Platelet Aggregating Activity Mediated by Thrombin Generation in the NCG Human Neuroblastoma Cell Line

Noriko Esumi, Shinjiro Todo, and Shinsaku Imashuku

Department of Pediatrics, Kyoto Prefectural University of Medicine, Kawaramachi, Kamikyoku, Kyoto, Japan 602

ABSTRACT

Platelet aggregating activity of the NCG human neuroblastoma cell line was compared with that of the HL-60 human promyelocytic leukemia cell line. NCG, in intact cell suspensions and ultracentrifuged pellets, induced platelet aggregation most significantly in hepatized platelet rich plasma (PRP) containing 2.5 units/ml of heparin, but not in the presence of higher concentrations of heparin or 5 mM ethylendiaminetetraacetate or in citrated PRP. NCG induced platelet aggregation was also inhibited by hirudin or (2R,4R)-4-methyl-1-[N(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)]-L-arginyl]-2-piperidinecarboxylic acid (MD 805) in the same manner as that of tissue thromboplastin induced platelet aggregation. HL-60 cells did not induce platelet aggregation in our heparinized PRP assay systems; however, after treatment with neuraminidase HL-60 cells became active in aggregating platelets in either hepatized or citrated PRP. NCG demonstrated high procoagulant activity by either intact cell suspensions or ultracentrifuged pellets. The procoagulant activity of NCG was reduced in Factor VII deficient human plasma as it was in the results obtained by tissue thromboplastin. These results suggest that NCG induces platelet aggregation via thrombin generated through procoagulant activity which is shed in association with microvesicles demonstrated in the ultracentrifuged pellets. This type of platelet aggregating activity found in NCG is significantly different from that of HL-60.

INTRODUCTION

Neuroblastoma is a common malignant solid tumor in childhood. Abnormal hemostasis is often noted in patients with neuroblastoma. Particularly, in advanced stages of neuroblastoma, an increase of plasma fibrinopeptide A or a disseminated intravascular coagulation has been reported (1, 2). Patients having multiple metastatic neuroblastomas result in a fatal outcome, suggesting the importance of preventing distant metastases in the treatment of this tumor. In the past decade, the role of hemostasis in cancer patients has been strongly implied as one of the triggering factors in metastasis progression. Accordingly, the management of hemostatic problems in neuroblastoma is mandatory from the viewpoint of tumor metastasis as well as hemorrhagic disorders. It is known that tumor cells produce substances enhancing blood coagulation and that they also interact directly with several host cells; among them, platelets play an important role (3-7) in the metastatic process of arrest and adhesion of the tumor cells to a vessel wall (4, 8, 9). However, little information is available about the interaction between neuroblastoma cells and platelets and/or the coagulation system (10, 11).

In terms of the interaction between tumor cells and platelets, several conflicting results have been reported in vivo and in vitro from different laboratories (12-18), and the exact role of platelets in metastasis still remains to be established. A positive correlation has been found between the number of pulmonary metastatic nodules in in vivo metastasis models and the ability of the tumor cells to induce platelet aggregation in vitro (4, 19). As artificial or spontaneous experimental metastasis models in vivo, the lung colonization method has commonly been used. Although no other alternate models are available in studying metastasis to other specific organs, many tumors form metastatic foci outside the lungs. In fact, neuroblastoma metastasizes to the bone marrow, bone, liver, and lymph nodes, but rarely to the lungs. However, the involvement of platelets and the coagulation systems appears to be inevitable in the hematogenous metastasis of neuroblastoma and other tumor cells. To date, at least three types of platelet aggregating mechanisms have been reported in different tumors: (a) membrane associated sialolipoprotein similar to the platelet aggregating material by Pearlstein et al. (20, 21); (b) ADP leaked from tumor cells (10, 22); and (c) thrombin generated through procoagulant activity of tumor cells (11, 23). Tumor cell procoagulants by themselves (11, 23-28) accelerate the coagulation system and induce hemostatic abnormalities, as well as being involved in one of the three mechanisms of platelet aggregating activity.

In this study, we investigated the platelet aggregating activity of NCG, a human neuroblastoma cell line (29), comparing with that of HL-60, a human promyelocytic leukemia cell line (30-32). NCG was found to induce platelet aggregation via thrombin associated with procoagulant activity shed in microvesicles. It was also found that procoagulant activity in NCG is exerted like a tissue factor dependent on Factor VII and is very potent compared with that in HL-60.

MATERIALS AND METHODS

Chemicals, Enzymes, and Reagents. All chemicals were reagent grade and the following were purchased from Sigma Chemical Company, St. Louis, MO: trypsin, type I from bovine pancreas (activity, 9200 BAEE units/mg protein); neuraminidase, type V from Clostridium perfringens (0.86 unit/mg protein); collagenase, type I from Clostridium histolyticum (180 units/mg solid); phospholipase A3 from bee venom (1260 units/mg protein); apyrase, grade I from potato (8.6 units/mg protein for ATP); hirudin, grade IV from leeches (1000 units/mg protein); DFP; PMSF; mercuric chloride; IAA; phosphoenol pyruvate trisodium salt; and pyruvate kinase, type II from rabbit muscle (515 units/mg protein). A highly specific thrombin inhibitor, MD 805, synthesized by Okamoto (33), was kindly provided by Mitsubishi Chemical Industries Ltd., Tokyo, Japan. Tissue thromboplastin from rabbit brain was purchased from Ortho Diagnostic Systems, Inc., Raritan, NJ. Coagulation factor deficient human plasma was obtained from Behringwerke AG, Marburg, West Germany, and Sepharose 2B was from Pharmacia, Uppsala, Sweden.

Tumor Cells and Cell Culture. The NCG human neuroblastoma cell line originally established by Brodeur et al. (29), and HL-60 human promyelocytic leukemia cell line established by Collins et al. (30) have been maintained in our laboratory. Cells were maintained with RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% heat inactivated fetal calf serum (Gibco, Ltd., Paisley, Scotland) supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml).

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1 The abbreviations used are: DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; IAA, iodoacetate; MD 805, (2R,4R)-4-methyl-1-[N(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)]-L-arginyl]-2-piperidinecarboxylic acid; HBSS, Hank's balanced salt solution; PRP, platelet rich plasma; PPP, platelet poor plasma; EDTA, ethylendiaminetetraacetate.

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To whom requests for reprints should be addressed.

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with 5 mM CaCl₂ 1 min prior to aggregation assays. The effect of incubated in PRP IV for 3 min at 37°C, which was then reconstructed involvement of thrombin in tumor cell induced aggregation, heparin to PRP II, and 2-20 mM CaCl₂ or 2-50 mM MgCl₂ were added to PRP thrombin inhibitors on platelet aggregation was evaluated for the pro were prepared and PRP II was used for standard aggregation studies. Gel filtered platelets were centrifuged at 150 x g for 10 min, the supernatant was then centrifuged in Ca²⁺-Mg²⁺ free HBSS, which was adjusted to 1 x 10⁶ cells/ml for aggregation assays and to 3 x 10⁵ cells/ml for clotting studies. In some experiments, the fresh cell suspensions were diazoyed overnight at 4°C against at least 100 volumes of Ca²⁺-Mg²⁺ free HBSS.

Cells were counted in a hemocytometer, and cell viability determined by the trypan blue exclusion method was more than 80% in all experiments.

Preparations of Ultracentrifuged Pellets and Treated Tumor Cells. The monolayers of NCG were treated with 5 mM EDTA in HBSS for 1 h at 37°C. The detached cell suspensions (50 ml) were centrifuged (100 x g, 10 min), the supernatant was then centrifuged (1000 x g, 10 min), and the final cell free supernatant was ultracentrifuged (50,000 x g, 2 h, 4°C) using the Beckman L8-55M ultracentrifuge with a type 50.2Ti fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA). HL-60 was treated with 5 mM EDTA in HBSS by gentle shaking for 1 h at 37°C, and the treated cell suspension (50 ml) was submitted for preparing the ultracentrifuged pellets. The ultracentrifuged pellets were resuspended in 500 µl of Ca²⁺-Mg²⁺ free HBSS. Protein determination was made by the method of Lowry et al. (34).

In the treatment of tumor cells with protease inhibitors or enzymes, cell suspensions were incubated with 5 mM DFP, 1 mM PMSF, 0.1 mM HgCl₂, or 1 mM IAA for 30 min at 37°C or were incubated with 1 mg/ml trypsin, 2 mg/ml neuraminidase, 1 mg/ml collagenase, or 50 µg/ml phospholipase A₂ for 1 h at 37°C. The treated cell suspensions were centrifuged at 100 x g for 10 min; the cell pellets were washed twice and resuspended in the same volume of HBSS as the primary suspensions.

Determination of ADP and ATP. Cellular ADP and ATP were measured by a modified luciferin-luciferase method using a TD-4000 lumiphotometer (Labo Science Co., Tokyo, Japan) following the procedure by Holmsen et al. (35) as described in Table 1.

Preparation of PRP. Blood was obtained from healthy laboratory staff members who had not taken anti-platelet agents for 10 days prior to the assay. Heparin Novo (final concentration, 5 units/ml) or sodium citrate (final concentration, 0.38%) was used. PRP was prepared by centrifugation at 150 x g for 10 min at room temperature. PPP was obtained by additional centrifugation of the remaining blood at 2000 x g for 20 min at room temperature. Platelet counts in PRP were adjusted to 2-4 x 10⁵ cells/ml with PPP or Ca²⁺-Mg²⁺ free HBSS. Gel filtered platelets were prepared from PRP by the method of Tangen et al. (36) using Column K 15/30 and Sepharose 2B.

Platelet Aggregation Studies. Platelet aggregation was measured turbidimetrically (37) with a Bio-Data Platelet Aggregation Profiler Model PAP-3 (Bio-Data Corporation, Horsham). PRP (450 µl) was warmed to 37°C for 2 to 3 min in a siliconized flat bottomed cuvet and stirring continuously at 1000 rpm in the aggregometer to obtain a baseline line on the recorder. Then, 50 µl cell suspensions or resuspended ultracentrifuged pellets were added, and aggregation was recorded as an increase in light transmission.

Heparinized PRP I and II and citrated PRP III and IV (Table 2) were prepared and PRP II was used for standard aggregation studies. In studies testing the effect of various compounds on tumor cell induced platelet aggregation, 5 mM EDTA or 60-480 µg/ml apyrase were added to PRP II, and 2-20 mM CaCl₂ or 2-50 mM MgCl₂ were added to PRP IV immediately prior to the addition of tumor cells. To investigate the involvement of thrombin in tumor cell induced aggregation, heparin (0.5-5.0 units/ml) or hirudin (0.4-40.0 units/ml) was incubated in PRP IV for 30 min at room temperature, or MD 805 (0.2-40 µM) was incubated in PRP IV for 3 min at 37°C, which was then reconstituted with 5 mM CaCl₂ 1 min prior to aggregation assays. The effect of thrombin inhibitors on platelet aggregation was evaluated for the prolongation of lag time and expressed as lag time (min)^-1. Tissue thromboplastin was used at a final concentration of 21.6 µg/ml (10.8 µg/assay) as a positive control producing thrombin.

Clotting Assays. Procoagulant activity was measured by the single stage recalcification time. PPP was freshly prepared as described above from citrated plasma. One hundred µl of the sample or HBSS were added into 100 µl of PPP prewarmed for 2 min at 37°C in a glass tube, and the mixture was incubated for another min. The reaction was initiated by adding 100 µl prewarmed 25 mM CaCl₂, and the clotting time was measured in seconds. Recalcification time by tumor cells was also measured in human plasma deficient in one of the following coagulation factors: V, VII, VIII, or X. Tissue thromboplastin (21.6 µg/assay; final, 72 µg/ml) was used in this system as a positive control activating the extrinsic coagulation pathway.

Electron Microscopy. Ultracentrifuged pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C, held in 0.1 M cacodylate buffer at 4°C overnight, and fixed in the cold with 1% osmium tetroxide in the same buffer for 1 h. Then, the fixed samples were dehydrated in a series of graded ethanol solutions and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined by a Hitachi-H-500 transmission electron microscope.

RESULTS

Platelet Aggregation by Fresh Cell Suspensions. Fresh cell suspensions of NCG induced platelet aggregation consisting of two different components without a lag period in heparinized PRP (Fig. 1, top). HL-60 showed only a small reversible aggregation curve without a second wave or a notch (data not shown).

The prompt aggregation wave by the fresh cell suspensions was inhibited by aprotinin, an ADP degrading enzyme, in a dose dependent manner. In addition, as time progressed, these aggregation waves became smaller after preparation of the cell suspensions. To exclude the probable effect of ADP, which may leak from the damaged cells during preparation, NCG fresh cell suspensions were further centrifuged, and the supernatant and the resuspended cell pellets were separately tested. Dialedized

Fig. 1. Platelet aggregation induced by NCG. Fifty µl of each sample were added (arrows) to 450 µl of prewarmed and stirring PRP (heparin, 2.5 units/ml) and aggregation was measured turbidimetrically with an aggregometer as an increase in light transmission. Top: fresh cell suspension (5 x 10⁶ cells/assay). Fresh cell suspension was further centrifuged, dividing into the supernatant and the resuspended cell pellets. Cell suspension dialyzed overnight at 4°C was also prepared. Bottom: Curve a, the supernatant (from suspension at 1 x 10⁶ cells/ml); Curve b, the resuspension of cell pellets (5 x 10⁵ cells/assay); Curve c, dialyzed cell suspension (5 x 10⁶ cells/assay). Data were taken from 1 of 6 separate experiments with similar results.
cell suspensions were also examined. The supernatant promptly induced platelet aggregation similar to that of ADP without a notch (Fig. 1, bottom, Curve a). By contrast, the resuspended tumor cell pellets and the dialyzed cell suspensions induced irreversible aggregation with a lag time (Fig. 1, bottom, Curves b and c), followed by a visible fibrin clot formation in a cuvet in 5 to 10 min after platelet aggregation.

Contents of ADP and ATP. To study the involvement of ADP in platelet aggregation by tumor cells, the contents of ADP and ATP were measured in both intact and surfactant-lyzed cell suspensions. Results are shown in Table 1; the ADP concentrations in fresh intact cell suspensions of NCG and HL-60 ranged from 3.4 to 4.0 μM per 1 × 10⁶ cells/ml, which were the concentrations of cell suspensions used for the aggregation assay. These concentrations of ADP were sufficiently high to induce platelet aggregation, because ADP at more than 0.2 μM aggregated platelets and ADP at more than 1.0 μM induced the maximum aggregation in our heparinized human PRP system (data not shown). Therefore, all of the results presented below were obtained using cell suspensions prepared by additional washing or dialysis to exclude the influence of ADP.

Platelet Aggregation by Cell Suspensions Depleted of ADP and by Ultracentrifuged Pellets. NCG cell suspensions and ultracentrifuged pellets demonstrated similar platelet aggregation with a lag period (Fig. 2). This aggregation was induced most significantly in PRP II [heparinized PRP diluted (1:1) with HBSS] but not in PRP I [heparinized PRP diluted (1:1) with PPP] (Table 2). In addition, platelet aggregation by NCG was not seen in citrated PRP except in a specific system as can be seen in Table 2. HL-60 did not induce platelet aggregation either by cell suspensions or by ultracentrifuged pellets. When the lower concentrations of cell suspensions (down to 2.5 × 10⁵ cells/assay) and ultracentrifuged pellets (down to 20 μg protein/assay) of NCG were used, prolongation of the lag time but no significant change in the maximum aggregation was observed.

Morphology of Ultracentrifuged Pellets. As shown in Fig. 3, transmission electron microscopy of the ultracentrifuged pellets of NCG disclosed numerous microvesicles with a granular background. These crude microvesicle pellets included cell debris and intracellular organelles to some degree. Similar microvesicles were also obtained by following the same procedures from the spent medium in which NCG cells were cultured for 24 h and from the serum free medium in which NCG cells were incubated overnight.

Effect of Heparin, Divalent Cations, and Apyrase on Platelet Aggregation. As indicated in Table 2, NCG induced platelet aggregation was observed in PRP II (heparin, 2.5 units/ml) at concentrations as follows. Top: Cell suspensions were NCG (5 × 10⁶ cells/assay) and HL-60 (5 × 10⁶ cells/assay). Bottom: Concentrations of ultracentrifuged pellets were NCG (50 μg protein/assay) and HL-60 (50 μg protein/assay). Data were taken from 1 of 4 separate experiments with similar results.

Table 1 Contents of adenine nucleotides in tumor cell suspension

<table>
<thead>
<tr>
<th>Specimens</th>
<th>ATP (nmol/10⁶ cells)</th>
<th>ADP (nmol/10⁶ cells)</th>
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<tbody>
<tr>
<td>NCG suspension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>10.1 ± 4.6</td>
<td>4.0 ± 1.1</td>
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<tr>
<td>Lysed cells</td>
<td>8.2 ± 4.3</td>
<td>8.8 ± 3.4</td>
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<tr>
<td>HL-60 suspension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact cells</td>
<td>6.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Lysed cells</td>
<td>7.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEP-PK, phosphoenolpyruvate-pyruvate kinase.
Table 2 Effect of heparin, apyrase, and divalent cations on NCG-induced platelet aggregation

<table>
<thead>
<tr>
<th>Platelet suspension</th>
<th>Additives</th>
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<th>Lag</th>
<th>Maximum</th>
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<tr>
<td></td>
<td></td>
<td>concentration (min)</td>
<td>time (min)</td>
<td>% of transmission</td>
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<tr>
<td>PRP I</td>
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<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PRP II</td>
<td>None</td>
<td>1.6</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>EDTA (mm)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Apyrase (mg/ml)</td>
<td>60</td>
<td>1.8</td>
<td>40</td>
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<td></td>
<td></td>
<td>120</td>
<td>1.6</td>
<td>40</td>
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<td>480</td>
<td>1.9</td>
<td>44</td>
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<tr>
<td>PRP III</td>
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</tr>
<tr>
<td>PRP IV</td>
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<td>2</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Ca2+ (mm)</td>
<td>5</td>
<td>0.6</td>
<td>25++</td>
</tr>
<tr>
<td></td>
<td>Mg2+ (mm)</td>
<td>10</td>
<td>0.6</td>
<td>15+++</td>
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<tr>
<td>Gel filtered</td>
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<td>0</td>
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</tbody>
</table>

A fibrin clot was formed in the middle of aggregation.

aggregation. A visible fibrin clot was noticed in the middle of the aggregation slope in PRP IV reconstructed with Ca2+ or Mg2+. NCG did not induce aggregation in gel filtered platelet suspension.

Effect of Enzyme Treatment on Platelet Aggregation. Platelet aggregation induced by NCG cell suspensions was inhibited only by phospholipase A2 but not affected by trypsin, neuraminidase, or collagenase (Fig. 4). NCG ultracentrifuged pellets treated with enzymes demonstrated similar results to cell suspensions (data not shown). HL-60, which did not induce platelet aggregation by intact cell suspensions, aggregated platelets with a lag period after treatment with 2 mg/ml neuraminidase in either heparinized or citrated PRP (PRP I–IV). Neuraminidase at the same concentration did not affect the activity of NCG.

Effect of Thrombin Inhibitors on Platelet Aggregation. We used citrated PRP reconstructed with 5 mM CaCl2 to exclude the possible interaction or additive or synergistic effect of heparin and other thrombin inhibitors. Heparin, hirudin, and MD 805 inhibited both NCG and thromboplastin induced platelet aggregation in the same manner. Fig. 5 demonstrates a dose dependency in prolongation of lag time by these three thrombin inhibitors. In the higher concentration of inhibitors, especially heparin at more than 2.5 units/ml, a fibrin clot was not observed in a cuvet of platelet aggregation even 5 h later.

Clotting Assays by Cell Suspensions and Ultracentrifuged Pellets. As shown in Fig. 6, NCG cell suspensions and ultracentrifuged pellets significantly shortened the single stage recalcification time in a dose dependent manner and HL-60 demonstrated moderate procoagulant activities. In either preparation of samples, NCG demonstrated higher procoagulant activity than HL-60. Table 3 summarizes the effect of protease inhibitors and enzyme treatment on the procoagulant activity of NCG. No significant reduction of the activity was observed by either DFP, PMSF, HgCl2, or IAA. These protease inhibitors did not affect the recalcification time by HL-60 (not shown). In NCG, only phospholipase A2 prolonged the recalcification time, but neither trypsin, nor neuraminidase, nor collagenase affected it in the same way as with platelet aggregation.

Clotting studies with coagulation factor deficient human plasma are summarized in Table 4. A significant shortening of the recalcification time by NCG was observed in normal and Factor VIII deficient human plasma, but a reduced procoagulant activity was noted in Factor VII deficient human plasma. HL-60 showed similar results but was less active than NCG. Thromboplastin showed conspicuously similar results to NCG in these human plasma. Accordingly, it was confirmed that the coagulation factors in the common pathway (Factors V and X) were essential, but the intrinsic coagulation factor (Factor VIII) was not necessary for the expression of these tumor cell associated coagulant activities. The data also suggested that procoagulant activities of NCG and HL-60 were expressed via activation of Factor VII in the extrinsic coagulation pathway and subsequently via activating the steps in the common pathway as well as tissue factor (tissue thromboplastin).

DISCUSSION

In this study of NCG, we screened several other cell lines and finally used HL-60 as a control. HL-60 was previously reported to have a tissue factor-like procoagulant activity (31, 32) and to induce platelet aggregation by a thrombin dependent mechanism (23). Using NCG and HL-60, we first observed that fresh cell suspension induced platelet aggregation did not have a lag phase and was completely inhibited by apyrase as seen in ADP induced aggregation. Determination of adenine nucleotide contents in tumor cell suspensions suggested that the concentration
of ADP in the assay system was sufficient to induce platelet aggregation, if ADP is leaked from tumor cells. An aggregation curve with a lag time was subsequently obtained by additional washing or dialysis of tumor cell suspensions. These results suggested that in the fresh cell suspension, ADP initiated platelet aggregation as suggested by Holme et al. (22), but other factors also contributed to aggregation. In subsequent studies, it was confirmed that the central mechanism of aggregation by these tumor cells is not mediated by ADP.

Platelet aggregation by NCG was induced only in heparinized PRP but not in citrated PRP and was most significant in PRP II (heparin, 2.5 units/ml) but not obtainable in PRP I (heparin, 5 units/ml). In addition, heparin inhibited platelet aggregation by NCG in a dose dependent manner and 5 units/ml of heparin were sufficient to inhibit the expression of this activity. To date, several investigators (4, 20, 38–40) have reported that tumor cell induced platelet aggregation was seen only in heparinized PRP but not in citrated PRP. In such reports, heparin at concentrations of 5 to 10 units/ml was commonly used as an anticoagulant, and no inhibitory effect of heparin on platelet aggregation was noted. However, Tohgo et al. (41) demonstrated that heparin completely prevented aggregation by B16 and 3LL cells at final concentrations of 1 and 10 units/ml, respectively, and that the platelet aggregating activity of these two cell lines was mediated by thrombin generated through tumor cell coagulants. Our results on NCG supported Tohgo’s data, emphasizing that heparinized PRP at the commonly used heparin concentrations is not suitable for detecting certain types of tumor cell induced platelet aggregation.
As for the requirement of divalent cations, platelet aggregation by NCG was completely inhibited by citrate or EDTA; however, the inhibition in citrated PRP was overcome by adding more than 5 mM CaCl₂ or 10 mM MgCl₂, resulting in platelet aggregation and subsequent fibrin clot formation. Thus, NCG required Ca²⁺ for the expression of platelet aggregating activity. To the contrary, platelet aggregation by neumaminidase treated HL-60 was equally induced in either citrated or heparinized PRP. Furthermore, hirudin or MD 805 similarly inhibited the platelet aggregations by NCG and by thromboplastin. These results suggest that thrombin is involved in NCG induced platelet aggregation and that the mechanisms of platelet aggregation by NCG or neumaminidase treated HL-60 are quite different.

The clotting studies further indicated the existence of procoagulant activity that was very potent in NCG and moderate in HL-60. Procoagulant activities associated with the ultracentrifuged pellets were in the same order as in the cell suspensions. In general, at least three different types of tumor cell procoagulants have been described: (a) a tissue factor-like substance dependent on Factor VII (11, 23, 26, 42–44); (b) protease activating Factor X to X₄ (27, 45); and (c) a phospholipid surface for prothrombinase complex formation (42, 46). Our studies on NCG in coagulation factor deficient plasma revealed a dependency on Factor VII and no dependency on Factor VIII. In addition, the tissue thromboplastin behaved quite similarly to NCG. The fact that phospholipase A₂ abolished NCG induced platelet aggregation as well as coagulation may reflect that phospholipids are required for the expression of tissue factor activity (47, 48) and for the acceleration of the coagulation cascade by providing a phospholipid surface, on which platelet aggregation takes place. HL-60 had a procoagulant activity showing characteristics similar to those of NCG but did not aggregate platelets unless treated with neumaminidase. This phenomenon was probably due to the insufficiency of procoagulant activity in HL-60. The results presented here suggested that intact cells and microvesicles of NCG induce platelet aggregation mediated by thrombin, which is generated through tissue factor-like substance. Although intact cells of HL-60 did not initiate platelet aggregation in our system, the fact that neumaminidase treated HL-60 became active in inducing platelet aggregation requires further investigation.

Several investigators have demonstrated that intact tumor cells are not necessarily essential to induce platelet aggregation or coagulation. Pearlstein et al. (19, 20) identified a sialolipoprotein extracted with 1 M urea as a platelet aggregating material, and Gasic et al. (49) demonstrated the platelet aggregating activity associated with cell membrane vesicles shed during culture. Dvorak et al. (42) identified the procoagulant activity associated with plasma membrane vesicles shed in the medium by cultured tumor cells, and Bastida et al. (23) also reported that procoagulant and platelet aggregating activities were found in microvesicles shed from U87MG human glioblastoma cell line and that platelet aggregating activity was mediated by procoagulant activity. Although NCG microvesicle pellets were not highly purified, platelet aggregating activity and procoagulant activity were both recovered in the ultracentrifuged pellets. Our results are compatible with the data of Bastida et al. and the present report is the first describing the shedding of platelet aggregating activity and procoagulant activity associated with microvesicles in human neuroblastoma.

Concerning the interaction between neuroblastoma cells and hemostatic systems, Hara et al. (38) reported the platelet aggregation by a mouse neuroblastoma cell line (Neuro-2a) via sialolipoprotein activating platelet secretory reaction, Bastida et al. (10) demonstrated that platelet aggregation induced by human neuroblastoma SKNMC was inhibited by apyrase and the phosphonoenolpyruvate-pyruvate kinase system but not by hirudin, suggesting the involvement of ADP in the first step, and Pearlstein et al. (11) described that four different neuroblastoma cell lines aggregated platelets and that one line (IMR) had appreciable procoagulant activity, but no studies were made to link both activities as we clarified in NCG. The discrepancy of the results between SKNMC and NCG may result from probable heterogeneity in human neuroblastoma cell lines, a difference in the ADP clearing system used, or the difficulty in detecting SKNMC induced platelet aggregation via thrombin as we first encountered in our heparinized PRP assay system.

It is obvious that several different mechanisms are present in inducing platelet aggregation from one tumor type to another. If platelet aggregating activity is mediated through very potent procoagulant activity, as in human neuroblastoma described here, the involvement of platelets and the coagulation system in metastasis is inevitable as one of the host factors. Platelets have been suggested to play an important role in the hematogenous metastasis of tumor cells to the lungs. On the other hand, the fact that NCG, one of the cell lines of neuroblastoma which is rarely seen to metastasize to the lungs, was also found to have strong platelet aggregating and procoagulant activities should be carefully evaluated, and the exact role of hemostatic components in metastasis to organs other than the lungs should be clarified in the future.

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PLATELET AGGREGATION BY NEUROBLASTOMA


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Noriko Esumi, Shinjiro Todo and Shinsaku Imashuku


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