 and SZ2 are directed against GPIX (CD42a) and the α chain of platelet GPIb (CD 42b), respectively (Immunotech, France). Mouse monoclonal antibodies P5 and P35 (clones FMC25 and GR-P, respectively) directed against platelet GPIX (CD42a), and mouse monoclonal antibody Gi27 directed against the β chain of platelet GPIb were obtained from the IVth and Vth International Workshop on Leucocyte Typing (18). Goat anti-mouse IgG conjugated with fluorescein isothiocyanate was obtained from Coulter Immunology.

**Platelet Aggregation.** Platelet-rich plasma was obtained by centrifugation of freshly drawn blood of healthy donors and patients [collected in heparin (10 units/ml)] at room temperature for 20 min at 160 x g. The remaining blood was centrifuged at 1500 x g for 15 min to obtain platelet-poor plasma. Platelet-poor plasma was used to calibrate light transmission at 100%. The platelet count in platelet-rich plasma was adjusted to 2 x 10^8 platelets/ml for each experiment. EDTA-treated osteosarcoma cells (10^6 cells/50 μl) in Hanks’ solution containing 0.2% (mass/volume) bovine serum albumin were either preincubated with an antibody and washed to remove unbound antibodies or directly added to the platelet-rich plasma. The aggregation pattern was then recorded in a Chrono-Log Dual Channel aggregometer at 37°C with continuous stirring.

**Electron Microscopy of Platelet-Osteosarcoma Cell Interaction.** At the peak of the aggregation curve, platelet-osteosarcoma cell aggregates were allowed to settle at the bottom of Eppendorf tubes. Cell aggregates were washed twice in phosphate-buffered saline, and fixed with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4°C. After incubation, fixed cells were washed 3 times in cacodylate buffer, pH 7.2, containing 0.2 M sucrose. Cells were then postfixed with 1% (mass/volume) osmic acid in 0.3 M cacodylate buffer, dehydrated with graded alcohols, and finally embedded in Epon. Ultrathin sections were cut with an ultramicrotome (UltraCut E Reichert), contrasted with uranyl acetate and lead citrate, and observed on a JEOL 1200EX electron microscope.

**RESULTS**

As previously observed (8), MG-63 and HOS cells in suspension induced aggregation of platelets in a dose-dependent fashion when added to platelet-rich plasma obtained from healthy donors. Increasing the number of MG-63 cells caused a progressive increase in platelet aggregation with a maximum effect at 2 x 10^8 cells/ml (Fig. **Fig. 2. Effect of human MG-63 osteosarcoma cells on aggregation of platelets from patients with Glanzmann's thrombasthenia, Bernard-Soulier syndrome, or May-Hegglin anomaly versus platelets from a normal donor (control).** EDTA-treated MG-63 cells (2 x 10^6 cells/ml) were directly added to the platelet-rich plasma (2 x 10^8 platelets/ml) and the aggregation pattern was recorded as described in the legend of Fig. 1. The addition of MG-63 cells is indicated by the short arrow.
Similar results were obtained with HOS cells. The aggregation patterns were usually biphasic, resembling those observed for ADP- and epinephrine-induced platelet aggregation. Since the aggregation response to osteosarcoma cells was found to be optimal at a concentration of $2 \times 10^6$ cells/ml, this cell concentration was therefore used in all subsequent experiments.

In order to assess the involvement of platelet GPIb/IX and GPIIb/IIIa during platelet aggregation induced by MG-63 and HOS osteosarcoma cells, platelets deficient in either GPIIb/IIIa (type I Glanzmann’s thrombasthenia) or GPIb/IX (Bernard-Soulier syndrome) were studied. In contrast to normal platelets, platelets from two unrelated patients with type I Glanzmann’s thrombasthenia did not aggregate with MG-63 cells (Fig. 2). Electron microscopy studies showed that aggregates of normal platelets closely interacted with MG-63 cells (Fig. 3A) while thrombasthenic platelets did not (Fig. 3B). Aggregometry and electron microscopy experiments performed with thrombasthenic platelets and HOS cells gave results similar to those obtained with MG-63 cells (results not shown). Using platelets from three patients with Bernard-Soulier syndrome, the aggregation response to MG-63 osteosarcoma cells was monophasic and reversible when compared to normal platelets (Fig. 2). Platelets from a parent (obligatory heterozygote) aggregated normally with MG-63 cells (results not shown). Because of the large size of Bernard-Soulier platelets, aggregation studies were also performed with platelets from a patient with May-Hegglin anomaly. This condition is also associated with giant platelets but there are no membrane glycoprotein abnormalities (19). These giant platelets were aggregated by MG-63 cells to an extent similar to that observed with normal sized platelets (Fig. 2).
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Electron microscopy studies revealed that aggregates of May-Hegglin platelets attached firmly to the surface of MG-63 cells (Fig. 3C), while Bernard-Soulier platelets did not interact at all with these osteosarcoma cells (Fig. 3D). Using May-Hegglin and Bernard-Soulier platelets, aggregometry and electron microscopy experiments performed with HOS cells gave similar results (results not shown).

Platelet GPIb/IX serves as a receptor for von Willebrand factor (9), and both von Willebrand factor (5) and GPIb are involved in platelet-tumor cell interaction (13, 15). In order to gain further insight into the role of GPIb/IX in the platelet-aggregating activity of human osteosarcoma cells, aggregation experiments were conducted with the platelet-rich plasma of two patients with type I von Willebrand's disease before and after DDAVP treatment. DDAVP treatment results in a moderate increase in plasma von Willebrand factor that is sufficient to protect a patient from hemorrhage. As shown on Fig. 4, platelets from patient P. S. were poorly agglutinated with ristocetin (2 mg/ml) because of the low level (30%) of plasma von Willebrand factor. Similar results were obtained with platelets from patient W. M. (50% decrease of plasma von Willebrand factor). By contrast, DDAVP treatment of these two patients restored normal ristocetin-induced platelet agglutination (Fig. 4). On the other hand, irrespective of the treatment of these two patients restored normal ristocetin-induced platelet agglutination (Fig. 4). On the other hand, irrespective of the treatment of these two patients, aggregation experiments with normal and gray platelets showed that platelet aggregation induced by MG-63 osteosarcoma cells resulted from platelet-osteosarcoma and platelet-platelet interactions (Fig. 6). However, aggregates of normal platelets closely interacted with MG-63 cells (Fig. 6A), while the interaction of gray platelets with osteosarcoma cells was looser and the size of the platelet aggregates was smaller (Fig. 6B). The aggregation response of gray platelets to HOS cells and the electron microscopic appearance of the platelet aggregates formed with HOS cells gave results similar to those obtained with MG-63 cells (results not shown).

DISCUSSION

Human MG-63 and HOS osteosarcoma cells induce biphasic platelet aggregation when added to normal platelet-rich plasma (Ref. 8 and this study). We have previously shown that the platelet-aggregating activity of these osteosarcoma cells depends at least in part upon tumor cell surface-associated thrombospondin, and suggested that osteosarcoma cell-induced platelet aggregation could occur through interactions with specific platelet membrane receptors (8). In the present study, using platelets from two unrelated patients with type I Glanzmann's thrombasthenia, we have shown that the first phase of the platelet-aggregating activity of osteosarcoma cells is initiated by the interaction of these tumor cells with platelet membrane glycoproteins GPIIib/IIIa. These results confirm and extend previous studies which showed that the platelet-aggregating activity of carcinoma and melanoma cells is mediated by platelet GPIIib/IIIa (12–15). Using GPIb/IX-deficient platelets from three patients with Bernard-Soulier syndrome, platelet aggregation induced by MG-63 and HOS cells was not inhibited in the presence of anti-GPIb/β antibody Gi27 or antibodies (SZ-1, P5, and P35) directed against platelet GPIIX.

Platelets in gray platelet syndrome are characterized by a selective deficiency in the α-granule secretory proteins (11). Using gray platelets, the second phase of the biphasic aggregation response to MG-63 cells was absent when compared to normal platelets (Fig. 5). Using platelet-rich plasma from a normal donor receiving aspirin (1 g/day during 3 days prior to the experiment), a monophasic aggregation response to MG-63 cells was also observed (Fig. 5). Electron microscopy studies with normal and gray platelets showed that platelet aggregation induced by MG-63 osteosarcoma cells resulted from platelet-osteosarcoma and platelet-platelet interactions (Fig. 6). However, aggregates of normal platelets closely interacted with MG-63 cells (Fig. 6A), while the interaction of gray platelets with osteosarcoma cells was looser and the size of the platelet aggregates was smaller (Fig. 6B). The aggregation response of gray platelets to HOS cells and the electron microscopic appearance of the platelet aggregates formed with HOS cells gave results similar to those obtained with MG-63 cells (results not shown).
syndrome, we have shown that the platelet aggregation response to MG-63 and HOS osteosarcoma cells is monophasic and reversible when compared to normal platelets. This aggregation pattern does not result from the large size of Bernard-Soulier platelets, since platelets from a patient with May-Hegglin anomaly (a condition associated with giant platelets but no membrane glycoprotein abnormalities) (19) are aggregated by MG-63 and HOS cells to an extent similar to that observed with normal-sized platelets. Moreover, the fact that platelet GP Ib/IX functions as a receptor for von Willebrand factor (9) cannot account for the defective aggregation pattern observed with Bernard-Soulier platelets. In type I von Willebrand’s disease, MG-63 and HOS cells induce aggregation of platelets irrespective of the concentration of plasma von Willebrand factor. In addition, monoclonal antibody SZ-2, which inhibits ristocetin-dependent binding of von Willebrand factor to platelet GP Ib (Ref. 20 and this study), does not inhibit the platelet-aggregating activity of human osteosarcoma cells, nor is there any effect of anti-GPIX antibodies. However, we cannot rule out the possibility that anti-GPIX antibodies could recognize nonfunctional epitopes. Platelet GP Ib also functions as a high-affinity \( \alpha \)-thrombin receptor on the platelet surface (9), and reduced thrombin binding is observed in Bernard-Soulier platelets (21). It has been shown that \( \alpha \)-thrombin binds to GP Ib through its anion-binding exosite (22). In addition, the specific thrombin inhibitor, hirudin, uses this locus for its initial binding during stoichiometric complex formation (23). Preliminary experiments performed with hirudin show that the platelet-aggregating activity of MG-63 and HOS cells is strongly impaired in the presence of this thrombin inhibitor, \(^{4}\) suggesting that the thrombin-binding domain of GP Ib \( \alpha \) plays an important role during platelet aggregation induced by osteosarcoma cells. Yamamoto et al. (24) have demonstrated that low concentrations of \( \alpha \)-thrombin induce platelet activation through a high-affinity pathway which is dependent on GP Ib. Moreover, Kroll et al. (25) have reported that GP Ib initiates signals for platelet activation through protein kinase C activation and an increase in ionized cytoplasmic calcium, which promotes platelet secretion and potentiates aggregation. In this regard, it is conceivable that the interaction of osteosarcoma cells with Bernard-Soulier platelets does not initiate signals for platelet activation and secretion because of the absence of the high-affinity thrombin-binding domain of GP Ib \( \alpha \). This is consistent with the fact that the secondary phase of the platelet aggregation process induced by osteosarcoma cells is absent when using Bernard Soulier, aspirin-treated, and gray platelets. Aspirin is a cyclooxygenase inhibitor which inhibits platelet secretion (3), and gray platelets are characterized by a selective deficiency in the \( \alpha \)-granule secretory proteins (11). In this regard, our results strongly suggest that the secondary phase of the platelet aggregation process induced by osteosarcoma cells involves the secretion of \( \alpha \)-granule proteins. Platelet \( \alpha \)-granule secretory proteins, including fibrinogen, fibronectin, thrombospondin, and osteonectin have been involved in the stability of platelet aggregates (17, 26). Moreover, thrombin-degranulated platelets are more readily reaggregated after reaggregation with thrombin than control platelets because they have lost \( \alpha \)-granule content (10). These findings are in agreement with our electron microscopic data on gray platelets showing the formation of small platelet aggregates attached to the tumor cell surface. Moreover, the second phase of the platelet-aggregating activity of osteosarcoma cells may also involve \( \alpha \)-granule membrane proteins such as GMP-140 (CD62, PADGEM) which is known to mediate the adhesion of thrombin-stimulated platelets to neutrophils, monocytes, HL60 promyelocytes, or U937 monoblastic cells (9).

In conclusion, the results presented in this study indicate that the first phase of the platelet-aggregating activity of human osteosarcoma cells is initiated by the interaction of these tumor cells with platelet membrane GP Ib/IIIa. The second phase, irrespective of the concentration of plasma von Willebrand factor, involves platelet membrane GP Ib and the participation of \( \alpha \)-granule proteins in membrane-mediated events, making aggregation irreversible.

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