Tumor gangliosides enhance $\alpha_2 \beta_1$ integrin-dependent platelet activation

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Abstract

Gangliosides, sialic acid-containing glycosphingolipids, enhance platelet adhesion to collagen and consequent platelet activation. For example, gangliosides shed by neuroblastoma tumor cells (NBTG) added to a subthreshold (non-activating) concentration (1 μg/ml) of collagen, cause platelet aggregation (59 $\pm$ 10%) and ATP release (2.3 $\pm$ 0.2 nmol) equivalent to that caused by 10 μg/ml collagen alone. Here we report further studies to characterize this effect. Platelet aggregation and ATP release were not induced by NBTG in combination with subthreshold concentrations of adenosine diphosphate, epinephrine, thrombin or arachidonic acid, suggesting that NBTG specifically influences collagen-mediated platelet activation. Maximal platelet aggregation and ATP release required extracellular magnesium and only a short (1 min) preincubation with NBTG, suggesting a collagen receptor-mediated mechanism of this ganglioside activity. Since gangliosides interact with several integrin receptors, we determined whether NBTG influences $\alpha_2 \beta_1$, a major integrin collagen receptor on platelets. Incubation of platelets with a monoclonal antibody directed against the α_2 chain (5E8) blocked the increase in platelet aggregation (9 $\pm$ 3% vs. 80 $\pm$ 2%) and ATP release (< 0.2 vs. 2.5 $\pm$ 0.1 nmol) induced by NBTG and 1 μg/ml collagen. Incubation with an antibody to the non-integrin collagen receptor, CD36, or with an isotype control antibody did not abrogate the effect of NBTG. Finally, NBTG and its major component, G$_{D2}$, enhanced $\alpha_2 \beta_1$-mediated platelet adhesion to immobilized collagen in an antibody 5E8-inhibitable manner. These findings implicate the $\alpha_2 \beta_1$-collagen interaction as a target of the effect of tumor-derived gangliosides.

Keywords: Ganglioside; Platelet; Integrin; Adhesion

1. Introduction

Tumor metastasis is a complex process consisting of a series of steps including local tumor growth, invasion and release of tumor cells into the lymphatic or vascular system, their transport in the circulation and interaction with platelets and coagulation proteins, subsequent arrest at a distant site, migration into the tissue, and finally growth at the secondary site. This process is dependent on a number of cell-cell interactions including homotypic platelet interactions [1,2] in which enhanced platelet activation may play an important role [3–7]. The mechanisms which underlie this enhanced platelet activation are not known [6]. However, certain tumor cells activate platelets in vitro [6], possibly by an action of membrane bound

sialic acid-containing complexes [8]. Gangliosides, sialic acid-containing glycosphingolipids found predominantly in cell membranes are candidates for such activity. These molecules have a water-soluble carbohydrate head group and a lipophilic tail, are shed by tumor cells in substantial quantities, and enter the circulation [9–11] where they are transported in association with lipoproteins [12]. We postulate that in the circulation, these molecules interact with host cells and modify their function in such a way as to promote tumor metastasis.

We previously reported that circulating tumor-derived gangliosides interact with platelets to enhance collagen-mediated activation [13]. Pre-incubation of platelets with the tumor-derived gangliosides resulted in platelet aggregation and ATP release following exposure to a sub-threshold (non-activating) concentration of collagen (i.e., the highest concentration of collagen that failed to induce $\geq$ 10% platelet aggregation or $\geq$ 0.2 nmol ATP release) [14]. In the present work, we further studied the role of these

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gangliosides in the process of platelet activation and delineate one possible mechanism for this activity. The evidence suggests that tumor gangliosides sensitize, or prime, platelets for the activating effects of collagen through an effect on $\alpha_2 \beta_1$ integrin-mediated platelet activation. We propose that this activity, modulation of integrin function, may be one mechanism by which tumor-derived gangliosides enhance tumor progression, by augmenting cell-cell interactions and subsequent metastasis.

2. Materials and methods

Materials. To assess platelet aggregation and ATP release, equine type I collagen from Chronolog Corp. (Havertown, PA) was used. Native collagen fibrils from equine tendons (Collagen reagent Horm, Hormon-Chemie, Munich, GMBH) were used to assess adhesion. Adenosine diphosphate, epinephrine, thrombin, arachidonic acid, aspirin and RGDS peptide were obtained from Sigma (St. Louis, MO). The anti-α_2 monoclonal antibody 5E8 [15] was generously provided by Richard Bankert, the OKM5 and OKM8 anti-CD36 antibodies [16] were a gift from Patricia Rao and the LA-N5 cells [17] were kindly provided by Robert Seeger.

Cell culture. LA-N5 human neuroblastoma cells were passaged without antibiotics in Leibowitz media supplemented with 15% fetal serum (Gibco) and 2 mM L-glutamine. The cells were maintained at 37°C with 5% CO_2 and fed every other day.

Ganglioside isolation. Total cellular gangliosides were isolated from LA-N5 neuroblastoma cells as previously described [18]. Briefly, the total lipids were extracted twice with 10 volumes of chloroform-methanol (1:1), the extracts combined, dried under a stream of N_2, re-dissolved in a small volume of chloroform-methanol (1:1), and stored overnight at $-20°C$. Insoluble glycoproteins were removed by centrifugation (1000 × g, 4°C) and the supernatant dried under a stream of N_2. The gangliosides were isolated by partitioning the dried total lipid extract in diisopropyl ether/1-butanol/water (6:4:5, v/v) [19], followed by Sephedex G-50 gel exclusion chromatography to remove traces of salts and other low molecular weight contaminants and further purified by normal phase high-pressure liquid chromatography [18,20] using a Hibar RT LiChrosorb Si100 NH$_2$ column (Merck, FRG). The total ganglioside fraction was collected, lyophilized and repurified by gel exclusion chromatography to remove salts. Gangliosides were quantified as nmol lipid bound sialic acid (LBSA) by a modification of the method of Svennerholm [21,22] and visualized by high performance thin-layer chromatography (HPTLC) [11,23]. Gangliosides were stained with resorcinol reagent and visualized as purple bands.

Platelet isolation. Platelet donors had abstained from all medications for a minimum of seven days, had fasted overnight, and provided written informed consent. Platelets were isolated from three donors according to the methods of Mustard et al. [24]. Briefly, blood was drawn into a plastic syringe containing 35 units/ml preservative-free heparin. Additional anticoagulant [ACD-A(1:6)] was added and the platelet-rich plasma (PRP) isolated by centrifugation (100 × g, 25 min at 22°C). For the aggregation and ATP release assays, the platelets were pelleted (3000 × g, 15 min at 22°C), resuspended in 5 ml ACD-A, washed twice (3000 × g, 15 min at 22°C), resuspended in citrated modified Tyrode's buffer (MTB) without Ca^{2+} or Mg^{2+} and washed two additional times prior to pooling for use. Platelets to study adhesion were prepared from platelet-rich plasma which was concentrated and gel-filtered in MTB without added Ca^{2+} or Mg^{2+} over Sepharose 2B (Pharmacia, Piscataway, NJ) according to the methods of Coller et al. [25].

Platelet aggregation. Platelet aggregation was measured with a Model 600, P.I.C.A.™ Chronolog lumi-aggregometer (Chrono-Log Corp., Havertown, PA) [26]. Washed platelets were re-suspended at $10^5/\mu l$ in purified gangliosides dissolved in citrated MTB, and incubated for one hour (unless otherwise indicated) at 37°C with gentle mixing. Following this preincubation, the platelets were washed three times, re-suspended to a concentration of $10^5/\mu l$ in MTB containing 2 mmol/l each of $CaCl_2$ and $MgCl_2$. 225 μl of the platelet suspension was added to siliconized glass cuvettes containing silicon-coated stir bars, and incubated for 30 min (37°C). Agonists were added in a 25-μl volume to achieve the indicated final concentrations. Aggregation was measured turbidometrically [27] as the change in light transmission compared to a reference cuvette containing only MTB and collagen. All experiments were performed in the absence of plasma. Neither fibrinogen nor other proteins were added to the reaction cuvette. Optimal and subthreshold concentrations of collagen were the lowest that induced maximal aggregation or ATP release and the highest that failed to induce ≥ 10% aggregation or ≥ 0.2 nmol ATP release, respectively. In these experiments, the platelet enhancing activity of NBTG was identified by utilizing a non-activating, sub-threshold concentration of collagen [14] and determining whether pre-exposure of platelets to gangliosides rendered them susceptible to activation by this subthreshold concentration of collagen.

ATP release. ATP release was measured simultaneously in the aggregometer by the Chrono-Lume luciferace-luciferin assay [26,28] with the gain set to produce a full-scale deflection upon complete ATP release according to the manufacturer's instructions. The amount of ATP released (nmol/2.5 × 10^7 platelets/250 μl) was quantified by comparison of peak heights generated in experimental cuvettes compared to those generated by known standard amounts of ATP.

Platelet adhesion assay. Platelet adhesion was determined according to the methods of Ill et al. [29] and

Santoro et al. [30] as modified by Coller et al. [25]. Native collagen fibrils from equine tendons (Collagen reagent Horm, Hormon-Chemie, Munich, GMBH) were diluted with isotonic glucose (pH 2.7–2.9) to a concentration of 33 μg/ml. 100 μl of this suspension was used to coat the wells of a polystyrene microtiter plate (Falcon 3915, Becton-Dickenson) overnight at 22°C. The wells were aspirated and blocked with 100 μl 0.5% bovine serum albumen (BSA) solution for one hour at 22°C then washed three times. Gel-filtered platelets were adjusted to $10^5/\mu$l in MTB containing 2 mmol/l $MgCl_2$ and 100 μl of the final platelet suspension added to each well. After a 60-min incubation at 37°C with gentle mixing, the wells were vigorously washed six times with MTB to remove non-adherent platelets and aggregates. The presence of platelet aggregates was assessed by light microscopy before and after washing. While occasional loose, large aggregates were present after the 60-min incubation period, in all experiments, minimal or no aggregates were present at the conclusion of the assay. The adherent platelets were removed with trypsin (0.01%) and counted automatically (CoulterSTKR, Coulter Electronics, Heighleia, FL).

3. Results

Effect of gangliosides on collagen-initiated platelet activation. The total cellular gangliosides isolated from human neuroblastoma tumor cells (NBTG) enhanced ATP release and platelet aggregation in response to non-activating concentrations of collagen. This is shown in Fig. 1, in which the activity of 5 μM NBTG is compared to that of gangliosides isolated from normal human brain (HBG) and

normal human serum (HSG). No ATP release (≤ 0.2 nmol) was observed when platelets were pre-incubated in 5 μM HSG, while a significant increase in ATP release (1.3 ± 0.1 nmol) was seen when platelets were preincubated in HBG ($P < 0.05$). Preincubation in NBTG caused the greatest ATP release (2.3 ± 0.2) ($P < 0.01$). Differences in potency among NBTG, HBG, and HSG were also evident in their effects on platelet aggregation (59 ± 10, 31 ± 12, and $< 10\%$ aggregation, respectively). These results confirm that circulating gangliosides of tumor patients enhance platelet activation [13] and support our conclusion that ganglioside molecules derived from tumor cells may underlie the activity of the circulating gangliosides in patients with neuroblastoma. The results also suggest that the specific molecular structure of the gangliosides influences their ability to activate platelets. The most active gangliosides, NBTG, were therefore chosen to conduct the experiments described below.

Effect of NBTG on platelet activation initiated by other agonists. The first step in determining the mechanism of the effects of NBTG on platelet activation was to examine the possible selectivity of NBTG preincubation, by testing its effect on platelet activation by other agonists. Optimal and subthreshold concentrations of adenosine diphosphate (5×10^{-4} M and 5×10^{-6} M), epinephrine (1×10^{-4} M and 1×10^{-6} M), thrombin (1 u/ml and 0.1 u/ml) and arachidonic acid (1000 ug/ml and 100 μm/ml) were determined for washed platelets resuspended in MTB containing 2 mmol/l each of $MgCl_2$ and $CaCl_2$. Next, preincubation of platelets with 5 μM NBTG was studied. This causes maximal ATP release following exposure of the platelets to a subthreshold concentration of collagen [13]. However, this same NBTG concentration, together with
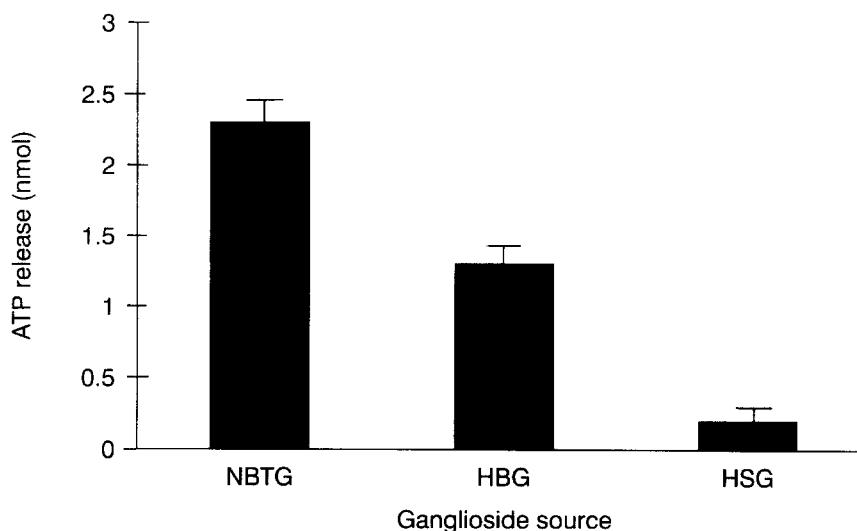

Fig. 1. Effect of gangliosides on platelet ATP release. Washed platelets were preincubated with 5.0 μM NBTG, HBG or HSG for 30 min at 37°C with gentle rocking. Unbound gangliosides were removed by centrifugation and the platelets resuspended to a concentration of $10^5/\mu$l in MTB containing 2 mmol/l $CaCl_2$ and $MgCl_2$. A subthreshold concentration of collagen (1 μm/ml) was added and ATP release determined. Data shown are the means ± S.E.M. of three experiments, each performed in duplicate. NBTG, neuroblastoma tumor gangliosides; HBG, human brain gangliosides; HSG, human serum gangliosides.
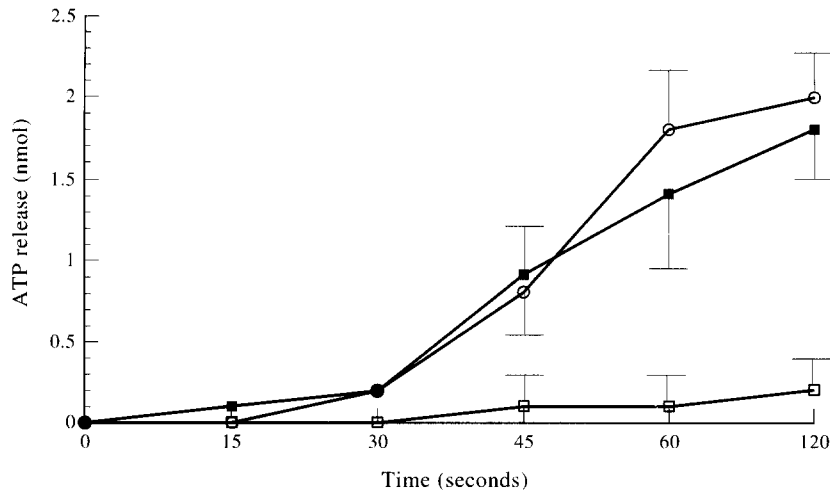
Fig. 2. Effect of NBTG on the lag phase of collagen-initiated platelet activation. Washed platelets were incubated with NBTG (0.05 μM) for 30 min at 37°C, washed twice to remove unbound gangliosides, resuspended to a concentration of $10^5/\mu$l in MTB with 2 mmol/l $CaCl_2$ and $MgCl_2$, and exposed to a subthreshold concentration of collagen (1 μg/ml) (solid squares). Tubes were rapidly chilled in an ice bath and ATP release determined at the indicated times after addition of collagen. Control platelets were incubated in buffer and then exposed to a standard (10 μg/ml) (open circles) or subthreshold concentration of collagen (1 μg/ml) (open squares). Data shown are the means ± S.E.M. of three experiments, each performed in duplicate.

sub-threshold concentrations of adenosine diphosphate, epinephrine, arachidonic acid or thrombin caused no ATP release (Table 1) and is contrasted to ATP release by optimal concentrations of each agonist. These results suggest that NBTG influences a specific, collagen-dependent, process rather than some less specific process leading to platelet activation.

Effect of NBTG on the lag phase of collagen activation. Collagen-mediated platelet activation is characterized by an initial receptor-ligand interaction, a lag phase, and a terminal response phase which can be measured as platelet

aggregation or secretion. Having shown that NBTG enhances the terminal response phase of platelet activation, we next determined the effects of NBTG on the lag phase of platelet activation. NBTG-incubated platelets were exposed to collagen for 15–120 s and ATP release measured (Fig. 2). Platelets preincubated in 0.05 μM NBTG and exposed to 1 μg/ml collagen (filled squares) release ATP in a manner indistinguishable from control platelets preincubated in MTB without gangliosides and exposed to a standard concentration (10 μg/ml) of collagen (open circles). In each case, ATP release did not occur until 45 s
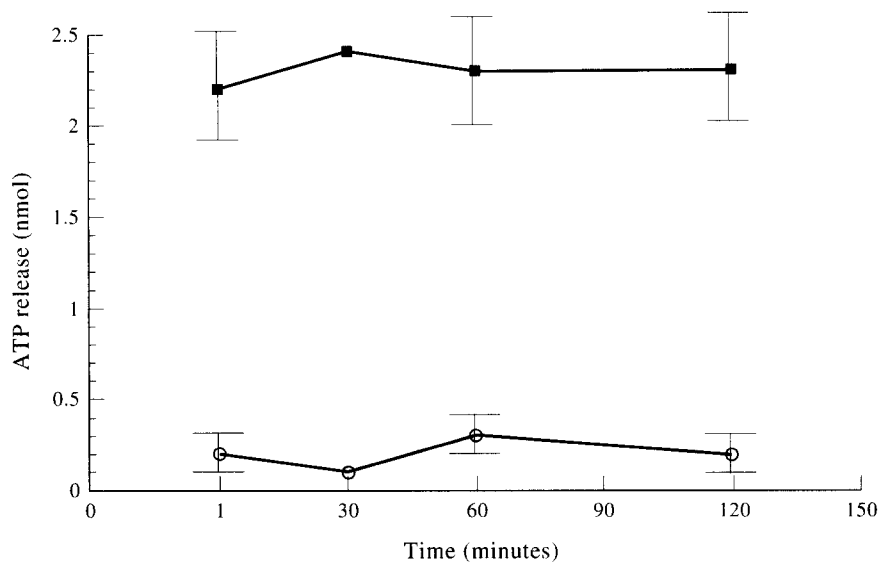

Fig. 3. Effect of duration of ganglioside preincubation on collagen-initiated platelet activation. Washed platelets were incubated with 5.0 μM NBTG for the time indicated, rapidly chilled (4°C), and unbound gangliosides removed by centrifugation. The platelets were resuspended to a concentration of $10^5/\mu$l in pre-warmed (37°C) MTB with 2 mmol/l $CaCl_2$ and $MgCl_2$. After a 30 min recovery period, a subthreshold concentration of collagen (1 μg/ml) was added and ATP release determined. Control platelets (open circles) were incubated in buffer and treated in parallel with NBTG-incubated platelets (solid squares). Each point represents the mean ± S.E.M. of three separate experiments, each performed in duplicate.

Table 1
Effect of NBTG on platelet ATP release

Platelet agonist	Concentration	ATP release $(nmol/2.5 \times 10^7/250 \, uL)$ [a]	
		Control	NBTG
Collagen	10 μg/ml	1.4±0.1	nt [b]
	1 μg/ml	< 0.2	1.7±0.1
ADP	5×10^{-4} M	1.6±0.2	nt
	5×10^{-6} M	< 0.2	0.2±0.1
Epinephrine	1×10^{-4} M	1.0±0.1	nt
	1×10^{-6} M	< 0.2	< 0.2
Thrombin	1 u/ml	2.0±0.1	nt
	0.1 u/ml	< 0.2	< 0.2
Arachidonic acid	1000 μg/ml	0.8±0.1	nt
	100 μg/ml	< 0.2	< 0.2

[a] Results are the average of duplicate determinations and standard error from three separate experiments.

[b] not tested.

after the addition of collagen, at which time the amount of ATP released was identical: (0.9 ± 0.3 vs. 0.8 ± 0.3 nmol ATP) and greater than the amount released from control platelets preincubated in MTB without gangliosides and exposed to the sub-threshold concentration of collagen (open squares) (< 0.2 nmol ATP). The preservation of the characteristic lag phase and the identical pattern of ATP release suggests that NBTG acts at a proximal step in the process of collagen-mediated platelet activation and furthermore does not appear to alter the normal physiological response of the platelet to collagen.

Effect of the duration of NBTG preincubation on platelet activation. We next determined the time course of the priming effects of NBTG on platelet activation. In these experiments, platelets were preincubated with 5 μM NBTG at 37°C for 1 to 120 min, then rapidly chilled to 4°C, and unbound NBTG removed by centrifugation. The platelet pellet was resuspended and equilibrated for 30 min in pre-warmed (37°C) MTB with 2 mmol/l $CaCl_2$ and 2 mmol/l $MgCl_2$, and ATP release measured (Fig. 3). Minimal ATP release (< 0.3 nmol), was observed in control experiments when platelets were preincubated in MTB for up to 120 min and then exposed to the sub-threshold concentration of collagen. In contrast, maximal activity (2.2 ± 0.2 nmol ATP released) was observed following preincubation in NBTG regardless of how long (120 min) or short (1 min) the preincubation time. The rapid onset of the enhancing effect of NBTG suggests that the effect is independent of platelet metabolism and may reflect rapid ganglioside binding to platelets. Combined with the specificity for collagen and the probable proximal site of action, the results suggested that NBTG might interact with and influence the binding of collagen to a surface collagen receptor.

NBTG interacts with platelets by an $\alpha_2 \beta_1$-dependent mechanism. While a number of receptors for collagen have been proposed (reviewed in Ref. [31]), one of these, the integrin collagen receptor $\alpha_2 \beta_1$ (very late antigen-2, VLA-2, GPIa/IIa), appears to be the predominant receptor mediating platelet-collagen interactions in the absence of plasma [25]. Since gangliosides are known to interact with several integrins [32–34], we studied the possibility that NBTG might exert its enhancing influence through an effect on the integrin collagen receptor, $\alpha_2 \beta_1$. In these experiments, we used an anti-α_2 monoclonal antibody, 5E8, which blocks this receptor. In the absence of antibody (control), platelets preincubated with 5.0 μM NBTG and activated with a subthreshold concentration of collagen
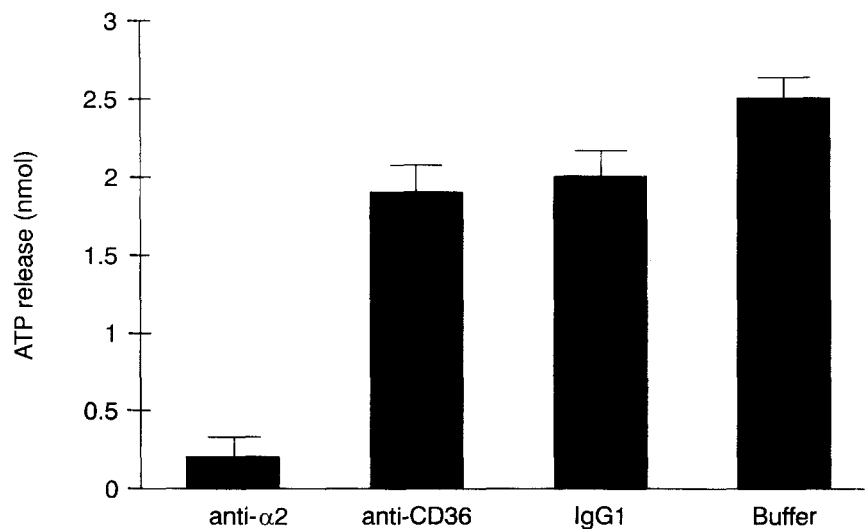

Fig. 4. Effect of blocking the integrin collagen receptor, $\alpha_2 \beta_1$, on NBTG-enhanced platelet ATP release. Washed platelets were preincubated for 30 min with 5.0 μM NBTG at 37°C with gentle rocking. Then, unbound gangliosides were removed by centrifugation. An excess amount of monoclonal antibody, 5E8 anti-α_2 (10 μg/ml), OKM5 or OKM8 anti-CD36 monoclonal antibody (40 μg/ml), or an equal amount of non-immune isotype control antibody was added. Following a 30-min incubation at 37°C, 2 mmol/l $MgCl_2$ and collagen (1 μg/ml) were added and ATP release determined. Data represent the means ± S.E.M. of three separate experiments, each performed in duplicate.

released 2.5 ± 0.1 nmol ATP and demonstrated $80 \pm 7\%$ aggregation, respectively (Fig. 4). In the presence of the α_2 blocking antibody, NBTG-enhanced ATP release and platelet aggregation were completely abrogated (Fig. 4). Incubation with OKM5 or OKM8 antibodies directed to CD36 (GP IV), which is a non-integrin collagen receptor on platelets [35], did not prevent NBTG-enhanced platelet activation (Fig. 4). Finally, no effect was observed when an irrelevant isotype control antibody was used (Fig. 4). These combined findings suggests that the effects of NBTG are specific for an $\alpha_2 \beta_1$-dependent mechanism leading to platelet activation.

Role of divalent cations ions in the priming effect of gangliosides. Since magnesium ions play a critical role in

the interaction of $\alpha_2 \beta_1$ with collagen [30], we determined the requirement of NBTG for Mg^{2+}. In these experiments, physical aggregation, rather than ATP release was used as the end-point. This is because the luciferase assay used to quantify ATP cannot be performed in the absence of Mg^{2+} ion. As shown in Table 2, only Mg^{2+} (2 mmol/l) was necessary for the effect of NBTG. Ca^{2+} alone (2 mmol/l) was insufficient, while the effect of the combination of Ca^{2+} and Mg^{2+} was not different from that of Mg^{2+} alone. These findings that NBTG-enhanced, collagen-initiated platelet activation is Mg^{2+}-dependent and does not require Ca^{2+} are consistent with an $\alpha_2 \beta_1$-mediated effect [30].

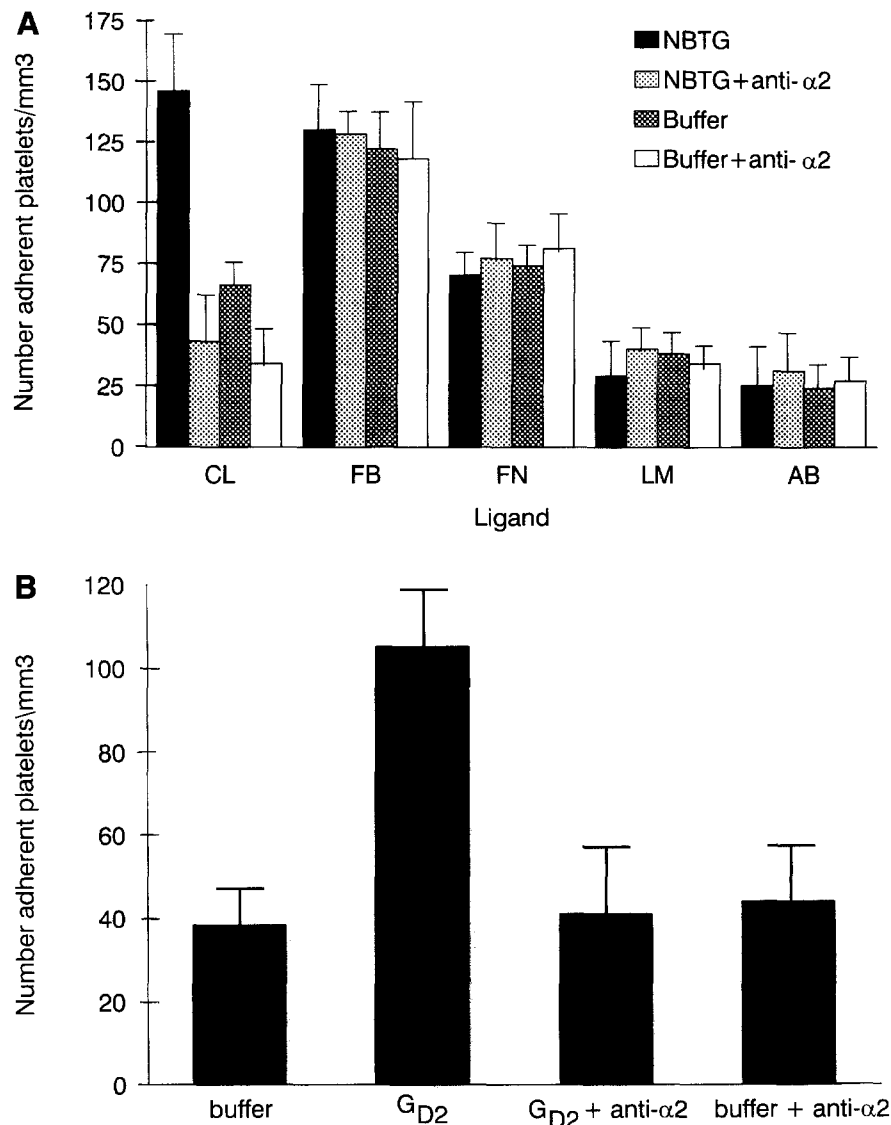*NBTG augments platelet adhesion to collagen.* Next,

Fig. 5. Effect of tumor-derived gangliosides on platelet adhesion to immobilized collagen. In panel A platelets were preincubated with NBTG (5 μM) (solid bars), NBTG followed by anti-α_2 monoclonal antibody (10 μg/ml) (shaded bars), buffer alone (open bars) or buffer followed by anti-α_2 monoclonal antibody (hatched bars) and allowed to adhere to plastic wells coated with collagen (CL), fibrinogen (FB), fibronectin (FN), laminin (LM), or albumen (AB). Non-adherent platelets were removed by washing and the adherent platelet number determined. Results represent the mean $\pm$ S.E.M. of three experiments, each performed in triplicate. In panel B the effects of G_{D2} (1 μM) and anti-α_2 monoclonal antibody on platelet adhesion to immobilized collagen were examined. Results represent the mean $\pm$ S.E.M. of four determinations in separate experiments.

we determined how NBTG influences platelet adhesion to immobilized collagen. We found that NBTG enhanced the adhesion of platelets to collagen more than two-fold (146 $\pm$ 25 $\times$ 10³/mm³ vs. 66 $\pm$ 10 $\times$ 10³/mm³) (Fig. 5A). There was no effect of pre-exposure of platelets to 5 μM NBTG upon platelet adhesion to fibrinogen, fibronectin, or laminin (130 $\pm$ 20 vs. 122 $\pm$ 15%, 70 $\pm$ 10 vs. 74 $\pm$ 10% and 29 $\pm$ 15 vs. 38 $\pm$ 10%, respectively) nor was there any effect upon non-specific adhesion of platelets to albumen (25 $\pm$ 15 vs. 24 $\pm$ 10%). Similar to the effects on aggregation, the effect of NBTG on platelet adhesion was specific for collagen and could be blocked by 5E8 (43 $\pm$ 20).

In these same experiments, the effects of a single tumor ganglioside, G_{D2}, on platelet adhesion were studied. Incubation of gel-filtered platelets with 1 μM G_{D2} enhanced adhesion (105 $\pm$ 14 $\times$ 10³/mm³ vs. 38 $\pm$ 9 $\times$ 10³/mm³). The increase could be prevented by preincubation with anti-α_2 monoclonal antibody 5E8 (41 $\pm$ 20 $\times$ 10³/mm³) (Fig. 5B). Thus, NBTG and its major component, G_{D2}, enhanced $\alpha_2 \beta_1$-mediated platelet adhesion to immobilized collagen in an antibody 5E8-inhibitable manner. These findings implicate the $\alpha_2 \beta_1$-collagen interaction as a target of the effect of tumor-derived gangliosides.

Finally, we conducted additional experiments to determine the contribution of platelet aggregation to the platelet adhesion, and particularly, the ganglioside enhancement of platelet adhesion observed in these experiments. Exposure of the platelets to 100 μM aspirin for 30 min prior to incubation with G_{D2} did not abrogate the enhancement in adhesion (86 $\pm$ 18 $\times$ 10³/mm³ vs. 105 $\pm$ 14 $\times$ 10³/mm³) nor did incubation with 40 μg/ml RGDS peptide (95 $\pm$ 14 $\times$ 10³/mm³) (Table 3). Performance of the assay at 22°C without mixing diminished the stimulatory effects of G_{D2} compared to experiments done at 37°C with mixing (66 $\pm$ 11 $\times$ 10³/mm³ vs. 105 $\pm$ 14 $\times$ 10³/mm³). Despite this reduction, the effect of G_{D2} under these experimental conditions (22°C without mixing) was still three-fold greater than control (29 $\pm$ 8 $\times$ 10³/mm³). These experiments suggest that neither arachidonic acid formation nor $\alpha_{IIb} \beta_3$ (glycoprotein IIb/IIIa) occupancy plays a role in

Table 2
Divalent cation requirements for NBTG-enhanced collagen platelet aggregation

NBTG (5.0 μM)	Magnesium (2 mmol/l)	Calcium (2 mmol/l)	% aggregation [a]
+	−	−	< 10
−	−	−	< 10
+	+	−	75 $\pm$ 14
−	+	−	< 10
+	−	+	< 10
−	−	+	< 10
+	+	+	68 $\pm$ 10
−	+	+	< 10

[a] Average of duplicate determinations and standard error from three separate experiments.

Table 3
Effects of G_{D2} ganglioside on platelet adhesion to collagen under conditions in which platelet aggregation is prevented

Experimental condition	Platelet adhesion [a]	
	G_{D2}	Buffer
Control	105 $\pm$ 14	38 $\pm$ 9
Conditions inhibiting aggregation		
Asprin (100 μM)	86 $\pm$ 18	42 $\pm$ 13
22°C, no mixing	66 + 11	29 $\pm$ 8
RGDS (40 μg/ml)	95 + 14	32 $\pm$ 11

[a] number adherent platelets $\times$ 10³/mm³.

ganglioside-enhanced platelet activation in response to collagen. Furthermore, they confirm that the interaction of gel-filtered platelets with an immobilized fibrillar collagen substrate in the presence of Mg^{2+} ions is due primarily to platelet adhesion and that secondary aggregation plays, at best, a minor role in this interaction. Together, these results (Table 3) confirm that the significant enhancing effect of the neuroblastoma tumor-derived ganglioside G_{D2} is on platelet adhesion, not platelet aggregation.

4. Discussion

Gangliosides, shed from the surface of tumor cells, enhance tumor progression [10,11]. One mechanism by which these acidic glycosphingolipids may accomplish this is by interacting with and modulating the immunologic activity of host immunocytes including lymphocytes [36], monocytes [37], and NK cells [38]. Another mechanism by which shed tumor gangliosides may enhance tumor progression and tumor metastasis is by enhancing platelet activation. Many steps in the metastic cascade involve changes in the ability of cells to adhere to each other, or to extracellular matrix proteins. Here we show that gangliosides shed by neuroblastoma tumor cells influence $\alpha_2 \beta_1$ integrin-mediated platelet activation in response to low (normally inactive) concentrations of collagen. This effect is rapid, Mg^{2+}-dependent, and specific for collagen. We suggest that this interaction of tumor-derived gangliosides with integrins may occur not only on platelets but also on other cells including host endothelial cells [39], and thereby promote tumor cell adhesion in the vascular system and contribute to the establishment of metastatic foci [40].

Platelet activation is thought to be an integral step in the complex, multi-step cascade of events which occur during metastasis [3–7]. The association of tumor cells with platelets is well described in animal models of metastasis [41] and certain tumor cells can activate platelets in vitro [42,43]. However, the mechanism by which this association occurs is not known. It is known that an initial extracellular receptor-ligand interaction leads to a series of intracellular receptor-coupled events which culminate in a cellular response [44–46]. When a sufficient number of

$\alpha_2\beta_1$ receptors on the surface of circulating platelets interact with collagen in the subendothelial matrix, a cascade of intracellular events are initiated which amplify the initial signal. They lead to Ca^{2+} influx and activation of protein kinase C. In turn, a multitude of cellular responses, including release of adenine nucleotides and other components of platelet granules, platelet adhesion, and aggregation [44].

Gangliosides interact with a number of cell surface receptors, including integrin receptors [32-34,47-49]. An initial demonstration that gangliosides can alter receptor function was provided by Bremer and Hakomori [50]. They showed that G_{M3} ganglioside inhibited cell growth by interacting with the cell surface receptors for platelet-derived growth factor and epidermal growth factor [50]. Neurite outgrowth and spreading [51], neuroblastoma cell adhesion [47] and germinal cell adhesion [52], all cell surface receptor-mediated processes, have subsequently been shown to be influenced by gangliosides, as is internalization of the CD4 epitope on T-lymphocytes [53]. Similarly, platelet adhesion is influenced by gangliosides [54,55]. Complex gangliosides inhibit the adhesion of thrombin-activated platelets to the $\alpha_{IIb}\beta_3$ substrates, fibronectin, fibrinogen and von Willebrand factor [55]. Interactions between specific gangliosides and integrin molecules are also known. For example, monoclonal anti-

bodies to the oligosaccharide portion of G_{D2} or G_{D3} inhibit melanoma cell attachment and spreading [32]. Gangliosides colocalize with the integrin vitronectin receptor, $\alpha_v\beta_3$, which in the presence of ganglioside and Ca^{2+} demonstrates enhanced interaction with vitronectin. Recently, it was also shown that depletion of complex gangliosides results in a reduction in tumor cell adhesion to vitronectin, and a decrease in affinity of $\alpha_v\beta_3$ for vitronectin, while supplementation with exogenous gangliosides restored the ability of the cells to adhere to vitronectin and enhanced the affinity of $\alpha_v\beta_3$ for vitronectin [49]. Another example of ganglioside modulation of integrin function is the enhancement by G_{M3} of the interaction between the integrin fibronectin receptor, $\alpha_5\beta_1$, and fibronectin [34].

The results of these experiments demonstrating enhancing effects of pathologic tumor gangliosides on collagen-mediated platelet activation (Fig. 1 and Table 1), abrogation of this enhancing effect in the presence of anti-α_2 monoclonal antibody (Fig. 4), those defining a requirement for Mg^{2+} ion (Table 2), and finally enhancement of platelet adhesion to immobilized collagen and abrogation of this enhancing effect in the presence of anti-α_2 monoclonal antibody (Fig. 5) provide several lines of evidence supporting the hypothesis that tumor gangliosides influence $\alpha_2\beta_1$-mediated platelet activation. The mechanism
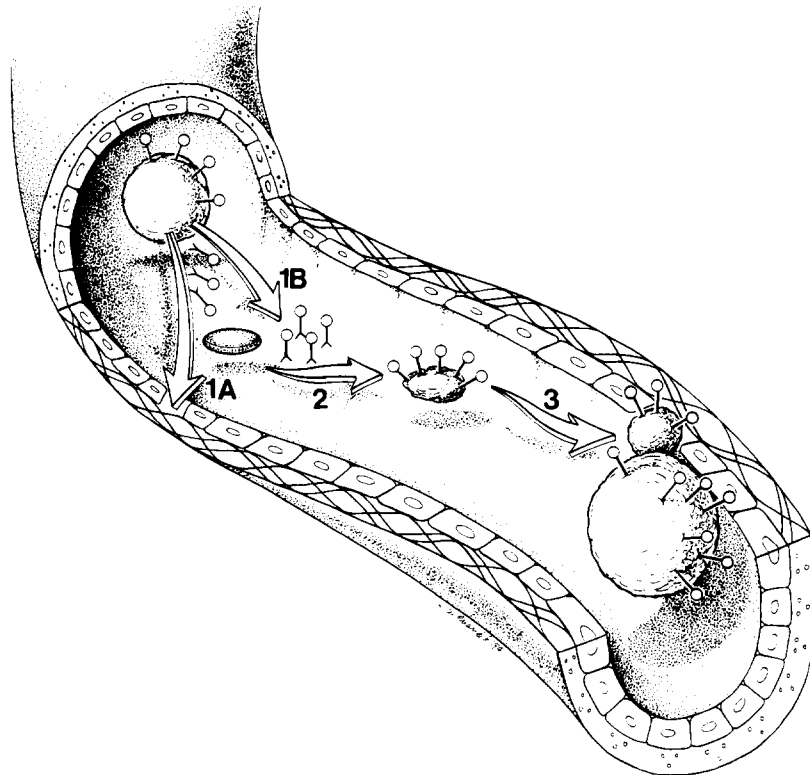

Fig. 6. Proposed schema of the influence of ganglioside shedding upon platelet activation and tumor metastasis. Tumor cells induce endothelial cell retraction (1A). Gangliosides are shed into the circulation from tumor cells (1B) and interact with resting platelets to prime them for subsequent activation [2]. The primed platelets may be activated by low, subthreshold concentrations of collagen present on intact blood vessels or as a result of tumor cell induced endothelial cell retraction [3]. Formation of platelet-endothelial cell-tumor cell aggregate promotes tumor metastasis.

by which pathologic, tumor-derived gangliosides alter integrin function [32,49,56], and more specifically, platelet $\alpha_2 \beta_1$ activity, is not known. What is known is that G_{M3} comprises 92% of total cellular gangliosides in the platelet [57], is the major ganglioside in the platelet membrane, and is highly enriched in the platelet dense granule membrane [58]. Other gangliosides, including G_{M2} [59] and complex polysialogangliosides are present in the platelet membranes, but comprise only a minor fraction ($< 2\%$) of the total and are absent from the dense granules [58]. NBTG is composed of two major gangliosides – G_{D2} and G_{M2} and a small amount of G_{T1b}; HBG is composed of over 20 different gangliosides with G_{M1}, G_{D1a} and G_{T1b} the major components. Finally, the major components of HSG are G_{M3} (which is absent in NBTG and a very minor component of HBG), G_{D3}, and G_{D1a}. Since qualitative or quantitative changes in gangliosides present in the platelet microenvironment have dramatic effects on platelet activity [55], incubation of platelets in NBTG or HBG may be introducing complex ganglioside molecules that are either absent or present only in very low concentrations in the platelet membrane. In turn, these complex ganglioside molecules may alter integrin-ligand affinity directly through interactions with $\alpha_2 \beta_1$ on the platelet surface or indirectly by influencing membrane fluidity and integrin clustering. Alternatively, their effects may be due to modulation of post-receptor signaling. With respect to the data presented here, we have two explanations for our observation that platelet adhesion is increased 2.2-fold by NBTG while aggregation is increased greater than 6-fold. First, NBTG may also influence downstream steps in the signal transduction pathway. Secondly, the difference in the magnitude of the effect of NBTG on adhesion and aggregation could simply be due to physiologic amplification which occurs following the initial receptor-ligand interaction which ultimately leads to platelet aggregation. The mechanism(s) by which pathologic tumor-derived gangliosides affect $\alpha_2 \beta_1$-mediated platelet activation (e.g., modulation of affinity and/or clustering and/or signal transduction) is under investigation.

Our data together with those of others begin to give a picture of how tumor gangliosides may influence the processes of integrin activation, cell adhesion, and metastasis. Schematically (Fig. 6): the intact endothelium normally prevents contact between platelets and collagen in the subendothelial matrix, but tumor cells induce endothelial cell retraction [60], exposing small amounts of collagen. Unless large areas of subendothelial matrix are exposed, however, this amount of collagen is insufficient in itself to cause platelet activation. But, tumor cells shed gangliosides [9–11] (Fig. 6, step 1B) which, we propose, can interact with circulating, resting platelets in vivo [13]. So, whereas in the physiological situation there are no tumor cells in the circulation and therefore neither 'exposed' collagen nor circulating tumor-derived gangliosides, when a tumor is present, platelets may be exposed to and bind tumor gangliosides. When platelets carrying such bound tumor gangliosides encounter 'insufficient' amounts of exposed collagen, they become activated, release ATP, aggregate, and adhere to collagen (Fig. 6). In turn, these tumor ganglioside-exposed platelets may promote the interaction of tumor cells with endothelial cells [40], a step in tumor metastasis. In conclusion, the findings that NBTG enhance $\alpha_2 \beta_1$-mediated platelet activation, combined with the observations by others that gangliosides modulate the activity of integrins, support the hypothesis that by enhancing cell adhesion, tumor gangliosides contribute to the process of tumor cell metastasis.

Acknowledgements

References

[1] Gasic, G.J., Gasic, T.B. and Stewart, C.C. (1968) Proc. Natl. Acad. Sci. USA 61, 46–52.
[2] Pearlstein, E., Ambrogio, C. and Karpatkin, S. (1984) Cancer Res. 44, 3884–3887.
[3] Mehta, P. (1984) Blood 63, 55–63.
[4] Gasic, G.J. (1984) Cancer Metastasis Rev. 3, 99–114.
[5] Honn, K.V., Steinert, B.W., Onoda, J.M. and Sloane, B.F. (1987) Biorheology 24, 127–137.
[6] Karpatkin, S., Pearlstein, E., Ambrogio, C. and Coller, B.S. (1988) J. Clin. Invest. 81, 1012–101.
[7] Ordinas, A., Diaz-Ricart, M., Almirall, L. and Bastida, E. (1990) Blood Coagul. Fibrinolysis 1, 707–711.
[8] Hara, Y., Steiner, M. and Baldini, M.G. (1980) Cancer Res. 40, 1217–1222.
[9] Ladisch, S. and Wu, Z.-L. (1985) Lancet I, 136–138.
[10] Ladisch, S., Wu, Z.-L., Feig, S., Ulsh, L., Schwartz, E., Floutsis, G., Wiley, F., Lenarsky, C. and Seeger, S. (1987) Int. J. Cancer 39, 73–76.
[11] Valentino, L., Moss, T., Olson, E., Wang, H.J., Elashoff, R. and Ladisch, S. (1990) Blood 75, 1564–1567.
[12] Valentino, L.A. and Ladisch, S. (1992) Cancer Res. 52, 810–814.
[13] Valentino, L.A. and Ladisch, S. (1994) Blood 83, 2872–2877.
[14] Pratico, D., Iuliano, L., Pulcinelli, F.M., Bonavita, M.S. Gazzaniga, P.P. and Violi, F. (1992) J. Lab. Clin. Med. 119, 364–370.
[15] Chen, F.A., Repasky, E.A. and Bankert, R.B. (1991) J. Exp. Med. 173, 1111–1119.
[16] Greenwalt, D.E., Lipsky, R.H., Ockenhouse, C.F., Ikeda, H., Tandon, N.N. and Jamieson, G.A. (1992) Blood 80, 1105–1115.
[17] Seeger, R.C., Danon, Y.L., Rayner, S.A. and Hoover, F. (1982) J. Immunol. 128, 983–989.
[18] Li, R. and Ladisch, S. (1991) Biochim. Biophys. Acta 1083, 57–64.
[19] Ladisch, S. and Gillard, B. (1985) Anal. Biochem. 146, 220–231.
[20] Ladisch, S., Sweeley, C., Becker, H. and Gage, D. (1989) J. Biol. Chem 264, 12097–12105.
[21] Svennerholm, L. (1958) Acta. Chem. Scand. 12, 547–554.
[22] Miettinen, T. and Takki-Luukkainen, I.T. (1959) Acta. Chem. Scand. 13(4), 856–858.

[23] Ando, S., Chang, N.-C. and Yu, R.K. (1978) Anal. Biochem. 89, 437–450.

[24] Mustard, J.F., Perry, D.W., Ardlie, N.G. and Packham, M.A. (1972) Br. J. Haematol. 22, 193–204.

[25] Coller, B.S., Beer, J.H., Scudder, L.E. and Steinberg, M.H. (1989) Blood 74, 182–192.

[26] Feinman, R.D., Lubowsky, J., Charo, I., Zabinski, M.P. (1977) J. Lab. Clin. Med. 90, 125–129.

[27] Born, G.V.R. (1962) Nature 194, 927–929.

[28] Feinman, R., Detwiler, T. and Ingerman-Wojenski, C. (1985) in The Platelets: Physiology and Pharmacology, pp.429–440, Academic Press, New York.

[29] Ill, C.R., Engvall, E. and Ruoslahti, E. (1984) J. Cell Biol. 99, 2140–2145.

[30] Santoro, S.A. (1986) Cell 46, 913–920.

[31] Kehrel, B., Kronenberg, A., Rauterberg, J., Niesing-Bresch, D., Niehues, U., Kardoeus, J., Schwippert, B., Tschope, D., Van de Loo, J. and Clemetson, K.J. (1993) Blood 82, 3364–3370.

[32] Cheresh, D.A., Pytela, R., Pierschbacher, M.D., Klier, G., Ruoslahti, E. and Reisfeld, R.A. (1987) J. Cell Biol. 105, 1163–1173.

[33] Burns, G.F., Lucas, C.M., Krissansen, G.W., Werkmeister, J.A., Scanlon, D.B., Simpson, R.J. and Vadas, M.A. (1988) J. Cell Biol. 107, 1225–1230.

[34] Zheng, M., Tsuruoka, T., Tsuji, T. and Hakomori, S. (1992) Biochem. Biophys. Res. Commun. 186, 1397–1402.

[35] Tandon, N.N., Kralisz, U. and Jamieson, G.A. (1989) J. Biol. Chem. 264, 7576–7583.

[36] Ladisch, S., Becker, H. and Ulsh, L. (1992) Biochim. Biophys. Acta 1125, 180–188.

[37] Ladisch, S., Ulsh, L., Gillard, B. and Wong, C. (1984) J. Clin. Invest. 74, 2074–2081.

[38] Grayson, G. and Ladisch, S. (1992) Cell. Immunol. 139, 18–29.

[39] Lauri, D., Martin-Padura, I., Biondelli, T., Rossi, G., Bernasconi, S., Giavazzi, R., Passerini, F., Van Hinsbergh, V. and Dejana, E. (1991) Lab. Invest. 65, 525–531.

[40] Menter, D.G., Hatfield, J.S., Harkins, C., Sloane, B.F., Taylor, J.D., Crissman, J.D. and Honn, K.V. (1987) Clin. Exp. Metastasis 5, 65–78.

[41] Hilgard, P. (1973) Br. J. Cancer 28, 429–435.

[42] Pearlstein, E., Salk, P.L., Yogeeswaran, G. and Karpatkin, S. (1980) Proc. Natl. Acad. Sci. USA 77, 4336–4339.

[43] Ferroni, P., Lentil, L., Guadagni, F., Martini, F., D'Agostino, F., Spila, A., Pontier, G.M., Gazzaniga, P.P. (1995) Eur. J. Cancer 31A:79–84.

[44] Holmsen, H. (1991) Proc. Natl. Sci. Counc. Repub. China [B.] 15, 147–152.

[45] Kroll, M.H. and Schafer, A.I. (1989) Am. J. Hematol. 74, 1181–1195.

[46] Ashby, B., Daniel, J.L. and Smith, J.B. (1990) Platelets in Health and Disease 4, 1–26.

[47] Mugnai, G., Lewandowska, K., Choi, H.U., Rosenberg, L.C. and Culp, L.A. (1988) Exp. Cell Res. 175, 229–247.

[48] Spiegal, S. (1988) in New trends in ganglioside research (Leeden, R.W., ed.), Vol. 14, pp.405–422, Liviana Press, Padova.

[49] Barletta, E., Mugnai, G. and Ruggieri, S. (1993) Biochem. Biophys Res. Commun. 192, 214–222.

[50] Bremer, E.G. and Hakomori, S. (1982) Biochem. Biophys. Res. Commun. 106, 711–718.

[51] Blackburn, C.C., Swank-Hill, P. and Schnaar, R.L. (1986) J. Biol. Chem. 261, 2873–2881.

[52] Stallcup, W.B., Pytela, R. and Ruoslahti, E. (1989) Dev. Biol. 132, 212–229.

[53] Morrison, W.J., Offner, H. and Vandenbark, A.A. (1989) Life Sci. 45, 1219–1226.

[54] Mazurov, A.V., Prokazova, N.V., Mikhailenko, I.A., Mukjin, D.N., Repin, V.S. and Bergelson, L.D. (1988) Biochim. Biophys. Acta 968, 167–171.

[55] Santoro, S.A. (1989) Blood 73, 484–489.

[56] Conforti, G., Zanetti, A., Pasquali-Ronchetti, I., Quaglino, D., Jr., Neyroz, P. and Dejana, E. (1990) J. Biol. Chem. 265, 4011–4019.

[57] Marcus, A.J., Ullman, H.L. and Safier, L.B. (1972) J. Clin. Invest. 51, 2602–2612.

[58] Chatterjee, D. and Anderson, G.M. (1993) Arch. Biochem. Biophys. 302, 439–446.

[59] Jolivet-Reynaud, C., Launay, J.-M. and Alouf, J.E. (1988) Arch. Biochem. Biophys. 262, 59–66.

[60] Abecassis, J., Millon-Collard, R., Klein-Soyer, C., Nicora, F., Fricker, J.P., Beretz, A., Eber, M., Muller, D. and Cazenave, J.-P. (1987) Int. J. Cancer 40, 525–531.