Involvement of Platelet Membrane Glycoprotein Ib and Glycoprotein IIb/IIIa Complex in Thrombin-dependent and -independent Platelet Aggregations Induced by Tumor Cells

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ABSTRACT

Involvement of platelet membrane glycoproteins (GP) in interactions between platelets and tumor cells was studied by using two human tumor cell lines and two monoclonal antibodies against platelet membrane GP. HMV-I cells derived from vaginal melanoma induced platelet aggregation in heparinized plasma, which was not followed by coagulation. M7609 cells derived from colon adenocarcinoma also induced platelet aggregation in heparinized plasma, which, on the contrary, was followed by coagulation. Aggregating activities of the HMV-I cells were abolished by pre-treatment with neuraminidase or trypsin, but M7609 activity was not labile to these enzymes. Aggregations induced by M7609 were inhibited by hirudin or MD805, while those by HMV-I were not. M7609 cells dose dependently shortened the recalcification time of normal as well as Factor IX-deficient plasmas, while they were not effective in shortening the time of Factor II- or Factor VII-deficient plasmas. The procoagulant activity of HMV-I cells was 1000 times less than M7609 on the basis of cell numbers. When human platelets were preincubated with monoclonal anti-GPIIb or anti-GPIIb/IIIa complex antibodies, neither cell line could cause aggregations.

These findings suggest that both GPIIb and the GPIIb/IIIa complex on the platelet surface are involved in the thrombin-dependent and -independent platelet aggregations induced by tumor cells.

INTRODUCTION

Since Gasic and coworkers (1) demonstrated that cells from a variety of tumors can induce platelet aggregation in vivo and cause thrombocytopenia when injected into animal models, several reports have suggested (1-4) that platelets are involved in the hematogenous metastasis of tumor cells from the primary tumor site through the circulation. Several investigators have reported different mechanisms through which tumor cells activate platelets: (a) production of platelet-aggregating materials like a sialolipoprotein of the tumor cell membrane (5, 6); (b) generation of thrombin by the procoagulant activity of the cells (7); (c) generation and release of ADP from the tumor cells (8); or (d) a trypsin-sensitive surface protein of tumor cells (9). In contrast, factors on the platelet surface which are involved in the interaction between platelet and tumor cells have yet to be identified and characterized.

In order to clarify the membrane components involved in the interaction between tumor cells and platelets, we used two monoclonal antibodies against platelet membrane glycoproteins which are believed to be directly involved in platelet aggregation (10-12).

MATERIALS AND METHODS

Tumor Cell Lines and Preparation of Cell Suspension. The HMV-I cell line was generously given to us by Dr. T. Kasuga, Tokyo Medical and Dental University, Tokyo, who established the cell line from human vaginal melanoma (13). The M7609 cell line derived from human adenocarcinoma of the colon (14) was obtained through the courtesy of Dr. I. Urushizaki, Sapporo Medical School, Sapporo. Cell lines were grown to confluency as monolayer cultures in 100-mm x 20-mm plastic dishes (Costar, Cambridge, MA) and maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. After monolayers were rinsed with PBS(-), the cells were added with a solution of 5 mM ethyl glycol bis(β-aminoethylthlycer)-N,N,N',N'-tetraacetic acid in PBS(-), allowed to stand for 10 min on ice, and then harvested. The harvested cells were washed and resuspended in PBS(-) with 1% bovine serum albumin (type V; Sigma) prior to use. The cells were counted in a hemocytometer under phase-contrast microscopy.

The cell membrane fractions were obtained by the method of Boone et al. (15).

Preparations of Platelet-rich and -poor Plasma. Blood was obtained from healthy volunteers who had not taken any drugs for at least 2 wk. Blood was anticoagulated by heparin (final concentration, 5 units/ml of whole blood; Novo Industry A/S Bagsvaerd, Denmark) or trisodium citrate (final concentration, 0.38%). PRP was obtained by centrifuging the blood at 160 x g for 15 min, and then PPP was obtained by centrifuging the remaining at 2000 x g for 10 min. Platelet numbers were determined by a Coulter Counter (Coulter Electronics, Hialeah, FL), and platelet concentration was adjusted to 3 to 4 x 10^8 platelets/ml with PPP.

In Vitro Platelet Aggregation Studies. Platelet aggregation was measured turbidimetrically with an aggregometer (Sienco, Houston, TX). In a typical experiment, 200 µl of PRP were prewarmed at 37°C in a flat-bottomed cylindrical cuvet with stirring at 1000 rpm, and then a 50-µl aliquot of tumor cell suspension was added.

Monoclonal Antibodies. TM60 and TM83, monoclonal antibodies against GPIIb and GPIIb/IIIa complex, respectively, were produced according to the methods of Koehler and Milstein (16) and have been described previously (10-12). The subclass of TM60 is IgG2a (10), and that of TM83 is IgG1 (11). Briefly, TM60 inhibited ristocetin- and thrombin-induced aggregations, but not ADP-, epinephrine-, collagen-, or A23187-induced aggregations. It immunoprecipitated GPIIb from surface-labeled platelets, thus showing GPIIb as its epitope (10). TM83 inhibited ADP-, epinephrine-, and collagen-, A23187-, and thrombin-induced aggregations (11). Radiolabeled TM83 inserted in the intermediate gel in crossed immunoelectrophoresis was incorporated into the immunoprecipitin lines of both GPIIb and the GPIIb/IIIa complex, showing that the epitope of TM83 is GPIIb, and TM83 can also recognize the GPIIb/IIIa complex (11).

Enzymatic Treatments of Tumor Cells. Washed tumor cells were incubated with neuraminidase (0.3 units/ml; Seikagaku Kogyo, Tokyo, Japan) at 37°C for 60 min or incubated with trypsin (500 BAEE units/ml; Difco, Detroit, MI) at 37°C for 20 min and used after they were washed. After the treatment with neuraminidase, cell electrophoretic mobility was measured with an automated instrument, Pamaquanti-L (Kureha Chem., Tokyo, Japan) (17).

Measurement of Procoagulant Activity. Procoagulant activities of the tumor cells were measured using the plasma recalcification time method described by other investigators (7, 18). Briefly, 100 µl of fresh normal citrated PPP were incubated with 100 µl of cell suspensions with...
sequentially diluted cell numbers for 1 min at 37°C; and then 100 μl of prewarmed 20 mM CaCl₂ were added, and the plasma clotting times in seconds were recorded. Recalcification times were also performed with human plasmas deficient for Factors IX, VII, or II (Organon Teknika Corporation, Morris Plains, NJ).

RESULTS

Platelet Aggregations Induced by Tumor Cells and Their Membrane Fractions. Both HMV-I and M7609 cells dose dependently induced platelet aggregation in heparinized plasma (Fig. 1, A and B). HMV-I cells at final concentrations of 2 × 10⁵ or more cells/ml caused platelet aggregations that were not followed by coagulation. M7609 cells at concentrations of more than 5 × 10⁴ cells/ml caused aggregation. The patterns of aggregations induced by 5 × 10⁴ or more M7609 cells/ml were deformed due to fibrin clot formations (Fig. 1B), which were also confirmed macroscopically. The membrane fractions obtained from the HMV-I cells (Fig. 1C) and the M7609 cells (Fig. 1D) also induced platelet aggregations at final concentrations equivalent to similar numbers of the intact cells.

Effects of Enzymatic Treatments of Tumor Cells on Their Platelet Aggregating Activities. Treatment of HMV-I cells with trypsin or neuraminidase abolished their aggregating activities (Fig. 2A), while M7609 cells were not affected by these treatments (Fig. 2B). After the treatment with neuraminidase, the cell electrophoretic mobilities of the HMV-I and M7609 cells were decreased, confirming that the neuraminidase removed sialic acid from the surfaces of both types of tumor cells (data not shown).

Effects of Hirudin and M7609 on Tumor Cell-induced Aggregation. As shown in Fig. 3B, platelet aggregation induced by 2 × 10⁵ M7609 cells/ml was completely inhibited by 5 units/ml of hirudin (Sigma) or 2 × 10⁻⁶ M MD805, an inhibitor of thrombin (19) (data not shown). HMV-I cell-induced aggregations of platelets were not inhibited by either of the compounds (Fig. 3A).

Effects of Tumor Cells on the Recalcification Time. M7609 cells strongly shortened the recalcification time of normal human citrated plasma in a dose-dependent manner, as shown in Fig. 4A. In contrast, HMV-I cells weakly shortened the recalcification time. The procoagulant activity of HMV-I cells was 1000 times less than that of M7609 on the basis of cell numbers (Fig. 4A).

As shown in Fig. 4C, M7609 cells equally shortened the recalcification times of normal and Factor IX-deficient plasmas; however, they could not shorten the recalcification times of either Factor VII- or Factor II (prothrombin)-deficient plasmas, suggesting that M7609 cells have a tissue factor-like activity in the extrinsic coagulation pathway. HMV-I cells could not shorten either the normal or the factor-deficient plasmas (Fig. 4B).

Effects of Monoclonal Antibodies, TM60 and TM83, on Tumor Cell-induced Aggregations. When heparinized PRP was preincubated with TM60 or TM83 for 4 min at 37°C, both aggregations induced by HMV-I or M7609 cells were inhibited dose dependently (Fig. 5). Platelet aggregations induced by 1 × 10⁶ HMV-I cells/ml were completely inhibited by preincubation with 12 μg/ml of TM60 (Fig. 5A) or 9 μg/ml of TM83 (Fig. 5B). The aggregations by 2 × 10⁵ M7609 cells/ml, an amount which could not induce coagulation of PRP within 12 min after the addition of the cells as shown in Fig. 1B, were completely inhibited by preincubating heparinized PRP with 50 μg/ml of TM60 (Fig. 5C) or 18 μg/ml of TM83 (Fig. 5D). When a final concentration of 1 × 10⁶ M7609 cells/ml, an amount which
Fig. 2. Effect of treatment of tumor cells with neuraminidase on platelet-aggregating activities. Tumor cells were incubated with 0.5 units/ml of neuraminidase for 60 min at 37°C, and after washing out the enzyme, 25 μl of the treated tumor cell suspension were added to 200 μl of fresh heparinized human PRP. A, HMV-I cells (final concentration, 1 x 10⁶/ml); B, M7609 cells (final concentration, 1 x 10⁶/ml).

Fig. 3. Effect of hirudin on tumor cell-induced platelet aggregation. Two hundred μl of heparinized human PRP were preincubated with 25 μl of hirudin in PBS(−) at 37°C for 3 min prior to the addition of 25 μl of tumor cells. A, HMV-I cells (final concentration, 1 x 10⁶/ml); B, M7609 cells (final concentration, 2 x 10⁶/ml). Final concentrations of hirudin are shown beside the aggregation curves.

Fig. 4. Effects of tumor cells on recalcification times. A, shortening of recalcification times of normal plasma by tumor cells. One hundred μl of HMV-I or M7609 cells were incubated with 100 μl of citrated plasma for 1 min at 37°C; then 100 μl of 20 mM CaCl₂ were added, and the clotting times of the plasma were recorded. B and C, effect of HMV-I or M7609 cells on the recalcification time of coagulation factor-deficient plasmas. One hundred μl of tumor cell suspension were incubated with 100 μl of each test plasma for 1 min at 37°C, and then 100 μl of 20 mM CaCl₂ were added. •, normal plasma; △, Factor II-deficient plasma; ●, Factor VII-deficient plasma; ■, Factor IX-deficient plasma.

DISCUSSION

It is well known that some tumor cells induce platelet aggregation in vitro and that the mechanisms for this phenomenon may be variable (4). It has been suggested that these aggregations might be involved in hematogenous metastatic processes (1–4). In the first half of this report, mechanisms of platelet aggregations induced by two human tumor cell lines, M7609 and HMV-I, were studied. Since M7609 cells showed potent procoagulant activity in shortening the recalcification times of normal and Factor IX-deficient plasmas but not Factor VII- or Factor II-deficient plasmas (Fig. 4), it was concluded that M7609 cell-induced aggregation was dependent on thrombin generation triggered by a tissue factor-like activity of the cells. Many other cell lines derived from human tumors have been shown to aggregate platelets by thrombin generated similarly by tissue factor-like activity of the cells (7, 18, 20–23; for review, see Ref. 4). It is notable that cancer procoagulants which directly activated Factor X have been reported for animal cell lines (24–26) but not for human cell lines.
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Inhibitory effects by monoclonal antibodies on tumor cell-induced platelet aggregations. Two hundred µl of heparinized human PRP were preincubated with 25 µl of monoclonal antibody at 37°C for 3 min prior to the addition of 25 µl of tumor cells. A and B, inhibitory effects of monoclonal antibodies on HMV-I (final concentration, 1 x 10^6/ml) cell-induced platelet aggregation; C and D, effects of antibodies on M7609 (final concentration, 2 x 10^6/ml) cell-induced platelet aggregation. Final concentrations of monoclonal antibodies are shown beside the aggregation curves.

Inhibitory effects of TM83 on M7609 cell-induced coagulations and platelet aggregations. Two hundred µl of heparinized human PRP were preincubated with 25 µl of TM83 at 37°C for 4 min prior to the addition of 25 µl of M7609 cells (final concentration, 1 x 10^6/ml). The arrowheads indicate the coagulations. Final concentrations of TM83 antibody are shown beside the aggregation curves.

In contrast, since HMV-I cells had negligible procoagulant activities (Fig. 4) and their aggregating activity was abolished by the treatment with neuraminidase (Fig. 2), HMV-I-induced platelet aggregation was not dependent on thrombin generation. It is probable that HMV-I cells activate platelets by a direct interaction between platelets and sialic acid-containing moieties, on the surfaces of HMV-I cells. Other human cell lines (9, 23, 27) have been shown to aggregate platelets by a mechanism resembling that of HMV-I cells. The thrombin-independent mechanisms of platelet aggregations by tumor cells may be heterogenous in nature and remain to be clarified (4, 28).

Using these two types of cell lines which have different mechanisms of platelet aggregation, we studied the effects of two monoclonal antibodies, the anti-GPIb antibody TM60 and the anti-GPIIb/IIIa complex antibody TM83, in order to clarify the involvement of platelet surface glycoproteins in the interaction between platelets and tumor cells. GPIb and GPIIb/IIIa complex are the major glycoproteins on the platelet membrane. GPIb binds both thrombin and von Willebrand factor, and the GPIIb/IIIa complex is the binding site for fibrinogen during platelet aggregation (for reviews, see Refs. 29 and 30). However, it has been still unknown how platelet membrane glycoproteins were involved in the tumor cell-induced aggregation, although Menter et al. recently reported that tumor cell adhesion to subendothelial matrix was enhanced by platelet membrane factors (31, 32).

We found that both TM60 and TM83 inhibited platelet aggregation induced by either HMV-I or M7609 (Fig. 5), suggesting that both GPIb and the GPIIb/IIIa complex may be involved in the thrombin-dependent and -independent interaction between platelets and the tumor cells. As described above, M7609 cells induce platelet aggregations via a mechanism involving thrombin generation. Therefore, the stimulus-response mechanism in this type of cancer cell-induced aggregation could be analogous to that in thrombin-induced aggregation, where GPIb is the receptor for thrombin and the GPIIb/IIIa complex exposes the binding sites for fibrinogen after the activation by thrombin (29, 30). By this analogy, it is understandable that both the monoclonal antibody against GPIb and that against the GPIIb/IIIa complex inhibited M7609 cell-induced aggregations. Additionally, M7609 cells caused clot formation in the final concentration of 5 x 10^5 or more cells/ml (Fig. 1), which always followed the onsets of aggregations, since platelet activation requires less thrombin than fibrinogen proteolysis does in PRP. Both TM60 and TM83 antibodies inhibited also M7609-induced coagulations (Fig. 6). Although exact mechanisms of the inhibitions are not known, inhibitions by these antibodies of platelet activation induced by the tumor cells may be associated with inhibition of thrombin generation on the platelet membrane surfaces (33).

In contrast, we could not directly identify the initial event in the thrombin-independent interaction between platelets and HMV-I cells. We found that sialic acid-containing moieties on
The HMV-I cell surface play a crucial role in triggering the stimulus to platelets, since the pretreatment of the cells with neumaminidase abolished their aggregating activity. Although there are conflicting reports on a correlation between the degree of cell surface sialylation of the tumor cells and their ability to metastasize (34, 35), several cell lines derived from humans (27) and animals (5, 6, 36) have been shown to lose their thrombin-independent aggregating activities after treatment with neumaminidase. The component on the platelet surface which interacts with the sialic acid-containing moieties is not directly known. The GPIb/IIa complex has been believed to expose the binding sites for the so-called adhesive proteins (29, 30), but is apparently not the site which first receives an extrinsic stimulus. However, since the monoclonal anti-GPIb TM60 antibody completely inhibited HMV-I-induced aggregation, it is most likely that the receptor for the sialic acid-containing moieties on HMV-I cells may be GPIb. TM60 also inhibited platelet aggregation induced by B16F10 mouse melanoma cells (37); the aggregation required the presence of heparin and cryoprecipitate. These results suggest the importance of adhesive proteins and GPIb as a receptor in tumor cell-induced platelet aggregations.

REFERENCES


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