Characterization of Platelet Aggregation Induced by the Human Melanoma Cell Line HMV-I: Roles of Heparin, Plasma Adhesive Proteins, and Tumor Cell Membrane Proteins

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ABSTRACT

We investigated the in vitro mechanism of platelet aggregation induced by HMV-I human melanoma cells. HMV-I cells, in the absence of exogenous plasma proteins, induced platelet aggregation, followed by the release reaction. Heparin at an anticoagulant concentration had no effect on the aggregation. Calcium ion was essential for this tumor cell-platelet interaction and could not be replaced by magnesium. Among the adhesive proteins containing RGD sequences that have been reported to enhance experimental metastasis, fibronogen and thrombospondin significantly enhanced the aggregation induced by HMV-I cells, fibronectin and von Willebrand factor inhibited it, and vitronectin had no effect. To identify the platelet-aggregating factor(s) of the tumor cells, we have developed a monoclonal antibody against HMV-I cells that can inhibit HMV-I cell-induced platelet aggregation. Immunoprecipitation analysis revealed that this antibody recognized an M₇₁,000 membrane protein. These results suggest that the association between the tumor cells and platelets is mediated by the M₇₁,000 membrane protein recognized by this monoclonal antibody.

INTRODUCTION

The capacity of tumors to metastasize is a vital feature of their malignancy potential. The metastatic cascade is a complex sequence of events involving multiple cell-cell (i.e., tumor cell-platelets, tumor cell-endothelial cells, etc.) and cell-matrix (i.e., tumor cell-subendothelium, etc.) interactions (1). The aggregation of host platelets by circulating tumor cells has been recognized as an important step in the metastatic process of tumor cells (2–4).

Platelet-platelet (i.e., aggregation) and platelet-subendothelial matrix interactions are mediated, in large part, by the multifunctional receptor GPIIb/IIIa, a member of the "integrin" family of adhesive protein receptors (5–7). GPIIb/IIIa exists as a Ca²⁺-dependent heterodimer complex on the platelet surface (5, 8, 9), serving as a receptor for Fbg and vWF (5, 8, 10). Other adhesive proteins such as Fn, Vn, and TSP bind to GPIIb/IIIa through RGD sequences in adhesive proteins (11–13). Recently, molecules on some tumor cells immunologically related to the platelet GPIIb/IIIa complex were identified as receptors that mediate platelet-tumor cell interactions (14–16), but whether the platelet-tumor cell interaction is actually mediated by ligands such as adhesive proteins that are known to bind to the GPIIb/IIIa complex remains to be determined.

Several mechanisms have been proposed to explain how various tumor cells activate platelets in vitro: (a) platelets are activated by thrombin generated through a tumor cell procoagulant activity (17–21); (b) platelets are activated by extracellular ADP released from tumor cells under metabolic control (22, 23); and (c) trypsin-sensitive proteins on tumor cells might participate in platelet aggregation (24, 25). Our previous study showed that HMV-I human melanoma cells activated platelets in heparinized PRP in a thrombin-independent manner and that trypsin- and neuraminidase-sensitive proteins on the tumor cells might participate in the aggregation (26). However, the requirements of divalent cations and plasma proteins for the platelet aggregation induced by HMV-I cells have not been identified.

In the present study, we investigated the in vitro mechanism of HMV-I cell-induced platelet aggregation. We report the effects of heparin and divalent cations, the roles of adhesive proteins in platelet aggregation, and the absence of GPIIb/IIIa-like proteins on the tumor cells. We also present evidence that the HMV-I cell factors which induce platelet aggregation are not well-known agonists, such as ADP, collagen, or PAF. To identify these factors, we have developed a monoclonal antibody against HMV-I cells that can significantly inhibit this tumor cell-induced aggregation. This antibody was found to recognize a trypsin-sensitive membrane protein with a molecular weight of 71,000.

MATERIALS AND METHODS

Reagents

Heparin was purchased from Novo Industry A/S (Bagsvaerd, Denmark); fibronogen, from Kab (Stockholm, Sweden); hirudin, collagenase, trypsin, soybean trypsin inhibitor, bovine serum albumin (BSA), cytochalasin D, colchicine, and apyrase, from Sigma Chemical Co. (St. Louis, MO); PAF, from Avanti (Alabaster, AK); and neuraminidase, from Seikagaku Co. Ltd. (Tokyo, Japan). WEB2086 was kindly provided by Boehringer Ingelheim KG (Ingelheim, Federal Republic of Germany). Other reagents were of analytical grade.

Melanoma Cell Lines

The HMV-I cell line was kindly provided by Dr. T. Kasuga, Tokyo Medical and Dental University, who established the cell line from human vaginal melanoma (27). The HMVTG Cap cell line derived from human skin melanoma was from the Japanese Cancer Research Resources Bank. The melanoma cells were cultured as described previously (26). Briefly, cells were grown in monolayers in plastic dishes (Costar, Cambridge, MA) and maintained at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 60 mg/liter kanamycin sulfate.

For platelet-aggregating experiments, the tumor cells were washed with PBS (pH 7.2), incubated with 5 mM EGTA in PBS (pH 7.2) at room temperature for 5 min, and then harvested. The cells were washed twice with PBS (pH 7.2) containing 1% BSA and suspended at a
concentration of 2 x 10⁷ cells/ml. The viability was always more than 90%.

Purification of Proteins

Fbg was purified from the commercial preparation as described by Lipinska et al. (28). SDS-PAGE analysis of purified Fbg in the presence of 2-mercaptoethanol indicated three close bands with molecular weights of approximately 64,000, 57,000, and 48,000 corresponding to the α, β, and γ chains of Fbg, respectively. vWF was purified from fresh frozen plasma according to the method of Thorell and Blombäck (29). Purified vWF had ristocetin cofactor activity and demonstrated a single band with the molecular weight of 200,000 on SDS-PAGE under the reducing conditions. Fn was also purified from fresh frozen plasma by affinity chromatography over gelatin-Sepharose and arginine-Sepharose (Pharmacon, Stockholm, Sweden) (30). TSP was purified from the supernatants derived from A23187-activated washed platelets using heparin-Sepharose (31), followed by ion-exchange chromatography on a Mono Q column. SDS-PAGE analysis indicated that purified Fn and TSP were homogenous and the molecular weights of their subunits were 220,000 and 170,000, respectively. Vn was isolated from fresh frozen plasma by the method of Yatohgo et al. (32) and gave two bands (M₀ 75,000 and 65,000) on SDS-PAGE under the reducing conditions.

Platelet Preparation and Aggregation

Venous blood from young healthy volunteers was collected in the presence of 5 units/ml of heparin (final concentration) or 0.38% sodium citrate (final concentration), and heparinized PRP or citrated PRP was prepared by centrifugation at 160 x g for 15 min at room temperature. The anticoagulant hirudin was used at the final concentration of 167 units/ml, and hirudin-anticoagulated PRP was prepared as described above. Washed platelets were prepared from citrated PRP according to the method of Mustard as modified by Kirby and Mills (33). The twice-washed platelets were finally suspended in Tyrode’s solution (pH 7.4) containing 0.35% BSA and 0.1% glucose without added Ca²⁺ to a concentration of 1 x 10⁷ platelets/μl. In some experiments, washed platelets were prepared with Tyrode’s/heparin buffer as described previously (34). Platelet aggregation induced by HMV-I cells was monitored by an NBS 8-channel platelet aggregometer, model PAC-8S (Niko Bioscience, Inc., Tokyo, Japan). PRP or suspensions of washed platelets (200 μl) were incubated at 37°C with stirring. After 2 min, the washed HMV-I cell suspension (22.2 μl) was added, and the changes in transmission were monitored. To examine the effects of heparin, citrate solution, and adhesive proteins, the reagents were added before the addition of HMV-I cells as described below.

Effects of Heparin and Sodium Citrate

Heparinized PRP or suspensions of washed platelets prepared using Tyrode’s/heparin buffer (200 μl) were incubated with heparin or sodium citrate solution (25 μl) for 1 min at 37°C with stirring, followed by the addition of HMV-I cells (25 μl), and the changes in transmission were monitored. HNBP-anticoagulated PRP was also used to examine the effect of heparin on HMV-I cell-induced platelet aggregation under the same conditions. The effects were evaluated by the maximal aggregation.

Effects of Adhesive Proteins

Suspensions of washed platelets prepared by the method of Kirby and Mills (33) (150 μl) were incubated with purified adhesive proteins (75 μl) for 2 min at 37°C with stirring, followed by the addition of HMV-I cells (25 μl), and the changes in transmission were monitored. In some cases, HMV-I cells were first incubated with an equal volume of purified adhesive proteins at 4°C for 30 min and then washed twice with PBS containing 1% BSA. The platelet-aggregating activities of these cells were examined as described above.

Preparation of Microvesicles

Microvesicles of the tumor cells were prepared as described by Bastida et al. (21). Briefly, the HMV-I cell monolayers were detached with and centrifuged to obtain the cell-free supernatant, which was then centrifuged at 50,000 x g for 2 h at 4°C, and the particulate material obtained was then resuspended in 50 μl of PBS containing 1% BSA for each 10 ml cell-free detachment solution.

Enzyme Treatment of HMV-I Cells

Trypsin Treatment. HMV-I cells were harvested as described above and washed twice with PBS. The cells were resuspended in PBS at a concentration of 1 x 10⁷ cells/ml and incubated with trypsin (0, 10, 50, and 125 benzoyl-L-arginine ethyl ester units/ml, final concentrations) at 37°C for 15 min. Soybean trypsin inhibitor (1000 units) was added to the reaction mixture, and the tumor cells were then washed twice with PBS containing 1% BSA. The resulting platelet-aggregating activity of HMV-I cells was observed in heparinized PRP.

Neuraminidase Treatment. HMV-I cells were harvested as described above and then washed twice with Tyrode’s/Hepes buffer (pH 6.7) containing 0.35% BSA (34). The cells were resuspended in the same buffer at 5 x 10⁶ cells/ml and incubated with neuraminidase (0, 0.01, and 0.04 units/ml, final concentration) at 37°C for 30 min. The treated cells were washed with PBS containing 1% BSA, and the platelet-aggregating activity was observed in heparinized PRP.

Collagenase Treatment. HMV-I cells were harvested and washed twice with PBS containing 1% BSA. The cells were resuspended at a concentration of 1 x 10⁷ cells/ml in the same buffer supplemented with 2.5 mM CaCl₂ and 100 units of soybean trypsin inhibitor, which inhibited any residual tryptic activity, and then incubated with collagenase (1875 units/ml, final concentration) at 37°C for 1 h. The tumor cells were washed twice with PBS containing 1% BSA. The resulting activity of HMV-I cells was observed in heparinized PRP. As a control, collagen (Hormon-Chemie Munchen GMBH, Munich, Germany, 20 μg/ml) was incubated with collagenase as described above.

Effect of PAF Antagonist, WEB2086

WEB2086 was dissolved in saline and sonicated. Heparinized PRP (200 μl) was incubated with WEB2086 (25 μl) for 1 min at 37°C with stirring, followed by the addition of HMV-I cells (25 μl), and the changes in transmission were monitored.

Crossed-Immunoelectrophoresis

Crossed-immunoelectrophoresis was performed using rabbit anti-human platelet glycoprotein IIb/IIIa antibody as described previously (35).

Effects of Inhibitors

The cytoskeletal inhibitors cytochalasin D and colchicine were dissolved in dimethyl sulfoxide and saline, respectively; the protein synthesis inhibitor cycloheximide was dissolved in absolute ethanol. HMV-I cells were harvested and washed twice with PBS containing 1% BSA. The cells in the same solution were incubated with cytochalasin D or colchicine at 37°C for 30 min and washed twice with PBS containing 1% BSA. The platelet-aggregating activity was compared with that of the cells incubated with dimethyl sulfoxide or saline. In the case of cycloheximide, the HMV-I cells grown in the monolayer were incubated with cycloheximide at 37°C for 16 h, and the aggregating activity then examined as described above.

Monoclonal Antibodies to HMV-I Cells

HMV-I cells washed with PBS were used as the immunogen for BALB/c mice. Three mice were immunized 3 times i.p. at 10-day intervals with 1 x 10⁷ cells, which were mixed with complete Freund’s adjuvant. Four days after the last injection, spleen cells were fused with NS1, mouse myeloma cells, using polyethylene glycol 4000 (36). Supernatants of the resulting hybridomas grown in selective media (hypoxanthine/aminopterin/thymidine) were tested for their ability to inhibit platelet aggregation induced by HMV-I cells. HMV-I cells were preincubated with the supernatants at 4°C for 15 min, and 50 μl of the mixture was added to 200 μl of PRP, which had been incubated at 37°C.
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Fig. 1. HMV-I cell-induced platelet aggregations of human heparinized PRP and washed platelets. (A) Platelets in heparinized PRP were aggregated by HMV-I cells at concentrations of more than 1.25 x 10⁵ cells/ml. (B) Washed platelets were aggregated by HMV-I cells at concentrations of more than 2.5 x 10⁶ cells/ml.

with stirring, and the changes in transmission were observed. Positive hybridomas were subcloned at limiting dilution at least twice. Positive clones were injected into pristane-primed BALB/c mice for the production of ascitic fluid, and immunoglobulins were purified by ammonium sulfate fractionation and chromatography on Q-Sepharose.

Immunoprecipitation

HMV-I cells harvested with EGTA as described above were washed twice with PBS and labeled with Na¹²⁵I (Amersham, Amersham Place, United Kingdom) by use of Iodogen (Pierce, Rockford, IL). The labeled HMV-I cells were solubilized with lysis buffer (10 mM Tris buffer, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamide chloride, 10 μM leupeptin, 100 kallikrein-inhibiting units/ml aprotinin) on ice for 30 min and centrifuged at 15,000 x g for 30 min at 4°C. Aliquots of the supernatant were incubated with mouse nonimmune serum and protein G-Sepharose (Pharmacia, Stockholm, Sweden) for 16 h at 4°C. After centrifugation at 15,000 x g for 20 min at 4°C, the aliquots of the supernatant were incubated with monoclonal antibodies for 2 h at 4°C, followed by the incubation with protein G-Sepharose for 1 h at 4°C. The immunoprecipitates were centrifuged for 5 min at 15,000 x g at 4°C and washed five times with the lysis buffer. The immunoprecipitates were immediately prepared for SDS-PAGE followed by autoradiography.

Flow Cytometric Analysis

Melanoma cells were harvested with EGTA and washed twice with PBS containing 1% BSA. After 1-h incubation with monoclonal antibodies at 4°C, the cells were washed three times with the same buffer and then resuspended in a solution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Coulter Immunology, Hialeah, FL), followed by incubation at 4°C for 1 h. The cells were washed as before, resuspended in the same buffer, and immediately analyzed on an EPICS PROFILE (Coulter).

Electron Microscopy

HMV-I cells were added to suspensions of washed platelets as described above, and the platelet aggregates were fixed with 0.1% glutaraldehyde at 30 s or 5 min after the addition of the cells. The samples were then separated by centrifugation and processed for routine electron microscopy as described previously (37). Sections were examined and photographed with a JEM 100C electron microscope at an accelerating voltage of 80 kV.

RESULTS

Aggregation of Washed Platelets Induced by HMV-I Cells.

As shown in Fig. 1, HMV-I cells could also induce the aggregation of washed platelets, although this required 10-fold more HMV-I cells than that required in heparinized PRP. This indicates that HMV-I cells can activate platelets without exogenous plasma proteins. However, adding heparinized plasma dose-dependently enhanced HMV-I-induced aggregation of washed platelets (data not shown).

Effect of Heparin. The effect of heparin on HMV-I cell-induced aggregation in heparinized PRP was dependent on the final concentration: More than 35 units/ml heparin reduced the aggregation, while 5–35 units/ml heparin did not alter the maximal aggregation induced by HMV-I cells (Fig. 2A). Furthermore, the low concentration of heparin (0.1–5 units/ml) also had no effect on the HMV-I cell-induced aggregation of washed platelets (Fig. 2B). In hirudin-anticoagulated PRP, the aggregation induced by 8.5 x 10⁵ HMV-I cells was inhibited by heparin with a dose dependency very similar to that of the washed platelet suspension.

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Effect of Sodium Citrate. The aggregation induced by HMV-I cells in heparinized PRP was reduced by the addition of sodium citrate at a final concentration of 13 mM (0.38%), the concentration usually used to anticoagulate whole blood (Fig. 2C). This inhibitory effect was also observed in the aggregation of washed platelets prepared by the method of Kirby and Mills (33). The addition of 32 mM sodium citrate completely inhibited the aggregation, although this effect was neutralized by subsequent addition of more than 0.45 mM Ca^{2+} prior to the addition of $4 \times 10^5$ HMV-I cells (data not shown). However, the addition of Mg^{2+} (up to 6 mM) could not reverse the effects of citrate in either the PRP or washed platelet suspension.

To examine whether chelation of divalent cations affects HMV-I cells, they were preincubated with 10 mM EDTA in PBS or 130 mM sodium citrate for 30 min at 22°C or 37°C, respectively, and washed twice with PBS containing 1% BSA without added Ca^{2+}. The platelet-aggregating activity of these cells was not affected under these experimental conditions.

Role(s) of Adhesive Proteins. Since heparinized plasma had an enhancing effect, we examined the role(s) of plasma adhesive proteins on platelet aggregation induced by HMV-I cells (Fig. 3). The HMV-I cell-induced aggregation of washed platelets was slightly enhanced by 190 µg/ml of Fbg and significantly enhanced by 770 µg/ml of Fbg. TSP at more than 2.8 µg/ml also promoted the aggregation. In contrast, Fn and vWF dose-dependently inhibited the aggregation, while Vn at concentrations up to 100 µg/ml had no effect (data not shown). When HMV-I cells ($1.7 \times 10^7$ cells/ml) were preincubated with Fbg (1.15 mg/ml), TSP (14.0 µg/ml), Fn (1.80 mg/ml), or vWF (65.0 µg/ml) at 4°C for 30 min and washed twice with PBS containing 1% BSA, the respective effects described above were not observed.

Treatment of HMV-I Cells with Several Enzymes and the Effect of a PAF Antagonist. The tumor cells were treated with several enzymes to characterize the platelet-aggregating factors of HMV-I cells. Trypsin or neuraminidase treatment reduced the platelet-aggregating activity (Fig. 4) as described previously (26). In addition, trypsin or neuraminidase-treated tumor cells (5 x 10^{6} cells/ml) also failed to aggregate washed platelets. In contrast, collagenase treatment did not affect the activity of HMV-I cells (Fig. 4). Moreover, 26.5 µM WEB2086, 10^{6} times higher concentration than that required to inhibit the aggregation induced by 10^{-6} M PAF, did not inhibit the tumor cell-induced aggregation.

Absence of Proteins Immunologically Related to GPIIb/IIIa on HMV-I Cells. Since a tumor cell membrane glycoprotein that is immunologically related to the platelet GPIIb/IIIa complex has been reported to be responsible for tumor cell-induced platelet aggregation (14-16), we determined whether the HMV-I cell surface contained a molecule immunologically related to GPIIb/IIIa by means of crossed-immunoelectrophoresis using polyclonal rabbit anti-platelet GPIIb/IIIa antibody (35). However, no such glycoproteins were detected (data not shown). In addition, flow cytometric analysis of the tumor cells with the monoclonal antibody PAX25 (38) further verified that such glycoproteins were absent (data not shown).

Effect of Inhibitors. Pretreatment of HMV-I cells with the cytoskeletal inhibitors cytochalasin D (200 µM) or colchicine (200 µM) did not affect the ability of the tumor cells to induce platelet aggregation, the maximal aggregation and lag time being unchanged by this treatment (data not shown). The aggregating activity of the HMV-I cells incubated with the monoclonal antibody TM83 (38) further verified that such glycoproteins were absent (data not shown).

To examine whether inhibition of platelet aggregation of human melanoma cell line. Among the six hybridomas producing antibodies that inhibited HMV-I-induced aggregation, the one producing the monoclonal antibody PAX25 (immunoglobulin subclass IgGl) was chosen for its efficient growth and immunoglobulin production. The HMV-I-induced aggregations of human platelets in heparinized PRP were weakly inhibited by 13.8-55 µg/ml of PAX25, while 100 µg/ml produced complete inhibition (Fig. 5); PAX25 similarly affected the aggregation of washed platelets. In addition, PAX25 also inhibited the aggregation induced by HMVTG Cap, which can induce aggregation without thrombin generation. However, PAX25 could not inhibit the thrombin-dependent platelet aggregation induced by the human colon carcinoma cell line M7609 (26). When ADP or collagen was used as an agonist instead of the tumor cells, addition of PAX25 to the heparinized PRP did not affect the aggregation (35).

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aggregation. Furthermore, flow cytometric analysis with PAX25 showed that both HMV-I cells and HMVTG Cap cells expressed PAX25 antigen (Fig. 6).

The antigen recognized by PAX25 was determined by immunoprecipitation from surface-labeled HMV-I cell lysate. PAX25 precipitated a 68,000 band on SDS-PAGE under reducing conditions (Fig. 7). However, Western blot analysis of PAX25 failed to reveal the antigen recognized by PAX25. In addition, flow cytometric analysis demonstrated that the reactivity of PAX25 to HMV-I cells decreased when the tumor cells were treated with trypsin, whereas neuraminidase treatment did not alter the reactivity (data not shown).

Ultrastructural Study of the HMV-I Cell-Platelet Interaction. Ultrastructural techniques were used to directly observe the HMV-I cell-platelet interaction. At 30 s after the addition of HMV-I cells to washed platelets, round platelets were observed, but their granules were not centralized. The platelets interacted with a limited area of the tumor cells (Fig. 8A). At 5 min, extensive platelet aggregates were present. Platelets directly in contact with the melanoma cell were almost all degranulated as compared with platelets at the periphery of the tumor-platelet aggregates, suggesting that the latter had undergone aggregation but had not completed the release reaction (Fig. 8B).

DISCUSSION

The present study characterizes the platelet aggregation induced by the human melanoma cell line HMV-I. Washed platelets aggregated in response to HMV-I cells, indicating that plasma proteins, particularly exogenous Fbg, are not essential for this process.

It has been generally assumed that in vitro platelet aggregation is induced by tumor cell-derived ADP, which nonspecifically leaked from the tumor cell cytoplasm because the cells were damaged during the harvesting procedure (22). Recently, Grignani and Jamieson (23) reported that release of ADP by tumor cells is under cellular control and not the result of tumor cell damage. Although we have observed that phosphate/creatine phosphokinase, an ADP-scavenging system, inhibited HMV-I cell-induced aggregation (24), we concluded that ADP did not participate in this aggregation, at least the early stage, because adding up to 10 \( \mu \text{M} \) ADP (final concentration) could not induce the aggregation of washed platelets (prepared by the method of Kirby and Mills) in the absence of exogenous Fbg (39). In addition, when platelet aggregation in PRP induced by HMV-I cells was monitored on a Lumiaggregometer, ATP release from platelets occurred as the aggregation began (data not shown). Therefore, ADP release from the platelet-dense granules seems to be important in the late stage of this reaction because the ADP scavenger inhibited the aggregation. Our current ultrastructural study also confirmed that the release reaction caused the late stage aggregation, because the observed platelet degranulation is consistent with the importance of platelet-degranulated ADP.

Because we observed that the addition of heparinized plasma enhanced the HMV-I cell-induced aggregation of washed platelets, we examined the effect of heparin on the platelet aggregation. High heparin concentrations (more than 35 units/ml) significantly reduced the aggregation in both PRP and washed platelet suspension. The mechanism by which this occurs is unclear since HMV-I cells activate platelets without thrombin generation (26), but perhaps high concentrations of heparin may interact with HMV-I cells and thereby reduce the aggregation through their positive charge. Low heparin concentrations (up to 35 units/ml) did not affect the aggregation in washed platelet suspension and hirudin-anticoagulated PRP, indicating not only that heparin itself did not promote the aggregation but also that heparin could not enhance the aggregation through an interaction with some plasma factors. There have been several discrepant reports concerning the effects of heparin on platelet aggregation (40-42), which may be due to the different sources and preparation methods for heparin (43).

Contradictory observations have been made on the divalent cation requirement of tumor cell-induced platelet aggregation (17-19). In the present study, sodium citrate addition to heparinized PRP (Fig. 2) or the washed platelet suspension completely inhibited HMV-I cell-induced aggregation. This could be reversed by Ca\(^{2+}\) addition but not by Mg\(^{2+}\). It is well known that pretreatment of platelets with EDTA at 37°C abolishes aggregation by dissociation of GPIIb/IIIa complex which functions as a Fbg receptor (8, 9, 38) and that the presence of 13 mm sodium citrate, the concentration usually used as an anticoagulant, has no inhibitory effect on platelet aggregation. Therefore, it is very interesting that HMV-I-induced platelet aggregation was found to be sensitive to the concentration of calcium ion. Since pretreatment of HMV-I cells with EDTA or sodium citrate did not reduce the platelet-aggregating activity of the cells, it appears that irreversible changes did not occur on the tumor cells by the chelation of divalent cations. Our data suggest that Ca\(^{2+}\) plays an essential role in platelet aggregation in this system, which cannot be substituted for by Mg\(^{2+}\). Because heparinized plasma enhanced the aggregation of washed platelets induced by HMV-I cells and platelet aggregation is believed to be dependent on the availability of adhesive proteins including Fbg (5, 9, 39), we investigated the possible

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Fig. 6. Flow cytometric analysis of melanoma cell lines with PAX25. PAX 25 antibody (bottom) bound to both HMV-I and HMVTG Cap cells in contrast to the control normal mouse IgG (top).

Fig. 1. Immunoprecipitation from the surface-labeled HMV-I cells by PAX25. A, Whole lysate of the surface-labeled HMV-I cells on SDS-PAGE under the reducing. B, The antibody immunoprecipitated a protein with a molecular weight of 71,000 on SDS-PAGE under the reducing conditions.

roles of adhesive proteins in the aggregation. Since enhancing effects of Fbg and TSP and inhibitory effects of Fn and vWF were not detected when HMV-I cells were preincubated with the proteins, these adhesive proteins appeared to interact with the platelets only. The effect of Fbg seems to be due to the binding of exogenous Fbg to GPIIb/IIIa of the platelets stimulated by HMV-I cells. Other investigators have also observed such an association of Fbg with platelets during activation (39, 44). This is one of the reasons why a small number of HMV-I cells can easily aggregate platelets in PRP because of the presence of a large amount of Fbg. It has been postulated that TSP cross-links platelet-Fbg (45). Tuszyński et al. (46) reported that TSP (4–40 µg/ml) potentiates both thrombin- and ADP-stimulated platelet aggregations. Thus, the effect of TSP (14 µg/ml, comparable to those used by Tuszyński et al.) on the tumor cell-induced aggregation observed here results from the interaction between TSP and platelets, and endogenous TSP released from platelet α granules may also play an important role in the platelet aggregation. The mechanism of the inhibitory effects of Fn and vWF are not known. Fn inhibited the secretion-dependent second phase of ADP-induced aggregation of washed platelets, suggesting that there is a similar inhibitory mechanism in these reactions. Furthermore, possible roles of the release reaction from platelets during HMV-I cell-induced aggregation should be investigated. Although the five adhesive proteins examined here have RGD sequences which are recognized by the platelet integrin, GPIIb/IIIa, and have been reported to bind to GPIIb/IIIa on stimulated platelets,5 they may have different effects on HMV-I cell-induced aggregation.

Many investigators have reported tumor cell factors that induce platelet aggregation. As mentioned above, we excluded possible mechanisms that involve platelet stimulation by ADP derived from HMV-I cells and the induction of release reactions (23). Bastida et al. (21) reported that membrane-derived microvesicles of U87MG human glioblastoma cells participate in platelet aggregation. We prepared microvesicle fractions from HMV-I cells according to their method but could not detect any aggregating activity. Since the aggregation curves of washed platelets induced by HMV-I appeared to be similar to those induced by collagen because of the characteristic lag time after

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were almost completely degranulated (arrows), while the platelets at the periphery
aggregates around a melanoma cell. All platelets directly in contact with the cell
the cells (A). However, at 5 min after the addition of the cells, the platelets formed
interaction in which a cell-associated lipid mediator, PAF,
addition of these agonists (18), the tumor cells were treated
that trypsin- and neuraminidase-sensitive molecules on the
induces a rapid and reversible intercellular adhesion. However,
cytoskeleton and a membrane glycoprotein immunologically
related to GPIIb/IIIa in tumor cell-induced platelet aggrega-
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celerate the platelet aggregation. The association between the
tumor cells and platelets may be mediated by an M\(_{71000}\) protein on the HMV-
I cells with trypsin decreased the binding of PAX25, indicating
that the antigen was sensitive to trypsin digestion.

Reportedly, factors responsible for platelet aggregation have
been purified from several murine cancer cell lines (20, 25, 48).
The M\(_{71000}\) protein identified in this study is apparently
distinct from those reported above in terms of both molecular
weight and platelet-aggregating ability.

In conclusion, our results indicate that HMV-I cells interact
with platelets in the absence of added heparin or exogenous
plasma proteins, followed by the release reaction from platelets.

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PLATELET AGGREGATION INDUCED BY MELANOMA CELLS


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