LIPID VESICLE INTERACTION WITH EMT-6 TUMOR CELLS AND EFFECT ON SUBSEQUENT CELL GROWTH*

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Received September 27,1976

SUMMARY. Artificial lipid vesicles of varying composition were incubated with EMT-6 tumor cells, after which the transfer of vesicle lipids to the cells and their growth in vitro were determined. Vesicles composed of phosphatidylcholine, cholesterol, and gangliosides could transfer phosphatidylcholine and cholesterol to the cells, but this transfer had no effect on the subsequent growth of the cells. However, preincubation of the cells with vesicles containing sterylamine or phosphatidylserine did inhibit the subsequent growth of the cells in a tissue culture assay system. It might be possible to deliver to certain cells growth inhibitory compounds carried in lipid vesicles.

INTRODUCTION. Artificial lipid vesicles can be made from a variety of lipid components using phosphatidylcholine as the primary component in the membrane system (1). These vesicles have been shown to interact with mammalian cells, and during this interaction lipid components of the vesicle can be transferred to the cells (2-4). Recently, there has been an increased focus on the role that membrane composition plays in determining the tumorigenic properties of a cell, and several groups of investigators have presented evidence which demonstrates that in certain cases the membrane composition of tumor cells differs from the corresponding normal cells (5-11). This paper describes interaction of lipid vesicles with EMT-6 tumor cells, and the resulting effect on the ability of these cells to grow in an in vitro tissue culture assay system.

MATERIALS AND METHODS

Formation of Vesicles. Artificial lipid vesicles were prepared according to the general procedures described by McDougall et al (1) Lipids, dried on the surface of a glass flask, were suspended in 2 cc Waymouth's medium

*This work supported by the Charles Deere Wyman Fund and N.I.H. Grant CA-10372.

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(MB 752/1 GIBCO, Oakland, CA) and sonicated at 50°C for 30 minutes using a Biosonik probe sonicator (1) or a Heat System 117 volt ultrasonic bath. Vesicles-A were formed from 44 micromoles 1,2-dipalmitoyl-sn-glycero-3 phosphocholine (Sigma Chemicals, St. Louis, MO, No. 8878), 44 micromoles cholesterol (Sigma Chemicals, CH-S) and 4 micromoles gangliosides (Supelco Inc., Bellefonte, PA, No. 94-0632). Vesicles-B contained the lipids of vesicles-A plus 1 mg sterylamine (octadecylamine)(K+K Laboratories, Plainview, N.Y., No. 4246) and vesicles-C plus 5 mg phosphatidylserine (Calbiochem, San Diego, CA, NO. 524641). Vesicles prepared for use in in vitro assays were prepared sterilely: sonication was done in a closed vial using the bath sonicator. Vesicles were labeled with either [^{14}C(U)]-phosphatidylcholine (New England Nuclear, Boston, MA, NEC-588) or [4-^{14}C]-cholesterol (New England Nuclear, NEC-018) by adding tracer levels (0.4μCi) of label lipid to the vesicle preparation before sonication. Vesicles were separated from free radiopharmaceuticals or labeled lipid by passage through a Sepharose 6B column.

The approximate number of vesicles formed from a known amount of lecithin was calculated, assuming that all the phosphatidylcholine goes into a bilayer structure and that each lecithin molecule occupies a surface area of 60Å^2 (9). Vesicles measured by light scattering techniques have been shown to be about 500 Å in daimeter (12). One can calculate the number of vesicles formed according to the following formula:

$$\mu \text{ moles lecithin } (6.2 \times 10^{23}) \ (60\text{Å}^2) \ / 4\pi r^2 \quad (13)$$

Because the wall of the vesicle is a bilayer structure, the denominator contains the factor 2; $4\pi r^2$ is the surface area of a sphere; and 4 is the mean radius of the vesicle. For example, 44 micromoles of physphatidylcholine would make 4.5×10^{15} vesicles.

Culture Conditions and Techniques for EMT-6 Cells. EMT-6 mammary sarcoma is the tissue culture-adapted derivative of the KHJJ mammary carcinoma (14). EMT-6 cells were maintained in glass milk dilution bottles (160 ml volume, Corning) in Waymouth's medium supplemented with 15% fetal bovine serum containing 200 u/ml penicillin and 0.2 mg streptomycin in a humid atmosphere containing 5% CO_2 and 95% air. Cells were recovered from stock cultures by incubating the cells in 0.05% trypsin at 37°C for ten minutes and then removing the cells from the glass surface by gentle pipetting. Further details on culture conditions are as described by Rockwell et al (14). Cells were counted in a hemocytometer.

Incubation of EMT-6 Cells with Vesicle Preparations. EMT-6 cells were incubated with vesicle preparations and lipid preparations, and then cell viability was determined using an in vitro tissue culture assay system. EMT-6 cells were incubated with the different vesicle preparations for a period of 15 minutes at room temperature: about 1×10^5 cells in 0.1 Waymouth's medium were incubated with 0.1 ml of test compound which is approximately five percent of a total vesicle preparation or 2×10^{14} vesicles. 1×10^5 EMT-6 cells were also incubated with 0.1 ml of sterylamine (1 mg/1 ml). In each experiment, the same number of EMT-6 cells was also exposed to Waymouth's medium alone: this served as the standard for determining the plating efficiency. The integrity of cells after exposure to test compound was determined by using the trypan blue exclusion test (15). Experimental exposure was stopped by diluting the incubation mixture in Waymouth's medium to 10 cc and thoroughly resuspending the cells. Three concentrations of diluted cells were plated: 0.01 ml (100 cells), 0.02 ml (200 cells), .03 ml (300 cells) in 60 mm plastic Petri dishes, five plates per dilution in a volume of 5 cc Waymouth's medium/plate. The cells were incubated undisturbed in a CO_2 incubator for 14 days. After the incubation period, the medium was aspirated off, the plates washed with salt buffer (1), and the cells stained with a crystal violet solution (2.5 gm crystal violet in 900 ml methanol and 100 ml formaldehyde). Colonies of greater than 50 cells were counted under a

Table 1

Number of EMT-6 cells forming colonies in an *in vitro* tissue culture assay system.

	Preexposure of EMT-6 Cells to:	Number of Cells Plated	Number of Colonies	Plating Efficiency %	% of Control Growth
Exp. 1	Waymouth Medium	60	38 ± 5.0	47	100
	Vesicles A	60	29 ± 6.1	48	100
Exp. 2	Waymouth Medium	100	52 ± 13.0	53	100
	Vesicles A	100	53 ± 11.0	53	100
Exp. 3	Vesicles A	100	49 ± 7.8	50	100
	Vesicles B (+ 1 mg sterylamine)	100	33 ± 3.9	33	66
	Vesicles C	100	14 ± 2.7	14	28
	Sterylamine (alone)	100	0	0	0

dissecting microscope. Plating efficiency was calculated as number of colonies/number of cells plated.

Vesicles, labeled with ^{14}C lipid, and cells were incubated in 1 ml of Waymouth's medium for 15 minutes at room temperature, and then centrifuged at 1000 g to obtain a cell pellet; the supernatant was aspirated and the cell pellet washed two times with 10 ml Waymouth's medium. Vesicles alone were centrifuged to determine nonspecific sedimentation of vesicles at 1000 g: this value was used as the background level of radioactivity for determining the activity of ^{14}C in the cell pellet. A Packard liquid scintillation spectrometer (model 3320) was used to count ^{14}C samples in Omniflor (New England Nuclear).

RESULTS AND DISCUSSION. EMT-6 cells could reproducibly be grown in a tissue culture system with a plating efficiency of 50% (Table 1). These results are similar to that reported by Rockwell et al (14). When EMT-6 cells are preincubated with artificial lipid vesicles formed from phosphatidylcholine, gangliosides and cholesterol, the plating efficiency of the cells is unaltered. Phosphatidylcholine, ganglioside and cholesterol are all normal constituents of the cell membrane (16,17), and even though incubation of vesicles with these

TABLE 2

Incubation of vesicles-A labeled with phosphatidylcholine-^{14}C or cholesterol-^{14}C with EMT-6 cells: determination of ^{14}C-marker transferred to cells.

	Number of EMT-6	Number of Vesicles	% of ^{14}C-marker Bound to Cells	Number of Vesicles*/Cell
a) Ph.choline-^{14}C - vesicles	1×10^7	1.5×10^{14}	5.8	8.5×10^5
b) Cholesterol-^{14}C - vesicles	1×10^7	1.0×10^{14}	1.3	1.3×10^5

*Assuming all ^{14}C-marker is incorporated in vesicle at beginning of incubation.

cells can result in lipid exchange between cell and vesicle (2), the exchange has no detectable effect on cell growth. However, the growth of the cells was reduced when they were preincubated with vesicles-B (sterylamine containing vesicles) or vesicles-C (phosphatidylserine vesicles) to 66 and 28 percent of control, respectively. Preexposure to sterylamine alone completely inhibited cell growth. Other workers have prepared vesicles with sterylamine (18) and sterylamine alone has been found to inhibit the growth of certain cells in tissue culture (19). Sterylamine is a positively charged lipid and phosphatidylserine a negative charged lipid: incorporation of these lipids into the cell membrane might result in altered membrane function which prevents the cells from multiplying efficiently.

Cell permeability was not affected by incubation of EMT-6 cells with vesicles or lipids alone: cells remained intact and impermeable to trypan blue. This impermeability to dye was maintained despite evidence that vesicular lipid (e.g., [^{14}C]-cholesterol and [^{14}C]-phosphatidylcholine) is transferable to the cells (Table 2).

Other workers have also reported that artificial lipid vesicles fuse with mammalian cells, and during this fusion process various lipids are transferred from vesicle to cell. Grant and McConnell using vesicles prepared from dipalmitoyl phosphatidylcholine present evidence that suggests that these vesicles fuse with the membranes with Acholeplasma laidawii (20). Huang and Pagano report that vesicles formed from dioleyl phosphatidylcholine and dimyristoyl phosphatidylcholine interact with Chinese hamster V79 cells and during this interaction lipid from the vesicle is transferred to the cells (3,4). Inbar and Shinitzky have also presented evidence that vesicles can transfer lipids to cells (8,11). Papahadjopoulos and his associates also show that vesicles can interact with cells (21). Previous studies in our laboratory show that vesicles can interact with spleen cells, thymocytes, lymphocytes, red blood cells, and ascites tumor cells (2).

The present studies show that when artificial lipid vesicles are formed from the natural membrane components phosphatidylcholine, cholesterol, and gangliosides and these vesicles contact EMT-6 cells, no inhibition of in vitro cell growth occurs. When sterylamine vesicles or phosphatidylserine vesicles contact an EMT-6 tumor cell, they inhibit the subsequent in vitro growth of the cell. Vesicles upon interaction with EMT-6 cells a) can transfer substances to the cells which decrease their growth or b) transfer vesicle components to the cells without altering their growth. The potential exists to deliver growth inhibiting compounds to tumor cells in vivo by carrying these inhibitory substances in lipid packages (1,22).

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