Inhibition of Platelet Adhesion to Fibronectin, Fibrinogen, and von Willebrand Factor Substrates by Complex Gangliosides

By Samuel A. Santoro

Gangliosides, which are complex glycosphingolipids containing sialic acid, are found in cell membranes and have been implicated in a variety of cell surface events including cellular adhesion. Complex gangliosides were observed to inhibit the adhesion of thrombin-activated platelets to substrates of fibronectin, von Willebrand factor, and fibrinogen. This adhesion, which is mediated by the glycoprotein Ib-IIa complex, was differentially inhibited by gangliosides depending on the number of sialic acid residues present within the ganglioside. The observed order of effectiveness was GTb > GDb > GM > asialo-GM. Another structurally related glycosphingolipid, globoside, exhibited little inhibitory activity. In contrast to the inhibition of platelet adhesion to von Willebrand factor mediated by the glycoprotein Ib-IIa complex, gangliosides had no detectable effect on the ristocetin-dependent adhesion of platelets to von Willebrand factor mediated by glycoprotein Ib. These results suggest that the function of the glycoprotein Ib-IIa complex may be modulated by gangliosides in a manner similar to that previously described for the closely related vitronectin receptor.

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MATERIALS AND METHODS

Materials. Na2 51CrO4 was obtained from ICN, Inc (Irvine, CA). Ristocetin was purchased from Sigma (St Louis). Highly purified human thrombin (3,300 U/mg) was a generous gift from Joseph P. Miletich (Washington University School of Medicine, St Louis). Mixed gangliosides, gangliosides GTb, GDb, GM, and globoside, all high performance liquid chromatography (HPLC) standard grade, were purchased from Supelco (Belleville, PA).

Immediately before use in adhesion assays, solutions of lipids in chloroform:methanol (1:1) were dried under N2 and then resuspended in 0.15 mol/L NaCl, 0.05 mol/L Tris - HCl (pH 7.4), 2 mmol/L CaCl2, 2 mmol/L MgCl2, 0.5% wt/vol bovine serum albumin and sonicated until clear (usually about five minutes at 50% power of a Virtis ultrasonic disrupter with the microprobe accesso).

Adhesive proteins. All adhesive proteins were purified and characterized as previously described in detail.15 Fibrinogen was obtained from Kabi (Stockholm) and freed from contaminating fibronectin by chromatography on gelatin-Sepharose (Sigma) as previously described.15 Fibronectin was purified from plasma by affinity chromatography on gelatin-Sepharose as described by Engvall and Ruoslahti.16 Von Willebrand factor was purified from the cryoprecipitate fraction of plasma as described.15

Platelet preparations. 51Cr-labeled platelets were prepared from freshly drawn human blood anticoagulated with citrate.15 The
platelets were radiolabeled with Na$_2$CrO$_4$ and washed by a combination of centrifugation and gel filtration exactly as recently described. For use in adhesion assays the platelets were suspended in 0.05 mol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, 5 mmol/L glucose, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, and 0.5% wt/vol bovine serum albumin (BSA) at 2.5 x 10$^8$ platelets/mL.

**Adhesion assays.** Substrates for platelet adhesion were prepared by incubating polystyrene dishes (35 mm diameter, Falcon no. 1008) with 1 mL of 0.05 mol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl (TBS) containing 100 µg/mL of protein for 30 minutes. The solutions were removed by aspiration, and the dishes rinsed twice with 1 mL aliquots of TBS containing 5 mmol/L glucose, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, and 0.5% (wt/vol) BSA. Control plates were coated only with BSA.

Aliquots of 0.5 mL of the platelet suspension were preincubated for five minutes with 1 U/mL of thrombin and adhesion to fibronectin substrates was determined in 30-minute adhesion assays performed in the presence of the indicated glycolipids each present at 500 µg/mL of mixed gangliosides. In the absence of added gangliosides, 23% to 28% of the initially added platelets adhered to the substrate, values that are in agreement with our earlier observations.

The inhibitory effect of the gangliosides was further examined with purified gangliosides GT$_{ib}$, GD$_{1b}$, GM$_1$, and asialo-GM$_1$. In this series of gangliosides, the sialic acid content decreases from three to zero sialic acid residues per molecule as shown in Table 1. All of the lipids were tested at 500 µg/mL. As shown in Fig 2, the effectiveness of gangliosides as inhibitors of the adhesion of activated platelets to fibronectin substrates was a function of the sialic acid content of the gangliosides. GT$_{ib}$, which contains three sialic acid residues, was the most effective inhibitor and reduced adhesion to approximately 65% of the control level. GD$_{1b}$, GM$_1$, and asialo-GM$_1$ were progressively less effective inhibitors, although asialo-GM$_1$ still reduced adhesion to approximately 65% of the control value. In contrast, in the presence of an
identical concentration of globoside, another complex glycolipid, adhesion was 90% of the control value.

The concentration dependence of the most effective ganglioside inhibitor, GT1b, was explored in more detail. As shown in Fig 3, the maximum extent of inhibition of adhesion of thrombin-activated platelets to fibronectin substrates was achieved by 250 μg/mL of GT1b. The extent of adhesion observed in the presence of this and higher concentrations of GT1b was approximately 25% of the control value and was equal to the extent of adhesion to the substrate observed with unactivated platelets.

Activated platelets can recognize and adhere not only to fibronectin via the IIB-IIIa complex but also to von Willebrand factor and fibrinogen substrates.13,16,17 As shown in Fig 4, adhesion of thrombin-activated platelets to these latter two substrates was also effectively inhibited by 250 μg/mL of GT1b. Adhesion to all three substrates was inhibited to comparable extents. Adhesion to the control substrate, bovine serum albumin, was <5% of the control values and was not significantly enhanced by thrombin activation (data not shown), findings that are in agreement with our earlier reports.13,16,17

Platelets can adhere to von Willebrand factor substrates by two distinct mechanisms.17 Thrombin activated platelets adhere via the glycoprotein IIB-IIIa complex, which recognizes the amino acid sequence arg-gly-asp-ser located approximately 300 amino acid resides from the carboxyterminus of the von Willebrand factor molecule.13,17,21 In the presence of ristocetin, platelets adhere to the von Willebrand factor substrate via the glycoprotein Ib-IX complex, which recognizes a domain located within the amino terminal one third of the von Willebrand factor molecule.22,23,25 The existence of these two distinct adhesive mechanisms was exploited to further examine the specificity of the inhibitory effect of gangliosides on platelet adhesion.

As shown in Fig 5 and consistent with the result shown in Fig 4 above, GT1b effectively inhibited the adhesion of thrombin-activated platelets onto von Willebrand factor substrates. The concentration dependence of the inhibition by GT1b was quite similar to that observed for adhesion to fibronectin substrates and shown above in Fig 3. In contrast to the results observed with thrombin-activated platelets, GT1b had no detectable inhibitory effect on the ristocetin-dependent adhesion of platelets to von Willebrand factor substrates.

**DISCUSSION**

Complex gangliosides, but not the structurally related glycosphingolipid globoside, have been shown to inhibit the adhesion of thrombin-activated platelets onto fibronectin, fibrinogen, and von Willebrand factor substrates. Previous studies by a number of laboratories have demonstrated that this adhesive process is mediated by the platelet surface glycoprotein IIb-IIIa complex. The observed order of effectiveness of the different gangliosides, GT1b > GD1a > GM1 > asialo GM1, is the same as observed in earlier studies demonstrating the ability of gangliosides to inhibit the hemagglutinating activity of fibronectin,2 the ability of fibronectin substrates to support cell spreading,24 the ability of fibronectin to facilitate cell adhesion to collagen,2 and the ability of fibronectin to restore the normal morphologic
sary divalent cations. Alternatively, complex gangliosides could inhibit the process by binding the necessary divalent cations. The gangliosides may bind to the ligand and thereby prevent the platelet surface receptor from recognizing the ligand. The gangliosides might have inhibited platelet activation. The gangliosides could bind rather nonspecifically to the platelet surface and interfere with the function of multiple receptors in a nonspecific manner. As adhesion mediated via the glycoprotein Ib-IX complex is dependent on the presence of divalent cations, gangliosides could inhibit the process by binding the necessary divalent cations. Alternatively, complex gangliosides may specifically bind and modulate the function of a single receptor or family of receptors on the platelet surface such as the glycoprotein Ib-IIIa complex.

Several laboratories have observed that gangliosides do, in fact, bind to fibronectin. The binding site has recently been localized to a 31 kilodalton (Kd) fragment derived from the amino terminus of fibronectin. This is a distinctly different region of the molecule than that containing the arg-gly-asp-ser sequence, which mediates adhesion via the Ib-IIIa complex. Studies have also revealed that gangliosides do not bind to von Willebrand factor. No published data is available concerning the binding of gangliosides to fibrinogen. In the aggregate, these data suggest that it is unlikely that the ability of gangliosides to inhibit the adhesion of activated platelets to fibronectin, von Willebrand factor, and fibrinogen substrates results from the binding of gangliosides to the adhesive proteins.

The possibility that the gangliosides inhibited activation-dependent adhesion by inhibiting platelet activation is excluded by the experimental design of the present studies. Platelets were activated with thrombin before any exposure to gangliosides.

As it is well established that the ability of the glycoprotein IIb-IIIa complex to bind its adhesive protein ligands is divalent cation-dependent, it was possible that the gangliosides inhibited the function of this receptor by binding the required divalent cations. We have previously shown that in adhesion assays identical to those used in this study, half maximal adhesion of thrombin activated platelets was achieved in a medium containing 25 nmol/L free Ca++. Near maximal adhesion was observed in media containing 0.5 mmol/L free Ca++. As near maximal inhibition was achieved with 250 μg/mL (125 amol/L) of GT Ib, and assays were conducted in media containing 4 mmol/L divalent cations, it is unlikely that binding of divalent cations by gangliosides could adequately account for the observed effects. The observations that the di- and monosialo-gangliosides, which should bind progressively less divalent cations, also possessed significant inhibitory activity argues against this mechanism of action.

The function of all platelet surface adhesive protein receptors was not inhibited in a nonspecific manner by the presence of gangliosides. This was clearly illustrated for von Willebrand factor to which platelets can adhere by at least two distinct mechanisms, which can be readily distinguished in adhesion assays. In the presence of ristocetin, unactivated platelets adhere to von Willebrand factor via the glycoprotein Ib-IX complex, whereas activated platelets in the presence of divalent cations adhere via the IIb-IIIa complex. Only the latter mechanism was inhibited by gangliosides.

A second fibronectin receptor, the glycoprotein Ic-IIa complex, which is identical to the previously described fibronectin receptor on fibroblasts, has recently been identified on platelets. It is unlikely that inhibition of the function of this receptor contributed significantly to the inhibition by gangliosides of adhesion of activated platelets to fibronectin, von Willebrand factor, and fibrinogen substrates observed in the present study. The Ic-IIa complex is functional on unactivated platelets and mediates the low level of adhesion of unactivated platelets to fibronectin, which did not exceed 20% to 25% of the extent of adhesion of activated platelets to fibronectin. Furthermore, since the extent of adhesion of unactivated platelets to fibronectin was only twice that to fibrinogen, for which only activation-dependent receptors have been shown to exist, it is unlikely that specific adhesion to fibronectin mediated by Ic-IIa actually exceeds 10% to 12% of the total adhesion to fibronectin observed in this study. Thus, even if adhesion to fibronectin mediated by Ic-IIa were completely inhibited by gangliosides, it could not account for greater than 10% to
15% of the observed inhibition of adhesion of activated platelets. Additionally, the fibroblast fibronectin receptor, and therefore Ic-IIa, specifically binds fibronectin, but does not bind either von Willebrand factor or fibrinogen.\(^\text{19}\) Activation-dependent adhesion to all three substrates was effectively inhibited to essentially identical extents by the gangliosides. The IIb-IIIa complex recognizes all three of these adhesive proteins in an activation-dependent manner.\(^\text{24}\)

The observations described in this report thus raise the interesting possibility that the activity of the glycoprotein IIb-IIIa complex is specifically modulated by gangliosides. This hypothesis is not without precedent. Cheres et al\(^\text{19}\) recently observed that the receptor for vitronectin isolated from human melanoma cells existed in a divalent cation-dependent association with gangliosides and that gangliosides modulated the binding activity of the purified receptor for synthetic peptides containing the arg-gly-asp sequence. In this regard, it is important to note that the glycoprotein IIb-IIIa complex and the vitronectin receptor share a common \(\beta\) subunit.\(^\text{11,12}\) Perhaps the common \(\beta\) chain mediates the specific interactions of these receptors with gangliosides, which in turn can modulate the activity of the heterodimeric receptors.

Since the structural differences between the gangliosides used in this study reside within the oligosaccharide head group, the inhibitory activity of the more active gangliosides is likely due to an interaction of the oligosaccharide portion of the gangliosides, rather than lipid tails, with the IIb-IIIa complex thereby modulating the functional activity of the adhesive protein receptor. Earlier studies in other cell systems lead to similar conclusions.\(^\text{23}\) The molecular details of these interactions await resolution. Alteration of ganglioside interactions with the IIb-IIIa complex may play an as yet undefined role in the poorly understood process by which the IIb-IIIa complex acquires ligand binding activity on platelet activation.

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