Gangliosides Interact Directly with Plasminogen and Urokinase and May Mediate Binding of These Fibrinolytic Components to Cells†

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ABSTRACT: Receptors for the fibrinolytic molecules plasminogen and urokinase are expressed at high capacity on a wide variety of peripheral blood cells and transformed cell lines. We have considered whether gangliosides, components of the outer leaflets of cell membranes, may modulate the interactions of these fibrinolytic ligands with cells. Radiolabeled plasminogen and urokinase bound directly to insolubilized gangliosides. The interactions were saturable and were 50% inhibited by 2.2 μM unlabeled plasminogen or 12 nM unlabeled urokinase, respectively. A panel of gangliosides inhibited binding of both ligands to U937 monocytoid cells, and the order of decreasing inhibitory effectiveness was $G_{D1a} > G_{M1} > G_{T1b} > G_{M2}$, while G_{M3} was minimally effective. The individual components of gangliosides, hexoses, hexosamines, sialic acid, G_{M1} pentasaccharide, ceramides, and glucocerebrosides were ineffective in inhibiting the binding of plasminogen and urokinase either to cells or to insolubilized gangliosides. Binding of both ligands to endothelial cells and granulocytes and binding of plasminogen to platelets were also inhibited by gangliosides. U937 cells were cultured with gangliosides to allow incorporation of these glycolipids into the cell membranes. After 3 days of culture, both urokinase binding and plasminogen binding to the cells became enhanced. These results suggest that gangliosides can directly bind to these fibrinolytic components and may mediate or modulate the interactions of plasminogen and urokinase with a variety of cell types.

The assembly of fibrinolytic components on cell surfaces provides a mechanism for control of the proteolytic functions of this system. Receptors for plasminogen have been identified on a variety of peripheral blood cells and on cultured cells [reviewed in Miles and Plow (1988)]. The majority of these cell types also bear receptors for the plasminogen activator urokinase [reviewed in Blasi (1988)]. This class of urokinase receptors interacts with the amino-terminal region of the molecule and, therefore, binds both active and diisopropyl fluorophosphate inactivated two-chain urokinase (M_r 55 000) and single-chain urokinase (M_r 55 000) but not low molecular weight urokinase (M_r 33 000) (Blasi, 1988). These receptors function to promote plasminogen activation and protect cell-bound plasmin from inactivation, thus localizing the proteolytic activity of plasmin at specific sites (Miles & Plow, 1988).

To understand the molecular mechanisms underlying these functions, identification of the cellular receptors for these components is essential. The capacity of cells for both plasminogen (10^4–10^7 sites/cell) and urokinase (10^3–10^5 sites/cell) is high. Therefore, the possibility that non-protein components of cell membranes, as well as cell surface proteins, may participate in binding of fibrinolytic molecules to cells must be considered. Gangliosides, sialic acid containing glycosphingolipids, are present at high density in the outer leaflet of plasma membranes (Karlsson, 1977). There is considerable evidence establishing that cellular gangliosides can serve as receptors for a variety of ligands [reviewed in Hakomori (1981)]. In addition, the ganglioside content of cells can change dramatically when they acquire a tumorigenic phenotype [reviewed in Hakomori (1986)], a situation in which the fibrinolytic potential of cells is frequently enhanced [reviewed in Dano et al. (1985)].

In this study, we have assessed whether gangliosides can bind directly to plasminogen and/or urokinase. To test whether gangliosides may mediate the interactions of fibrinolytic ligands with cells, their ability to inhibit the binding of plasminogen and/or urokinase to cell surfaces has been assessed. In addition, the effect of culturing cells with gangliosides in order to incorporate these glycolipids into cell membranes, on the binding of the fibrinolytic ligands to cells, has been examined.

Experimental Procesures

Proteins. Urokinase (u-PA)[1] was purchased from Calbiochem, La Jolla, CA. Only the high molecular weight (M_r 55 000) two-chain form was used for radiolabeling and was subsequently inactivated with diisopropyl fluorophosphate (DFP). Glu-Plasminogen was isolated from fresh plasma (prepared from blood drawn into 50 μg/mL soybean trypsin inhibitor, 100 kallikrein inhibitor units/mL trasylol, 3 mM benzamidine, and 3 mM EDTA) by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970) in the presence of 1 mM benzamidine, 0.02% NaN_3, and 3 mM EDTA. The protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 16.8 (Wallen & Wiman, 1972). Glu-Plasminogen and u-PA were radiolabeled by using a modified chloramine T procedure as described (Miles & Plow, 1985; Plow et al., 1986). Plasminogen labeled by using this method is 99% activatable to plasmin (Miles &

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[1] Abbreviations: u-PA, urokinase; DFP, diisopropyl fluorophosphate; Glu-plasminogen, the native form of plasminogen with N-terminal Glu; ACD, acid/citrate/dextrose; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; 6-AHA, 6-aminohexanoic acid.

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Plow, 1985). ^{125}I-Labeled human factor X was a gift from Dario Altieri, M.D., Research Institute of Scripps Clinic. Goat anit-mouse IgG and goat anti-rabbit IgG were purchased from Boehringer Mannheim, Indianapolis, IN, and radiolabeled by the chloramine T method as for plasminogen. Human fibrinogen was isolated (Doolittle et al., 1967) and radiolabeled (Marguerie et al., 1980) as described.

Cells. The human monocytoid cell line U937 was grown in RPMI 1640 containing penicillin, streptomycin, and 5% fetal calf serum. Human umbilical vein endothelial cells (second passage) were cultured as described (Levin et al., 1984). Platelets were prepared by differential centrifugation and gel filtration of acid/citrate/dextrose (ACD) blood, as previously described (Marguerie et al., 1980). Granulocytes were separated from ACD blood on Ficoll-Hypaque followed by brief hypotonic lysis of residual RBC as described (Plow & Edgington, 1975).

Platelet concentrations were determined by counting in a Coulter Model II counter (Coulter Electronics, Inc., Hialeah, FL). Other nonadherent cells were counted microscopically using a hemocytometer. Endothelial cells were counted after removal of cells from culture wells by trypsinization.

Ligand Binding Assays. For binding assays, leukocytes (1.5×10^6–1.5×10^7 cells/mL) in suspension were incubated in Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA) (Behring Diagnostics, La Jolla, CA) with ^{125}I-plasminogen or ^{125}I-u-PA and buffer, unlabeled ligand, or test reagent in a final volume of 200 μL in 1.5-mL polypropylene snap cap tubes. After incubation at 37 °C for 1 h, triplicate 50-μL samples were layered over 300 μL of 20% sucrose in HBSS and centrifuged for 2.5 min in a Beckman microfuge (Beckman Instruments, Inc.). The tube tips were cut off and counted in an Iso-Data γ counter (Iso-Data, Inc., Palatine, IL). With platelets, the procedure was the same, with the exception that divalent ion free Tyrode's buffer containing 0.1% BSA was used in the reaction mixture.

Binding experiments with endothelial cells were performed as previously described (Miles et al., 1988). Briefly, confluent cell layers in 12-well tissue culture dishes were washed 3 times with HBSS/0.05 M HEPES containing 0.1% BSA. Radiolabeled ligands and test reagents or unlabeled ligands were added to the wells in a final volume of 400 μL. Following incubation at 37 °C, the reactions were stopped by aspirating the fluid from the wells and washing the wells twice with HBSS. The cell-associated radioactivity was extracted with 0.1% SDS. Cultures were examined microscopically to ensure that the test reagents did not cause detachment of the cells.

Molecules of ligand bound per cell were determined from the specific activity of the radiolabeled ligand. Saturable binding was determined by subtracting the counts bound in the presence of a 100- or 5000-fold molar excess of unlabeled plasminogen or u-PA, respectively, from the counts bound in the presence of buffer.

Solid-Phase Ganglioside Binding Assays. A modification of the method of Holmgren et al. (1980a) for binding of ligands to gangliosides coupled to polystyrene was used. Briefly, mixed gangliosides (as described below) [100 μL at 2.5 μg/mL in phosphate-buffered saline (0.01 M sodium phosphate/0.15 M NaCl, pH 7.3) (PBS)] were incubated in 96-well round-bottom Falcon microtiter plates at 4 °C overnight. The plates were washed 6 times with PBS and coated with 200 μL of 3% gelatin or 10 mg/mL BSA in PBS for 1 h at 22 °C followed by 20 min at 37 °C when using gelatin. Test reagents were added to the wells in a volume of 100 μL and incubated at 37 °C for 90 min. The wells were washed 6 times, and the individual wells were counted for radioactivity. The quantity of ligand bound per well was determined from the specific activity of the radiolabeled ligand.

Statistics. Data are reported as mean ± standard deviation unless otherwise noted. ID_{50} values were determined by linear regression analyses of semilogarithmic plots.

Reagents. Bovine gangliosides with chemical structures identical with human gangliosides, G_{M2}, G_{M3}, and mixed bovine gangliosides consisting of 21% G_{M1}, 40% G_{D1a}, 19% G_{T1b}, and 16% G_{D1b} were from Calbiochem (La Jolla, CA). The purity of these preparations was >98% by thin-layer chromatography, according to the manufacturer's specifications. Bovine brain gangliosides, G_{M1}, G_{T1b}, G_{D1a}, and G_{M3}, were obtained from Sigma and were ≥95% pure according to the manufacturer. Ceramides, glucocerebrosides, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetylneuraminic acid, galactose, and soybean trypsin inhibitor were also from Sigma. G_{M1} pentasaccharide was from Biocarb Chemicals, Biomol Research Laboratories, Plymouth Meeting, PA, and was sonicated before use in binding assays. The gangliosides were dissolved in PBS for use in ligand binding assays. Ganglioside G_{M1} (Sigma) was purified by preparative HP-TLC (Alufolien Kieselgel 60, 20 × 20 cm plates) (Ledeen & Yu, 1982) using chloroform/methanol/0.02% $CaCl_2$ (50:45:10, v/v) (Ando et al., 1981). In one lane, the G_{M1} was visualized by spraying with 4 mM periodic acid in tetrahydrofuran, dried, and sprayed with resorcinol reagent. One spot was observed. The corresponding spots in the remaining lanes were scraped from the TLC plates and mixed with 3 mL of H_2O and 1 g of Bio-Rex 70. Chloroform/methanol 1:1 was added, followed by periodic sonication. The mixture was centrifuged, and the top layer was removed, dried under N_2, and resuspended in 0.5 mL of PBS. The G_{M1} concentration was measured by quantitating the total sialic acid in a chromogenic assay as described (Jourdian et al., 1971) using G_{M1} as a standard. The control was a lane not containing G_{M1} which was scraped from the TLC plate. Ceramides, sphingomyelin, and glucocerebrosides were suspended in PBS and sonicated prior to each assay as recommended by Yamada et al. (1981). The solutions of sphingomyelin and ceramides remained cloudy after sonication, as previously reported for the ceramides (Yamada et al., 1981), while the sonicated glucocerebroside solution was clear. Trasylol was from FBA Pharmaceuticals (New York, NY). The cell line secreting the monoclonal antibody W6/32 was from American Type Culture Collection, and the purified IgG was a generous gift from Dr. Dario Altieri, Department of Immunology, Research Institute of Scripps Clinic.

Results

Direct Binding of u-PA and Plasminogen to Gangliosides. In order to mediate receptor function, a candidate molecule must directly bind to the ligand in question. Therefore, we tested whether u-PA or plasminogen could bind directly to gangliosides. The approach used was to insolubilize a mixture of gangliosides, as has been previously described to measure the interaction of cholera and tetanus toxins (Holmgren et al., 1980a), Sendai virus (Holmgren et al., 1980b), and fibronectin (Perkins et al., 1982) with these glycolipids. ^{125}I-u-PA, at 4 nM, bound directly to the insolubilized gangliosides. The binding was saturable, as total binding was 85% inhibited by 200 nM unlabeled u-PA. As shown in Figure 1A, inhibition of ^{125}I-u-PA binding by unlabeled u-PA was dose-dependent, and saturable binding was 50% inhibited by 11 nM unlabeled u-PA. In three experiments, the mean ID_{50} was 12 ± 1.4 nM. This binding was not inhibited by unrelated proteins, BSA,

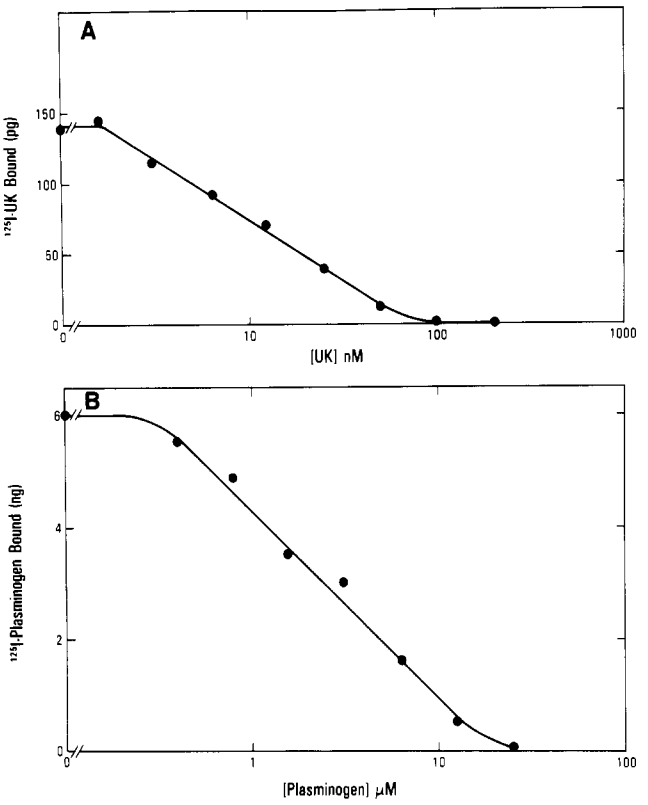

FIGURE 1: Inhibition of saturable u-PA and plasminogen binding to insolubilized gangliosides by unlabeled ligands. In panel A, ^{125}I-u-PA (4 nM) was mixed with the indicated concentrations of unlabeled u-PA in a volume of 350 μL. Then triplicate 100-μL samples were added to microtiter wells coated with mixed gangliosides and postcoated with 3% gelatin as described under Experimental Procedures. Incubations were carried out for 90 min at 37 °C. Saturable binding was determined by subtracting counts bound in the presence of 200 nM unlabeled u-PA from the total counts bound at each test point. The same protocol was followed in panel B, but 0.2 μM ^{125}I-plasminogen was used and saturable binding was determined with 25 μM unlabeled plasminogen.

or transferrin (Table I). A panel of representative gangliosides inhibited ^{125}I-u-PA binding to insolubilized gangliosides. This inhibition was observed with the mixed gangliosides used to coat the microtiter wells as well as with individual purified gangliosides. The individual hexose and hexosamine moieties contained in the carbohydrate portions of the gangliosides or the entire carbohydrate portion of a representative ganglioside, G_{M1} (G_{M1} pentasaccharide), were noninhibitory. Ceramides (composed of sphingosine in an amide linkage with a fatty acid) or glucocerebrosides (composed of glucose linked to ceramide) were also noninhibitory. Thus, both the lipid and the complex carbohydrate moieties of gangliosides are required for the inhibitory activity.

^{125}I-Plasminogen (0.2 μM) also bound directly to insolubilized gangliosides. Unlabeled plasminogen (25 μM) inhibited total ^{125}I-plasminogen binding by ≥74%. In the inhibition curve shown in Figure 1B, the ID_{50} for plasminogen was 2.5 μM. In three experiments, a mean ID_{50} of 2.2 ± 0.6 μM was obtained. BSA and transferrin also did not compete with plasminogen for binding to the insolubilized gangliosides (Table I). The binding of plasminogen to the gangliosides was inhibited by 6-aminohexanoic acid (6-AHA), with a 1 mM concentration inhibiting the interaction by 50–85% (three experiments). This inhibitory activity suggests that the lysine binding sites of plasminogen must be unoccupied for interaction with the gangliosides. Soluble gangliosides inhibited ^{125}I-plasminogen binding to the insolubilized gangliosides, and the same ones that inhibited u-PA binding also inhibited

Table I: Specificity of Plasminogen and Urokinase Binding to Insolubilized Gangliosides[a]

test competitor	urokinase (% bound)[b]	plasminogen (% bound)[c]
none	100	100
G_{D1a}	2.5	0
G_{M1}	19	19
G_{T1b}	0	0
mixed gangliosides	0	0
G_{M2}	18	0
G_{M3}	6, 23[d]	23, 78[d]
galactose	88	100
N-acetyl-D-galactosamine	100	100
N-acetylglucosamine	100	92
N-acetylneuraminic acid	100	97
ceramide	100	100
glucocerebrosides	100	100
BSA	100	100
transferrin	100	100
plasminogen	81	0
urokinase	0	0
G_{M1} pentasaccharide	100	97

[a] Gangliosides, carbohydrates, and lipids were present at 250 μM (glucocerebroside at 1 mg/mL). BSA, transferrin, and plasminogen were at 20 μM. Urokinase was present at 1.7 μM. The data represent triplicate determinations and are representative of six to eight experiments. [b] Saturable binding was determined by subtracting counts bound in the presence of 2000 Ploug units/mL (0.9 μM) urokinase. [c] Saturable binding was determined by subtracting counts bound in the presence of 6-AHA (0.2 M). [d] G_{M3} from two different manufacturers yielded variable results. The two values indicate the levels of binding observed with the two preparations in the same experiment. The preparation from Calbiochem was more effective than the preparation from Sigma with both ligands.

plasminogen binding. As with u-PA, the individual hexose and hexosamine components of gangliosides, G_{M1} pentasaccharide, ceramides, and glucocerebrosides did not compete for binding to the insolubilized gangliosides.

As shown in Table I, plasminogen produced minimal inhibition of ^{125}I-u-PA binding to the insoluble gangliosides while u-PA effectively competed with ^{125}I-plasminogen. This difference may be due to the approximately 200-fold greater affinity of u-PA as compared with plasminogen for the gangliosides (as determined by comparison of their ID_{50} values in Figure 1A,B).

Influence of Gangliosides on Binding of Fibrinolytic Proteins to Cells. The ability of gangliosides to inhibit ^{125}I-u-PA and ^{125}I-plasminogen binding to U937 monocytoid cells was assessed. ^{125}I-u-PA was mixed with varying concentrations of representative gangliosides and added to cells. As shown in Figure 2A, G_{D1a}, G_{M1}, G_{T1b}, and G_{M2} inhibited ≥92% of the saturable binding of ^{125}I-u-PA to the cells. This inhibition was dose-dependent. G_{M3} showed minimal inhibition at the concentrations tested. The same gangliosides were also effective inhibitors of ^{125}I-plasminogen binding (Figure 2B). All inhibited binding of this fibrinolytic ligand to U937 cells by ≥93% while G_{M3} was minimally effective. The ID_{50} values for inhibition of ligand binding to the U937 cells by gangliosides are summarized in Table II. The effectiveness of the mixed gangliosides was similar to that of G_{D1a}, G_{M1}, and G_{T1b} which are contained within the mixture and suggests that another component of the mixture, G_{D1b}, may also be an inhibitory ganglioside. The order of inhibitory potency of the individual gangliosides is similar for both ^{125}I-u-PA and ^{125}I-plasminogen. However, the inhibitory gangliosides were all more effective in blocking u-PA binding to the U937 cells. The potency of the gangliosides is also similar to the range (8–130 μM) reported for 50% inhibition of fibronectin-mediated cell spreading and attachment (Yamada et al., 1981).

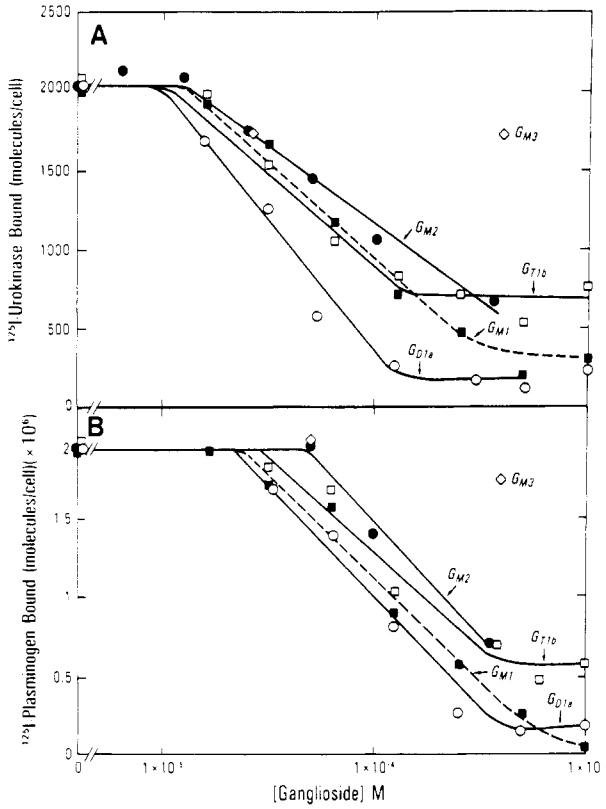

FIGURE 2: Effect of gangliosides on u-PA and plasminogen binding to U937 cells. Gangliosides at the indicated concentrations and either ^{125}I-u-PA (1 nM) (panel A) or ^{125}I-plasminogen (0.2 μM) (panel B) were added to U937 cells and incubated for 60 min at 37 °C. The gangliosides are (●) G_{M2}, (■) G_{M1}, (○) G_{D1a}, (□) G_{T1b}, and (◇) G_{M3}. (Results with both G_{M3} products were similar.)

Table II: Gangliosides as Inhibitors of ^{125}I-Urokinase and ^{125}I-Plasminogen Binding to U937 Cells[a]

ganglioside	ID_{50} (μM)[b] urokinase	plasminogen
G_{D1a}	56 ± 23	130 ± 39
G_{M1}	91 ± 1.7	190 ± 90
G_{T1b}	130 ± 67	320 ± 180
mixed gangliosides[c]	130 ± 16	150 ± 140
G_{M2}	250 ± 160	410 ± 270
G_{M3}	>360	>400

[a] Average of two separate experiments with triplicate determinations in each. ^{125}I-u-PA (1 nM) or ^{125}I-plasminogen (0.2 μM) was incubated with cells at 37 °C for 60 min. [b] Concentration that inhibited saturable binding by 50%. [c] Mixed gangliosides contain 21% G_{M1}, 40% G_{D1a}, 16% G_{D1b}, and 19% G_{T1b}.

Inhibition by the gangliosides was not due to cell lysis, based on microscopic counting of cells at the end of the incubation period. In addition, the highest concentration of the gangliosides tested did not cause cell death, as assessed by trypan blue exclusion (≥89% cell viability in the presence of gangliosides compared to 92% viability in their absence). A generalized perturbation of cell membranes by the gangliosides was excluded by the following experiment. G_{D1a}, at a concentration that inhibited ^{125}I-plasminogen binding by ≥80% and ^{125}I-u-PA binding by ≥89% (250 μM), did not affect binding of radiolabeled W6/32, a monoclonal antibody directed against the major histocompatibility complex (Barnstable et al., 1978). This experiment also excludes an effect of the gangliosides on cell recovery in the binding assays. To verify that the effects of gangliosides were not due to contaminants within the preparations, several steps were undertaken. After either boiling for 5 min in PBS or dialysis

Table III: Effect of Structural Components of Gangliosides on Saturable Plasminogen and Urokinase Binding to U937 Cells[a]

compound	urokinase binding (%)	plasminogen binding (%)
none	100	100
mixed gangliosides	1	2
G_{D1a}	4	4
galactose	100	100
N-acetyl-D-galactosamine	100	100
N-acetylglucosamine	100	100
N-acetylneuraminic acid	91	100
ceramides	100	100
glucocerebrosides	100	100
G_{M1}	1	13
G_{M1} pentasaccharide	99	100

[a] G_{M1} and G_{M1} pentasaccharide were at 0.5 mM and glucocerebrosides at 1 mg/mL. All other compounds were present at ≥1 mM. ^{125}I-u-PA (1 nM) and ^{125}I-plasminogen (0.2 μM) were incubated with cells at 37 °C for 60 min.

in M_r 1000 cutoff dialysis tubing vs PBS (2 L, three changes for 24 h at 4 °C), the representative ganglioside, G_{M1}, retained >98% of its ability to inhibit both ^{125}I-plasminogen and ^{125}I-urokinase binding to cells. In addition, G_{M1} was further purified by preparative HP-TLC as described under Experimental Procedures. The eluted material retained >78% potency in inhibiting binding of ^{125}I-plasminogen to cells.

In order to determine whether the carbohydrate or lipid components of gangliosides could inhibit u-PA or plasminogen binding to the cells, these compounds were tested individually. As shown in Table III, none of the neutral carbohydrates tested or *N*-acetylneuraminic acid inhibited ^{125}I-u-PA or ^{125}I-plasminogen binding to the cells by ≥9%, while equivalent concentrations of G_{D1a}, G_{M1}, or the mixture of gangliosides inhibited ligand binding by ≥87%. Ceramides or glucocerebrosides also did not inhibit either u-PA or plasminogen binding to the cells. When the complex oligosaccharide moiety of G_{M1} (G_{M1} pentasaccharide) was tested, it was also noninhibitory, suggesting that both the lipid and oligosaccharide moieties are required for function. It was not technically feasible to test the effects of asialogangliosides. A representative one, asialo-G_{M1}, precipitated both ^{125}I-plasminogen and ^{125}I-u-PA in the absence of cells. This precipitation did not appear to be specific as four other nonrelated radiolabeled proteins, human factor X, human fibrinogen, goat anti-mouse IgG, and goat anti-rabbit IgG, were precipitated to a similar extent in the presence of asialo-G_{M1}.

The fibrinolytic receptors, expressed by U937 cells, are representative of plasminogen and u-PA receptors expressed by a variety of cells (Miles & Plow, 1988; Blasi, 1988). We tested whether the gangliosides also affected binding of these ligands to other cell types. As shown in Table IV, the individual gangliosides, as well as the mixture of gangliosides, that inhibited u-PA binding to U937 cells also inhibited its binding to endothelial cells and granulocytes. The order of potency of the individual gangliosides was similar with granulocytes and U937 cells. However, G_{T1b}, rather than G_{D1a}, was the most inhibitory compound with endothelial cells. These differences may reflect differences in the ganglioside composition of the different cell membranes. In addition, the gangliosides that inhibited plasminogen binding to the U937 cells also inhibited its binding to granulocytes, endothelial cells, and both resting and stimulated platelets. The order of potency and extent of inhibition were similar with granulocytes, endothelial cells, and U937 cells, although G_{M2} was relatively more effective with the first two cell types. With platelets, the gangliosides were more effective on resting than on stimulated cells. This reinforces our previous postulate that the recog-

Table IV: Effect of Gangliosides on Saturable Binding of Fibrinolytic Ligands to Several Cell Types[a]

	^{125}I-plasminogen bound (%)				^{125}I-urokinase bound (%)	
	platelets					
ganglioside	resting[b]	stimulated[c]	granulocytes	endothelial cells	granulocytes	endothelial cells
none	100	100	100	100	100	100
G_{D1a}	7.5	35	27	54	41	8.5
G_{M1}	46	88	35	54	54	25
G_{T1b}	26	31	63	73	75	4.5
G_{M2}	43	90	21	40	74	26
G_{M3}	100	100	>97	100	100	ND[d]
mixed gangliosides	0	13	11	83	24	7.5

[a] Gangliosides at 250 μM. [b] Reaction mixtures contained 9 μM PGE_1 and 2.5 mM theophylline. [c] Platelets were stimulated with 1 unit/mL human α-thrombin, followed by neutralization with Phe-Pro-Arg chloromethyl ketone. [d] ND, not determined.

Table V: Culturing U937 Cells with Gangliosides Enhances Plasminogen and Urokinase Binding[a]

treatment	^{125}I-urokinase bound (molecules/cell × 10^3)	^{125}I-plasminogen bound (molecules/cell × 10^6)
none	2.5 ± 0.8	1.2 ± 0.3
gangliosides	5.4 ± 1.2	7.2 ± 0.9

[a] U937 cells, ~1 × 10^5 cells/mL, were cultured with or without 50 μg/mL mixed gangliosides in 50 mL of RPMI 1640, 10% FCS, 100 units/mL penicillin, 1000 μg/mL streptomycin, and 2 mM L-glutamine. After 3 days of culture, cells were washed 4 times with HBSS. ^{125}I-u-PA was at 1 nM, and ^{125}I-plasminogen was at 0.2 μM. Both ligands were bound for 60 min at 37 °C.

nition of plasminogen by resting and stimulated platelets involves distinct mechanisms (Miles et al., 1986).

Influence of Cellular Ganglioside Content on Ligand Binding to Cells. Coculturing of cells with gangliosides results in their incorporation into membranes (Moss et al., 1976; Fishman et al., 1976; Kanda et al., 1982), and we tested whether coculturing of U937 cells with gangliosides influenced ^{125}I-u-PA and ^{125}I-plasminogen binding. U937 cells were cultured with the mixture of inhibitory gangliosides, and the results of binding analyses performed after 3 days of exposure of the cells to the gangliosides are summarized in Table V. Saturable u-PA binding was enhanced 2-fold, and saturable plasminogen binding was enhanced 6-fold by incubation of the cells with gangliosides. Thus, incorporation of gangliosides into cell membranes enhanced binding of both ligands to the cells. The enhancement was time-dependent as no increase in ligand binding was observed after 1 day of culture. The time dependence is more extended than reported for other systems (Moss et al., 1976; Fishman et al., 1976), suggesting that additional interactions of the gangliosides with other membrane components may be required to induce competency for enhanced binding of fibrinolytic ligands, as contrasted to other ligands.

Discussion

A role for gangliosides in cellular receptor function for the fibrinolytic ligands plasminogen and u-PA has been assessed. Support for their possible involvement is provided by the following observations: (1) plasminogen and u-PA bound directly to gangliosides; (2) gangliosides inhibited binding of plasminogen and u-PA to several cell types; and (3) culturing of U937 cells in the presence of gangliosides, which results in their incorporation into cell membranes (Moss et al., 1976; Fishman et al., 1976), via their lipid moieties (Kanda et al., 1982), enhanced both plasminogen and u-PA binding to these cells. Thus, our study adds plasminogen and u-PA to the group of bioactive molecules that can bind directly to gangliosides. Included in this group are fibronectin, cholera toxin, Botulinus toxin, glycoprotein hormones, interferon, Sendai virus, and serotonin [reviewed in Hakomori (1981)]. Different structural specificities distinguish these various protein–ganglioside interactions. With both plasminogen and u-PA, the degree of sialylation of the gangliosides did not determine their ability to inhibit binding of these ligands to the cells; G_{D1a} and G_{M1}, with two and one sialic acid residues, respectively, were more effective than G_{T1} and G_{M2}, with three and two residues, respectively. A preference for a tetrose carbohydrate structure and an apparent requirement for an *N*-acetylgalactosamine moiety were noted as the three most inhibitory gangliosides had tetrose oligosaccharide moieties. Also, all gangliosides tested that contained *N*-acetylgalactosamine inhibited binding of plasminogen and u-PA to cells, while G_{M3}, lacking this moiety, did not. In contrast, the more highly sialylated gangliosides are most effective in binding fibronectin when incorporated into cells (Yamada et al., 1983), in inhibiting fibronectin-mediated cell spreading and hemagglutination (Yamada et al., 1981), and in inhibiting fibronectin-dependent cell adhesion to collagen (Kleinman et al., 1979). The latter two interactions are also inhibited by oligosaccharide moieties of gangliosides (Kleinman et al., 1979) in contrast to the lack of an effect of the G_{M1} pentasaccharide on plasminogen and u-PA binding to cells. G_{M1} is most potent in inhibiting cholera toxin mediated effects on cells (Cuatrecasas, 1973; Holmgren et al., 1973; King & Heyningen, 1973). Thus, the structural requirements for the interaction of plasminogen and u-PA with gangliosides appear to entail unique recognition specificities.

The direct interaction of plasminogen with the gangliosides was inhibited by the lysine analogue 6-AHA at a concentration suggesting that lysine binding sites in plasminogen may be involved in its interaction with gangliosides. The lysine binding sites of plasminogen are associated with the kringle structures of plasminogen (Sottrup-Jensen et al., 1988). u-PA also contains a kringle (Gunzler et al., 1982), and so the kringle structures may be the common feature of these proteins that establish their capacity to interact with gangliosides.

The inhibitory effects of gangliosides were observed with all cell types tested which bear plasminogen and/or u-PA receptors. Gangliosides may, therefore, be sufficient to provide receptor function for each of these ligands. This is supported by the similarities in K_d values for interaction of both plasminogen (0.3–2.8 μM) (Miles & Plow, 1988) and u-PA (0.2–2 nM) (Blasi, 1988) with cells and the ID_{50} values determined for inhibition of binding of both ligands to gangliosides by the respective unlabeled ligands, 2 μM for plasminogen and 12 nM for u-PA. In addition, gangliosides are present in cells which bind plasminogen and u-PA, including U937 cells (Nojiri et al., 1984), endothelial cells (Gillard et al., 1987), neutrophils (Macher et al., 1981; Nojiri et al., 1984), and lymphocytes (Hildebrand et al., 1972; Stein & Marcus, 1977; Schwarting & Marcus, 1979; Macher et al., 1981). Finally, culturing the cells with gangliosides enhanced the binding of

these ligands to the cells. As exogenous gangliosides are incorporated into cell membranes, this may be explained by a direct binding of the ligands to the gangliosides. However, gangliosides are known to induce other changes in cellular function, and coculturing may have changed the capacity of cells for these ligands by more complex mechanisms. We did, indeed, observe a 3-fold decrease in cell growth in the presence of the gangliosides, similar to the described effect of G_{M3} on U937 cells (Nojiri et al., 1986).

While the data could suggest a direct role for the gangliosides in binding these fibrinolytic ligands, the interrelationships of gangliosides with protein receptors must be considered in view of recent reports of cell surface proteins which bind u-PA (Needham et al., 1987; Nielsen et al., 1988) and plasminogen (Plow & Miles, 1988). A similar situation exists for fibronectin; cellular protein receptors have been identified [e.g., see Pytela et al. (1986)], but yet fibronectin also binds to cellular gangliosides (Perkins et al., 1982). To account for the role of both protein and ganglioside moieties in receptor function for the fibrinolytic ligands, three models can be considered. First, protein receptors may bear oligosaccharide moieties with structures similar to those found in gangliosides. The u-PA receptor appears to be heavily glycosylated (Behrendt et al., 1988), and cell surface glycoproteins reactive with antibodies to gangliosides have been identified on 3T3 and transformed 3T3 cells (Tonegawa & Hakomori, 1977). As a second possibility, an initial interaction of fibrinolytic ligands with cellular gangliosides may concentrate the ligands at cell surfaces or initiate changes in the cell membranes such as modulation of fluidity, which allow protein receptors to become competent to subsequently interact with the ligands. As a third model, the gangliosides may mediate receptor function while in association with a cellular protein. The existence of such ganglioside–protein receptor complexes has been described between the melanoma vitronectin receptor protein and G_{D2} (Cheresh et al., 1987). The present data would appear to be most compatible with this third model in which both gangliosides and proteins are involved in receptor function. Although u-PA and plasminogen appear to react with gangliosides with similar structural specificity, the capacity of cells for plasminogen is higher, and some cell types bind plasminogen but not u-PA. Thus, cellular structures, in addition to the gangliosides, must account for difference in the binding of these two ligands to cells. In addition, coculturing cells with gangliosides enhanced plasminogen more than u-PA binding, suggesting that additional cellular components must limit u-PA binding to the gangliosides. Thus, as the cellular membrane proteins that bind plasminogen and u-PA become identified, an assessment of their interaction with gangliosides becomes a key issue.

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Registry No. G_{D1a}, 12707-58-3; G_{M1}, 37758-47-7; G_{T1b}, 59247-13-1; G_{M2}, 19600-01-2; G_{M3}, 54827-14-4; u-PA, 9039-53-6; L-lysine, 56-87-1; plasminogen, 9001-91-6; plasminogen activator, 105913-11-9.

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^{31}P and ^{1}H NMR Studies of the Structure of Enzyme-Bound Substrate Complexes of Lobster Muscle Arginine Kinase: Relaxation Measurements with Mn(II) and Co(II)†

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ABSTRACT: The paramagnetic effects of Mn(II) and Co(II) on the spin–lattice relaxation rates of ^{31}P nuclei of ATP and ADP and of Mn(II) on the spin–lattice relaxation rate of the δ protons of arginine bound to arginine kinase from lobster tail muscle have been measured. Temperature variation of ^{31}P relaxation rates in E·MnADP and E·MnATP yields activation energies (ΔE) in the range 6–10 kcal/mol. Thus, the ^{31}P relaxation rates in these complexes are exchange limited and cannot provide structural information. However, the relaxation rates in E·CoADP and E·CoATP exhibit frequency dependence and ΔE values in the range 1–2 kcal/mol; i.e., these rates depend upon ^{31}P–Co(II) distances. These distances were calculated to be in the range 3.2–4.5 Å, appropriate for direct coordination between Co(II) and the phosphoryl groups. The paramagnetic effect of Mn(II) on the ^{1}H spin–lattice relaxation rate of the δ protons of arginine in the E·MnADP·Arg complex was also measured at three frequencies (viz., 200, 300, and 470 MHz). These ^{1}H experiments were performed in the presence of sufficient excess of arginine to be observable over the protein background but with MnADP exclusively in the enzyme-bound form so that the enhancement in the relaxation rates of the δ protons of arginine arises entirely from the enzyme-bound complex. Both the observed frequency dependence of these rates and the $\Delta E \leq 1.0 \pm 0.3$ kcal/mol indicate that this rate depends on the ^{1}H–Mn(II) distances. From the frequency dependence of the relaxation rate an effective τ_C of 0.6 ns has also been calculated, which is most likely to be the electron spin relaxation rate (τ_{S1}) for Mn(II) in this complex. The distance estimated on the basis of the reciprocal sixth root of the average relaxation rate of the δ protons was 10.9 ± 0.3 Å.

Knowledge of the structures of substrates and their relative dispositions at the active sites of enzyme-bound complexes is essential to the understanding of the mechanism of catalysis of multisubstrate enzymes. Paramagnetic enhancement of spin–lattice relaxation rates provides a useful method of determination of distances of various ligand nuclei from the paramagnetic probe in an enzyme–substrate complex (Mildvan et al., 1980; Villafranca, 1984; Jarori et al., 1985; Ray & Nageswara Rao, 1988; Ray et al., 1988). These distances can then be used to characterize the substrate conformation at the active site. This method is particularly attractive for ATP[1]-utilizing enzymes that require a divalent cation, Mg(II), as an obligatory component in vivo but can accept paramagnetic cations such as Mn(II) and Co(II) in most cases. Recently, ^{31}P relaxation measurements in the presence of Mn(II) and Co(II) were used to characterize the enzyme–substrate complexes of creatine kinase (Jarori et al., 1985), 3-P-glycerate kinase (Ray & Nageswara Rao, 1988), and adenylate kinase (Ray et al., 1988). These studies reveal that considerable care

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; E·M·S, enzyme–metal–substrate; E·S, enzyme–substrate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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