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THE ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE FROM CULTURED CELLS

V. THE CHEMICAL COMPOSITION OF PLASMA MEMBRANES ISOLATED FROM CHICKEN TUMORS INITIATED WITH VIRUS-TRANSFORMED CELLS

JAMES F. PERDUE, DAVID WARNER and KATHERINE MILLER

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

Sarcomas were initiated in chicken breast muscle and wing web by Bratislava 77 and *morph*^{r} Fujinami virus. Plasma membrane was isolated from the virus-induced tumor cells by differential centrifugation and flotation equilibrium centrifugation. The levels of neutral sugar and sialic acid in these isolated plasma membranes were very similar to the levels found in cultured chick embryo fibroblasts transformed *in vitro* with the same oncogenic viruses and differed markedly from the levels found in uninfected and leukosis virus-infected fibroblasts.

The phospholipid content of the isolated cell membranes from tumors was less than the quantity of lipid found in the plasma membrane of cultured cells and differed with the site of the tumor. Breast muscle tumors contained less plasma membrane phospholipid than did wing tumors.

The similarities in the neutral sugar and the sialic acid content of these two different sources of plasma membrane indicate that oncogenic transformation in cell culture reproduces *in situ* neoplastic change to a large extent, for at least this one parameter of cell surface change.

INTRODUCTION

Study of cultured cells has greatly advanced the knowledge of altered plasma membrane chemistry and altered cell surface functions in cancer cells. Chicken embryo fibroblasts and their oncogenic RNA virus-transformed counterparts represent one such experimental system. The results of our studies of these cells with the "C"-type avian RNA virus, B_{77} (RBA), and *morph*^{f} and *morph*^{r} Fujinami viruses have established the following relationships: (a) infection and transformation of chick embryo fibroblasts by oncogenic RNA virus results in a 25% decrease in the sialic acid content of the plasma membrane and (b) fibroblasts that have been transformed by RNA viruses that change cell shape from fusiform to round have a 40% increase in the neutral sugar content of the isolated cell membrane[1,2].

The carbohydrate and lipid content of the isolated plasma membrane from cultured cells was high compared with values reported for these components for cell membrane isolated from tissues such as liver and hepatomas[3]. The values for neutral sugar and sialic acid content of plasma membrane in virus-transformed cells may be unique for cultured cells and *in vitro* oncogenic transformation. To examine this possible artifactual change, plasma membrane was isolated from chicken sarcomas that were initiated by cells transformed *in vitro* by the RBA, and *morph*^{r} Fujinami viruses. The neutral sugar and sialic acid contents of these membranes were found to be similar to the values obtained for cell membranes isolated from the cultured, virus-transformed cells[1,2]. Cholesterol and phospholipid contents of the tumor cell plasma membrane were different from those of the cultured fibroblasts and were unique for different sites of the tumor. Density of the membrane differed also and was site-specific. Tumors grown in the breast muscle had less phospholipid in their plasma membrane than those grown in the wing web.

MATERIALS AND METHODS

Fertilized chicken eggs, culture medium, serum and reagents were the same as used in previous studies[1,2]. The avian RNA sarcoma-producing viruses, B_{77} and *morph*^{r} Fujinami[4] viruses were kindly supplied by H. M. Temin. The B_{77} virus that had been passaged through the rat and back into the chicken was used in these studies and is designated RBA.

Cell culturing and infection of fibroblasts with virus

The methods employed in this study were the same as those used in previous studies[1,2].

Tumor promotion

Chick embryo fibroblasts which had been transformed by specific RNA oncogenic viruses were suspended by trypsinization and washed in phosphate-buffered saline by resuspension and sedimentation. The cells were counted, and different amounts ($1 \cdot 10^3$–$2 \cdot 10^7$) were injected into the left breast muscle of 2-day-old White Leghorn chickens. Tumors were palpable in the breast muscle by 2 weeks after the injection of $1 \cdot 10^5$ RBA and *morph*^{r} Fujinami virus-transformed cells. These tumors have been designated as fibrosarcomas*. In other experiments, the cells in a volume of 0.2 ml, were injected into the web of the wing of the chicken and tumors were evident within a week. The sarcomas, weighing up to 3 g, were removed from the breast, and blood, necrotic areas, and muscle were separated from the clear, more solid, yellow-colored tissue.

It must be acknowledged and stressed that the tumors from which plasma membrane were isolated are composed of many cell types: white blood cells, mesenchymal cells, degenerating muscle cells, and sarcoma cells. Therefore, the values we have obtained for CTPase, sialic acid, neutral sugar, *etc.*, are the mean values which are distinctive for each of these cell types. However, since the tumors were dissected

* Diagnosed by Dr Stanley Goldfarb, Dept. of Pathology, University of Wisconsin Medical Center, Madison, Wisc.

free of necrotic tissue and since microscopy indicated we were dealing with predominantly transformed cells, the contribution of cells other than sarcoma cells to the isolated membrane preparation was not major.

The isolation of plasma membrane

The tumor tissue was cut into small pieces, weighed, and made to a 10% suspension (w/v) with 0.25 M sucrose. The tissue was homogenized in the cold with a Potter–Elvehjem homogenizer until a majority of the cells were broken up, as determined by phase microscopy. Tumors obtained from the breast contained small bundles of muscle which were not broken up by homogenization. However, tumors of the wing were much softer and broke up completely during homogenization.

Differential centrifugation was used to remove muscle, intact cells, and nuclei from the homogenate prior to continuous density gradient centrifugation. The homogenate was centrifuged at $750 \times g$ for 10 min in the SS-34 head of a Sorvall RC2-B centrifuge. The supernatant was decanted, and the pellet of whole cells and nuclei resuspended in one-fifth vol. of 0.25 M sucrose. The resuspended pellet was centrifuged along with the supernatant at $750 \times g$ for 15 min. Following this centrifugation, the supernatants were removed and combined; then they and the resuspended and combined pellets were centrifuged a final time at $750 \times g$ for 15 min. The pellets of nuclei and mitochondria were separated from the supernatant fractions. These supernatants were combined and centrifuged at $200000 \times g$ for 60 min. The pellet designated as the particulate homogenate was resuspended in 65% (w/v) sucrose and brought to a refractive index of 1.430 (68% w/v) with a saturated solution of sucrose. Over this suspension was layered a linear 25–65% (w/v) sucrose in water gradient, and the material was centrifuged according to the previously published method[5]. The bands A and B were purified on a second sucrose gradient[5], the bands removed, washed with saline or water, and saved for enzymatic or chemical analysis.

Chemical procedures

The methods for determining protein, RNA, phospholipid, cholesterol, and neutral sugar expressed as glucose equivalents were the same as given in a previously published study[5]. In the present study, sialic acid was determined by the method of Jourdian *et al.*[6]. The quantity of membrane-bound sialic acid obtained by this procedure was 12% greater than that obtained by the thiobarbituric acid procedure of Warren[7], with equivalent amounts of membrane.

Enzyme assays

The activities of nucleotide phosphohydrolase, succinate dehydrogenase–coenzyme Q reductase (EC 1.3.99.1) and NADH–cytochrome *c* reductase (EC 1.6.2.1) of the particulate homogenate and gradient fractions were determined by methods given previously[5].

RESULTS

Isolation of plasma membrane

The methods developed for the isolation of plasma membrane from cultured cells, flotation equilibrium centrifugation of the cell particulates on continuous

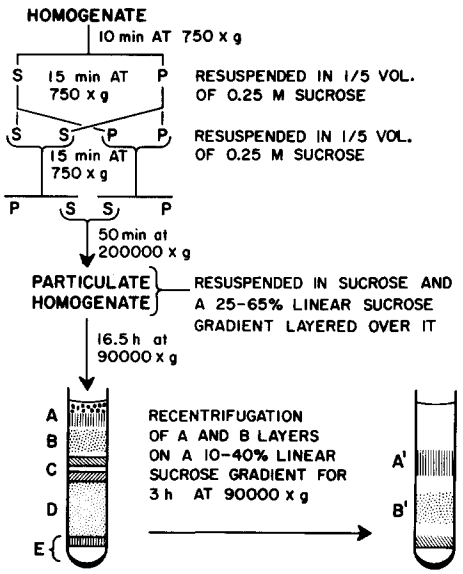

Fig. 1. Procedures used to isolate plasma membrane from chicken tumors of breast muscle and wing. P, pellet; S, supernatant.

sucrose density gradients, were applicable for the isolation of plasma membranes from tumors (Fig. 1). Differential centrifugation was employed to remove whole cells and muscle from the homogenate, but nuclei were also removed by this treatment. Mitochondria and some of the intracellular membranes were sedimented by differential centrifugation, as evidenced by the enzymatic activity of succinate dehydrogenase–coenzyme Q reductase and NADH–cytochrome *c* reductase in the pellets. However, this latter enzyme was present in the starting material at a level that was 2.5 times higher in specific activity than we had found for the *in vitro* RBA virus-transformed cells[1]. This is due in large measure to the concentration of the intracellular membranes in this final supernatant. It is very likely that plasma membrane was also sedimented during differential centrifugation. However, for the quantification and calculation of recoveries of enzymes and chemical components in the various fractions on the gradients, the high-speed pellet obtained from the final supernatant fraction, designated particulate homogenate, was considered the starting material.

The overnight centrifugation of this material resolved its components into bands, designated A–E (Fig. 1). The position of these bands on the 25–65% (w/v) sucrose gradient was similar to that observed for cultured cells, but the appearance of these bands was different. The C band was resolved into two fine bands; the lower band was slightly pink in color. The previously observed bands of particulate material in the broad D band were missing or they were markedly reduced in amount. The pellet in the combined E fraction was generally very small. As in our previous studies, the A and B bands were removed and recentrifuged on a 10–40% sucrose gradient. Flotation equilibrium was achieved in 3 h. The position of the A′ band was lower than we had observed previously for cultured cells and the density of the membranes in this band was also greater (Table I). The position and density of the membranes

in the B′ band were not significantly different from those of membranes obtained from cultured cells.

TABLE I

THE DENSITY OF PLASMA MEMBRANE IN THE A′ AND B′ BANDS FROM UNINFECTED AND VIRUS-TRANSFORMED CULTURED CELLS AND CHICKEN TUMORS

Cells or tissue	*Band A′*	*Band B′*
Uninfected fibroblasts*	1.060 ± 0.001**	1.108 ± 0.006
RBA virus-transformed fibroblasts*	1.066 ± 0.003	1.108 ± 0.007
Virus-induced breast muscle tumor***	1.099 ± 0.014	1.120 ± 0.009
Virus-induced wing tumor***	1.093 ± 0.009	1.123 ± 0.001

* Data from Perdue[15].

** The mean ± the S.D.

*** These results are the mean ± S.D. from seven preparations of breast muscle tumor and five preparations of wing tumor.

Enzymatic and chemical composition of membrane fractions

Those chemical components and enzymes which were previously shown to be associated with the plasma membranes of cultured cells, (*e.g.* CTPase activity, sialic acid, neutral sugar, phospholipid, and cholesterol) were concentrated in the A′ and B′ bands from the tumor cells (Table II). The specific activities of other nucleotide phosphohydrolases were also increased in the A′ band membranes compared with the values of the homogenate (Fig. 2). The membranes in the A′ band were free of mitochondrial contamination, but this fraction did have about 2% of the cytochrome *c* reductase activity of the particulate homogenate and 30 μg of RNA/mg membrane protein. This increase of 3-fold in the specific activity of the nucleotide phosphohydrolases and the specific concentration of sialic acid and the decrease in RNA and cytochrome *c* reductase compared with that of the particulate homogenate does not reflect the actual purification of plasma membrane that was achieved by this procedure. The differential centrifugation steps have removed nuclei, mitochondria and whole cells. The pellet, obtained by centrifuging the final supernatant, is already concentrated 2–2.5 times more in RNA, cytochrome *c* reductase, and neutral sugar, and by 20% in sialic acid when compared with the particulate homogenate from *in vitro* transformed cells[1]. Furthermore, the cells soluble proteins, which constituted about 60% of the protein in a homogenate, have been discarded and were not considered in the calculation of purification. The membranes in the B′ band were contaminated to a greater extent with mitochondria and reticulum, but the specific contents of sialic acid, phospholipid, and cholesterol and the high CTPase activity indicate that this fraction is a subfraction of plasma membrane. The recovery of enzyme and chemical components among the isolated fractions was 60–80%.

The sialic acid content of the membrane in the A′ band isolated from the chicken tumor, 72 nmoles/mg protein (Table II), was similar to that of plasma membrane isolated from *in vitro* virus-transformed cells: 79 ± 3 for RBA virus-transformed cells[1] and 64 ± 5 for *morph*^{r} Fujinami virus-transformed cells[2]. The neutral sugar

TABLE II

THE DISTRIBUTION OF MEMBRANE-ASSOCIATED ENZYMES AND CHEMICAL COMPONENTS AMONG BANDS A′, B′, C, D AND E, FOLLOWING CENTRIFUGATION OF HOMOGENATES OF CHICKEN BREAST MUSCLE TUMORS ON CONTINUOUS DENSITY GRADIENTS OF SUCROSE

Component or enzyme	*Homogenate Units/mg protein*	*Band A′*		*Band B′*	
		Units/mg protein	%*	*Units/mg protein*	%
CTPase (μmoles P_i released/30 min)	2.8±0.7**	8.1±1.2	7±3	8.6±1.3	17±7
Cytochrome *c* reductase (μmoles reduced/min)	0.064±0.010	0.018±0.008	2±1	0.036±0.012	4±2
Succinic dehydrogenase (μmoles indophenol reduced/min)	0.008±0.001	0.00	0	0.003	2 (2)***
Sialic acid (nmoles)	24.7±1.5	72.0±8.5	10±2	51.8±6.8	17±6
Glucose equivalents (μg)	180±2	322±27	6±2	164±11	6±2
Phospholipid (μg)	222±18	780±73	10±3	502±42	6±1
Cholesterol (μg)	63±6	273±43	14±4	192±26	12±3
RNA (μg)	140±39	30±16	1±1	31±17	2±1
Protein (mg)	22±2†	0.5±0.1†	3±1	1.0±0.2†	7±2

* The percent distribution of the membrane components present in the homogenate (100%).

** The calculated means ±S.E. are based upon the combined results of 5–7 experiments with the exception of CTPase activity and the RNA content which were determined for three preparations.

*** The number in parentheses refers to the number of experiments when less than three.

† These values represent the total recovery of protein in the fraction.

content of the membranes isolated from the tumor cells, 322 μg/mg protein, was also very similar to the values obtained with virus-transformed cultured cells. The RBA virus-transformed cell membrane contained 358±12 μg of neutral sugar, and the *morph*^{r} Fujinami virus-transformed cell membrane had 331±14[1,2].

The phospholipid and cholesterol contents of the membrane isolated from the breast muscle tumors (Table II) were significantly smaller than those of cultured cells. The plasma membrane of RBA virus-transformed cells contained 1979±98 and 613±46 μg of phospholipid and cholesterol, respectively, per mg of membrane protein.

The carbohydrate and lipid content of plasma membrane from tumors of the wing

The marked difference in the phospholipid and cholesterol contents of plasma membrane of breast muscle tumors *versus* that of *in vitro* virus-transformed cells[1], could be owing to our isolation of membrane from cells which were not fibroblast-like in origin (*e.g.* skeletal muscle plasma membrane). However, the previously observed high level of lipid in cultured cells could also be a special property of *in vitro* growth conditions. RBA and *morph*^{r} Fujinami virus-transformed cell tumors were initiated in the wing web. The morphology of these tumors was not different from the breast muscle tumors and the plasma membrane isolated from these tumors was similar in

Band C		*Band D*		*Band E*		*Recovery*
Units/mg protein	%	*Units/mg protein*	%	*Units/mg protein*	%	%
3.0±1.0	26±5	1.1±0.4	10±4	0.4±0.2	3±1	63
0.038±0.014	9±5	0.074±0.026	29±14	0.046±0.004	21±3	65
0.017±0.003	25±7	0.013±0.002	41±17	0.004±0.001	15±6	83
36.8±4.9	18±5	23.2±0.5	25±4	12.4±2.1	13±2	83
143±21	10±2	142±10	19±4	155±10	24±3	65
308±20	16±5	234±20	22±4	88±10	7±1	61
105±18	24±7	54±10	19±3	11±4	4±2	73
152±18	18±13	271±13	24±10	333±117	40±14	85
2.8†±0.8	16±3	3.9†±0.7	25±3	3.5±0.5†	23±3	74

its sialic acid and neutral sugar content to that isolated from the latter tumors (Table III). However, the phospholipid content of plasma membranes isolated from the wing tumors was significantly higher ($P=0.05$) than that isolated from breast muscle tumors. The quantity of cholesterol in these two sources of plasma membrane was similar.

DISCUSSION

The neutral sugar and sialic acid contents of the plasma membrane from breast muscle and wing tumors were essentially the same as had been found for plasma membrane isolated from RBA and *morph*^{r} Fujinami virus-transformed cells[1,2]. Taking into consideration the two different methods used to determine sialic acid – the Jourdian *et al.*[6] procedure gave 12% higher estimates – the plasma membrane from the breast muscle and wing tumor would contain 64 and 72 nmoles of this carbohydrate per mg protein, respectively. The cell membranes from uninfected fibroblasts and from fibroblasts infected with the non-tumor-producing leukosis virus, RAV-49, were significantly different from those of transformed cells in their neutral sugar and sialic acid content (Table III and ref. 1).

The difference in the phospholipid content of plasma membrane from the

breast muscle and wing tumors may result because there are different populations of cells in the tumors. Siegler[8] has studied tumor development in virus-induced murine sarcomas of mice and separated the process into descriptive groups: (a) intense inflammatory phase characterized by edema and cellular infiltration; (b) granulomatous development with necrosis of striated muscle; and (c) proliferation of

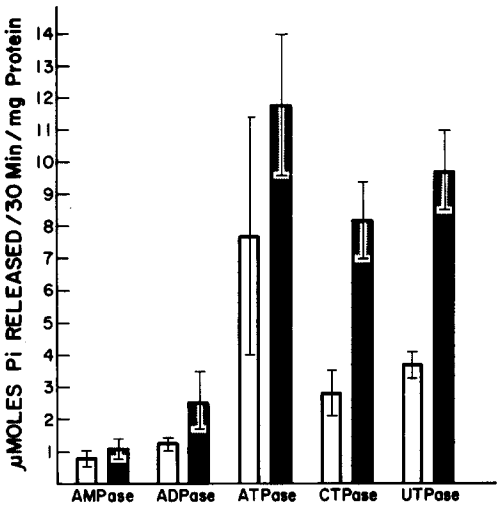

Fig. 2. The nucleotide phosphohydrolase activities of the particulate homogenate and membrane in the A′ band isolated from breast muscle tumors. Membrane fractions from the particulate homogenate (white column) and A′ band (shadowed column) were incubated for 30 min at 37 °C in a 1-ml volume containing 3 μmoles $MgSO_4$, 3 μmoles of nucleotide substrate, 18 μmoles Bicine (pH 7.8), and sucrose to 250 mosM. The quantity of P_i released was determined and the data expressed as μmoles P_i released per 30 min per mg protein. The calculated means ±S.E. are based on the results of four experiments.

TABLE III

THE CARBOHYDRATE AND LIPID CONTENT OF MEMBRANES IN THE A′ AND B′ BANDS FROM TUMORS OF THE WING COMPARED WITH THAT FROM CULTURED FIBROBLASTS

Component	*Homogenate*		*Band A′*		*Band B′*	
	Wing tumor	*Cultured fibroblast*	*Wing tumor*	*Cultured fibroblast*	*Wing tumor*	*Cultured fibroblast*
Sialic acid (nmoles)	30.3±1.3*	21±3**	83.2±7.6*	106±3**	59.5±5.4*	79±3**
Neutral sugar (μg)	161 ±10	127±19	292 ±33	243±8	150 ±10	153±6
Phospholipid (μg)	282 ±5	320±15	1134 ±97	1998±63	639 ±44	916±43
Cholesterol (μg)	81 ±6	105±8	345 ±39	613±46	247 ±32	403±23

* The calculated means ±S.E. are based upon the combined results of 3–5 wing tumor isolation experiments.

** Data from Perdue *et al.*[1].

mesenchyme-like cells with subsequent differentiation to mature-appearing fibroblasts. There could be chemical differences between the mesenchyme-like cells which give rise to tumors in the breast muscle and those cells which give rise to tumors of the wing.

Kaighn *et al.*[9] working with *in vitro* cultured skeletal muscle myoblasts and fibroblasts from chicken embryos, established that the myoblasts and fibroblasts were equally susceptible to transformation by the Rous Sarcoma virus. The transformed myoblasts were altered in shape and produced in infectious virus. Using the same cultured cells, Lee *et al.*[10] found that fluorescent antibodies to Rous Sarcoma virus were associated with the cell surface of the myoblasts as well as differentiated myotubules. It was assumed for the latter cell type that transformation occurred in the myoblasts with subsequent fusion of those transformed cells to form myotubes.

It is conceivable that tumorigenesis in the breast muscle of 2-day-old chickens could involve infection and transformation of undifferentiated mesenchyme-like cells as well as of cells already on a pathway of differentiation leading to muscle. By contrast, tumors developing in the wing web would arise from cells brought in during the inflammatory response, as well as those within the connective tissue. It is possible that the phospholipid content of the cell membrane from these different sources of cells is unique for each cell and that these differences in lipid content are maintained in the sarcomas.

The carbohydrate content of the cell membrane was not dependent on the site of the tumor. Moreover, tumors and *in vitro* virus-transformed cultured fibroblasts have about the same levels of sialic acid and neutral sugar in their plasma membrane. The similarity in carbohydrate content of the *in vitro* and *in vivo* virus-transformed cells indicates that, with respect to this one parameter of cell surface change, oncogenic transformation in cell culture resemble to a large extent neoplastic change *in situ*.

In our use of the term content (*e.g.* phospholipid content, sialic acid and neutral sugar content, *etc.*), the variable parameter has been expressed in terms of amount per mg of protein, based on the assumption that the amount of protein within the isolated plasma membrane is constant. These parameters have been examined in cultured fibroblasts[1], virus-transformed fibroblasts[1,2], cultured rat liver cells and murine sarcoma virus-transformed liver cells[11], and now in tumors. In most instances, the parameters of CTPase activity and sialic acid and neutral sugar content of these membranes were constant irrespective of the source of plasma membrane. There were variations in the density and the lipid content of the isolated membranes, depending upon the source of the cell. Another variation was in the lipid and carbohydrate content of the membranes in the A′ band and the B′ band, but this was independent of the cell source. The chemical and enzymatic composition and subunit composition[12] of the membrane in the A′ and B′ band indicate that both are plasma membrane fractions.

A conclusion that can be drawn from this work is that the plasma membrane is a composite of protein, lipid, and carbohydrate entities whose relative composition varies. The A′ band, for example, contains more phospholipid and cholesterol than the B′ band. What we have discerned as variations in the lipid content of these membranes may instead be variations in the protein and glycoprotein content of the plasma membrane.

The recently proposed fluid mosaic membrane model[13] is useful to explain our

results. The model proposes that a membrane is a phospholipid bilayer interspersed with globular proteins in a random array. With this model, then, the plasma membrane of the A′ band, with its lower density, would contain less protein per unit of surface area than the membrane in the B′ band. But the proteins within these two membranes might be the same. Some of it is associated with carbohydrate and interacts with the external milieu. Other proteins are not accessible to the external environment, as indicated by their failure to be labeled by ^{125}I (ref. 12), and they lie either within the lipid bilayer or on the intracellular side of the plasma membrane.

If these assumptions of plasma membrane structure and organization approximate the real situation, then the chemical differences in the plasma membranes between cells and tissues can be explained simply by an increase or decrease in the glycoprotein and protein content per unit of surface area. Furthermore, the difference in the sialic acid and neutral sugar content of plasma membrane in the A′ and B′ fractions can be accounted for by the greater content of glycolipids in the A′ band (Wray, V. P. (1972), unpublished). The decrease in sialic acid that we have observed in the A′ band membranes after transformation with oncogenic viruses may result from the synthesis of incomplete glycolipids, as observed by Hakomori and Murakami[14]. The increase in neutral sugar resulting from virus transformation, however, probably reflects changes in both glycoproteins and glycolipids (Wray, V. P. (1972) unpublished). Studies are now in progress to clarify this point.

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