Synergism between Membrane Gangliosides and Arg-Gly-Asp-directed Glycoprotein Receptors in Attachment to Matrix Proteins by Melanoma Cells

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Abstract. The identification of specific cell surface glycoprotein receptors for Arg-Gly-Asp-containing extracellular matrix proteins such as fibronectin has focused attention on the role of gangliosides in this process. Is their involvement dependent or independent of the protein receptors? In attachment assays with cells from a human melanoma cell line, titration experiments with an antibody (Mel 3) with specificity for the disialogangliosides GD2 and GD3, used together with a synthetic peptide containing the cell binding sequence Arg-Gly-Asp, show that their joint effect is synergistic. Both the Mel 3 antibody and the synthetic peptide individually cause rapid detachment of melanoma cells from fibronectin substrate but, when used together, much smaller concentrations of both are required to achieve the same effect. The Mel 3 antibody was not nonspecifically reducing receptor binding to the Arg-Gly-Asp sequence since, in binding assays with radiolabeled peptide performed with cells in suspension, very little peptide is bound by the melanoma cells under these conditions but addition of Mel 3, an antibody of IgM isotype, causes a two- to threefold increase in specific binding. The simplest interpretation of these data is that the Mel 3 antibody is causing sufficient clustering of membrane gangliosides in local areas and producing a favorably charged environment to facilitate peptide binding by specific glycoprotein receptors.

An understanding of cell attachment to extracellular matrix proteins will provide insights into such processes as cell migration and spreading, and tissue invasion by tumor cells. For this reason, the mechanisms by which different cells attach to fibronectin have been much studied and it is now clear that more than one interaction is involved. Gangliosides have been suggested as candidate receptors for fibronectin since their addition to cultures caused rounding up and detachment of cells from the fibronectin substrate (24, 29, 47). Also, the insertion of gangliosides into the membranes of ganglioside-deficient cells enabled them to bind fibronectin on the cell surface (40), and mAbs directed against determinants on the carbohydrates of the gangliosides GD2 and GD3 (41) prevented the attachment of melanoma cells to extracellular matrix proteins including fibronectin (12, 45).

In another approach, Pierschbacher and colleagues identified a peptide within the cell-binding domain of fibronectin that mediated the cell attachment activity of this molecule (31–33), and Hayman et al. (18) and Yamada and Kennedy (46) went on to demonstrate that peptides containing the sequence arginyl-glycyl-aspartic acid (Arg-Gly-Asp) caused detachment of cultured cells from their substratum and prevented baby hamster kidney and Chinese hamster ovary cells from spreading on fibronectin substrates. From this information, Pytela et al. (35) were able to identify a specific glycoprotein receptor of ~140 kD on osteosarcoma cells and rat fibroblasts. It is now apparent that the fibronectin receptor thus isolated is but one of a family of structurally similar proteins that bind to Arg-Gly-Asp sequences contained within a variety of extracellular matrix proteins (reviewed in references 21 and 37).

Within the fibronectin molecule there is an additional domain that also appears to be involved in cell attachment. This domain is located towards the carboxy-terminal end of the A chain, which contains heparin binding activity (27), and high levels of Arg-Gly-Asp in solution have little effect on cells adhering to fragments containing this heparin-binding domain (27). Binding of this fragment, however, did not promote melanoma cell motility (27), indicating that cell binding to different domains of the fibronectin molecule may result in different functions. The relationship between the ganglioside “receptor” and other receptors is uncertain. The negative charges on the sialic acid moieties of GD2 and GD3 could...
bind fibronectin by interacting with the clusters of positively charged residues present in the type III homologies of the fibronectin molecule (30, 39). Alternatively, Cheres and Klier (10) have suggested that, for optimal cell adhesion, the active redistribution of gangliosides into discrete areas of cell attachment may cause synergism with cell surface receptors by creating an appropriate electrostatic environment.

The present study presents evidence supporting this concept. We show that an mAb that binds to GD2 and GD3 inhibits attachment and, when used in relatively high concentrations, causes detachment of melanoma cells from fibronectin substrates. At low concentrations the antibody acts synergistically with a synthetic peptide containing the Arg-Gly-Asp sequence such that concentrations of antibody or peptide, which individually had no effect on attachment, together caused rapid loss of adhesion by the melanoma cells. In addition, we used a synthetic peptide containing the Arg-Gly-Asp sequence and a radiolabeled tyrosine residue to demonstrate that melanoma cells in suspension bound only weakly to the peptide. However, when the cells were reacted with the IgM mAb with specificity for GD2/GD3, this binding was increased severalfold. We favor the interpretation that the IgM antibody against GD2/GD3 was causing localized clustering (patching) of the gangliosides to provide favorable areas for attachment of the peptide to specific glycoprotein receptors.

Materials and Methods

Cell Cultures

The melanoma cell lines used in this study have been maintained in our laboratory for several years and have been demonstrated to be free of mycoplasma contamination. The LiBr line was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland), glutamine (2 mM), 2-mercaptoethanol (5 x 10^-5 M), and penicillin (100 µg/ml), and streptomycin (100 µg/ml). The cells were periodically passaged at subconfluence by detaching the adherent cells by vigorous pipetting.

Reagents

Fibronectin was prepared from human plasma as described below or was purchased from Sigma Chemical Co. (St. Louis, MO). Iodoheads were from漕 (Cranfield, U.K.). Glutathione (GSH) was from Silius Laboratories Pty. Ltd. (Melbourne, Australia).

mAbs

The preparation and tissue specificity of the Mel 3 antibody has been described in detail elsewhere (44, 45). Detailed analysis of the epitope recognized by Mel 3 revealed this to be in the sugar sequance, GalNAc 1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer, and the antibody binds to the gangliosides GD2, GD3, GT3, and GGp but not to other gangliosides such as GM1, GM2, GM3, Gp3, GDta, Gp1b, and GT1b (17). The mAb is an IgMκ. The anti-β, microglobulin mAb, HB28, was a gift from Dr. Heddy Zola (Flinnders Medical Centre, Adelaide, Australia). The CA2 antibody was raised by conventional fusion after immunization with activated human T lymphocytes and was selected for its ability to inhibit lysis of tumor target cells by activated lymphocyte killer cells by a method described elsewhere (7). The mAb, an IgMκ, immunoprecipitated a glycoprotein heterodimer of 170/85 kD from peripheral blood mononuclear cells and details of its specificity have been given elsewhere (5). A complete list of anti-melanoma antibodies of every isotype that we have tested for inhibition of melanoma cell attachment and others kindly donated from several laboratories has been given elsewhere (44). None of these anti-glycoprotein mAbs affected adhesion. In the present experiments, the Ns4B mAb (from Dr. H. Koprowski, Wister Institute, Philadelphia, PA) was used routinely as a control.

Preparation of Synthetic Peptides and Radiolabeled Peptides

The peptides GRGDSP, KYGRGDSP, and GRGESP were synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) by a modified Merrifield Boc solid-phase peptide chemistry procedure (2). On completion of each synthesis, the crude peptide was cleaved from the resin support by treatment with hydrogen fluoride (95% liquid HF/9% anisole, 0°C for 30 min) and purified by gel-permeation chromatography on a Biogel P2 column (1 x 100 cm) in 0.5 M aqueous acetic acid.

The peptide KYGRGDSP (150 µg) was mixed with 1 µCi 125I and incubated at room temperature for 12 min with Iodoheads as described by the manufacturer. The free 125I was fractionated from bound 125I on a 35-ml Biogel P2 Column (Bio-Rad Laboratories, Richmond, CA) equilibrated with 25 mM NaOH. The fractions containing radiolable were further purified by reverse-phase HPLC on a gradient system (model 334; Beckman Instruments, Inc., Fullerton, CA) coupled to a variable wavelength detector (model 163; Beckman Instruments, Inc.) set at 220 nm and a radioisotope detector (model 170; Beckman Instruments, Inc.) connected in series. Samples were chromatographed on an Ultrasphere C18 column (4.6 x 150 mm) with a gradient from 10 to 50% acetonitrile in phosphate buffer (10 mM, pH 7.0) over a duration of 10 min at a flow rate of 1 ml/min. The retention time of unlabeled peptide was 8.5 min, and that of the labeled peptide 12 min.

Attachment and Detachment Assays

Fibronectin was purified from human plasma by affinity chromatography on gelatin-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) as described by Wight et al. (40). The purity and yield were determined by SDS-PAGE with Coomassie Blue staining and by a protein assay (Bio-Rad Laboratories). The fibronectin was added to the wells of flat-bottomed micortiter plates (Flow Laboratories, Inc., McLean, VA) as 0.1 mg/ml for 2 h at 25°C and the wells were washed twice with PBS and twice with medium. LiBr melanoma cells from subconfluent cultures were resuspended in RPMI 1640 medium containing 0.2% BSA (Sigma Chemical Co.) to 106 cells/ml. To each of the fibronectin-coated wells was added 100 µl of the cell suspension together with the antibody or peptide being tested (10 µl) and the plates were incubated for 2 h at 37°C in 5% CO2 before assay. Detachment assays were performed in essentially the same way but in these tests the cells were incubated alone for 2 h at 37°C to enable attachment, then the agents under test were added in a volume of 10 µl and the cells incubated for a further 30 min.

A colorimetric assay modified from Chong and Parish (14) was used to quantitate the attached cells. After the period allowed for detection, the nonattached cells were washed off and residual cells were stained with 0.25% (wt/vol) Rose Bengal dye (Koch-Light Ltd., London, England) in PBS for 10 min at room temperature. Excess dye was then washed off and the plate inverted to drain. The wells were then washed twice in PBS and the dye released by adding 100 µl of 50% ethanol in PBS. Released dye was quantified as absorbance at 570 nm on a microplate reader (Dynatech Laboratories Inc., Alexandria, VA), blanked against a well containing only medium, and the percent inhibition of attachment and percent detachment were calculated from the OD570 values of test vs. control wells. A series of preliminary experiments demonstrated that the OD570 values were linear for cells plated at 103-106 cells/well, and in every experiment triplicate wells were used for each test substance; the SDS of these triplicate values were generally very small.

Radioligand Binding Assay

LiBr cells from subconfluent cultures were harvested, washed, and resuspended in cold incubation buffer (Hanks’ balanced salt solution [HBSS], 1.6 mM CaCl2, 10 mM NaCl, 5 µg/ml cytochalasin B, 0.1% BSA), pH 7.2, to 4 x 105 cells/100 µl. 100 µl suspended cells were incubated at 4°C, 2 h with 10 µM 125I-GRGDSP ± 1 nM CRGDSP in the presence or absence of the mAb under test. In a series of preliminary experiments, various concentrations of Mel 3 or control antibodies were preincubated with the cells for various times on ice, at room temperature, or at 37°C before performing the binding assay (see Results). Bound ligand was separated from free by filtration through GF/C filters (Whatman, Inc., Clifton, NJ) presoaked for 15 min in 0.5% (wt/vol) 3H-thymidine (specific activity 105-106 Ci/mmol) in sterile HBSS and counted for cpm in a gamma counter (Packard Instrument Co., Downers Grove, IL). In every assay, each value was calculated as...
specific binding by subtracting the mean of duplicates performed in the presence of excess cold peptide from the mean of triplicates without cold; the SD of replicates was generally <10% of the specific binding. An excess of the control peptide GRGESP did not reduce the binding of [125I]
GRGDSP.

Immuno precipitation and Western Blot Analysis

To analyze receptor proteins coprecipitated by the Mel 3 antibody that specifically binds gangliosides, 10⁶ LiBr melanoma cells were lysed in Triton X-100 buffer and the clarified supernatant quantitated for protein content. A 50-μl volume containing 100 μg of protein was incubated overnight at 4°C with 200 μl Sepharose 4B beads covalently coupled to the Mel 3 antibody. The beads were washed three times in PBS and boiled in non-reducing buffer. The supernatant was then boiled in reducing buffer and subjected to electrophoresis in 7.5% SDS–polyacrylamide gels; 100 μg of total lysate was run in an adjacent track. At the completion of the run the immunoprecipitated proteins and total proteins were electrophoretically transferred to nitrocellulose paper, a strip containing the high molecular weight markers (Bio-Rad Laboratories) excised for Coomassie Blue staining, and the remaining tracks probed with rabbit antibodies (diluted 1:1,000) specific for the IIIα β chain of the vitronectin receptor on melanoma cells. Bound antibody was detected by an indirect enzyme assay using horseradish peroxidase as described previously (25).

Results

The Effects of Anti-GD2/GD3 and of Synthetic Fibronectin Peptides Are Synergistic in Preventing Attachment and Inducing Detachment by Melanoma Cells

The mAb Mel 3 behaved in a similar way to other anti-GD2 and anti-GD3 antibodies in preventing the attachment of melanoma cells to fibronectin-coated substrates, and also to dishes coated with laminin, fibrinogen, or thrombospondin (Lucas, C. M., unpublished observations). In addition, it was found that Mel 3 caused rapid detachment of melanoma cells that were already attached to fibronectin (data not shown). It has been shown by others that the synthetic peptide GRGDSP, but not GRGESP, prevents spreading on and causes detachment from fibronectin substrate by a number of human and mouse cell types (18, 46). This was shown also to be the case for the human melanoma cell line LiBr. We found that GRGDSP at 200 μg/ml (300 μM) specifically prevented 50% of newly plated cells from attaching to fibronectin, and at 50 μg/ml (75 μM) specifically caused detachment of 50% of cells that had been allowed to attach to a fibronectin substrate for 2 h before addition of GRGDSP or the control peptide (Figs. 1 and 2). The above experiments present data from trials carried out with cells in serum-free medium supplemented with 2 mg/ml BSA. Essentially similar data were obtained in the presence of 5 or 10% FCS but higher concentrations of mAb or peptide were required and the majority of experiments were performed with defined serum-free medium.

Used alone, Mel 3 prevented attachment to a titer of 1:30 ascites and in separate experiments it was found that 100 μg/ml of purified antibody caused half-maximal inhibition (not shown). In the presence of GRGDSP at 100 μg/ml, a concentration which by itself caused no inhibition of attachment, half-maximal inhibition was obtained at a dilution of 1:160 of ascites (Fig. 1). The same synergistic effect between Mel 3 and GRGDSP was seen in detachment assays (Fig. 2).

Ganglioside Co-distributed with the Vitronectin Receptor Does Not Inhibit Binding of the Synthetic Peptide GRGDSP

When melanoma cells are allowed to attach and spread onto plastic or fibronectin substrate, there is a rapid redistribution of the complex gangliosides, and GD2, at least, becomes preferentially localized into microprocesses that make direct contact with the fibronectin substrate (10). We have confirmed this observation at the level of light microscopy by staining with Mel 3, and have shown that this antigen is also left behind in focal attachment processes after removal of the cells from the coverslip (data not shown). In addition, Cheresh et al. (13) have shown that the vitronectin receptor on human melanoma cells exists as a functional complex with a GD2 ganglioside; thus, the antigens were demonstrated to colocalize in focal adhesion plaques as demonstrated by double-label transmission EM and indirect immunofluorescence, and purification of the vitronectin receptor on affinity columns caused copurification of GD2. We have found that the Mel 3 antigen is also physically associated with the vitronectin receptor on LiBr melanoma cells by immunoprecipitating LiBr lysates with Mel 3 and probing Western blots of the immunoprecipitates with rabbit antibodies to the vitronectin receptor. Moreover, such specific physi-
Figure 2. Detachment of LiBr cells from a fibronectin substratum by Mel 3 and GRGDSP peptide. Cells were incubated for 2 h in serum-free medium to enable attachment and various concentrations of Mel 3 ascites and GRGDSP were added for a further 30 min incubation. Attached cells were quantitated as in Fig. 1 legend and percent detachment calculated from controls.

The anti-ganglioside antibody used, Mel 3, binds to both GD2 and GD3 (17) and might therefore be expected to be very effective in preventing melanoma cell attachment, since antibodies individually directed against GD2 or GD3 are additive in their effect (12). Nevertheless, when used together with the synthetic peptide GRGDSP in detachment assays, the effect on melanoma cell attachment was clearly synergistic. These data suggest that gangliosides and the specific receptors for the cell attachment sequence of fibronectin are operating at distinct but related levels in the attachment process. In this regard, it is of interest that, in similar assays performed with the CA2 antibody which identifies a glycoprotein molecule and also prevents cell attachment to fibronectin (5), the addition of synthetic peptide was additive whereas the mAbs CA2 and Mel 3 were synergistic in causing detachment (Burns, G. F., and C. M. Lucas, unpublished observations). Radiolabeled peptide binding assays carried out with melanoma cells in suspension indicated a possible mechanism of action for the gangliosides. We have shown previously (45) that melanoma cells maintained in suspension exhibit reduced levels of gangliosides on their surface membrane. As they reattach, the cells redistribute the gangliosides into the microprocesses that make direct contact with extracellular matrix proteins (10) and these are also laid down in the adhesion plaque (11). Treatment of melanoma cells with anti-ganglioside antibodies prevents this redistribution of gangliosides to the basal surface causing the micro-

Table I. Inhibition of Binding of Iodinated Synthetic Peptide KYRGDSP to Melanoma Cells in Suspension by Different Antibodies

<table>
<thead>
<tr>
<th>Pretreatment of cells</th>
<th>Specific cpm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>637 ± 330 (280-965)</td>
</tr>
<tr>
<td>Anti-β2 microglobulin</td>
<td>494 ± 380 (244-934)</td>
</tr>
<tr>
<td>Mel 3</td>
<td>2,661 ± 1,796 (1,104-5,452)</td>
</tr>
<tr>
<td>CA2</td>
<td>69 ± 6 (62-74)</td>
</tr>
</tbody>
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Melanoma cells from the LiBr line were harvested from subconfluent cultures maintained in RPMI medium supplemented with 10% FCS. The harvested cells were washed and 4 × 10⁶ cells in 100 μl medium mixed with 10 μl of 1:10 ascites of the antibody under test in incubation buffer (BSS, 1.6 mM CaCl₂, 10 mM NaN₃, 5 μg/ml cytochalasin B, 0.1% BSA). Cells with antibody were incubated at 37°C for 15 min, and cooled on ice; and 10 μM (~150,000 cpm) ¹²⁵I-GRGDSP was added to each tube ± 1 nM unlabeled GRGDSP, and binding was allowed to occur for 180 min on ice. Specific cpm were determined as described in Materials and Methods. The results are tabulated from four independent experiments.

* Mean ± SD. Numbers in parentheses represent the range of the results.

Discussion

Since the discovery of specific glycoprotein cell surface receptors for extracellular matrix proteins (6, 34, 35), the role of gangliosides has undergone a reappraisal. In the present study we present data substantiating the suggestion (10, 12) that gangliosides do not bind directly to fibronectin but facilitate specific receptor–ligand binding at points of attachment.

The anti–ganglioside antibody used, Mel 3, binds to both GD2 and GD3 (17) and might therefore be expected to be very effective in preventing melanoma cell attachment, since antibodies individually directed against GD2 or GD3 are additive in their effect (12). Nevertheless, when used together with the synthetic peptide GRGDSP in detachment assays, the effect on melanoma cell attachment was clearly synergistic. These data suggest that gangliosides and the specific receptors for the cell attachment sequence of fibronectin are operating at distinct but related levels in the attachment process. In this regard, it is of interest that,
processes to detach and the cells to cluster (11). Cheresh et al. (12) have recently demonstrated that anti-ganglioside antibodies effectively prevented the attachment of melanoma cells to solid substrates of synthetic hexapeptides containing the Arg-Gly-Asp sequence, and that such peptides were not bound directly by melanoma gangliosides separated on thin layer chromatograms. The same group (12) also found that mAbs directed to gangliosides on tumor cells failed to induce significant capping of the antigen. We have observed, however, that certain antigens are rapidly synthesized as they are capped, and that capping is masked by the constant redistribution of newly produced antigen unless cyclohexamid is included during the capping process (Burns, G.F., unpublished observations), and we have found that the Mel 3 antibody caused prominent clustering of the surface gangliosides. Hence, the simplest interpretation of our binding data is that the anti-ganglioside antibody, which is of IgM isotype, causes sufficient clustering of gangliosides in localized areas to produce artificial processes with an electrostatic environment conducive to ligand binding. Because such binding is not localized to microprocesses on the basal surface of the cell, attachment as such does not ensue, but we would predict that other events consequent upon specific ligand binding (42) would be observed. We are currently attempting to isotype switch the Mel 3 hybridoma to determine whether IgG antibodies with the same specificity require further cross-linking to produce the same effect.

The physical relationship between the ganglioside GD2 and Arg-Gly-Asp-directed receptors on the surface of melanoma cells has recently been the subject of study by Cheresh et al. (13). Immunostaining with antibodies to the vitronectin receptor and to GD2 revealed association of the two antigens, and affinity purification of the vitronectin receptor in the presence of Ca**+ enabled copurification of GD2. We have confirmed these results by demonstrating biochemical coassociation after immunopurification in the opposite direction, whereby immunoprecipitation of GD2 and GD3 by the Mel 3 antibody coprecipitated vitronectin chain antigen as detected by Western blotting (data not shown). That the ganglioside–receptor complex is stable on the cell membrane was illustrated in two-color fluorescent staining studies, where it was shown that ganglioside clustering induced by the Mel 3 antibody also caused colocalization of the vitronectin receptor. Hence, both components of the complex appear to migrate together within the membrane, perhaps suggesting that the fluidity of the membrane gangliosides may provide a mechanism for the rapid redistribution of receptor proteins during attachment. The formation of such ganglioside–receptor clusters induced by Mel 3 would also explain the increased attachment of synthetic peptide seen with melanoma cells maintained in suspension.

The role of gangliosides is clearly of more general significance than the binding of extracellular matrix proteins. The mAb, Mel 3, inhibited cytolysis of melanoma cells by cytotoxic lymphocytes by preventing conjugate formation (44), perhaps indicating a role for gangliosides in cell–cell adhesive processes; in support of this rat macrophages have a membrane receptor for gangliosides (36). Also, while the Mel 3 antibody alone had no effect on cell viability, it inhibited by 50% colony formation in agar by anchorage-dependent melanoma cells (Burns, G.F. and J.A. Werkmeister, unpublished observations), an event which is unaffected by Arg-Gly-Asp–containing peptides (20). Gangliosides have also been implicated in a number of cell types in such diverse receptor-mediated processes as the binding of various growth factors (4, 23) and the binding of lipopolysaccharide (8), various bacterial toxins (15, 43), viruses (26), thyroid stimulating hormone (1), transferrin (28), and interferon (3). With the identification of specific glycoprotein receptors for many of these factors, it is now becoming apparent that the role of gangliosides is that of an accessory molecule to facilitate binding or to modulate receptor function rather than functioning themselves as specific receptors. Whether or not the gangliosides simply confer a charged environment conducive to ligand binding or are involved in the regulation of biochemical events such as receptor phosphorylation (9) awaits further study.

The present report in describing synergy between an mAb to gangliosides and synthetic peptides containing the cell-binding region of fibronectin in preventing melanoma cell attachment has immediate practical implications. It has been known for some time that preventing cells from adhering and spreading can interfere with their growth in vitro (16). Houghton et al. (19) reported some success in treating patients with malignant melanoma with an antibody to the ganglioside GD3. Attempts at treatment with other anti-melanoma mAbs of different specificity have been less successful, and it was suggested that the peculiar success of this antibody could be attributable to the fact that it was of IgG3 isotype and particularly effective in mediating complement- and cell-mediated cytotoxicity (19). This interpretation was made less tenable by a recent report (22) of treatment with a human mAb to GD2 inducing regression of cutaneous malignant melanoma since the antibody used was of IgM isotype and would not mediate cell-mediated cytotoxicity. A complement-mediated effect for these antibodies remains a possibility but it is probably significant that the only recorded successes in this area are with anti-ganglioside antibodies. In support of alterations in the adhesive properties of melanoma cells being responsible for successful treatment is the finding that injection of the synthetic peptide GRGDS together with murine melanoma cells dramatically inhibited the formation of experimental lung metastasis in mice (20). Our findings reported here suggest that future strategies should incorporate treatment with anti-ganglioside mAb and peptide given together.

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