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GANGLIOSIDES DEPLETED IN PLASMA MEMBRANE ARE DIRECTED TO INTERNAL MEMBRANES OF RAT HEPATOMAS: EVIDENCE FOR A GLYCOLIPID SORTING DEFECT IN HEPATOCARCINOGENESIS

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Ganglioside compositions of plasma membrane fractions highly purified from rat liver and hepatomas by phase partitioning were compared with those of fractions composed of internal membranes, free of plasma membrane. With liver, 70-80% of the the lipid bound sialic acid were accounted for by a plasma membrane location. In hepatomas this percentage was reduced to 50-65%. More pronounced was the distribution of the simple monosialoganglioside G_{M3}. In the hepatomas, 60-80% of the G_{M3} was found associated with internal membranes as compared to liver where only 35% of the G_{M3} was present in internal membranes. The findings suggest a glycolipid sorting defect in hepatocarcinogenesis where gangliosides, and especially monosialogangliosides, are diverted to internal membranes rather than being correctly transported to the cell surface. Since G_{M3} is synthesized exclusively in the Golgi apparatus of both liver and hepatomas, the basis for the sorting defect may reside in a functionally altered Golgi apparatus.

Glycolipid alterations of the cell surface now are regarded as among the more general of the many features of tumorigenesis (1-9). Analyses of the chemical composition of the cell surface comparing normal and tumor cells have revealed that glycosylated lipids, especially the sialic acid-containing sphingolipids (gangliosides), and their precursors show patterns of alteration as one consequence of the tumorigenic transformation (1, 2, 9). Not infrequently, the alterations appear as deletions or reductions of the more complex glycoconjugates concomitant with increases in precursor molecules including both gangliosides (1, 2, 9, 10, 11) and neutral glycolipids (12, 13).

Mitogen and alloantigen stimulation of human lymphocytes resulted in differential glycolipid synthesis (14, 15). When transformed cells were treated with butyrate, plasma membrane levels of G_{M3} increased (16, 17). In contrast, when cell growth became unregulated after oncogenic transformation there was a decrease in G_{M3} (18). These findings provided evidence that G_{M3} and related monosialogangliosides somehow may be involved in growth control.

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The findings that elevated G_{M3} in the plasma membrane correlated with a reduced growth rate and contact inhibition does not agree with observed elevations in G_{M3} in hepatomas (19) where growth rate most often was accelerated and normal growth controls were lost (5-8). Gangliosides are generally believed to be localized predominantly on the outer leaflet of the plasma membrane. However, studies of gangliosides in hepatomas were either with tissues or unfractionated membranes and did not determine whether or not the elevated G_{M3} was associated with the plasma membrane as is the situation with liver (20).

New methods developed for preparation of plasma membranes (21) facilitated our study of the plasma membrane localization of G_{M3} in hepatomas. Results obtained point to a sorting defect in hepatomas which results in gangliosides being directed away from the cell surface to internal membranes.

MATERIALS AND METHODS

Animals and Diets: Male Fischer 344 rats weighing 100 to 125 g were from Harlan Animal supply (Indianapolis, IN). Hepatocellular carcinomas were propagated *in vitro* as described by Kloppel and Morré (22).

Isolation of Plasma Membranes: Livers and hepatomas were homogenized using a Polytron 10ST tissue homogenizer (Kinematica, Lucerne, Switzerland) for 45 s in 2 ml/g tissue of a medium containing 37 mM maleate, pH 6.4, 0.5 M sucrose, 1% dextran (Sigma; average molecular weight 225,000), 5 mM $MgCl_2$, and 5 mM mercaptoethanol (23). The homogenate was centrifuged at low speed (5000 x g) for 15 min to concentrate the plasma membrane vesicles. The supernatant and the top one-fourth of the pellet (the cream-colored upper layer enriched in Golgi apparatus) were removed and the middle one-third to one-half of the pellet (avoiding the underlying nuclei and unbroken cells) was resuspended in 0.4 to 0.6 ml/g liver of 1 mM sodium bicarbonate using 20 up and down strokes with a Duragrind tissue homogenizer (Wheaton, Millville, NJ). Another 15 to 20 ml of sodium bicarbonate was added after the pellet was resuspended and the mixture was centrifuged for 15 min for 5000 x g. The supernatant was discarded and the light brown, top portion of the pellet was used for preparation of plasma membranes by aqueous two-phase partition using 16 g systems consisting of 6.4% (w/w) dextran T500 (Pharmacia), 6.4% polyethylene glycol 3350 (Fisher), 0.25 M sucrose, and 5 mM potassium phosphate, pH 7.2 as described (21). The contents of the two-phase systems were mixed by 40 inversions of the tubes in the cold (4° C) and the two phases were separated by low speed centrifugation. The upper phase, enriched plasma membranes, was diluted with 1 mM bicarbonate and the plasma membranes were collected by centrifugation for 20 min at 80,000 g.

Isolation of gangliosides: Gangliosides were isolated from pooled membrane fractions from 4 to 20 rat livers or hepatomas by a modification of the procedure of Ledeen et al. (24). Lipids were extracted with 1:1, 1:2 and then 2:1, mixtures of chlorform/methanol (v/v), each overnight. Solvents were then evaporated and the samples were applied to DEAE-Sephadex columns in chloroform/methanol/water (30:60:8, v/v), washed with the same solvent mixture followed by 100 ml of methanol. Gangliosides were eluted together with other acidic lipids with chloroform/methanol/0.08 M sodium acetate (30:60:8, v/v). Following saponification with 0.2 N KOH in methanol to remove glycerolipids, the gangliosides were desalted either by dialysis against water or chromatography on Sep-Pak C18 columns according to Williams and McCluer (25). Finally, the gangliosides were purified on Unisil columns as described (24).

Sialic acid was determined using periodate-resorcinol (26) or thiobarbituric acid (27) with N-acetylneuraminic acid as standard. Gangliosides (5 to 10 nmol sialic acid) were spotted on HPTLC plates and the plates were developed with chloroform/methanol/0.2% calcium chloride in water (25:20:5, v/v) or with chloroform/methanol/ammonium hydroxide/water (60:35:7:3, v/v). Gangliosides were detected using resorcinol-HCl (26). For quantitation, plates were scanned using a densitometer (Photovolt Corp., New York, NY), fitted with a No. 33 gelatin filter (Eastman Kodak Co., Rochester, NY) to reduce interference from non-resorcinol-positive chromogens and compared to standard plates spotted with known quantities of gangliosides.

RESULTS AND DISCUSSION

Total gangliosides of two transplantable rat hepatomas were elevated 3- to 4-fold compared to rat liver (Table I). Similar elevations were found when data were expressed on a protein basis. The ganglioside content per mg protein was 3-fold greater for the hepatomas RLT-N and 5-fold greater for RLT-28 as compared to liver.

When hepatoma plasma membranes, highly purified by aqueous two-phase partition, were analyzed, the gangliosides were 2- to 3-fold elevated over those of liver plasma membrane on the basis of tissue weight, but only 1.7- to 1.9-fold elevated over liver plasma membranes on the basis of protein (Table I). The more marked 3- to 4-fold elevations were seen only in the lower phase membrane fractions depleted in plasma membranes.

As expected, the dominant ganglioside of liver and hepatomas, approximately 60% of the total, was the monosialoganglioside G_{M3}. With liver,

Table I. *Distribution of gangliosides of rat liver and two hepatomas comparing plasma and internal endomembranes*

Cell fraction	Ganglio-sides, nmoles/10g tissue	Total protein, mg/10g tissue	Ganglioside sialic acid, nmoles/mg protein	G_{M3} nmoles per 10g tissue	G_{M3} nmoles/mg protein
Rat liver					
Total homogenate	390	1300	0.3	234	0.2
Plasma membrane	317	52	6.1	193	3.7
Endomembranes	150	750	0.2	83	0.1
RLT-N hepatoma					
Total homogenate	1100	1100	1.0	550	0.5
Plasma membrane	749	70	10.7	112	1.6
Endomembranes	390	650	0.6	351	0.5
RLT-28 hepatoma					
Total homogenate	1800	1200	1.5	720	0.6
Plasma membrane	885	75	11.8	133	1.8
Endomembranes	640	710	0.9	575	0.8

approximately 80% was present in the upper phase of the two-phase separations associated with plasma membrane with the remaining 20% being associated with internal membranes of the lower phase.

With hepatomas, the opposite result was obtained. Approximately 3- to 4-fold greater amounts of G_{M3} were found in the lower phase than in the upper phase. When calculated on a protein basis, the plasma membrane content of G_{M3} in the hepatoma was found to be less than that of the plasma membrane of liver. In contrast, the endomembrane content of G_{M3} from the hepatomas was markedly increased to 4-fold for RLT-N hepatoma and to 7-fold for RLT-28 hepatoma as compared to liver.

The presence of G_{M3} in the lower phase (internal membranes) was verified by development of the G_{M3} zone from the first solvent system in a second solvent system (Fig. 1). The only resorcinol-positive material present was a zone with a mobility corresponding to authentic G_{M3}. The amounts present (total G_{M3} recovered from 3 to 6 mg total fraction protein) were in approximate proportion to the specific activities given in Table I as nmoles G_{M3}/mg protein. G_{M3} content was greatest in liver plasma membrane and least in liver internal membranes. The amounts of G_{M3} associated with internal membranes of the hepatoma were increased over that for liver and were present in amounts nearly equal to those observed in the plasma membranes from the hepatomas.

The findings provide evidence for an altered distribution of gangliosides between the plasma membrane and internal membranes comparing rat liver and

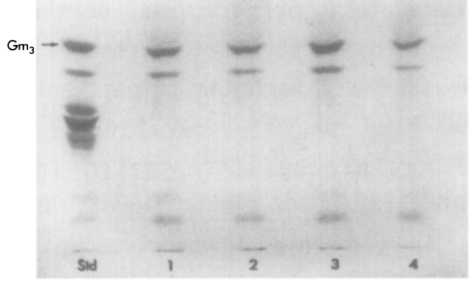

Figure 1. Thin-layer chromatograph of G_{M3} gangliosides isolated from rat liver and hepatoma membrane fractions. Gangliosides were isolated by the procedure of Ledeen (27) from pooled membrane fractions. After saponification, the gangliosides were purified from a Sep-Pak column (28), a Unisil column (27) and 3 more times from Sep-Pak columns (28). Primary separation was on HPTLC plates, developed in chloroform/methanol/0.2% $CaCl_2$ in water (25:20:5, v/v). Material comigrating with G_{M3} was finally developed in chloroform:methanol:ammonium hydroxide:water (60:34:7:3) and visualized with resorcinol. Hepatoma lower plasma membrane, hepatoma lower phase and liver plasma membrane were equivalent to 3 mg protein. Liver plasma membrane was equivalent to 9 mg protein. Std = bovine brain gangliosides with G_{M3} added. Lane 1. Plasma membrane of rat RLT-N hepatoma (upper phase). Lane 2. Internal membranes of RLT-N rat hepatomas (lower phase). Lane 3. Rat liver plasma membrane. Lane 4. Internal membranes of rat liver (lower phase).

hepatoma. The increased G_{M3} of the tumor membranes, in contrast to expectation, was primarily in the fraction comprised of endoplasmic reticulum and other internal membranes and not in the plasma membrane-enriched fraction.

Matyas and Morré (20) previously had shown the presence of gangliosides in highly purified endoplasmic reticulum fractions of rat liver. However, higher ganglioside homologs were poorly represented and the principal ganglioside present was G_{M3}. Yet endoplasmic reticulum gangliosides represented only 7.4% of the total cellular ganglioside pool, an amount comparable to that observed for internal membranes of rat liver in the present study.

The endoplasmic reticulum fractions analyzed previously (20) were shown by analyses of marker enzymes and by electron microscopy to be less than 2% contaminated by plasma membranes. This amount would have been insufficient to account for the gangliosides present, and bulk plasma membrane contamination could not account for the unique ganglioside composition for the endoplasmic reticulum fraction (20). Similarly, in the present study, the plasma membrane-(upper phase), and internal membrane- (lower phase) enriched fractions have been characterized extensively using both marker enzymes and electron microscopy (21). For the RLT-N hepatoma, the lower phase fractions consisted of 60% mitochondria, 30% endoplasmic reticulum and 10% other membranes. Plasma membranes were found to be less than 2% of the total (21).

The major ganglioside biosynthetic enzymes have been systematically characterized, and most are located in the Golgi apparatus (20, 28, 29). From the Golgi apparatus, gangliosides normally are delivered to the cell surface and not to internal membranes. In the hepatomas there appears to be a sorting defect affecting either the exit of G_{M3} from the Golgi apparatus, the targeting of G_{M3} carrier vesicles or molecules, or the recycling of G_{M3} from the plasma membrane to internal membrane compartments. The net result is an accumulation of ganglioside in a membrane compartment partitioning with the lower phase of the two phase separation. The findings that monosialogangliosides may not be concentrated in plasma membranes in hepatomas, despite their overall increased levels in hepatoma tissue, has important implications in the role of gangliosides in growth control in transformed cells and tissues generally.

One possibility, based on the work of Matyas et al. (30) with ras transfected 3T3 cells, is that the transformed cells might actually have increased levels of G_{M3} compared to that of nontransformed cells but considerably less expressed at the cell surface. Furthermore, the synthesis and delivery of G_{M3} to the cell surface in the transformed cells would be unresponsive to contact. Therefore, the normally growth-inhibitory levels of ganglioside at confluency would never be reached after transformation, i.e. the failure to accumulate gangliosides might result from a sorting defect similar to that encountered in hepatomas.

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REFERENCES

1. Brady, R.O. and Fishman, P. (1974) Biochim. Biophys. Acta 355, 121-148.
2. Hakomori, S.-I. (1981) Ann. Rev. Biochem. 50, 733-764.
3. Radin, N.S. and Inokuchi, J.-I. (1988) Biochem. Pharmacol. 37, 2879-2886.
4. Curatolo, W. (1987) Biochim. Biophys. Acta 906, 137-160.
5. Nicolson, G.L. (1984) Exptl. Cell Res. 150, 3-22.
6. Rapin, A.M.C. and Burger, M.M. (1974) Adv. Cancer Res. 20, 1-9.
7. Nicolson, G.L. (1976) Biochim. Biophys. Acta 458, 55-72.
8. Hynes, R.O. (1979) Surfaces of Normal and Malignant Cells. John Wiley, New York.
9. Hakomori, S. (1975) Biochim. Biophys. Acta 417, 55-89.
10. Richardson, C.L., Baker, S., Morré, D.J., and Keenan, T.W. (1975) Biochim. Biophys. Acta. 417, 175-186.
11. Fishman, P.H. and Brady, R.O. (1976) Science, 194, 906-915.
12. Dnistrian, A.M., Skipsky, V.P., Barclay, M., and Stock, C.C. (1977) Cancer Res. 37, 2182-2178.
13. Walter, V.P., Kloppel, T.M., Deimling, I.G., and Morré, D.J. (1980) Cancer Biochem. Biophys. 4, 145-151.
14. Vakirtzi-Lemonias, C., Evangelatos, G.P., Kapoulas, V.M., and Levis, G.M. (1980) Eur. J. Biochem. 109, 541-551.
15. Kniep, B., Hünig, T.R., Fitch, F.W., Heuer, J., Kölsch, E., and Mühlradt, P.F. (1983) Biochemistry 22, 251-255.
16. Fishman, P.H., Simmons, J.L., Brady, R.O., and Freese, E. (1974) Biochem Biophys Res. Commun. 59, 292-299.
17. Macher, B.A., Lockney, M., Moskal, J.R., Fung, Y.K., and Sweeley, C.C. (1978) Exp. Cell Res. 117, 95-102.
18. Hakomori, S. (1970) Proc. Natl. Acad. Sci. USA 67, 1741-1748.
19. Merritt, W. D., Richardson, C. L., Keenan, T. W., and Morré, D. J. (1978) J. Natl. Cancer Inst. 60, 1329-1337.
20. Matyas, G. and Morré, D.J. (1987) Biochim. Biophys. Acta. 921, 599-614.
21. Navas, P. Nowack, D.D., and Morré, D.J. (1989) Cancer Res. 49, 2147-2156.
22. Koppel, T. M. and Morré, D. J. (1980) J. Natl. Cancer Inst. 64, 1401-1411.
23. Morré, D. J. (1971) Meth. Enzymol. 22, 130-148.
24. Ledeen, R.W., Yu, R.K. and Eng, L.F. (1973) J. Neurochem. 21, 829-839.
25. Williams, M. A., and McCluer, R. H. (1980). J. Neurochem. 35, 266-269.
26. Jourdian, G.W., Dean, L. and Roseman, S. (1971) J. Biol. Chem. 246, 430-435.
27. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975.
28. Keenan, T.W., Morré, D.J., and Basu, S. (1974) J. Biol. Chem. 249, 310-315.
29. Van Meer, G. (1989) Ann. Rev. Cell. Biol. 5, 247-275.
30. Matyas, G., Aaronson, S.A., Brady, R.O., and Fishman, P.H. (1987) Proc. Natl. Acad. Sci. 84, 6065-6068.