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Neutrophil-mediated transfer of polar substances from liposomes to mammary tumor cells in vitro

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Summary

Little accumulation of fluid-phase markers, lucifer yellow CH (LY) and [^{14}C] sucrose, was observed in 13762NF rat mammary adenocarcinoma clone MTF7, when a cell monolayer was incubated at 37°C for 60 min with reverse-phase evaporation vesicles (REVs) containing the markers. However, time-dependent accumulation was observed when the REVs were preincubated with polymorphonuclear neutrophils (PMNs) and added to the tumor cell layer. In contrast, accumulation of liposomal lipid marker, [^{3}H]dipalmitoylphosphatidylcholine (DPPC), was independent of PMNs and approximately 10 times faster than those of the fluid-phase markers. The PMN-mediated transfer of LY and sucrose from REVs to MTF7 cells is attributed to PMN binding to the tumor cells and possibly high concentrations of the marker molecules in the unstirred layer of the tumor cells. The latter could have resulted from continuous phagocytosis and exocytosis of REV contents by PMNs. DPPC transfer must be spontaneous and could be simply collision-mediated. Significance of these in vitro observations is related to the role of PMNs in metastasis in vivo and the potential for drug delivery targeted to metastatic cells.

Introduction

Metastasis is a multistep process whereby a selected subpopulation of tumor cells invades from the primary tumor into surrounding normal tissues. Malignant cells eventually enter the lymphatics or vasculature where they are passively transported to nearby or distant secondary sites. In blood-borne metastases, tumor cells either arrest non-specifically as multicell homotypic (tumor cell–tumor cell) or heterotypic (tumor cell–platelet, leukocyte etc.) emboli in capillaries or recognize and adhere specifically to organ-specific small vessel endothelial linings. After traversing the endothelial and subendothelial basement membrane using a variety of proteinases and motile activities, tumor cells, if the milieu is satisfactory, will again proliferate and establish a secondary lesion.

Recent evidence suggests that the polymorphonuclear neutrophils (PMNs), which comprise approximately 60% of circulating leukocytes in hu-

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mans, may contribute to the ability of some tumor cells to metastasize. Crissman and colleagues, using several different metastatic murine melanoma cell lines, identified close tumor cell–platelet–PMN association in the vasculature until the integrity of the basal lamina was comprised (Crissman et al., 1988). Using several cell clones of differing spontaneous and experimental metastatic potentials from the 13762NF rat mammary adenocarcinoma, we have recently shown that circulating PMN levels increase in proportion to the metastatic potential of the cell line growing in the mammary fat pad (Aeed et al., 1988). Tumor-induced neutrophilia occurred concomitant with an increased ability of PMNs to secrete 50% more type IV collagenase and heparan sulphate endoglycosidase (heparanase) suggesting that PMNs were assisting tumor cells in the extravasation step of metastasis. Hence, we postulate that modulation of PMN function may be useful in reducing metastatic potential. Also, by virtue of the often close association between tumor cells and PMNs, we postulate that PMNs may be helpful in targeting tumor cytotoxic agents to more effectively eliminate malignant tumor cells before they have the opportunity to form secondary tumors.

Recently, we have demonstrated the efficiency of using liposomes to deliver the polar markers lucifer yellow CH (LY) and [^{14}C] sucrose to circulating PMNs in vitro (Scieszka and Cho, 1988). The present study is concerned with the mass transfer of phagocytosed liposome contents from PMNs to moderately metastatic mammary tumor cells following spontaneous attachment of the PMNs to those cells. Enhanced transfer of fluid-phase markers mediated through liposomes and PMNs would affirm the potential of this approach for targeted delivery of anti-metastatic drugs.

Materials and Methods

Mammary adenocarcinoma cell lines and tissue culture

13762NF mammary adenocarcinoma clone MTF7 was isolated from a cell line established from a tumor growing in the mammary fat pad of syngeneic Fischer 344/NHsd (F344) rats as previously described (Neri et al., 1982; Welch et al., 1983). MTF7 has an intermediate metastatic potential by both the experimental and spontaneous metastasis assays (Welch et al., 1983).

Cells were grown in α-modified minimal essential medium (Irvine Scientific) supplemented with 5% fetal bovine serum (Biocell) and no antibiotics (cAMEM). All cells were maintained in a 37 °C humidified atmosphere containing 5% CO_2 in air. Cultures were routinely grown on Corning tissue plastics (Corning) and subcultured at a split ratio of 1 : 50 when cells became approximately 80% confluent using a 0.25% trypsin solution (Gibco). All tumor cells were routinely screened and found to be free of *Mycoplasma* spp. and viral contamination. For kinetic uptake experiments, cells were seeded into 35-mm 6-well culture plates (Corning) at either 5, 3.5, or 1×10^4 cells per well in order to achieve $> 90\%$ confluence in 3, 4, or 5 days respectively.

Isolation of rat peritoneal PMNs

Syngeneic F344 rats (Harlan Sprague–Dawley) were injected i.p. with 5 ml of sterile 10% proteose peptone solution (Difco). Four to eight hours later, they were sacrificed by Metofane (Pitman-Moore) inhalation and peritoneal exudate was collected. PMNs were obtained from the cell pellet following centrifugation of peritoneal exudate over Ficoll-Paque (Pharmacia) gradient. Contaminating red blood cells were removed by lysis with a solution containing 0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM disodium EDTA at pH 7.4. The PMNs were then washed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS; Gibco) counted on a hemacytometer and adjusted to the desired concentration. This method routinely yielded $> 98\%$ viable and functionally active PMNs, as determined by exclusion of Trypan blue and pinocytic uptake of LY followed by epi-fluorescence microscopy, respectively.

Preparation and characterization of REVs

The method used for preparation and characterization of reverse-phase evaporation vesicles (REVs) was previously described in detail (Scieszka et al., 1988). Typically, a lipid mixture of 20 μmol phosphatidylcholine, 20 μmol phosphati-

dylglycerol, and 10 μmol cholesterol was processed in 1.0 ml of DPBS containing the fluid phase markers [^{14}C] sucrose (New England Nuclear) and LY (Molecular Probes). In many instances, the lipid mixture contained [^{3}H]dipalmitoylphosphatidylcholine (DPPC; NEN). Unentrapped LY and sucrose were separated from REVs in a Sephacryl S-1000 (Pharmacia) size exclusion column using DPBS as an eluent. The average diameter of REVs used was 0.45 μm when measured on a Nicomp model 200 laser particle sizer (Nicomp Instruments).

Accumulation of REV markers in MTF7 mammary tumor cells

Uptake kinetic experiments were done using two different incubation mixtures: REVs (2.5 μmol total lipid/ml) with 10% syngeneic F344 rat serum, and REVs with 10% rat serum and PMNs (1.0×10^7 cells/ml). The experiments were started by first preincubating the incubation mixture in separate tubes at 37° C for 15 min. Following preincubation, 1.0 ml of the appropriate mixture was added in triplicate to the culture plates containing the tumor cell monolayers. Due to instability of REVs in the presence of serum (Scieszka et al., 1988), the uptake experiments lasted only 60 min. At 5, 10, 20, 30, 45, and 60 min the incubation mixtures were aspirated and washed in four consecutive liters of saline at 4° C. A 1.0-ml aliquot of 0.5% trypsin-0.02% EDTA solution (Flow Laboratories) was added to the wells containing cells and incubated at 37° C for 2–3 min to detach the PMNs which adhered to the tumor cells and to remove the tumor cells from the plate. The rounded cells were then brought up into a uniform cell suspension by trituration followed by addition of 3.0 ml of cAMEM. The resulting tumor cell or tumor cell-PMN suspension was layered over 3.0 ml of Ficoll-Paque and centrifuged at 750 g for 25 min. After centrifugation, a tumor cell band was formed above the Ficoll-Paque layer, and in the case of the PMN-containing samples, the PMNs pelleted to the bottom of the tube. The tumor cell band was placed in a 1.5-ml microfuge tube and centrifuged at 16,000 *g* for 2.0 min using an Eppendorf Model 5415 microcentrifuge (Brinkmann). The bottom of the tube containing the cell pellet was cut away and placed into another microfuge tube containing 1.0 ml 0.02% sodium deoxycholate and shaken 14–18 h at 4° C to dissolve the cells.

Assay procedures

Protein was analyzed using the method of Lowry by removing 0.1 ml from the lysed cell suspensions. Radioactivity of both [^{3}H]DPPC and [^{14}C]sucrose was measured by removing 0.5 ml, diluting in 10 ml ACS (Amersham), and analyzing on a Packard Tri-Carb 4640 liquid scintillation counter. The remaining contents of the tube were centrifuged at 16,000 *g* for 2.0 min to pellet the cell debris. A 0.3 ml aliquot was then removed from the supernatant, added to an isopropyl alcohol (IPA) diluent to yield a final concentration of 75% IPA in water, and analyzed for fluorescence as previously described (Scieszka and Cho, 1988). Fluorescence intensity was measured using an SLM-Aminco SPF-500C spectrofluorometer with excitation and emission wavelengths and bandpasses at 430 and 540 nm, and 10 and 20 nm respectively.

Antibody labeling of PMNs for establishing tumor cell band purity

Purity of the tumor cells (void of PMNs) isolated from Ficoll-Paque separation was determined in a set of experiments separate from the kinetic studies. PMNs at a concentration of 1×10^7 cells per well were added to a 35-mm well containing a confluent monolayer of tumor cells and allowed to adhere. The PMNs were then labeled by adding a 50:1 dilution of fluoresceinated (FITC) rabbit anti-rat PMN antibody (Accurate Antibodies). The Ficoll-Paque separation procedure was performed as above and the isolated tumor cells were plated and monitored under fluorescence microscopy for any punctate fluorescence due to the presence of contaminating PMNs.

Results and Discussion

Several pieces of evidence appear to indicate that circulating PMNs may aid tumor cells at the early steps of cell attachment and extravasation in

the metastatic cascade (Starkey et al., 1984; Crissman et al., 1985; Orr and Warner, 1987; Aeed et al., 1988). Although the exact nature of PMN–tumor cell–endothelial cell interaction is not clearly understood (Crissman et al., 1988), from a drug delivery viewpoint the cell interaction provides a unique opportunity to test if PMNs can serve as drug carriers. Extravasation of PMNs under normal physiological conditions (Harlan, 1985) will be altered by metastasizing tumor cells and the PMN-binding of tumor cells may provide means of targeted drug delivery to the tumor cells with minimum spillover to normal (endothelial) cells. In loading PMNs with model probe molecules, we utilized phagocytic uptake of liposomes with serum as a source of opsonin(s). The procedure was found to be much more effective than simple pinocytosis (Scieszka and Cho, 1988). Specifically, we are comparing accumulation of fluid-phase markers LY and [^{14}C]sucrose and the lipid marker [^{3}H]DPPC in tumor cells when they are presented in REVs with and without PMNs. Incubation of PMNs with REVs and subsequent attachment of PMNs to the tumor cell layer would ensure a higher total analytical concentration of these marker molecules in the vicinity of the tumor cells than in the case of simple interactions between REVs and the tumor cells. A critical observation to make is whether a higher accumulation of the probe molecules in the tumor cells would occur.

As shown in Figs. 1 and 2, the accumulation of LY and sucrose in the tumor cells was significantly higher over a 60-min period when the incubation medium contained PMNs. If we assume a steady-state accumulation at $t > 20$ min, the rates are 140 ng LY mg protein^{-1} h^{-1} for LY and 540 DPM mg protein^{-1} h^{-1} for sucrose. The ratio of these rates (sucrose: LY 3.9) is slightly smaller than the starting mass ratio of sucrose to LY 5.9 (data not shown). If the mechanism of accumulation is the same for both molecules, these values should be identical. However, it is probable that the starting sucrose to LY ratio is somewhat reduced by preferential leakage of sucrose from REVs upon exposure to serum just prior to and during phagocytosis by PMNs (Scieszka and Cho, 1988). It is conceivable that negatively charged LY

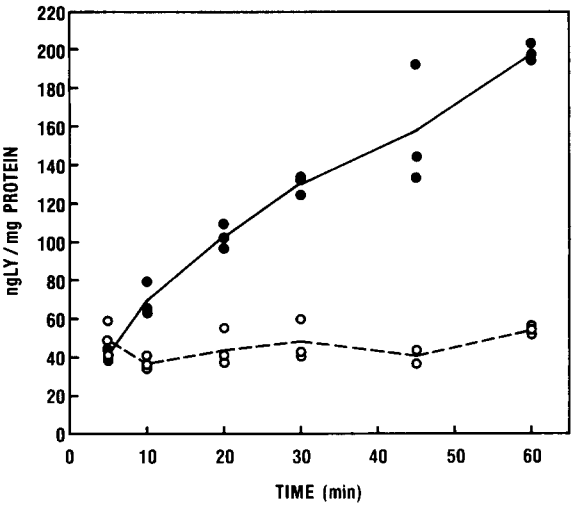

Fig. 1. Accumulation of LY in the tumor cells at 37° over a 60-min period when the cell layer was incubated with LY-containing REVs with (closed circles connected with a solid line) and without PMNs (open circles connected with a dotted line). The LY concentration found was normalized per milligram of the tumor cell protein. Lines pass through the average values of triplicate determination.

molecules pass through negatively charged lipid layers of REVs at a slower rate than neutral sucrose molecules. Similarly, the percent accumulated in the tumor cell layer for the 60-min incubation were 0.024% and 0.015% of the total for LY and sucrose, respectively. In contrast, when PMNs were absent in the system, little accumula-

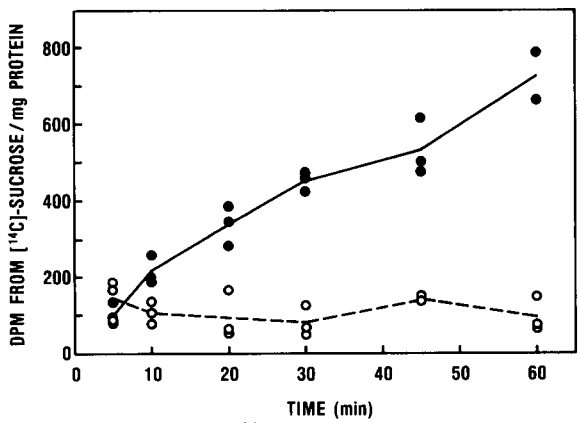

Fig. 2. Accumulation of [^{14}C]sucrose in the tumor cells when the cell layer was incubated with [^{14}C]sucrose-containing REVs with (closed circles) and without PMNs (open circles).

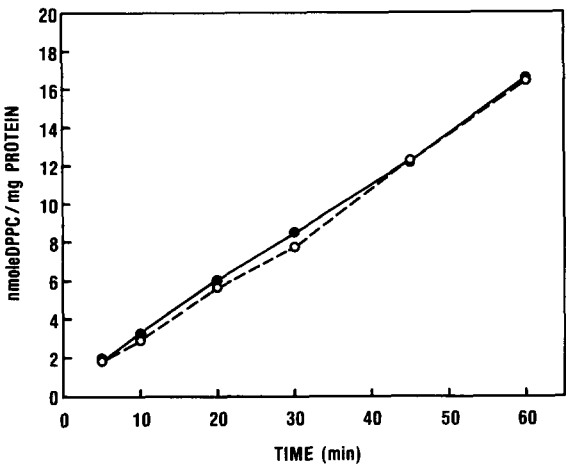

Fig. 3. Accumulation of [^{3}H]DPPC in the tumor cells when the cell layer was incubated with REVs labelled with [^{3}H]DPPC with (closed circles) and without PMNs (open circles). For simplicity, individual triplicate assay data are not shown. Instead, only the average values are displayed.

tion occurred. That is, there was little direct transfer of the fluid-phase markers from REVs to the tumor cells. When tested for the null hypothesis that the slope of the open circles is equal to zero, we obtained $t = 1.225$ with $P = 0.239$ for Fig. 1 (df = 16) and $t = -0.612$ with $P = 0.550$ for Fig. 2 (df = 16).

However, as shown in Fig. 3, the appearance of a liposomal lipid marker [^{3}H] DPPC was independent of PMNs. When tested for the null hypothesis that the slopes are equal (i.e., uptake rates are equal regardless of PMNs), we obtained $t = 0.645$ with $P = 0.527$ (df = 18). The steady-state rate of DPPC accumulation in the tumor cells was 18 nmol DPPC mg protein^{-1} h^{-1}. If we ignore the different expressions of units for the sake of comparison, this accumulation rate can be expressed as approximately 13% of LY accumulation rate, previously given as 140 ng mg protein^{-1} h^{-1}. This percentage difference is in stark contrast to the total amounts of the markers present at $t = 0$ of which DPPC is only 1.2% of the LY. Similarly, percent DPPC recovered at $t = 60$ min, 0.19%, was approximately 8 times higher than that of LY, 0.024%. Based on this difference, we conclude that the lipid transfer mechanism is different from that for LY and sucrose.

In the foregoing analysis, it was implicitly assumed that the assayed tumor cells were indeed free from contaminating REVs or PMNs. Since the amount of the markers recovered from the tumor cells even after 60 min of incubation was so small (see above), any small amount of contamination with either REVs or PMNs can drastically affect the experimental results. Little accumulation of the fluid-phase markers in the tumor cells from REVs in the absence of PMNs, open symbols in Figs. 1 and 2, strongly supports that the tumor cells were not contaminated with free REVs. Time-dependent accumulation of LY and sucrose in the presence of PMNs also supports that the tumor cells recovered were void of bound PMNs. It would be very unlikely to observe the time-dependance, had the accumulation been simply due to binding of PMNs onto the tumor cells. Finally, we were not able to detect any significant fluorescence on the tumor cells recovered when the latter was incubated with FITC-conjugated rabbit anti-rat PMN antibody (Fig. 4). From these observations, we concluded that the tumor cell band separated for the marker assay was free of any contamination and that the accumulation reported in Figs. 1–3 represents true uptake rather than loosely bound REVs or PMNs.

The faster accumulation of the lipid marker [^{3}H]DPPC we observed is in agreement with similar findings by others (Blumenthal et al., 1982). Spontaneous transfer of DPPC from REVs to the tumor cells could well be collision-mediated, as was the case of Nile red transfer from O/W emulsion droplets to plated macrophages (Haynes and Cho, 1988). The mechanism by which the fluid-phase markers accumulated in the tumor cells only in the presence of PMNs cannot be clearly established with the existing data alone. We postulate that the marker molecules released from PMNs which have phagocytosed the REVs can provide an extremely high concentration in the immediate vicinity of the tumor cells, thereby resulting in a high uptake via pinocytosis. Release of doxorubicin from macrophages which had phagocytosed doxorubicin-containing liposomes has been firmly established (Storm et al., 1988), supporting at least part of the above speculation.

On the other hand, the pinocytic uptake of the

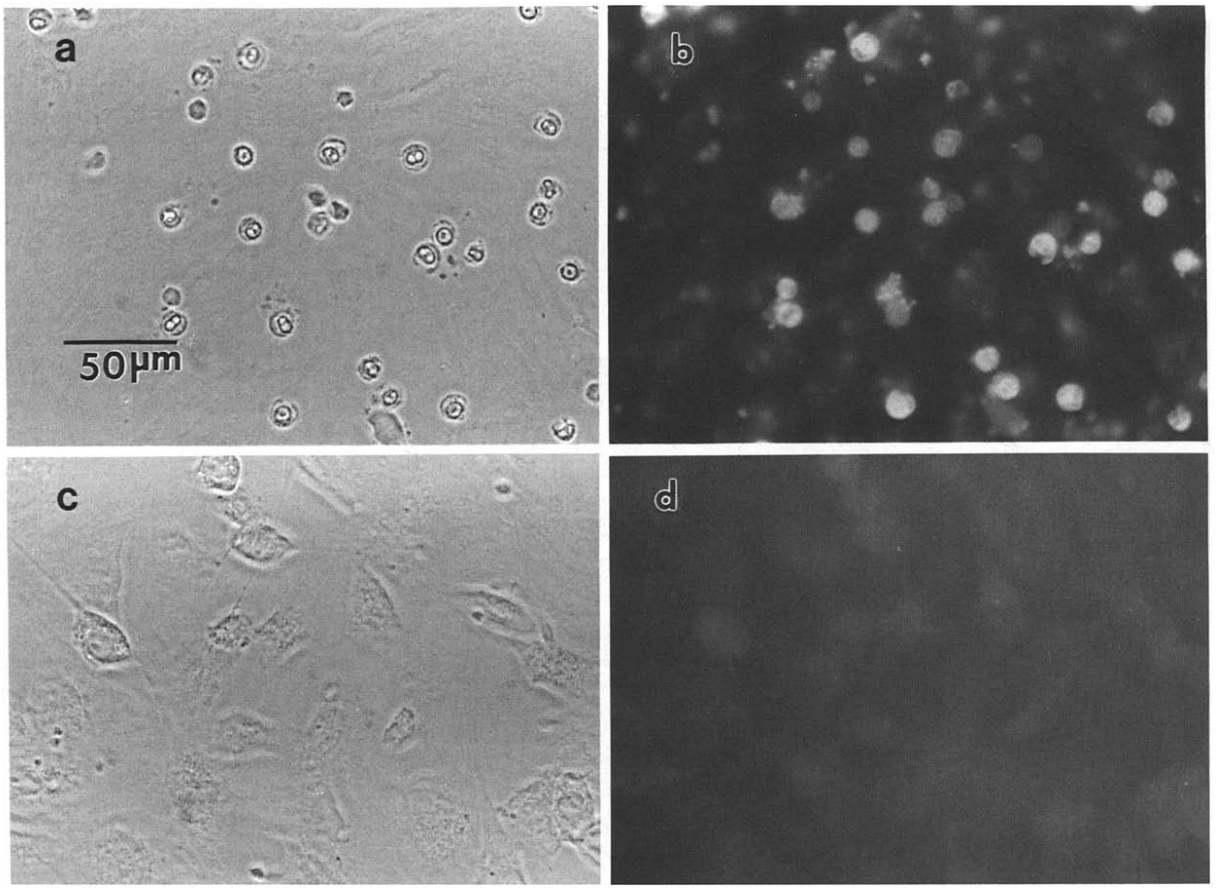

Fig. 4. Phase contrast (*a*) and corresponding fluorescence micrograph (*b*) showing PMNs adhered to the MTF7 tumor cell monolayer. The PMNs were labelled with FITC conjugated rabbit anti-rat PMN antibody. Phase contrast (*c*) and corresponding fluorescence micrograph (*d*) of tumor cells separated from attached PMNs as described in the text.

marker molecules released from PMNS by the tumor cells before diffusion out of the unstirred layer remains to be established. Interestingly, such a mechanism was ruled out for the facilitated uptake of [^{3}H]sucrose by murine tumor cells from bound liposomes (Blumenthal et al., 1982). One main difference in the present study from the latter is the presence of PMNs. Phagocytosis and exocytosis were perhaps occurring in a steady state manner throughout the experimental period, thus continuously providing a high concentration in the unstirred layer. Further studies are warranted for establishing the transport mechanism. It would be also interesting to test if the present finding is of any bearing to various in vivo metastasis model studies in which liposomal delivery was superior to simple solution preparations. Mayhew et al., for example, reported that doxorubicin-containing liposomes were significantly more effective than free doxorubicin in increasing life-spans of mice bearing two different liver metastases (Mayhew et al., 1987). It is tempting to speculate involvement of PMNs in their findings.

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