Expression of a M, 41,000 Glycoprotein Associated with Thrombin-independent Platelet Aggregation in High Metastatic Variants of Murine B16 Melanoma

Masahiko Watanabe, Yoshikazu Sugimoto, and Takashi Tsuruo

Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-ikebukuro, Toshima-ku, Tokyo 170 [M. W., Y. S., T. T.], and Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113 [T. T.], Japan

ABSTRACT

In the previous study, we generated a monoclonal antibody, 8F11, against NL-17, a high metastatic clone derived from a metastatic variant of murine colon adenocarcinoma 26. 8F11 inhibited platelet aggregation induced by NL-17 and recognized a M, 44,000 membrane protein as antigen. In the present study, the reactivity of 8F11 to murine B16 melanoma and its metastatic variants was examined, and the antigen recognized by 8F11 on the cell surface was characterized. 8F11 was found to strongly react with 3 metastatic variants of B16 melanoma. In contrast, only slight reactivity was observed with parent B16 melanoma. The reactivity of the antibody to these cells was in the order B16F10 > B16BL-6 > B16F1 > B16. Western blot analysis showed a M, 41,000 protein as the antigen recognized by 8F11 on the cell surface of B16F10 cells. The M, 41,000 antigen appeared to be a glycoprotein that bound to wheat germ agglutinin as has been observed for the M, 44,000 antigen of NL-17. To elucidate the functional role of the M, 41,000 antigen in B16 melanoma, platelet aggregation induced by B16 and B16F10 was compared. B16 was reported to stimulate platelet aggregation by the generation of thrombin, whereas B16F10 was found to activate platelet by at least 2 mechanisms: one dependent on thrombin and the other independent on thrombin. The activity of B16 and its metastatic variants to induce platelet aggregation in the presence of MD805, a synthetic antagonist of thrombin, well correlated with the reactivity of 8F11 to these cells. 8F11 blocked platelet activation by B16F10 under conditions preventing thrombin activity such as enzymatic formation of lysoselctinin through the treatment of the cell surface with phospholipase A2 or in the presence of MD805. These data indicate that M, 41,000 glycoprotein recognized by 8F11 on metastatic variants of B16 melanoma is involved in the thrombin-independent platelet aggregation. A positive correlation was observed between the levels of M, 41,000 glycoprotein expression of B16 and its metastatic variants and their pulmonary metastasis after i.v. injection, suggesting M, 41,000 glycoprotein, as well as other factors reported previously, may play an important role in the hematogenous spread of B16 melanoma.

INTRODUCTION

Interaction between platelet and tumor cells has been widely recognized to play an important role in the hematogenous spread of certain tumor cells (1, 2). Tumor cells inoculated i.v. into experimental animals have been demonstrated to activate platelets in vitro and induce thrombocytopenia (3-5). Some agents affecting platelet aggregation in vitro or inducing experimental thrombocytopenia have been reported to decrease lung colony formation of tumor cells (1, 6-9), however, this is not a universal finding (1, 10). Furthermore, antibodies that inhibit binding of tumor cells to platelet have been demonstrated to decrease pulmonary metastasis of some murine tumor cells (11). Interactions between platelets and tumor cells have been believed to facilitate the attachment and the extravasation of tumor cells to endothelial cells of specific organs and to promote tumor growth (2).

A number of tumor cells from various organs and species have been reported to induce platelet aggregation in vitro (1, 2). Several mechanisms by which tumor cells activate platelets have been proposed through investigations using inhibitors of platelet aggregation and/or enzymatic treatment of the cell surface. For example, generation of thrombin through a procoagulant activity of tumor cells (12-18), transmembrane efflux of ADP occurring as a consequence of tumor cell metabolism (18-20), and direct interaction with platelets via a membrane moiety (19, 21-25) have been demonstrated. However, the identification of factors responsible for the induction of platelet aggregation by tumor cells has not been fully studied with the exception of several reports (12, 13, 25), and a correlation between platelet aggregation and tumor metastasis has not been fully elucidated.

In previous studies, we established several metastatic clones derived from a high metastatic variant of murine colon adenocarcinoma 26 (26) and demonstrated that platelet aggregation induced by the tumor cells was an integral event for lung metastasis of colon 26 (27, 28). Recently, we reported that NL-17, a high metastatic clone of colon 26, activates platelets by a trypsin-sensitive and phospholipase A2-insensitive protein in a thrombin-independent manner and generated a mAb1 8F11, which inhibited platelet aggregation caused by NL-17. This mAb recognized a M, 44,000 membrane protein expressed on NL-17 (25). The amount of 8F11 bound to metastatic clones of colon 26 was found to correlate with platelet-aggregating activity of these clones, indicating the antigen recognized by 8F11 was a platelet-aggregating factor of colon 26.

In the present study, we examined the reactivity of 8F11 to murine B16 melanoma and its metastatic variants (29). 8F11 reactivity with metastatic variants of B16 melanoma; recognized a M, 41,000 glycoprotein as antigen on the surface of a high metastatic variant of B16 melanoma, B16F10; and inhibited platelet aggregation induced by B16F10 under conditions preventing thrombin activity. These data suggest that M, 41,000 glycoprotein may be a metastatic-related factor responsible for the induction of platelet aggregation in metastatic variants of B16 melanoma.

MATERIALS AND METHODS

Chemicals and Enzymes. The source of chemicals used in this work were as follows: NaCl [231] from Amersham Japan Ltd., Tokyo, Japan; apyrase (from potato, grade III, 55 units/mg for ATP) and phospholipase A2 (from bee venom, 560 units/mg) from Sigma Chemical Co., St. Louis, MO; Con A, Concanavalin A; Ricinus communis agglutinin I; WGA, wheat germ agglutinin; HSSS, Hanks' balanced salt solution without calcium and magnesium; HEPES, N-2-hydroxyethyl-l-piperazine-N'-2-ethanesulfonic acid; PRP, platelet-rich plasma. [3] The abbreviations used are: mAb, monoclonal antibody; Con A, concanavalin A; RICA-I, Ricinus communis agglutinin I; WGA, wheat germ agglutinin; HBSS, Hanks' balanced salt solution without calcium and magnesium; HEPES, N-2-hydroxyethyl-l-piperazine-N'-2-ethanesulfonic acid; PRP, platelet-rich plasma.

1 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 To whom requests for reprints should be addressed, at Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-iikebukuro, Toshima-ku, Tokyo 170, Japan.

[Received 4/23/90; accepted 7/12/90.]
galactose, and α-methyl-D-mannoside from Nacarai Tesque Inc., Kyoto, Japan; and HBSS and HEPES buffer (1 mM solution, pH 7.3) from Gibco Laboratories, Detroit, MI. MD805, a synthetic thrombin inhibitor (30), was kindly provided by Mitsubishi Kasei Co., Ltd., Tokyo, Japan. All other chemicals and reagents were of the highest purity available.

Tumor Cells and Animals. Murine B16 melanoma and the metastatic variants, B16F1, B16F10, and B16BL-6, kindly provided by Dr. I. J. Fidler (29), were used in this study. A high metastatic clone of murine colon adenocarcinoma 26, NL-17 was established in this laboratory (26). These cells were maintained in Eagle’s minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum, 2% fetal bovine serum, and kanamycin (100 mg/ml) and incubated at 37°C in a humidified atmosphere of 5% CO2. These cell lines were freshly cultured from frozen stocks every 2 months and were confirmed to retain their metastatic abilities as described previously (31).

Female BALB/c × DBA/2 F1 (hereafter called CD2F1) mice were obtained from Charles River Japan, Inc., Tokyo, Japan.

mAb 8F11. mAb 8F11, the immunoglobulin subclass of which was IgG2a, was generated from spleen cells of rats immunized with the crude membrane preparations of NL-17 and purified from ascitic fluid of athymic nude mice as described previously (25). The protein amount was determined by the method of Lowry et al. (32).

Radioimmunoassay and Western Blot Analysis. Radioiodination of mAb 8F11, radioimmunoassay, and Western blot analysis was described as previously (25). The specific activity of 125I-labeled 8F11 was 3.0 × 106 cpm/μg protein.

Lectin Affinity Chromatography. Crude membrane fractions isolated from NL-17 and B16F10 as described previously (25) were solubilized with 2% 3-[3-cholamidopropyl]dimethylammonio]-l-propanesulfonate in 50 mM Tris-HCl (pH 7.5)-150 mM NaCl (S-buffer) at 4°C for 2 h and centrifuged at 100,000 × g for 1 h at 4°C. The supernatant fractions were applied to Con A, RCA-I, and WGA lectin-agarose columns. Each column was washed with S-buffer and eluted with 0.1 M concentrations of the following specific sugars or with S-buffer: α-methyl-D-mannoside for Con A; galactose for RCA-I; and N-acetyl-D-glucosamine for WGA. Starting material, flowthrough fractions, and sugar-eluted fractions were solubilized with sodium dodecyl sulfate-sample buffer of Laemmli (33) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (33) and subjected to Western blot analysis. The presence of the antigen in the flowthrough and sugar-eluted fractions from each column and starting material were analyzed by Western blot assay (Fig. 3, A and B). Both the M, 44,000 antigen of NL-17 and the M, 41,000 antigen of B16F10 were detected in the fraction of WGA column eluted with sugar, indicating a binding of the antigen to WGA, whereas no binding occurred with Con A and RCA-I column.

Effect of Thrombin Inhibitor and Phospholipase A2 on Platelet Aggregation Induced by B16 and Its Metastatic Variants. Fig. 4, A and B, illustrates the representative tracing of platelet aggregation caused by B16 and B16F10 at a tumor concentration of 5 × 105 cells/ml in the presence or absence of 10 μM MD805, a synthetic thrombin inhibitor (30). MD805 completely blocked

RESULTS

Reactivity of 8F11 to Murine B16 Melanoma and Its Metastatic Variants. The specific binding of mAb 8F11 to B16 melanoma; a low metastatic variant, B16F1; and 2 high metastatic variants, B16F10 and B16BL-6, was determined by cell-binding radioimmunoassay. In preliminary experiments, the binding of 125I-labeled 8F11 to B16F10 was saturated at 1 μg/ml, and complete inhibition by unlabeled 8F11 occurred at 1 mg/ml. Thus, the binding experiments were performed at 1 μg/ml of the labeled mAb and the amounts of 8F11 bound to these cell lines were calculated from the difference between the amount of the bound 8F11 in the presence of 1 mg/ml of unlabeled mAb and that in the presence of the same concentration of normal rat immunoglobulin. A high metastatic variant, B16F10, reacted well with 8F11 compared with other variants, and the amounts of 8F11 bound to 5 × 105 cells of B16F10, B16BL-6, and B16F1 were 4.2, 3.1, and 2.2 fmol, respectively (Fig. 1). In contrast, 8F11 showed a marginal binding to parent B16 and the amount of mAb bound B16 was 0.068 fmol/1 × 105 cells, which was 60-fold lower than that bound to B16F10. The amount of 8F11 specifically bound to B16 and its metastatic variants was in the order B16F10 > B16BL-6 > B16F1 > B16.
platelet aggregation induced by B16, but not that induced by B16F10. Since the viability of both cell lines following the phospholipase A2 treatment was >90%, the disappearance of the platelet-aggregating ability in B16 cells was not due to cell death. In addition, ADP was not involved in the activation of platelet by both cells, because all experiments were performed with PRP containing 1 unit/ml of apyrase, an ADP-degrading enzyme.

Platelet aggregation induced by the parental B16 and its metastatic variants in the presence of 10 µM MD805 was compared. Fig. 5 demonstrates the representative tracing of platelet aggregation caused by B16, B16F1, B16F10, and B16BL-6 at a tumor concentration of 5 x 10^6 cells/ml. Two high metastatic variants, B16F10 and B16BL-6, induced platelet aggregation within 10 min, and B16F1 showed weak aggregation, whereas B16 could not activate platelets under these conditions. Taking lag time into consideration, the platelet-aggregating activities of the 4 cell lines appeared to be in the order B16F10 > B16BL-6 > B16F1 > B16. This order correlated well with that of the levels of 8F11 bound to these cell lines.

Effect of 8F11 on Platelet Aggregation Induced by B16F10. In the previous study, preincubation with 1 mg/ml of 8F11 completely prevented platelet aggregation induced by NL-17 (25). The effect of 8F11 on platelet aggregation caused by B16F10 was examined by pretreating with 1 mg/ml of mAb. Although B16F10 preincubated with 1 mg/ml of 8F11 induced platelet aggregation in the absence of MD805, the same cells could not stimulate platelets in the presence of 10 µM MD805 (Fig. 6A). The enzymatic treatment of the cell surface with phospholipase A2 offered a similar result (Fig. 6B). 8F11 prevented platelet aggregation activated by the phospholipase A2-treated cells, but not by untreated cells. These observations indicate that B16F10 cells possess 2 platelet-aggregating mechanisms: one is thrombin dependent and the other is thrombin independent. The latter was inhibited by pretreatment of 8F11, suggesting that the Mr, 41,000 antigen is involved in platelet-aggregating mechanisms of B16F10.

DISCUSSION

In the present study, we demonstrated that metastatic variants of murine B16 melanoma expressed a Mr, 41,000 glycoprotein that was recognized by mAb 8F11 and might be involved in platelet aggregation induced by a thrombin-independent mechanism. The amounts of the antigen on the cell surface of B16 and its metastatic variants correlated well with the activity of these cell lines to induce platelet aggregation in a thrombin-independent manner, and the ability to form metastatic nodules in lung following i.v. injection of these cells into mice.

Several mechanisms have been proposed to explain tumor cell-induced platelet aggregation in vitro based on specific enzymatic treatment and/or inhibitors of platelet aggregation. In this study, platelet aggregation induced by murine B16 melanoma was completely blocked with MD805 (30), a specific antagonist of thrombin (Fig. 4A). A similar result was obtained from the enzymatic treatment of tumor cell surface with phospholipase A2, which produces lyssolecithin from phosphatidylcholine (Fig. 4C). Lyssolecithin has been demonstrated to inhibit platelet aggregation caused by thrombin and/or ADP derived from tumor cells (18, 34). Both data confirmed previous reports indicating that B16 melanoma activated platelets by the generation of thrombin through a procoagulant activity (35, 36).
Thrombin inhibitor and the enzymatic treatment of the cell surface with phospholipase A₂ completely inhibited platelet aggregation induced by B16, whereas a high metastatic variant, B16F10, possessed platelet-aggregating activity under the same conditions (Fig. 4, B and D). These data suggest that B16F10 activates platelets by at least 2 mechanisms: one is thrombin dependent and another induces platelet aggregation in a thrombin-independent mechanism via a phospholipase A₂-insensitive protein. The latter mechanism is in agreement with the findings reported by Lerner et al. (24). They showed that B16F10 activated platelets in a thrombin-independent mechanism with a trypsin-sensitive and phospholipase A₂-insensitive protein. However, they could not observe the former mechanism (24). This contradiction may occur by the difference of the concentration of heparin contained in PRP. It is well known that heparin forms a complex with antithrombin III, a native potent thrombin inhibitor, and enhances its inhibiting activity (37). We performed all experiments with 2.5 units/ml of heparin, thereby eliminating the danger of missing thrombin activity. An increase in the concentration of heparin in PRP was observed to prolong the lag time until the onset of platelet aggregation, as was previously reported (37). This is in good agreement with the findings reported by Lerner et al. (24).

It is of great interest that 8F11 showed specific binding to B16F10 cells and prevented platelet aggregation induced by these cells under conditions inhibiting the activity of thrombin (Fig. 6, A and B). 8F11 recognized a M, 41,000 glycoprotein as an antigen on the cell surface of B16F10, the molecular weight of which was slightly lower than that expressed on NL-17 (Fig. 2). The amount of the antigen expressed on metastatic variants of B16 melanoma was the order B16F10 > B16BL-6 > B16F1 > B16 (Fig. 1). This order was in good agreement with that of

---

Fig. 4. Effect of thrombin-inhibitor or phospholipase A₂ on platelet aggregation induced by B16 and B16F10. Platelet aggregation induced by B16 (A) and B16F10 (B) was measured in PRP containing 10 μM MD805 and 0.01% dimethyl sulfoxide (b) or 0.01% dimethyl sulfoxide (a). The cell suspensions (2 x 10⁶ cells/ml) of B16 (C) and B16F10 (D) were incubated with (b) or without (a) 25 μg/ml of phospholipase A₂ at 37°C for 30 min. After 3 washes, platelet aggregation induced by the modified cells was measured.

Fig. 5. Platelet aggregation induced by B16 and its metastatic variants in the presence of 10 μM MD805. Platelet aggregation of these cells was measured as described in Fig. 4. a, B16; b, B16F1; c, B16BL-6; d, B16F10.

Fig. 6. Effect of 8F11 on platelet aggregation induced by B16F10. a, B16F10 cells were incubated with or without 1 mg/ml of 8F11 at 37°C for 3 min. Then, platelet aggregation induced by the treated cells was measured in the presence or absence of 10 μM MD805. a, Control; b, 8F11; c, MD805; d, 8F11 and MD805. B, Cell suspension (2 x 10⁶ cells/ml) was incubated with 25 μg/ml of phospholipase A₂ at 37°C for 30 min. After 3 washes, the modified cells were treated with or without 8F11 and platelet aggregation was measured as described above. a, Control; b, 8F11; c, phospholipase A₂; d, 8F11 and phospholipase A₂.
platelet-aggregating activity of these cell lines in the presence of MD805 (Fig. 5). In the previous study, we reported that 8F11 inhibited platelet aggregation caused by NL-17, which activates platelets through a trypsin-sensitive and phospholipase A2-insensitive protein. The levels of the antigen expressed on metastatic clones of colon 26 were in good agreement with platelet-aggregating activities of these clones (25). Collectively, these data indicate that the M, 41,000 glycoprotein recognized by 8F11 in metastatic variants of murine B16 melanoma is involved in the thrombin-independent platelet-aggregating activity. This factor resembles a trypsin-sensitive and phospholipase A2-insensitive protein.

A number of investigators have reported that high metastatic variants of B16 melanoma differ from low metastatic variants in several fashions including increased amount of a TSP-180 antigen, which was assumed to be associated with a growth-regulation mechanism (40, 41); increased levels of membrane bound-protein C (42) and pertussis toxin-sensitive G-protein (43), which related to signal transductions; and disappearance of Aα actin, which was identified as a component of microfilaments (44). In this study, B16F10 was found to possess an increased amount of M, 41,000 glycoprotein compared with B16F1, and the levels of the antigen expressed on B16 and its metastatic variants correlated well with the metastatic ability of these cells after intravenous inoculation into mice (29, 42). These data suggest that this protein is also one of the metastasis-related proteins in B16 melanoma. As far as we know, the M, 41,000 antigen revealed with 8F11 is a new protein related to pulmonary metastasis of B16 melanoma. Of particular interest is the near absence of this protein in parent B16 compared with metastatic variants. Such a phenomenon was not observed in colon 26; both parent colon 26 and its metastatic variant expressed a similar level of the 8F11 antigen as NL-17 and induced platelet aggregation in a thrombin-independent manner (data not shown). Expression of the M, 41,000 glycoprotein may intimately correlate with the hematogenous spread of B16 melanoma variants.

We currently observed that 8F11 and its F(ab')2 fragment inhibited the hematogenous spread of NL-17. As described above, platelet aggregation induced by NL-17 occurs in a thrombin-independent manner. It is mainly mediated through the similar antigen (M, 44,000) described here. The high metastatic variants of B16 melanoma, however, possess 2 platelet-aggregating mechanisms, one related to the generation of thrombin and the other related to the M, 41,000 antigen described here. It might be difficult to inhibit completely the hematogenous spread of B16 melanoma variants solely by inhibiting the M, 41,000 antigen with 8F11 and its F(ab')2 fragment. Experimentation along these lines, however, still bears enough relevance to be carried out in the future.

In conclusion, the precise functions of M, 41,000 glycoprotein and thrombin-independent platelet aggregation induced by metastatic variant of B16 melanoma remain to be solved, but our results indicate that the M, 41,000 glycoprotein, being associated with platelet aggregation in a thrombin-independent mechanism, plays a pivotal role in pulmonary metastasis of B16 melanoma. Further characterization of M, 41,000 glycoprotein, which is immunologically related M, 44,000 glycoprotein expressed on colon 26, will provide more detailed understanding of the role of platelet aggregation in pulmonary metastasis of these cell lines.

ACKNOWLEDGMENTS

We are grateful to Drs. T. Yamori and H. Hamada for their helpful suggestions throughout the study.

REFERENCES


Expression of a $M_r$ 41,000 Glycoprotein Associated with Thrombin-independent Platelet Aggregation in High Metastatic Variants of Murine B16 Melanoma

Masahiko Watanabe, Yoshikazu Sugimoto and Takashi Tsuruo


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/20/6657

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.