Study on the Proposed Role of Phospholipid in Tumor Cell Membrane

James K. Selkirk, 2 J. C. Elwood, and H. P. Morris

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706 [J. K. S.]; Department of Biochemistry, Upstate Medical Center, State University of New York, Syracuse, New York 13210 [J. C. E.]; and Department of Biochemistry, College of Medicine, Howard University, Washington, D. C. 20001 [H. P. M.]

SUMMARY

This study compares plasma membranes from solid hepatoma cells to liver cell plasma membranes for comparison of phospholipid profile, degree of fatty acid unsaturation, and amount of calcium and magnesium bound to membrane. These parameters are based on proposed roles in membrane structure.

Whole-tumor analysis showed the presence of the major phospholipids with no unidentifiable components. The Reuber H-35 hepatoma plasma membrane had the same percentage of the individual phospholipids as liver, whereas the Morris 3924A hepatoma cell membrane exhibited a 4-fold increase in sphingomyelin and the appearance of a significant increase in choline plasmalogen.

Phospholipid fatty acid analysis of whole tumor and plasma membrane showed the 3924A tumor plasma membrane to have the largest number of unsaturated fatty acids. The ratio of stearic acid to oleic acid in the two tumors was less than 1.

Calcium as measured by atomic absorption spectrophotometry exhibited increased membrane-bound calcium in both tumors with the 3924A tumor containing the largest amount.

INTRODUCTION

One of the distinguishing characteristics that sets a malignant tumor apart from benign tumors and normal tissues is its ability to invade adjacent normal tissue and produce secondary tumors in distant parts of the body. The movement of malignant cells to secondary sites via the lymphatic and vascular system as well as solid tissue tunneling is well known. However, the mechanism whereby a tumor can shed viable embolic cells has not been extensively investigated.

There are a number of publications in the literature that suggest that the invasive quality of tumor tissue may be related to the cell surface and intercellular adhesion (2, 4, 8, 30, 32). Coman (7) determined the relative differences in cell adhesiveness between joined tumor cell pairs as compared to joined normal cell pairs. He found there was a marked reduction in the intercellular adhesion between joined tumor cells. Beebe (1) was the first to report decreased calcium in human carcinomas, and Clowes and Frisbie (6) confirmed this result with Jensen mouse adenocarcinoma. In a comprehensive review of the literature to that time, Shear (23) in 1933, found the concept of reduced calcium content of tumor versus normal tissue to be valid. Shah and Shulman (20—22) have indicated the interrelationship of unsaturation of the phospholipid hydrocarbon moiety and the cation to which the glycerophosphate is bound. They measured the collapsing pressure and electrical potential of lecithin monolayers with varying degrees of sidechain unsaturation and several divalent cations. They concluded that calcium binding varied inversely with fatty acid unsaturation. A hypothesis was proposed to explain this observation, based on steric hindrance imposed by olefin linkages.

The object of this study was to analyze the phospholipids of hepatoma and liver cell plasma membranes and to compare the phospholipid profiles for structural alteration, degree of membrane fatty acid unsaturation, and the amounts of calcium and magnesium in whole tissue and cell membranes.

Two hepatomas were chosen from opposite ends of the growth spectrum. The Morris 3924A hepatoma is one of the more morphologically altered of the transplanted hepatomas; it exhibits a rapid growth rate and high ploidy and is histologically anaplastic. Such a tumor possesses great metastatic potential, capable of producing lung metastases during the 3 weeks of growth phase before host death (H. P. Morris, personal communication). The second tumor was the Reuber H-35 hepatoma, chosen for its classification as a minimal deviation type with a relatively slow transplantation time of 2 months.

MATERIALS AND METHODS

Isolation of Membranes. All membranes were isolated according to the procedure of Neville (17) as modified by Emmelot et al. (9). Yields were 10 to 15 mg of plasma membranes, dry weight, per 20 g of tissue, wet weight.

Extraction of Lipids. Lipids were extracted according to the method of Folch et al. (10). A 20-fold tissue volume of chloroform:methanol (2:1) was added to the tissue and shaken for 5 min. The solution was centrifuged at low speed to precipitate
the solid material, and the chloroform:methanol was pipetted off. The precipitate was then reextracted with a 5-fold volume of boiling chloroform:methanol mixture for 5 min, and both of these extractions were combined. The combined extracts were washed with 0.2 volume of water, which maintains over 99% of the lipid in the organic phase (10). The upper water layer was discarded, and methanol was added until the water:chloroform interface disappeared.

Lipids were then separated into neutral lipid and phospholipid classes by addition of silicic acid (1 g/25 mg lipid). The slurry was mixed intermittently for 10 min and then filtered. The filtrate was treated in the same way with an equal quantity of silicic acid and the silicic acid residues were combined. Neutral lipids were eluted from the silicic acid by shaking for 10 min with twice its volume of chloroform. The slurry was filtered and the residue was washed with one-half the previous volume of chloroform. Phosphorus analysis of the neutral lipid fraction was routinely performed. If the fraction was positive for phosphorus, it was reslurried with silicic acid, heated until charred. The spots were scraped off the plate, and the chloroform wash was repeated. The silicic acid was dried, made into a slurry with twice its volume of absolute methanol, and shaken for 10 min.

The slurry was filtered and washed twice with one-half the previous volume. The combined filtrates contained the phospholipids. A third filtration was usually negative for phosphorus. Borgstrom (3) has shown quantitative removal of phospholipid by this method, and we have found only traces remaining in the silicic acid after 2 washings. The solutions were stored under nitrogen in the freezer.

Preparation of Thin-Layer Chromatography Plates. Thin-layer plates were prepared according to the method of Stahl (28) with glass plates (200 x 200 mm). Plain silica gel (Warner-Chilcott Laboratories, Morris Plains, N. J.) was used to avoid the “load effect,” as described by Skipski et al. (26, 27). Plates were activated for 30 min at 120°immediately before use, and lipid samples were spotted under nitrogen at room temperature.

The plates were developed in chloroform:methanol:acetic acid:water (25:14:4:2) and then placed in iodine vapor for visualization of phosphatide spots. Plasmanolagen was identified by the method of Hack and Ferrans (11) and confirmed with the phenylhydrazine technique of Marinetti (14).

Phosphorus Analysis. The microdetermination of phosphorus was done according to the method of Chen et al. (5), as modified by Rouser et al. (19). Lipid spots were sprayed with 55% sulfuric acid containing 0.6% sodium dichromate and heated until charred. The spots were scraped off the plate, added to a flask containing perchloric acid, and heated until the solution became clear. Two undeveloped areas of each plate were treated in the same manner and used as controls. The amount of phosphorus was determined colorimetrically at 820 μm, with molybdate as the chromophore reagent.

Fatty Acid Analysis. The phosphatide fatty acids were cleaved from the glycerol by saponification with hot alkali. Pretreatment of the phospholipid fraction with acetic acid to release alkaline-resistant plasmanolagen fatty acids did not significantly increase fatty acid yields; consequently, Tables 3 and 4 may not include plasmanolagen fatty acids. Neutral lipids were removed from the saponification mixture by extraction with petroleum ether. The mixture was then acidified, and the free fatty acids were also extracted into ether. Fatty acid methylation was done with boron trichloride:methanol (Analabs Inc., Hamden, Conn.) in a sealed ampul at 100° for 5 min.

Methylated fatty acids were injected into an F-M Model 720 dual-column, temperature-programmed gas chromatograph. Columns were stainless steel (10 feet, 0.25 inch in diameter), packed with 20% diethylene glycol succinate. Programming was at 1°/min between 150 and 220°.

Cation Analysis. Calcium and magnesium levels of liver and plasma membrane were assayed on the Perkin-Elmer 303 atomic absorption spectrophotometer. The tissue was dried to constant weight at 104° and then ashed at 600° for 46 hr.

RESULTS

Table I lists whole-tumor and liver phosphatides as percentages of total phosphorus. The relative proportions of the liver phospholipids are in agreement with those in the literature (15, 16). A notable difference from normal liver is the elevated phosphoinositide in the Morris hepatoma 3924A. The phosphorus remaining at the origin on the thin-layer plate is presumed to be a phospholipid degradation product since additional acetic acid or methanol in the solvent system failed to remove the spot from the origin.

Table 2 lists phosphatides present in the plasma membranes of cells from liver and both tumors. Hepatoma 3924A contains 3 times more sphingomyelin than normal cell membranes and also shows the presence of choline plasmanolagen.

| Table 1 Whole tumor and liver phosphatides (% of total phosphorus) |
|---|---|---|
| Liver (10) | H-35 (10) | 3924A (10) |
| Solvent front | 2.9 ± 0.3 | 3.6 ± 1.7 | 6.0 ± 2.1 |
| Phosphatidylethanolamine | 18.8 ± 1.0 | 16.3 ± 1.3 | 21.2 ± 0.7 |
| Phosphatidylserine | 3.0 ± 0.2 | 2.5 ± 0.5 | 3.0 ± 0.05 |
| Phosphatidylcholine | 9.3 ± 0.4 | 8.0 ± 0.8 | 16.0 ± 1.0 |
| Plasmanolagen (choline) | +b | + | + |
| Lecithin | 52.6 ± 6.5 | 43.9 ± 4.2 | 41.7 ± 1.7 |
| Sphingomyelin | 4.1 ± 0.3 | 7.2 ± 0.5 | 4.5 ± 0.05 |
| Lysolecithin | 0.9 ± 0.1 | 1.2 ± 0.3 | 1.1 ± 0.1 |
| Origin | + | 11.0 ± 2.4 | + |

b No. of analyses. b +, <1%.

| Table 2 Tumor and liver membrane phosphatides (% of total phosphorus) |
|---|---|---|
| Liver (10) | H-35 (10) | 3924A (10) |
| Solvent front | 6.3 ± 2.8 | 1.8 ± 0.35 | +b |
| Phosphatidylethanolamine | 25.6 ± 4.6 | 14.2 ± 3.2 | 17.1 ± 1.3 |
| Phosphatidylserine | 6.8 ± 2.2 | 7.0 ± 1.0 | 6.1 ± 1.4 |
| Phosphatidylcholine | 6.9 ± 2.1 | 8.1 ± 1.0 | 6.0 ± 1.5 |
| Plasmanolagen (choline) | — | — | 7.2 ± 1.0 |
| Lecithin | 41.4 ± 3.5 | 54.1 ± 2.2 | 45.6 ± 0.8 |
| Sphingomyelin | 9.1 ± 0.8 | 8.3 ± 1.0 | 21.2 ± 5.7 |
| Lysolecithin | 6.1 ± 3.6 | — | 1.4 ± 0.3 |
| Origin | — | 2.2 ± 1.0 | 3.5 ± 1.7 |

b No. of analyses. b +, <1%; —, not present.
neutral lipid fatty acids from the tumor plasma membranes, which were 8% for Reuber hepatoma H-35 and 16% for Morris hepatoma 3924A. The neutral lipid fatty acids from liver tissue were contained more 18:1 than liver membranes, and the 18:0 to 18:1 ratio is reversed in tumor tissue phospholipid fractions.

Table 3 lists the fatty acid analyses of liver and tumor cell membranes. The neutral lipid fatty acids from liver tissue were 75% saturated. About 35% of the fatty acids were 18:0, which is in marked contrast to the neutral lipid fatty acids from the tumor plasma membranes, which were 8% for Reuber hepatoma H-35 and 16% for Morris hepatoma 3924A. The neutral lipid fatty acids from the tumor plasma membranes contained more 18:1 than liver membranes, and the 18:0 to 18:1 ratio is increased. Hepatoma H-35 also contained a high percentage of 18:2 and 20:4 fatty acids. A small amount of 22:1 was observed in both the H-35 and 3924A tumor plasma membranes that was not seen in liver tissue.

Fatty acids from the phospholipid fraction of liver plasma membranes contained 34% 18:0 as contrasted to 14 and 18% in tumor membranes. The H-35 hepatoma plasma membrane phospholipid fatty acids contained an inordinately high amount of C16 (43%). As in the neutral lipid fraction, the 18:0 to 18:1 ratio is reversed in tumor tissue phospholipid fatty acids as compared to liver membranes.

Table 4 lists the fatty acid profile in whole tissue of neutral lipid and phospholipid from hepatomas H-35 and 3924A. Greater than 60% of the neutral lipid fatty acids from hepatoma H-35 contained 18 carbon atoms, and most were unsaturated. The remainder of the fatty acids were C16 and C16:1 with the exception of 6% C24 saturated fatty acid. The fatty acids from the neutral lipid fraction of hepatoma 3924A did not contain any of the C24 but had significantly greater amounts of C18:0 and less C18:1 than the H-35 tumor. The phospholipid fraction of both tumors contained approximately 10% 22:1 fatty acid. In fact, the phospholipid fatty acids of these 2 tumors were quite similar.

Table 5 lists the results of analyses for calcium and magnesium from whole tissue and plasma membranes of liver and hepatomas H-35 and 3924A. The calcium and magnesium content of normal liver and host livers did not vary, which implied that the presence of transplanted hepatoma in the animal did not influence the amount of these 2 ions in the liver. Data from whole-tissue analyses of both hepatomas are given mainly to show by means of the standard deviation the difficulty encountered in separating membrane-bound calcium from necrotic tissue.

The calcium content of the 3924A plasma membrane was high compared to those in H-35 tumor and normal liver membranes (3924A > H-35 > normal liver). The magnesium content of the cell membranes of all 3 tissues was low and follows the reverse order with respect to calcium (normal liver > H-35 > H-35 host liver).
3924A). Therefore, the variability in our whole-tumor calcium analyses was due to necrosis not grossly visible when the tissue was dissected from analyses. However, since the isolated plasma membranes were not contaminated with necrotic calcium because of the numerous washing steps and sucrose density isolation, the increased amount of calcium for both tumor membranes is a reliable value.

**DISCUSSION**

This study was designed to investigate any relationship between phospholipid profiles, the degree of unsaturation of phosphatide fatty acid, and the calcium content of whole tissue and isolated plasma membranes of liver and hepatoma. These 3 factors are likely to be involved in tumor metastasis. Comparing 2 hepatomas of differing growth rate and morphology to normal liver as the control may clarify how these parameters vary between the normal and cancerous state. Physical models of biological membranes have explained how different phospholipids and cations affect surface pressure curves and molecular contraction (20—22). It was felt that these forces are probably active in the structural matrix and in membrane permeability.

The lipids were separated into the 2 major classes, neutral lipids and phospholipids (Tables 3 and 4) to differentiate the membrane fatty acids, since it is the phospholipids that figure significantly in maintaining the matrix. Neutral lipids have been postulated to act as a micellar packing substance by Kavanau (13) and to modulate changes from disc to pillar in the micellar membrane theory. There is no experimental evidence as yet to substantiate this hypothesis. Veerkamp et al. (30) concluded that the major difference between tumor and liver fatty acids was a reverse of the ratio of oleic acid to stearic acid. Our findings concur with those of these authors for both 3924A and H-35 hepatomas. We also find this to be true at the level of the cell membrane of the 2 tumors studied. This can be seen in Table 6.

The significance of the change in ratios of stearic acid to oleic acid is unknown at this time. However, on a theoretical basis, we do know that increasing unsaturation in the phospholipid fatty acid changes the physical characteristics of the molecular and could very easily have a profound effect on the stability and/or adhesive characteristics of the plasma membrane. Certainly, further work is needed to elucidate the actual mechanism and/or physiological significance of these findings.

The appearance of plasmalogen is fundamental to the proposed membrane model. This phosphatide is present in measurable quantities in the rapidly growing 3924A and is characterized by a vinyl ether-linked, unsaturated fatty acid. Veerkamp et al. (29) have found high levels of plasmalogen in tumors induced with p-dimethylaminoazobenzene.

\[
\text{CH}_2-\text{OCH}_{2}\text{CH}_2-\text{R}
\]

Minder and Abelin (16) found that adult rat tissues contained less plasmalogen than tissues from 3-week-old rats undergoing rapid growth.

Liver contains the lowest plasmalogen content of all rat tissues (18), while a substantial quantity is found in rapidly growing hepatoma. One may speculate that this phenomenon is related in some fashion to the unique proliferation kinetics of tumors and likewise to reduction in intercellular adhesiveness.

For a more accurate ascertainment of the role of phospholipid and unsaturated fatty acids in tumor membrane with respect to metastases, many more tumors of varying metastatic capabilities must be investigated (25). However, by selecting 2 radically differing hepatomas with respect to growth rate, ploidy, and histological appearance, it seems that the phospholipid of the more deviated tumor tends to be generally more olefinic in its carbon skeleton. This would agree with the lower membrane strength predicted in the model proposed by Shah and Schulman (20—22).

The findings of decreased calcium content of tumor tissue, although well documented, has largely been determined by flame photometry. Calcium is one of the more difficult elements to measure with this method due to overlap absorption of sodium lines. However, Hickie and Kalant (12), using atomic absorption spectrophotometry, which is of much greater sensitivity and at the same time unaffected by sodium lines, have found greater amounts of calcium in hepatoma 5123tc. Our findings, obtained by this same procedure, are in agreement with those of these authors for both 3924A and H-35 hepatomas. We also find this to be true at the level of the plasma membrane.

This would indicate a direct proportionality between unsaturated fatty acids and calcium content in the plasma membrane of rapidly growing tumor. Although the calcium results are not in accordance with the membrane model, we cannot account for the placement of neutrolipid and glycoprotein. It is possible that membrane lipids and sialic acid glycoproteins work in conjunction in maintaining intercellular adhesion as shown by Simon-Reuss et al. (24).

**ACKNOWLEDGMENTS**

We thank Dr. Charles Heidelberger for his assistance in preparing the manuscript.

**REFERENCES**


---

**Table 6**

<table>
<thead>
<tr>
<th>Cell membrane</th>
<th>C18:C18:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.76:1</td>
</tr>
<tr>
<td>H-35</td>
<td>0.85:1</td>
</tr>
<tr>
<td>3924A</td>
<td>0.78:1</td>
</tr>
</tbody>
</table>
Study on the Proposed Role of Phospholipid in Tumor Cell Membrane

James K. Selkirk, J. C. Elwood and H. P. Morris


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/31/1/27