Disialoganglioside GD_2 Distributes Preferentially into Substrate-associated Microprocesses on Human Melanoma Cells during Their Attachment to Fibronectin

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Abstract. Human melanoma cells (M21) actively attach and spread on a fibronectin substrate. Indirect immunofluorescence assays with specific monoclonal antibodies directed to the disialoganglioside GD_2, the major ganglioside expressed on M21 melanoma cells, indicate that during the cell attachment process this molecule redistributes into microprocesses that make direct contact with the fibronectin substrate. Scanning and transmission immunoelectron microscopic studies with anti-GD_2 monoclonal antibodies and immunogold staining demonstrate that GD_2 preferentially localizes into substrate-associated microprocesses that emanate from the plasma membrane of the M21 cells. Staining with monoclonal antibodies directed to other melanoma surface antigens fails to demonstrate a similar distribution pattern on these cells. Direct evidence is provided that GD_2 is involved in M21 cell attachment to fibronectin, since treatment of these cells with anti-GD_2 monoclonal antibodies causes cell rounding and detachment from a fibronectin substrate. Moreover, scanning electron microscopy demonstrates that this loss of attachment of fibronectin is characterized by a perturbation of the cell attachment–promoting microprocesses that in the presence of these antibodies lose contact with the fibronectin substrate.

A number of studies have indicated that the carbohydrate moiety of gangliosides plays a direct role in cell–substratum interactions, thus implicating them as putative cell surface receptors for fibronectin and collagen (8, 18, 29, 49). In a previous paper, we used monoclonal antibodies (Mabs)[1] that specifically recognize carbohydrate determinants on the gangliosides GD_2 and GD_3[2] (8) to localize them on the surface of human melanoma cells and their focal adhesion plaques. The contention that gangliosides are indeed important molecules in cell–substratum interactions is strengthened by the fact that pretreatment of melanoma cells with specific Mabs directed to either GD_2 or GD_3 significantly decreased their ability to attach and spread on a variety of extracellular matrix proteins, which include fibronectin, vitronectin, laminin, and collagen (10).

The attachment and spreading of cells on extracellular matrix components undoubtedly involve very complex interactions which include not only specific receptors (1, 4, 5, 13, 15, 27, 31, 33, 45, 46) on the surface of the membrane but also cytoskeletal components beneath it (22, 35, 36). Components of substrate-attached cell adhesion plaques include cytoskeletal proteins (22, 35, 36) and proteoglycans (14, 21, 22), as well as gangliosides (8, 26). A chemical analysis of such adhesion plaques demonstrated the presence of ~1% of cell proteins and phospholipid but as much as 5–15% of their carbohydrate (11). Taken together, these results suggest that complex carbohydrates, whether on a protein or lipid backbone, may play a role in cell attachment processes. At this time, it is unclear whether such glycoconjugates interact independently or in a synergistic manner with various other cell surface components to promote the attachment and spreading of cells. The use of specific Mabs directed to defined complex carbohydrates may make it possible to dissect the specific role of these molecules in the cell attachment process. We describe here experiments designed to investigate the role of the disialoganglioside GD_2 in the attachment and spreading of M21 human melanoma cells on a fibronectin substrate through the use of Mabs directed specifically to the carbohydrate portion of GD_2. Using indirect immunofluorescence as well as scanning and transmission immunoelectron microscopy, we demonstrate that this molecule specifically localizes within microprocesses on these cells when they are attached to fibronectin. Scanning electron microscopic studies also reveal that when M21 cell monolayers are overlayed with anti-GD_2, they round-up as the GD_2-containing microprocesses lose contact with the fibronectin substrate. Gangliosides may therefore be among the important glycoconjugates associated with the cell surface that are involved in the very complex process of

1. *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; Mabs, monoclonal antibodies; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TLC, thin layer chromatography.

2. Gangliosides are termed according to the nomenclature of Svennerholm (43).

human melanoma cell attachment to extracellular matrix components.

Materials and Methods

Cell Lines

The M21 human melanoma cell line was kindly provided by Dr. D. L. Morton (University of California, Los Angeles). These cells were propagated at 37°C in 7.5% CO_2/92.5% air in RPMI 1640 media, supplemented with 10% fetal calf serum, and were shown to be free of mycoplasma by repeated testing during the course of these studies.

Mabs

The various sources of Mabs used in these studies are listed with their isotype denoted in parentheses. Mabs 126 (IgM) and 14.18 (IgG3) produced in our laboratory (38) and Mab 3F8 (IgG3) provided by Dr. N. K. Cheung of Case Western Reserve School of Medicine (37) were shown to be directed to the carbohydrate portion of ganglioside GD_2. Mab 9.2.27 (IgG2a) produced in our laboratory was shown to be directed to a 250-kD core glycoprotein of a melanoma-associated chondroitin sulfate proteoglycan (2). All IgG Mabs were isolated from murine ascites fluid and purified by using a protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) immunoadsorbent procedure as previously described (28). Mab of the IgM isotype was isolated from murine ascites as previously described (44). The antibodies directed to GD_2 used in this study were shown not to react with carbohydrate determinants on glycoproteins. Specifically, these Mabs did not immunoprecipitate a protein from metabolically labeled melanoma cells, nor did they react by Western immunoblot analysis (Fig. 1). Furthermore, M21 human melanoma cells pretreated with mild trypsin actually reacted better with these Mabs, yet failed to react with a Mab directed to the protein antigen recognized by Mab 9.2.27 (data not shown).

Purification of Melanoma-associated Gangliosides, and Thin Layer Chromatography (TLC)

Gangliosides associated with M21 human melanoma cells were purified as previously described (8). M21 gangliosides (1 μl packed cell equivalent) were resuspended in chloroform/methanol (2:1), spotted per lane on plastic backed silica gel TLC plates (Merck & Co., Inc., Rahway, NJ), and developed for 2 h in a solvent that contained chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:10). The ganglioside standards GM_2, GM_1, GD_3, and GD_2 were spotted on each chromatogram and either visualized with resorcinol spray reagent as described (43) or reacted with anti-GD_2 as described below.

Immunostaining of Gangliosides Separated by TLC

The reactivity of Mabs 126 and 14.18 with authentic ganglioside standards or M21 gangliosides separated by TLC was determined by using an indirect immunostaining ELISA detection system as previously described (9).

Western Blot Analysis

Detergent lysates of M21 human melanoma cells were prepared as previously described (2). Lysates were electrophoresed on 5% polyacrylamide gels in the presence of SDS according to the method of Laemmli (23). Electrotransfer of the antigen from the polyacrylamide gels to nitrocellulose paper and development of the blots were done according to Towbin et al. (47). Mabs 126, 14.18, and 9.2.27 were used as primary antibody in the form of spent culture media, and goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) was used as secondary antibody.

Indirect Immunofluorescence

M21 melanoma cells were allowed to attach for 60–90 min to glass coverslips previously coated for 24 h with human plasma fibronectin at 5 μg/ml in phosphate-buffered saline, pH 7.2 (PBS). After the cells had attached they were fixed with either 3% paraformaldehyde or 0.5% glutaraldehyde for 15 min at room temperature. Each coverslip was then individually washed several times in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY), followed by an additional wash in HBSS that contained 0.1 M glycine. The cells were then overlayed with an appropriate Mab at a concentration of ~5 μg/ml for 1 h at room temperature. After three washes in HBSS that contained 1% bovine serum albumin (BSA), the cells were overlayed with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (Bio-Rad Laboratories) diluted 50-fold in HBSS that contained 1% BSA for 1 h at room temperature. After three additional washes, the coverslips were inverted and mounted on slides in a drop of fluoromount-G (Fischer Scientific, Springfield, NJ). The stained cells were observed through a Zeiss epifluorescent microscope and photographed. To examine focal-adhesion plaques, cells were removed from the fibronectin-coated coverslips with EDTA as previously described (8).

Scanning and Transmission Electron Microscopy

M21 cells were seeded on fibronectin-coated glass coverslips (for scanning electron microscopy [SEM]) or 35-mm culture dishes (for transmission electron microscopy [TEM]) as described above (in immunofluorescence) and fixed using 0.5% glutaraldehyde in PBS for 30 min at room temperature. After a brief wash in HBSS that contained 0.1 M glycine, the appropriately diluted murine Mab was added to each coverslip or dish for 3 h to allow penetration of substrate-attached zones. After three washes each sample was overlayed with goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) coupled to colloidal gold (16). Gold was prepared using the ether phosphorus (5–7 nm) or the Na-citrate (20–30 nm) reduction of aurochloric acid methods (40). After conjugation each preparation was centrifuged to remove aggregates and then tested for its ability to specifically stain the IgG band of immunoblotted mouse serum after separation by polyacrylamide gel electrophoresis (Pytela, R., and F. G. Klier, unpublished observation). After incubation with gold-labeled secondary antibody, the cells were washed three times and fixed with 2.5% glutaraldehyde in PBS, washed, and postfixed in 1% OsO_4. For TEM studies thiocarbohydrazide-osmium double fixation was done to increase contrast. For SEM, tannic acid was used to prevent excessive shrinkage as previously described (48). All samples were dehydrated using a graded series of ethanols from 50 to 100%. All 35-mm dishes were embedded in Epon 812 and cured in vacuo for 48 h at 60°C (19) and sectioned on an LKB ultrotome V (LKB Instruments, Inc., Gaithersburg, MD). Wet coverslips were transferred to Freon 113 and dried after substitution with liquid CO_2 in a Polaron 3000 critical point dryer. Dried coverslips were sputtered using a gold-palladium (80:20) source in an E5100 Polaron sputtercoater (Polaron Instruments Inc., Hatfield, PA). All samples were viewed in an Hitachi H-600 STEM with a LaB6 or pointed-W filament.

Results

Mabs 126 and 14.18 React Specifically with GD_2 on TLC

To demonstrate anti-GD_2 antibody-binding specificity, we attempted to directly visualize the reactivity of Mabs 126 and 14.18 on TLC plates that contained total glycolipid extracts from either M21 human melanoma or various ganglioside standards. As shown in Fig. 1*A*, Mabs 126 and 14.18 reacted with authentic GD_2 (lanes *1* and *2*) as well as a co-migrating doublet from the M21 glycolipid extract (lanes *3* and *4*). These Mabs did not react with any of the other known ganglioside standards, i.e., GM_2, GM_1, or GD_3. In addition, no reactivity was observed at the origin (arrow) of any of these lanes, which indicates the absence of reactivity with potential contaminating glycoproteins in the glycolipid preparation. The specificity of Mabs 126 and 14.18 for the GD_2 ganglioside is further demonstrated by their inability to react with a glycoprotein extract from M21 cells. As shown in Fig. 1*B*, these Mabs failed to react with a glycoprotein extract from these cells by Western immunoblot analysis (lanes *5* and *6*). Mab 9.2.27, known to react with a glycoprotein antigen produced by these cells, reacted with a band of 250 kD (lane *7*).

GD_2 Localization and Redistribution on the Surface of M21 Human Melanoma Cells Is Associated with Cell Attachment to Fibronectin

Surface localization of GD_2 on fixed M21 human melanoma cells was assessed by indirect immunofluorescence with various Mabs directed to the oligosaccharide portion of this

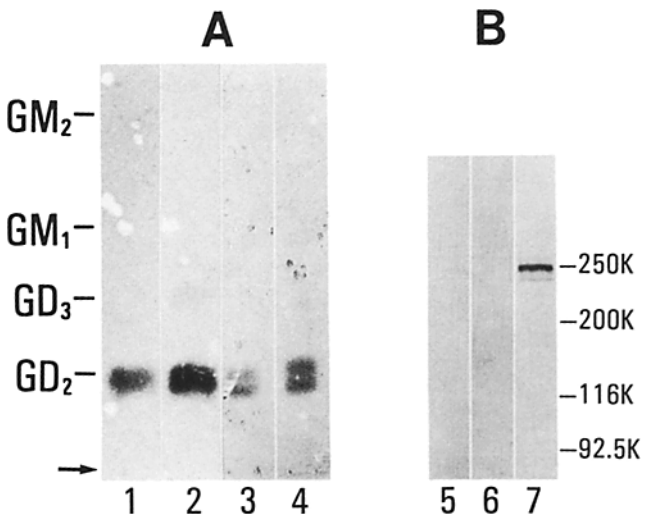


Figure 1. Anti-GD_2 Mabs specifically recognize the GD_2 ganglioside. (*A*) Gangliosides purified from M21 human melanoma cells from a 1 μl packed cell equivalent (lanes *3* and *4*) were separated by TLC along wih ~0.2 μg of authentic GM_2, GM_1, GD_3, and GD_2 (lanes *1* and *2*). Visualization of the standards was achieved by either resorcinol spray (as depicted on left) or by immunostaining with Mab 14.18 (lanes *1* and *3*) or 126 (lanes *2* and *4*). Arrow corresponds to the origin of the TLC plate. (*B*) M21 human melanoma protein lysate was separated by SDS polyacrylamide electrophoresis, transferred to nitrocellulose paper, and stained by immunoblot analysis, using Mabs 14.18 (lane *5*), 126 (lane *6*), and 9.2.27 (lane *7*) as primary antibody as described in Materials and Methods. The position of molecular weight standards ($\times 10^{-3}$) appears to the right.

ganglioside. As shown in Fig. 2, *A–C*, GD_2 localized in discrete foci on the cell surface as well as on a number of microprocesses, which emanated from the main cell body when M21 cells were attached and spread on a fibronectin substrate. In contrast, Mab 9.2.27, directed to a chondroitin sulfate proteoglycan on these same cells (Fig. 2, *D–F*), localized in an extracellular matrix pattern that is above the plane of the substrate attachment domains of the cell. It is quite apparent that this proteoglycan antigen did not localize in the microprocesses that made contact with the substrate.

To determine whether any redistribution of GD_2 on the M21 cell membrane is associated with cell attachment, cells were allowed to attach and spread on a fibronectin substrate and subjected to fixation and indirect immunofluorescence with or without prior exposure of anti-GD_2 Mab for 1 h at 4°C. As shown in Fig. 3, *A–C* M21 cells that are stained with anti-GD_2 after attachment for various times and not pretreated with anti-GD_2 attach and begin to spread at 5 min and at 20 min are completely spread with a number of GD_2-containing processes. In contrast, nonfixed viable cells treated with anti-GD_2 for 1 h at 4°C and then allowed to attach for 5–20 min (Fig. 3, *D–F*) show no spreading at 5–10 min with minimal spreading at 20 min.

When spread cells are treated for 1–2 min with EDTA to chelate divalent cations, the cells begin to round up as shown by SEM in Fig. 4*A*. Cells treated in this manner initially lose contact with the substrate leaving behind focal attachment processes that stain positively for GD_2 by indirect immunofluorescence (Fig. 4*B*). After complete cell removal (Fig. 4*C*),

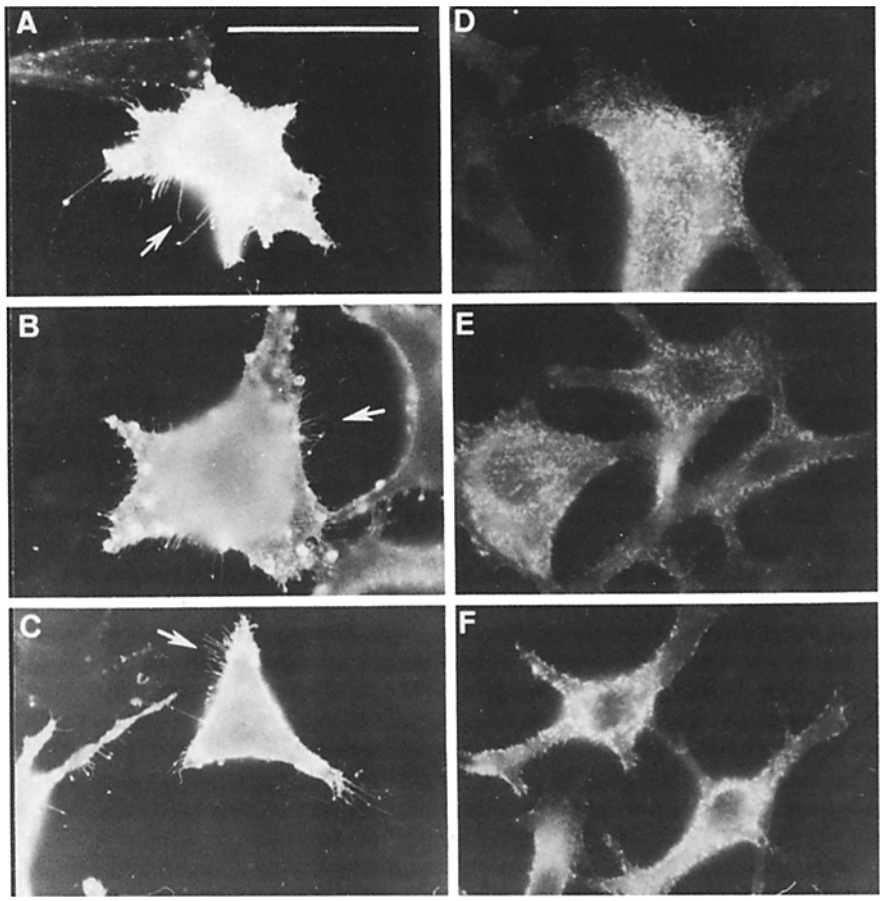


Figure 2. Immunolocalization of GD_2 and chondroitin sulfate proteoglycan antigens on the surface of M21 human melanoma cells. M21 cells were allowed to attach to fibronectin-coated coverslips for 60 min, fixed, and stained by indirect immunofluorescence with Mab 126 (*A–C*) and Mab 9.2.27 (*D–F*) as described in Materials and Methods to visualize the surface expression of GD_2 and the chondroitin sulfate core glycoprotein, respectively. The cells were photographed through a Zeiss microscope equipped with epifluorescence. Bar, 25 μm.

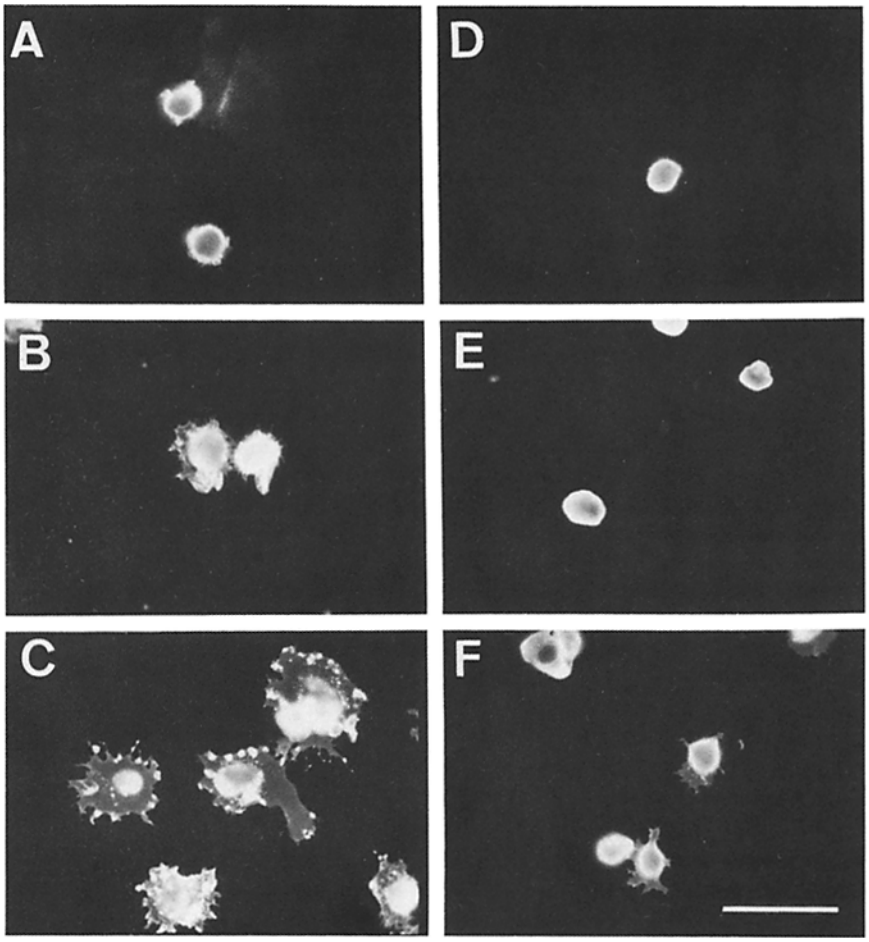


Figure 3. Distribution change of GD_2 surface expression during M21 cell attachment to fibronectin. M21 human melanoma cells were either preincubated at 4°C for 1 h in the presence of growth media (*A–C*) or the anti-GD_2 Mab 14.18 (10 μg/ml) in growth media (*D–F*) and allowed to attach to fibronectin-coated coverslips for either 5 (*A* and *D*), 10 (*B* and *E*), or 20 min (*C* and *F*). After fixation, cells were stained by indirect immunofluorescence using Mab 14.18 to localize GD_2 on the cell surface as described in Materials and Methods. The cells were photographed through a Zeiss microscope equipped with epifluorescence. Bar, 25 μm.

this ganglioside localizes in these adhesion domains which remain firmly associated with the fibronectin substrate. The cells, which ultimately are removed from the substrate with EDTA, now express GD_2 uniformly dispersed around the membrane as shown in Fig. 3*A*, which indicates that the focal distribution of GD_2 on M21 cells is dependent upon their attachment and spreading on a solid substrate.

Detection of GD_2 Preferentially Expressed on M21 Cell Microprocesses Making Direct Contact with the Fibronectin Substrate

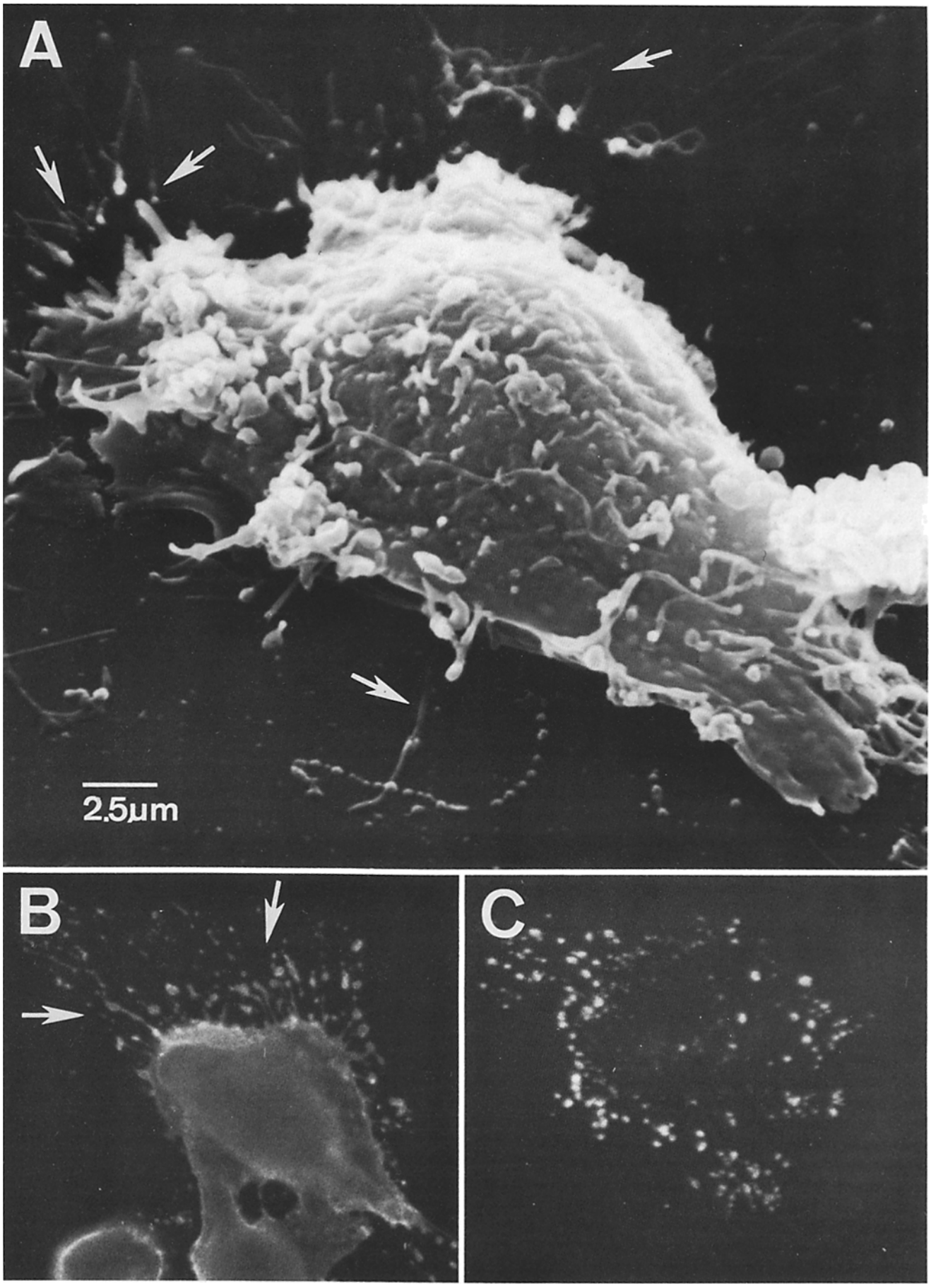
Scanning immunoelectron microscopy demonstrates the presence of GD_2 on microprocesses involved in the attachment of M21 cells to a fibronectin substrate. As shown in Fig. 5, *B–D*, GD_2 can be detected most heavily on microprocesses which project from the main cell body. The actual cell surface also expresses the antigen, however, at a much lower density. In contrast, Mab W6/32, which detects a common determinant of HLA histocompatibility class I antigens, is uniformly expressed on the M21 cell surface (Fig. 6, *B* and *D*), showing no particular preference for either cell body or microprocesses. A melanoma-associated chondroitin sulfate proteoglycan, identified by Mab 9.2.27, cannot be detected on adhesion processes of M21 cells but rather appears on the apical surface of these cells in association with short bleblike structures on the plasma membrane (Fig. 6*C*).

In support of the above observations TEM also demonstrates that GD_2 is localized directly on the microprocessess that make contact with the fibronectin substrate (Fig. 7, *A–C*). As shown in Fig. 7, *A* and *B* cross-sections of these microprocesses are heavily labeled with colloidal gold as compared to the cell body as seen in Fig. 7*A* (upper right). On a number of these cross-sections, GD_2 can be localized directly at the points of substrate contact (Fig. 7, *B* and *C*). In contrast, Mabs 9.2.27 primarily localizes to the apical surface of these cells when attached and fails to react with the microprocesses associated with the basal surface of these cells (data not shown).

Ultrastructural Effects of Anti-GD_2 on M21 Cells Attached to Fibronectin

SEM was used to analyze the ultrastructural effects of anti-GD_2 on M21 cells. The cells were allowed to attach and spread

Figure 4. Ultrastructural and immunofluorescence analysis of M21 focal adhesion plaques. M21 human melanoma cells were allowed to attach to fibronectin-coated coverslips, treated with EDTA for 1 min, fixed, and examined by SEM (*A*) or indirect immunofluorescence (*B* and *C*) using Mab 14.18 as described above. Arrows correspond to the focal adhesion plaque that remains on the fibronectin substrate.


A
2.5µm
B
C

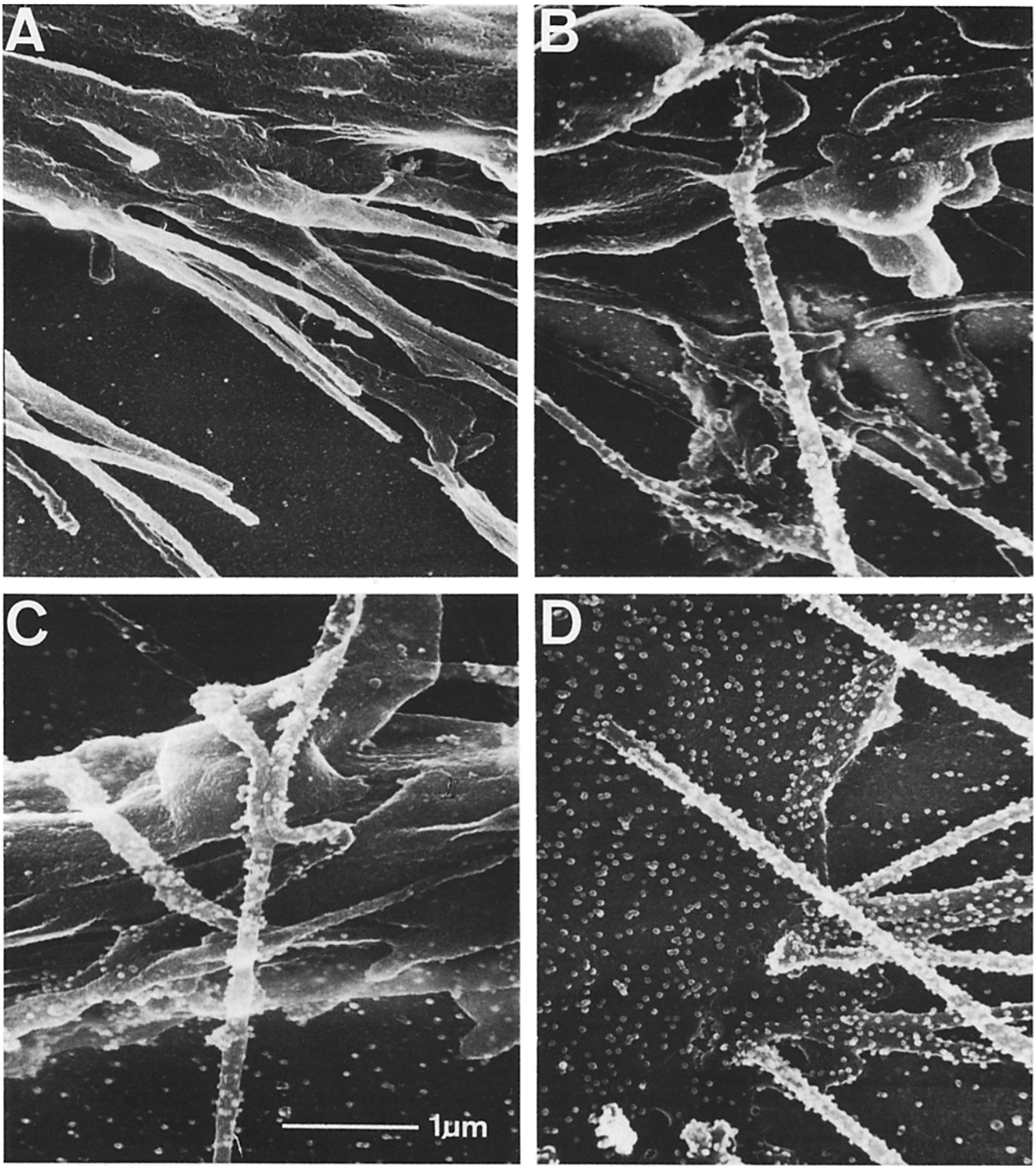


Figure 5. Immunolocalization of GD_2 on M21 microprocesses by scanning immunoelectron microscopy. M21 cells were allowed to attach and spread on fibronectin-coated coverslips, fixed with 0.5% glutaraldehyde, and stained using either irrelevant Mab KS1/4 (*A*) or anti-GD_2 Mabs 126 (*B*), 3F8 (*C*), and 14.18 (*D*) as primary antibodies followed by anti-mouse conjugated to colloidal gold (20–30 nm) as described in Materials and Methods.

on a fibronectin substrate and then overlayed with purified anti-GD_2 for 3–4 h. Most (90%) became rounded in appearance, whereas controls overlayed with Mab 9.2.27 or a nonbinding irrelevant Mab revealed no perturbation of the cell monolayer. Control cells, when washed, fixed, and analyzed by SEM remained firmly attached and spread with multiple attachment points which involved many microprocesses that made direct contact with the cell surface (Fig. 8 *A*). In contrast, M21 cells overlayed with anti-GD_2 not only caused cell rounding but also detachment of the microprocesses from the fibronectin substrate (Fig. 8 *B*), again suggesting that the specific localization of GD_2 in these structures on the melanoma

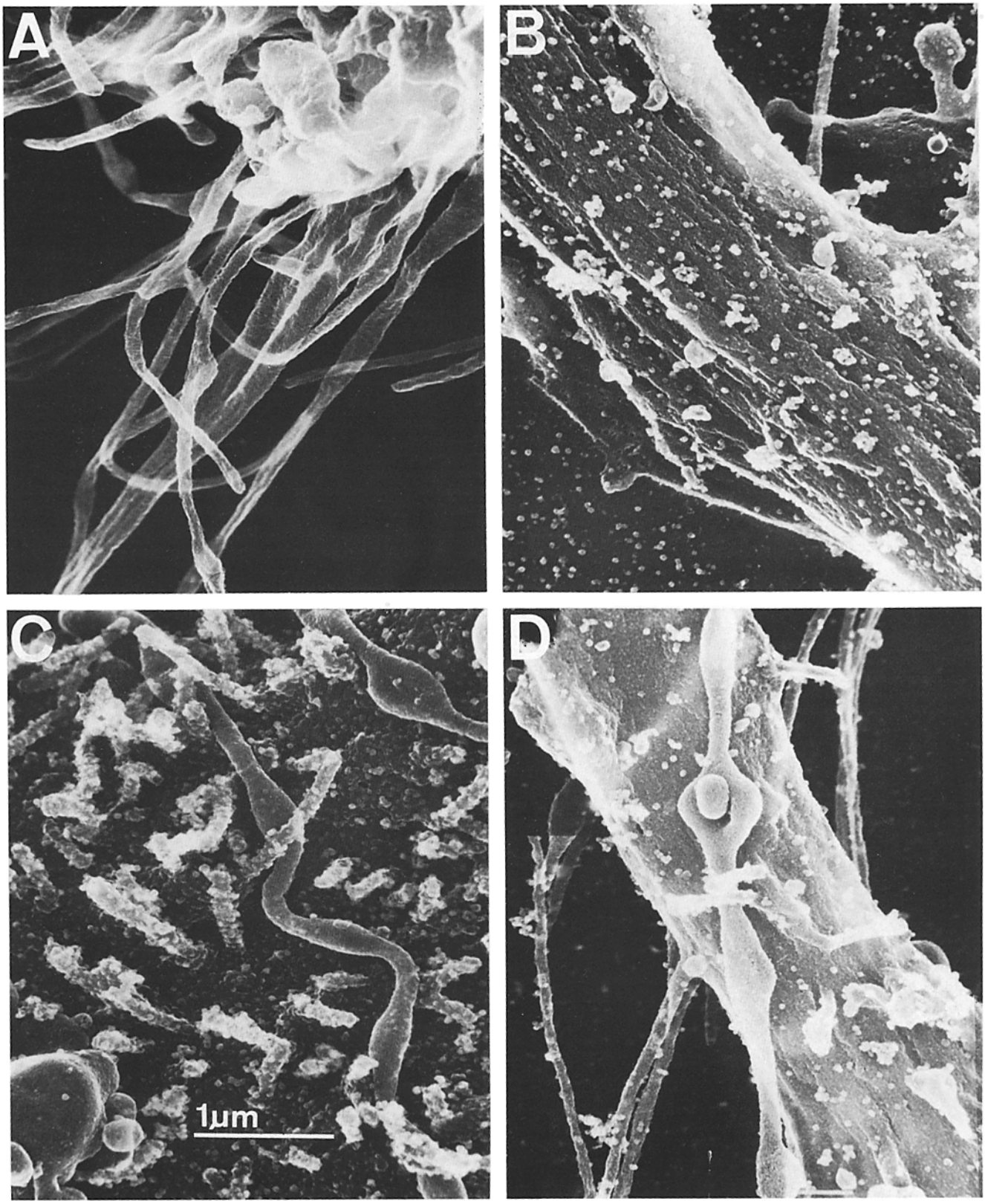


Figure 6. Immunolocalization of a chondroitin sulfate proteoglycan and class I histocompatibility antigen by scanning immunoelectron microscopy. M21 cells were treated identically as in Fig. 5, however, the primary Mabs were changed, i.e., (*C*) Mab 9.2.27 and (*B* and *D*) Mab W6/32.

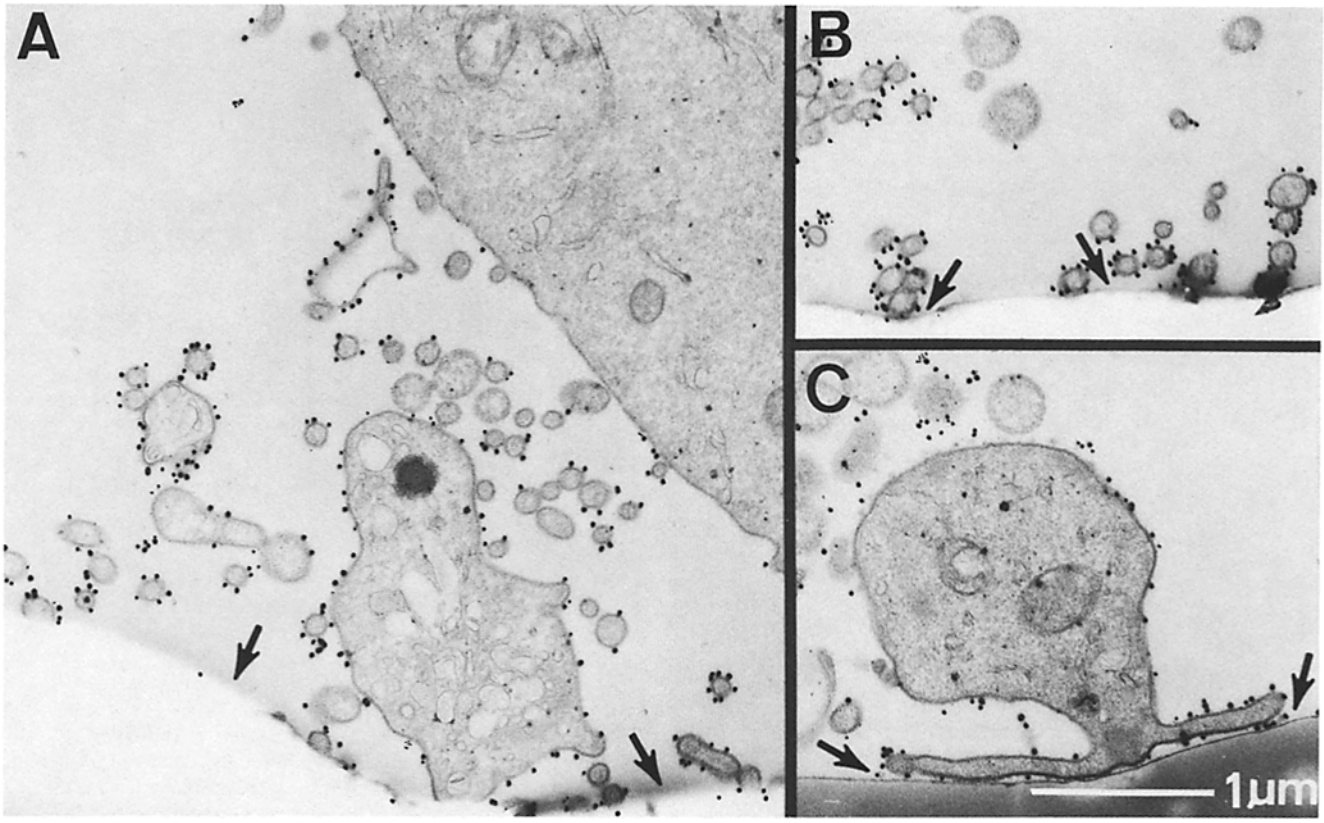


Figure 7. TEM immunolocalization of GD_2 on M21 microprocesses and cell-fibronectin contact points. M21 cells were allowed to attach to fibronectin-coated coverslips and stained with Mab 14.18 and (7–9 nm) gold-labeled anti-mouse Ig and prepared for TEM as described in Materials and Methods. Arrows correspond to substrate. (*A*) Includes a cross-section of the cell body (upper right) as well as microprocesses. (*B*) Demonstrates cross-sections of microprocesses that make direct contact with the substrate. (*C*) Demonstrates a longitudinal section of two microprocesses that make direct contact with the substrate.

cell surface are directly involved in cell–fibronectin interactions.

Discussion

A novel approach to study the functional properties of gangliosides is facilitated by antibodies that bind to the carbohydrate portion of the molecule when naturally exposed on the cell surface. In the present paper, we used specific Mabs directed to GD_2, the major ganglioside on M21 human melanoma cells, to demonstrate that this molecule plays a significant role in M21 cell attachment and spreading on a fibronectin substrate.

The production of highly specific Mabs directed to a variety of ganglioside antigens (3, 7–9, 12, 20, 25, 30, 37, 38) has aided considerably in the identification of new ganglioside structures previously unidentified and has been helpful in determining the specific tissue localization of certain gangliosides (6, 9, 17, 34, 38). We previously characterized several Mabs directed to gangliosides present on the surface of human tumors of neuroectodermal origin (6, 7–9, 38). Using Mabs directed specifically to gangliosides GD_3 and GD_2 we could localize these molecules on the surface of human melanoma cells and in their focal adhesion plaques after their attachment to glass coverslips (8). We also demonstrated that pretreatment of human melanoma cells with either anti–GD_2 or anti–GD_3 Mabs inhibited their ability to attach to a number of extracellular matrix proteins (10). These data are in support of the studies that implicate gangliosides in cell–substratum interactions (18, 29, 49). The conclusion reached in these reports were based upon the exogenous addition of gangliosides to cultured cells under the assumption that the exogenously added gangliosides could embed appropriately in the plasma membrane of the cells being examined.

In this paper, we provide direct evidence that gangliosides are molecules involved in M21 human melanoma cell attachment to the extracellular matrix. In this regard, we demonstrated by immunolocalization with anti–GD_2 Mabs that GD_2 rapidly distributes into cell attachment domains of M21 human melanoma cells almost immediately after their attachment and spreading on a fibronectin substrate. GD_2 was shown to preferentially localize within microprocesses that extend from the cell body and make direct contact with the fibronectin substrate. This was clearly indicated both by indirect immunofluorescence and scanning immunoelectron microscopy. Although the experiments in this study included an examination of GD_2 on M21 cells after 60 min of adhesion, we observed a similar distribution of this antigen after 24 h of adhesion (data not shown). The preferential distribution of GD_2 was demonstrated by the lack of redistribution of two other M21 cell surface antigens. Thus, identical treatment of M21 cells with Mab 9.2.27, which identifies a chondroitin sulfate proteoglycan core glycoprotein, localized this antigen primarily on the apical surface of these cells, whereas Mab

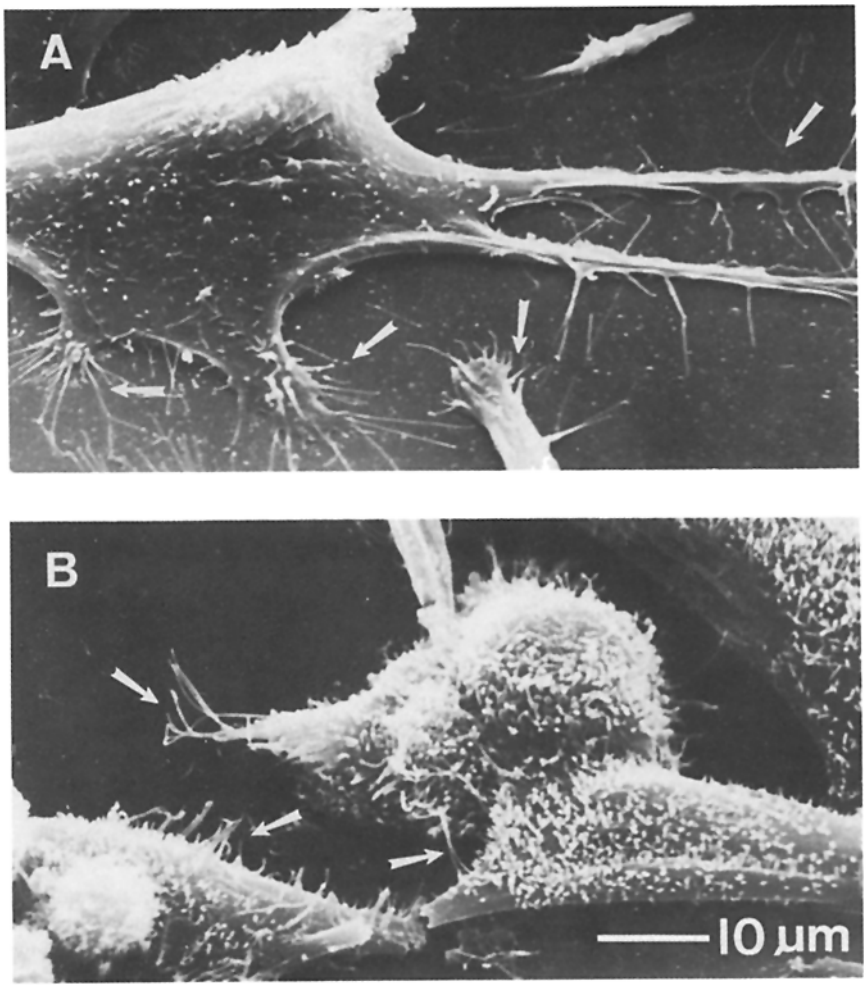


Figure 8. Detachment of M21 cells from fibronectin substrate by anti-GD_2 Mab. M21 cells were allowed to attach and spread on a fibronectin-coated coverslip for 1 h at 37°C. The cells were overlayed with 100 μg/ml irrelevant Mab (*A*) or Mab 14.18 (*B*) for 3 h at 37°C. The cells were fixed and prepared for SEM as described in Materials and Methods. Arrows correspond to attachment-promoting microprocesses.

W6/32, directed to HLA class I antigens, was distributed uniformly over their entire surface rather than localized preferentially on microprocesses.

The use of anti-GD_2 Mabs also facilitated monitoring of the redistribution of GD_2 on these cells during morphological changes associated with their rapid attachment and spreading on a fibronectin substrate. Specifically, GD_2 was shown to redistribute from a focal to a uniform pattern during cell rounding induced by chelation of divalent cations with EDTA. Moreover, M21 cells undergoing mitosis lose their spread appearance while simultaneously demonstrating a uniform GD_2 expression (data not shown). Additionally, treatment of fibronectin-attached M21 cells with anti-GD_2 Mabs resulted in a similar cell rounding and detachment of the cell membrane-associated microprocesses from the substrate. Collectively, these data implicate GD2 on M21 human melanoma cells as playing a direct role in their ability to attach and spread on fibronectin.

The M21 cell line was used as a model in these studies for two reasons. First, this cell line shows very rapid binding to immobilized fibronectin since in 30 min >75% of the cells are attached (10). Second, M21 cells contain GD_2 as their major ganglioside (>60% of the total), thus allowing the use of two available anti-GD_2 Mabs of different isotype to probe the system. Moreover, we demonstrate that the anti-GD_2 Mabs used in this study recognize the oligosaccharide on a lipid backbone only, since these reagents could not recognize a determinant by Western blot analysis (Fig. 1). It is tempting to speculate that a variety of gangliosides on other cell types may indeed have similar properties as GD_2 on M21 cells. In fact, Mabs directed to the GD_3 ganglioside inhibited M21 cell attachment ot fibronectin (10), however, significantly less than that observed with anti-GD_2 Mabs, which suggests that either ganglioside can serve a similar role in cell attachment depending on its relative surface expression. Moreover, Okada et al. demonstrated by biochemical means that GM_3, a major ganglioside on the surface of baby hamster kidney cells, can be localized on their detergent-insoluble substrate attachment matrix (26). In another study (24), it was found that the substrate-attached material from either normal or virally transformed BALB/c 3T3 cells was relatively rich in the disialoganglioside GD1a. These findings are consistent with our previous results (10), which indicate that the substrate-attached gangliosides do not represent indiscriminant membrane fragments.

A more direct interaction between gangliosides and fibronectin was indicated by Spiegel et al. who showed that exogenous addition of flourescence-labeled, complex gangliosides to transformed mouse fibroblasts caused retention of fibronectin into fibrillar networks on the cell surface (41, 42). A number of reports have indicated that the exogenous addition of polysialogangliosides to fibronectin-attached cells caused them to round up and detach from the substrate (18, 29, 49). These studies led to the conclusion that gangliosides may act

as specific fibronectin receptors. However, this conclusion may now be doubtful because of more recent reports of specific membrane-associated glycoprotein receptors for fibronectin (31), vitronectin (32), and laminin (33). Alternatively, these data suggest that gangliosides may augment cell attachment and spreading by acting synergistically with such cell surface receptors. In this case, depending on the oligosaccharide moiety, individual gangliosides may redistribute into discrete cell attachment domains, thereby creating an appropriate electrostatic environment for optimal cell adhesion. Our data are consistent with either hypothesis since we can preferentially localize the GD_2 gangliosides in cell attachment domains on human melanoma cells that contain this ganglioside.

The fact that GD_2 contains two sialic acid moieties, thus carrying two negative charges, argues against the possibility that the distribution we observed is due to random mobility of this molecule in the membrane of M21 cells, since such highly charged molecules in the membrane would tend to repel one another. However, it is conceivable that divalent cations, known to be required for fibronectin-mediated cell attachment (5, 27), are involved in a complex that contains the negatively charged ganglioside on the cell surface, thereby allowing them to distribute preferentially into certain domains on the cell surface. This contention is consistent with the data reported by Sharom and Grant who demonstrated that physiological levels of Ca^{+2} and Mg^{+2} lead to cross-linking and condensing of ganglioside headgroups in membranes by complexing carboxyl residues of sialic acid (39). Therefore, in the presence of divalent cations, it is possible that laterally mobile carbohydrate-bearing components such as gangliosides may show a tendency to cluster about complex glycoproteins that contain one or several carboxyl groups. This hypothesis may help to explain the numerous reports that implicate gangliosides and other glycoconjugates in the cell attachment process despite the recent convincing data that demonstrate the presence of specific cell surface protein receptors for components of the extracellular matrix.

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