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Membrane vesicles shed into the extracellular medium by human breast carcinoma cells carry tumor-associated surface antigens

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We have compared the pattern of surface antigen expression, as detected by monoclonal antibodies (mAbs), in plasma membranes vs shed membrane vesicles of two human breast carcinoma cell lines, MCF-7 and 8701-BC. Antigen expression was detected on cells by immunofluorescence (IF) analysis, whilst, due to their small dimensions, the same technique was not applicable to vesicles. For these structures dot-blot analysis and immunoelectron microscopy (IEM) were employed. When applicable, both cell membranes and membrane vesicles were immunoprecipitated and the precipitate (IP) was analyzed by SDS–PAGE. Cells of both lines expressed HLA class I antigens, epithelial cytokeratins, β1 integrins, CEA and the glycoprotein detected by mAb 19.9, but only MCF-7 cells expressed Lewis Y, episialin and globo-H antigens and only 8701-BC cells expressed folate receptor. Membrane vesicles of both cell lines appeared to be rich in β1, α3 and α5 integrin chains, expressed HLA class I antigens and carried most of the plasma membrane antigens found in the cell membranes. Overall we have analyzed 17 antigens on the two cell lines and on their vesicles. The results obtained for cells (IF and IP) and those for vesicles (dot-blot and IP) were generally concordantly positive or concordantly negative. We obtained a total of 26 clearly concordant combinations on 34 analyses. In three cases we found discordant results, whereas in the remaining combinations we observed slight reactivity and we found difficulties in determining concordance. Discordant results concerned the expression of the following antigens: folate receptors, which were clearly expressed in 8701-BC cells but not detected by dot-blot analysis or IEM on their shed membrane vesicles; *neu* (c-erb-B2) receptor found in MCF-7 cell membranes but not in their vesicles; and the globo-H antigen recognized by mAb MBr1, detected at low levels on 8701-BC plasma membranes but undetectable on their membrane vesicles. Like vesicles shed *in vitro* by cultured cells, the vesicles shed *in vivo* by human breast carcinoma cells could be tagged with several antibodies against tumor-associated antigens. The vesicles shed *in vivo* were found in association with a fiber network. Some of the fibers had the characteristic fibrin periodicity. These data suggest that tumor markers detected in the circulation of carcinoma patients, at least in part, are carried by shed membrane vesicles. Moreover the observation that membrane vesicles carry both tumor-associated antigens and HLA class I molecules indicate that these structures could in principle present antigens to the immune system. Together with our previous demonstration that membrane vesicles shed by breast carcinoma cells contain TGF-β, these results suggest an important role for vesicles in the immunological escape of these cells. The presence in membrane vesicles of integrins, together with the previous observation that they are rich in gelatinolytic activities, also points to a possible role of these structures in the metastatic behavior of tumor cells.

Keywords: antigen shedding, breast cancer, membrane vesicles, tumor-associated antigens

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Introduction

Tumor cells are known to shed membrane vesicles into the extracellular environment both *in vitro* and *in vivo* [1]. Although the shedding phenomenon has been widely investigated in the past, very few recent data are available despite several findings suggesting the potential importance of this phenomenon in tumor immunology and progression. For example, highly metastatic variants of the B16 murine melanoma were found to shed more membrane vesicles than did poorly metastatic variants [2], and weakly metastatic cells became more metastatic and colonized similar organs, after fusion with membrane vesicles from the highly metastatic variant [3]. Membrane vesicles appear to contain several enzymes which could be involved in the metastatic cascade. Vesicles released by murine melanoma cells were reported to contain a metalloproteinase activity (Mr 59 000) [4], and to carry the plasminogen activator (uPA) [1]. The presence of collagenolytic activity in vesicles shed by human rectal carcinoma cells has also been reported [5].

In our previous analysis of the gelatinase content of membrane vesicles shed *in vitro* by two human breast carcinoma cell lines, MCF-7 and 8701-BC [6], we demonstrated the presence of proMMP-9, an enzyme which is frequently found to be increased during tumor invasion. We also observed that both the amount and the enzymatic content of vesicles were influenced by growth conditions, especially in 8701-BC cells, where release of vesicles was strongly inhibited when cells were grown in the absence of serum [6], a stress condition in which the possibility of cell death is increased. Those results confirmed previous conclusions [1] that vesicle shedding is not a consequence of cell death, and suggest that shedding is regulated by extracellular signalling events.

Recent observations in our laboratory indicate that 8701-BC vesicles contain TGF-β and the ability of these vesicles to inhibit proliferation of peripheral blood lymphocytes is blocked by the addition of anti-TGF-β antibodies (manuscript in preparation).

Here we describe our analyses of the expression of some surface antigens on vesicles and on membranes of human breast carcinoma cell lines MCF-7 and 8701-BC and on vesicles recovered from the pleural effusion of a mammary carcinoma. These analyses confirm the plasma membrane origin of vesicles and their ability to carry tumor-associated antigens.

Materials and methods

Cell culture

The 8701-BC cell line, derived from a ductal infiltrating human breast carcinoma, was a kind gift from

Table 1. Characteristics of the monoclonal antibodies used in this study

mAb (isotype)	Antigen	Source	Immunoprecipitation ability	Reference
CAM.5(γ)	cytokeratins 8, 18, 19	CAM.5 clone, BD	ND	11
W6/32(γ2a)	HLA class I	ATCC	yes	12
MBr1(γ)	globo-H	produced by INT	no	13
MBr8(γ2)	Fucosyl-Lewis	produced by INT	no	14
MLuC1(γ2a)	Le^y	produced by INT	no	15
MLuC8(γ1)	CEA-like	produced by INT	no	16
MAM6(γ1)	episialin	provided by Dr J. Hilgers	no	17
B72.3(γ)	TAG-72 sialyl Tn	provided by Dr J. Schlom	no	18
19.9(γ)	mucin sialyl Le^a	provided by Centocor	no	19
MOV18(γ1)	folate receptor	produced by INT	yes	20
Mint5(γ1)	EGF receptor	produced by INT	yes	21
MGR6(γ2a)	c-erbB-2	produced by INT	yes	22
66IG10(γ1)	transferrin receptor	provided by Dr J. Hilgers	yes	23
MAR4(γ)	integrin β1 chain	produced by INT	yes	24
Anti-α2(γ)	integrin α2 chain	P1E6 clone, TP	yes	25
Anti-α3(γ)	integrin α3 chain	P1B5 clone, TP	yes	25
Anti-α5(γ)	integrin α5 chain	P1D6 clone, TP	yes	25
MAR6(γ)	integrin α6 chain	produced by INT	yes	26

BD = Becton Dickinson, San Jose, CA; TP = Telios Pharmaceuticals Inc, San Diego, CA; INT = Istituto Nazionale Tumori, Oncologia Sperimentale E, Milano.
ND = not determined.

S. Minafra [7]. MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and have recently been reported to be a useful model system for identifying factors that influence metastasis [8]. Cells were cultured in tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cultures were consistently negative for mycoplasma contamination (routinely tested with the Hybricomb Mycoplasma Test Kit, Biological Industries, Kibbutz Beth Haemek, Israel).

Monoclonal antibodies

Table 1 lists the mAbs used for phenotypic analysis of cell lines and vesicles. Most of the mAbs were used in purified form, obtained either directly from commercial sources or purified from ascitic fluids by affinity chromatography on Protein A for IgG, and gel filtration for IgM. Unless otherwise stated, purified mAbs were used at 10 μg/ml and ascitic fluid at 1:100 dilution.

Preparation of membrane vesicles from the cell culture medium and from a pleural effusion

Vesicles were prepared essentially as described [6]. Conditioned medium obtained from 48 h tumor cell cultures was centrifuged at 600 ***g*** for 15 min and then at 1500 ***g*** for 15 min. The pleural effusion from a breast carcinoma patient was centrifuged at 600 ***g***, diluted with phosphate buffered saline (PBS), and centrifuged at 1500 ***g*** for 15 min and at 2000 ***g*** for 15 min. All supernatants were centrifuged at 100 000 ***g*** for 1 h at 4°C. Pelleted vesicles were resuspended with PBS (pH 7.5) and analyzed for protein content using the Bradford [9] method (Bio-rad). Vesicle recovery was 3.5 μg/10^6 cells, and 7 μg/ml from the pleural effusion.

Immunoelectron microscopy

Vesicles resuspended in PBS were applied to collodion-coated grids, reacted with mAbs in PBS (50 μg/ml), and samples were incubated for 1 h at room temperature. After washing, samples were further incubated with a gold-conjugated anti-mouse antibody (Sigma) for 1 h. Samples were fixed, negatively stained with 1% phosphotungstic acid, brought to pH 7.0 with NaOH, and examined by transmission electron microscopy.

Dot-blot assay

Vesicles (2 μg of protein) were adsorbed to nitrocellulose squares (Hoefer), treated with a blocking solution (5% dry milk in PBS), washed and incubated overnight with mouse mAbs (10 μg/ml in blocking solution). After washing, a biotinylated rabbit anti-mouse IgG (Amersham) was added. Color was developed using avidin-peroxidase and diaminobenzidine.

Immunofluorescence analysis

Cells were harvested and washed twice at 4°C with PBS, 0.03% bovine serum albumin (BSA; Sigma). Viability of cells was assessed by Trypan-blue exclusion and was always greater than 95%. Cells were incubated with the mAbs (10 μg/ml) for 30 min on ice. After three washings with PBS + 0.03% BSA, cells were incubated with fluorescein-labeled goat anti-mouse IgM + IgG (Kirkegaard & Perry Laboratories Inc, Gaithersburg, USA) for 30 min on ice. For cytokeratin evaluation, cells were permeabilized by incubation for 10 min on ice with cold 70% ethanol and washed three times in PBS + 0.03% BSA before incubation with the relevant antibody. Cell-associated fluorescence was analyzed using a flow cytometer (FACScan, Becton-Dickinson) and with a Zeiss microscope. Total fluorescence (TF) was calculated as (mean fluorescence minus background) × total number of positive cells/100.

Radiolabeling and solubilization of cells and vesicles

Suspensions of viable MCF-7 and 8701-BC cells were radioiodinated by membrane lactoperoxidase-catalyzed iodination. Cells were washed three times with serum-free medium and twice with PBS, and incubated for 10 min at room temperature in a total volume of 1 ml PBS containing 10 μl ^{125}I (1 mCi), 195 μg lactoperoxidase and 25 μl of 0.03% H_2O_2. After 5 min, further aliquots of 25 μl of H_2O_2 were added. The reaction was stopped by adding 10 ml of cold PBS, and cells were then washed three times with the same buffer. Membrane vesicles (150 μg) were resuspended in 50 μl of PBS and lactoperoxidase catalyzed iodination was carried out as above. Free iodine was removed by several washings and centrifugation on Ultrafree-MC 10 000 NMWL filters (Millipore, Bedford, MA, USA). Solubilization was carried out at 0°C for 40–60 min by adding 1–2 ml of 50 mM Tris–HCl (pH 7.4) containing 1% NP40, 1% Antagosan (Behring, L'Aquila, Italy) and 0.001 M phenylmethylsulfonylfluoride (PMSF) (Sigma, St Louis, MO). Cell lysates were cleared by centrifugation at 10 000 ***g*** and the soluble extracts were used for immunoprecipitation analysis.

Immunoprecipitation

Conjugation of mAbs to protein A-Sepharose CL4B was performed as follows: protein A-Sepharose equilibrated in PBS containing 1% BSA and 0.5% NP40 was incubated with shaking for 30 min at

room temperature with rabbit anti-mouse antibodies (100 μg/ml); after three washings, aliquots (50 μl) of protein A-Sepharose were incubated overnight at 0°C with 500 μl of the different mAbs (10 μg/ml).

Cell or vesicle extracts (1 ml) were precleared by incubation with 40 μl of normal mouse serum and 40 μl Antagosan for 30 min on ice, followed by incubation with shaking for 30 min at 4°C with 400 μl Sepharose conjugated with goat anti-mouse IgG serum and equilibrated in PBS containing 0.5% NP40. Precleared extracts were recovered by centrifugation. Aliquots (50 μl) containing about 2×10^6 cpm were incubated with the different Sepharose-mAbs equilibrated in PBS containing 0.5% NP40 and 1% BSA. After incubation for 45 min at room temperature and an additional 45 min at 0°C, the Sepharose-conjugated mAbs were washed three times with radioimmunoprecipitation (RIP) buffer 1 (1 M NaCl, 0.02 M Tris–HCl, pH 7.4, 0.001 M EDTA, 0.5% NP40, 1% Antagosan, 0.001 M PMSF) and three times with RIP buffer 2 (0.002 M Tris–HCl, pH 7.4, 0.5% NP40, 1% Antagosan, 0.001 M PMSF). The precipitated molecules were then extracted from Sepharose with sample buffer [10] and heated for another 10 min at 90°C. Samples obtained were analyzed by SDS–PAGE in 4–15% linear gradient or 7.5% slab gel under reducing conditions.

Results

Expression of cell surface markers on cells and vesicles from tumor cell lines

Tumor cell lines 8701-BC and MCF-7 were both of epithelial origin, as indicated by expression of cytokeratins 8, 18 and 19 in the cytoplasm (Figure 1). Both cell lines also showed cell surface expression of HLA-class I antigens at different levels (Figure 1). Antigen expression was analyzed in MCF-7 (Table 2) and 8701-BC (Table 3) cells and vesicles using a panel of mAbs that detect adhesion molecules, tumor-associated antigens and membrane receptors. Antigen expression was detected on cells by immunofluorescence, whilst, due to their small dimensions, the same technique was not applicable to vesicles. For these structures dot-blot analysis and/or IEM was therefore employed. When applicable, both cell membranes and membrane vesicles were immunoprecipitated and the precipitate was analyzed by SDS–PAGE. Figure 2 shows the immunoprecipitation results obtained for MCF-7 (panel A) and 8701-BC (panel B) vesicles and cells using anti-HLA class I (MHC-I) and anti-β1 integrin mAbs. In the case of radiolabelled vesicle extracts, in spite of a high background also present in immunoprecipitates obtained using control mAbs (lanes 1 and 5), faint

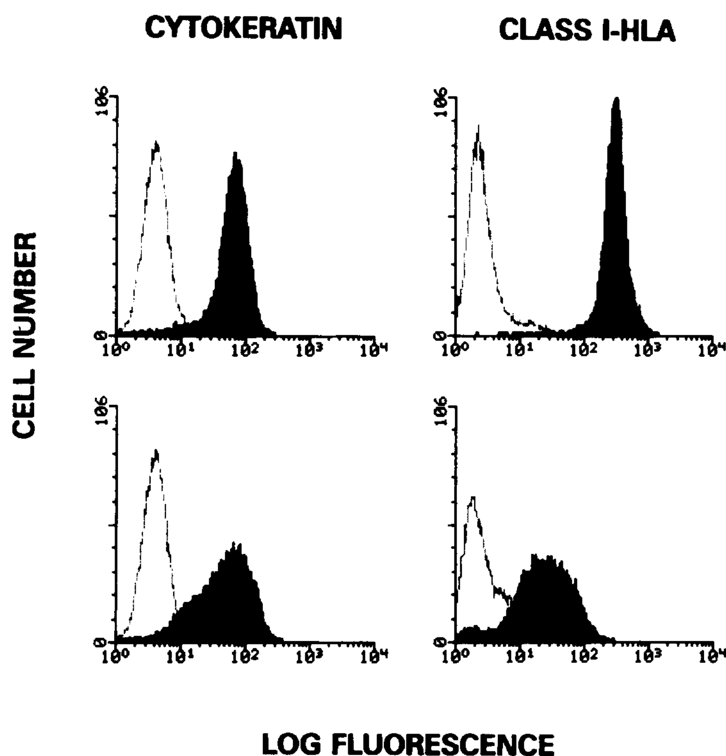

Figure 1. Cytofluorimetric analysis of the expression of cytokeratins 8, 18, 19 and HLA class I antigens on 8701-BC (A) and MCF-7 (B) cells. For cytokeratin analysis, cells were permeabilized with 70% cold ethanol. Shaded profiles represent fluorescence staining with CAM5 (anti-cytokeratins) and W6/32 (anti-HLA class I) mAbs. Unshaded profiles represent background fluorescence in the presence of fluorescein-labeled goat anti-mouse IgG + IgM.

Table 2. Phenotypic expression of surface antigens on MCF-7 breast carcinoma

mAb	Cells		Vesicles	
	IF[a]	IP[b]	Dot-blot[c]	IP[b]
Able to immunoprecipitate				
W6/32	25749 (+ +)	+	(+)	+
66IG10	9828 (+)	+/−	(+ +)	
MOV18	476 (−)	−	(−)	−
MAR4	3918 (+)	+	(+ +)	+/−
Anti-α2	14280 (+ +)	+ +	(+ +)	+/−
Anti-α3	7805 (+)	+	(+ +)	+/−
Anti-α5	7348 (+)	+	(+ +)	+/−
MAR6	1744 (+/−)	−	(+/−)	−
Unable to immunoprecipitate, or not tested				
Mint5	564 (−)		(−)	
MGR6	5538 (+)		(−)	
MLC8	10633 (+ +)		(+)	
MAM6	18178 (+ +)		(+ +)	
B72.3	382 (−)		(−)	
19.9	24793 (+ +)		(+ +)	
MBr1	47606 (+ +)		(+ +)	
MBr8	28505 (+ +)		(+ +)	
MLuC1	8517 (+)		ND	
Background	100–900			

[a] Immunofluorescence (IF); data are reported as total fluorescence (TF). −, TF < 1000; +/−, 1000 < TF < 2000; +, 2000 < TF < 10 000; + +, TF > 10 000.
[b] Immunoprecipitation (IP). +/−, faint; +, evident; + +, strong band at the expected M.W. after 24 h of autoradiographic exposure at −70°C using an intensifying screen.
[c] Data refer to intensity of staining in a dot-blot assay. −, Staining similar to that of negative control (second-antibody alone); + +, maximal staining.

bands at the expected molecular weights were visible in the immunoprecipitates produced by anti-HLA class I and by anti-integrin $\beta 1$ chain antibodies (lanes 3 and 7 respectively). Both cells and vesicles of the two cell lines were positive for reactivity with W6/32 anti-HLA ABC and were also positive for $\beta 1$,

Table 3. Phenotypic expression of surface antigens on 8701-BC breast carcinoma

mAb	Cells		Vesicles	
	IF[a]	IP[b]	Dot-blot[c]	IP[b]
Able to immunoprecipitate				
W6/32	53287 (+ +)	+	(+)	+
66IG10	1095 (+/−)	+/−	(+/−)	−
MOV18	10609 (+ +)	−	(−)	−
MAR4	7163 (+)	+	(+ +)	+
Anti-α2	2682 (+)	+	(+)	+/−
Anti-α3	22100 (+ +)	+ +	(+ +)	+
Anti-α5	24819 (+ +)	+ +	(+ +)	+
MAR6	1525 (+/−)	−	(+/−)	−
Unable to immunoprecipitate, or not tested				
Mint5	3393 (+)		(+/−)	
MGR6	830 (−)		(−)	
MLuC8	4311 (+)		(+/−)	
MAM6	189 (−)		(−)	
B72.3	98 (−)		(−)	
19.9	14061 (+ +)		(+ +)	
MBr1	2490 (+)		(−)	
MBr8	1176 (+/−)		(+/−)	
MLuC1	82 (−)		ND	
Background	100–900			

Key as Table 2.

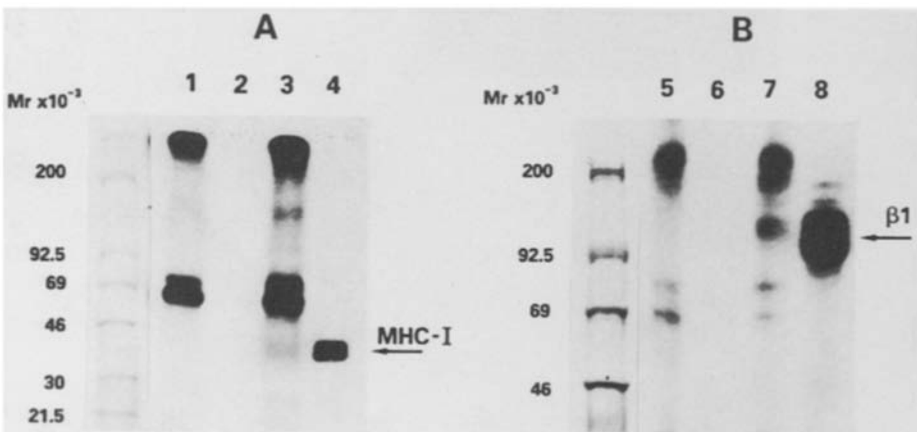

Figure 2. ^{125}I autoradiography of MCF-7 (panel A) and 8701-BC (panel B) cell membrane extracts (lanes 2, 4, 6, 8) and membrane vesicle extracts (lanes 1, 3, 5, 7), after radioimmunoprecipitation and SDS–PAGE. Panel A: 4–15% SDS–PAGE. Immunoprecipitation with control anti-folate receptor mAb MOV18 (lanes 1, 2) and with anti-HLA class I (MHC-I) mAb W6/32 (lanes 3, 4). Panel B: 7.5% SDS–PAGE. Immunoprecipitation with control anti-α6 integrin chain mAb MAR6 (lanes 5, 6) and with anti-$\beta 1$ integrin chain mAb MAR4 (lanes 7, 8).

α2, α3 and α5 integrin subunits. but negative for α6 (Tables 2 and 3).

The expression of adhesion molecules provides evidence that membrane vesicles could interact with extracellular matrix components and therefore support the hypothesis that they could be involved in the metastatic behavior of tumor cells. 8701-BC and MCF-7 cells and their vesicles were negative for reactivity with mAb B72.3 which recognizes the oncofetal mucin Tag 72, whereas the blood-group associated antigen detected by mAb MBr8 was strongly expressed on cells and vesicles of MCF-7 and only weakly on 8701-BC cells and vesicles. The CEA like molecule, recognized by MLuC8, was also expressed at higher levels on MCF-7 than on 8701-BC cells and vesicles. MCF-7 cells and vesicles were strongly positive for episialin, a mucin-like glycoprotein targeted by MAM6, and for the Le^y epitope recognized by MLuC1, whereas 8701-BC cells and vesicles did not express these antigens.

The saccharidic antigen recognized by mAb MBr1 was strongly expressed on MCF-7 cells and vesicles, detected at relatively low levels on 8701-BC cells but not at all on their vesicles. With the exception of the latter, total fluorescence on the cell surface was in good agreement with staining intensity of vesicles in dot-blot analysis for the antigens analyzed. These results confirm the cell membrane origin of vesicles and demonstrate their ability to carry tumor markers to the extracellular medium. On the other hand, there was less concordance between cells and vesicles in expression of membrane receptors. Both cell lines and their vesicles expressed transferrin receptor as detected by mAb 66IG10; however, MCF-7 cells were weakly and homogeneously stained with an anti-c-erbB-2 mAb, whereas the MCF-7-derived vesicles were negative. 8701-BC cells were clearly labelled with the anti-folate receptor mAb MOV18 but the shed vesicles were negative. Only a very limited number of breast carcinoma cell lines (about 5%) express the folate receptor. Expression of this receptor by 8701-BC cells was however confirmed by immunoblot analysis (data not shown).

Immunolocalization of tumor-associated antigens on MCF-7 and 8701-BC breast carcinoma cells and vesicles

To confirm the results obtained for vesicles using dot-blot analysis, immunoelectron microscopic localization

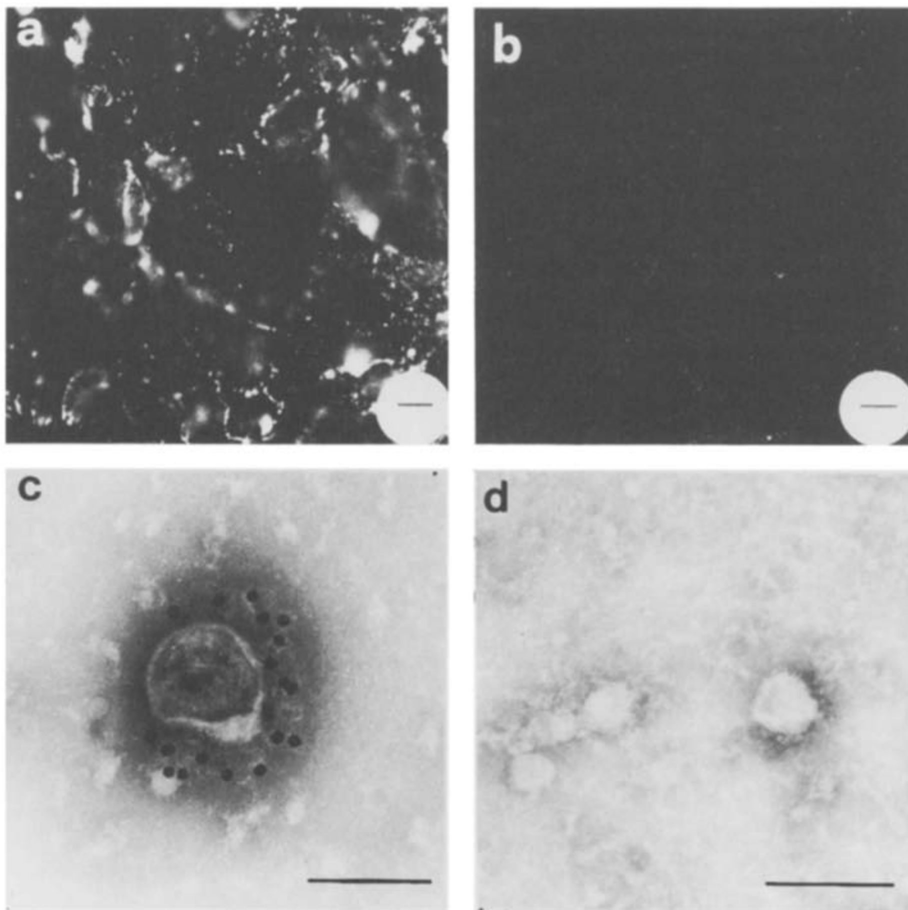

Figure 3. Reactivity of mAb MBr1 with the cell surface and membrane vesicles of 8701-BC (right panel) and MCF-7 (left panel) as determined by immunofluorescence on cells (a and b) and TEM immunolocalization on vesicles (c and d). In a and b, bars = 5 μm; in c and d, bars = 0.1 μm.

(IEM) of several mAbs was performed with vesicles from both breast carcinoma cell lines (Table 4). A general agreement between dot-blot and IEM was found both for positive cases (W6/32 and MAR4 reactivity on vesicles from both cell lines and MBrl reactivity on MCF-7 vesicles) and negative cases (MOV18 and MAR6 on vesicles of both cell lines, and MBrl on 8701-BC vesicles). IEM analysis of vesicles reactivity with mAb MLuCl, which had not been tested by dot-blot, revealed results that were in accord with the immunofluorescence results for cells. Figures 3 and 4 show the reactivity of mAbs MBrl and MLuCl respectively, on vesicles shed by both cell lines.

Table 4. TEM immunolocalization and dot-blot reactivity of tumor associated antigens on vesicles

Cells mAb	MCF-7		8701-BC	
	Dot-blot	IEM	Dot-blot	IEM
W6/32	+	+	+	+
MOV18	–	–	–	–
MAR4	+	+	+	+
MAR6	+/–	–	+/–	–
MBrl	++	++	–	–
MLuCl	ND	++	ND	–

TEM = transmission electron microscopy.
IEM = immunoelectron microscopic analysis.
ND = not determined.

TEM immunolocalization of tumor-associated antigens on vesicles recovered from the pleural effusion of a mammary carcinoma patient

The membrane shedding phenomenon is known to occur both *in vivo* and *in vitro*. Indeed, electron microscopic analysis of membrane vesicles isolated from the pleural effusion of a mammary carcinoma patient (Figure 5) showed that the vesicles were similar to those recovered from the tissue culture medium of breast carcinoma cells grown *in vitro*. Analysis of the vesicles shed *in vivo* for the presence of two tumor-associated antigens, i.e. the CEA-like molecule recognized by MLuC8 and the saccharidic antigen recognized by MBrl, revealed expression of both

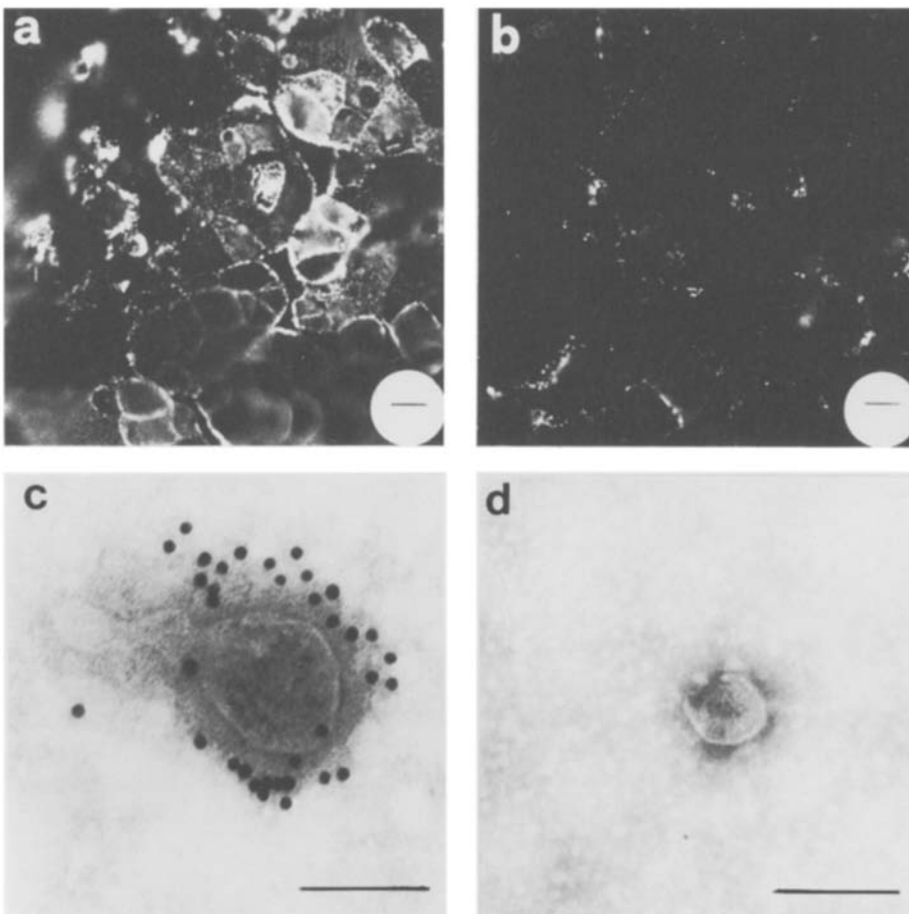

Figure 4. Reactivity of mAb MLuCl on cell surface and membrane vesicles of 8701-BC (right panel) and MCF-7 (left panel) cells as determined by immunofluorescence on cells (a and b) and TEM immunolocalization on vesicles (c and d). In a and b, bars = 5 μm; in c and d, bars = 0.1 μm.

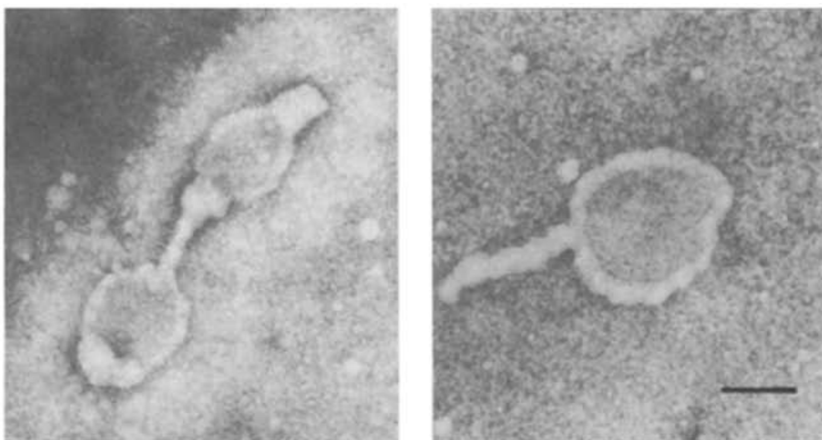

Figure 5. Isolated vesicles from pleural effusion observed by electron microscopy with negative staining. Bar = 0.1 μm.

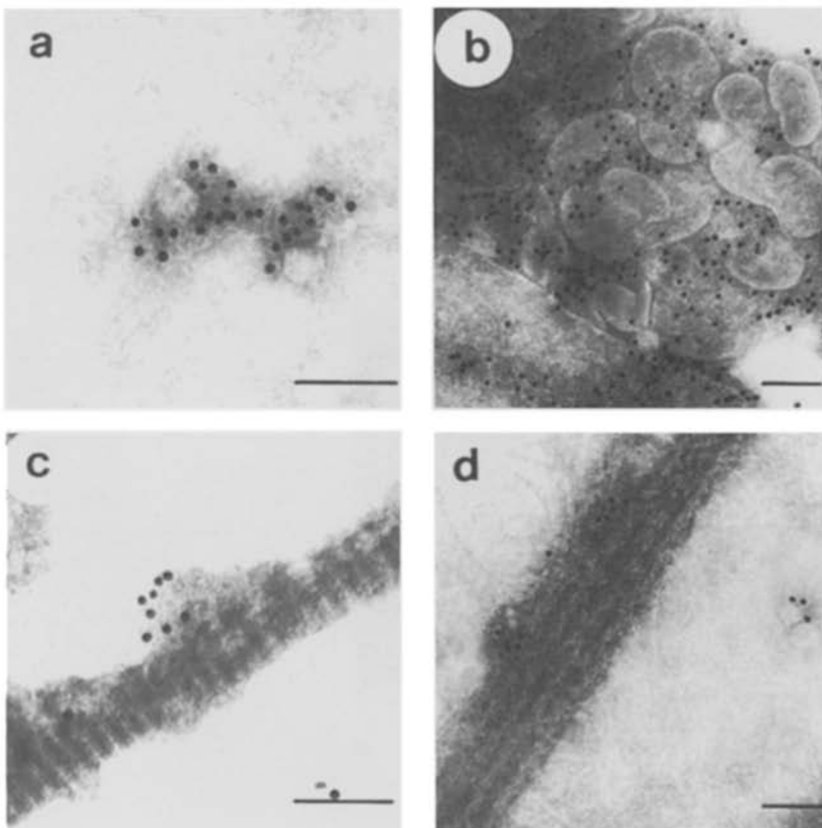

Figure 6. Immunolabeling of membrane vesicles obtained from pleural effusion of a mammary carcinoma patient. mAb MBr1 labeling is shown in isolated vesicles (a), and in a vesicle trapped in a fiber network (c). MLuC8 labeling is shown in a group of vesicles (b), and in a vesicle trapped by a fibrin fiber (d). Bars = 0.1 μm.

antigens and showed that most vesicles are trapped in a fiber network (Figure 6). Some of these fibers might be comprised of fibrin, based on the typical periodicity of these fibers (Figure 6c). The composition of the fibers shown in Figure 6d is as yet unknown.

Discussion

Early reports indicating that intracellular membrane markers are absent from vesicle membranes [28] suggested that vesicles probably originate from plasma membrane. Our studies on breast carcinoma cells confirm this origin, and indicate that most markers expressed on cell plasma membranes, including HLA class I molecules and tumor-associated surface antigens, are also present on membrane vesicles. It has been suggested that membrane vesicles shed by tumor cells may represent selected portions of the plasma membrane. Vesicles from ascitic fluids of murine leukaemia-bearing mice had a lower content of phospholipid and an increased content of proteins,

cholesterol and sphingomyelin compared to that of the plasma membrane of tumor cells [29]. In another study the activity of γ-glutamyltranspeptidase and protein-kinase was found to be high in plasma membranes and absent, or decreased, in shed membrane vesicles, whereas the activity of alkaline phosphatase was found to be increased in vesicles [28].

In the present study, we investigated whether differences in antigenic profile exist between plasma and vesicle membranes. Overall 17 antigens were analyzed on two cell lines in a total of 34 combinations. Only three discordant results were recorded in which the antigens seemed to be absent from the vesicle membranes. Two of the excluded antigens were receptors; 8701-BC cells were strongly labeled with the anti-folate receptor mAb MOV18 whereas their shed vesicles were negative; MCF-7 cells were stained with an anti-erb-B2 mAb, whereas their shed vesicles were negative. We also found differences in the expression of the globo-H antigen recognized by mAb MBr1: whilst both MCF-7 cells and vesicles were clearly positive, 8701-BC cells were only weakly reactive with this monoclonal antibody and their vesicles were negative. On the other hand, we observed no differences in the phenotypic expression of a large variety of surface antigens tested, although the qualitative nature of our assays leaves open the possibility of quantitative differences even amongst those antigens that are present on both plasma membranes and vesicles. More precise evaluation is needed to address this point.

Several previous observations have suggested that vesicles shed by tumor cells might be involved in the metastatic cascade [3, 30]. Our analysis of integrin expression on the cell membranes and vesicles designed to investigate the putative interaction of membrane vesicles with extracellular matrix components, revealed the presence of $\beta 1$ integrins. Membrane vesicles could therefore adhere to matrix components, release enzymes rich in gelatinolytic activities [6], and thus contribute to the metastatic behavior of tumor cells. Support for this hypothesis awaits analysis of the shedding phenomenon in a wider range of tumor cell lines.

We have shown that vesicles morphologically similar to those shed *in vitro* by breast carcinoma cells, were present in the pleural effusion of a breast carcinoma patient. These data suggest that tumor markers detected in the circulation of carcinoma patients, at least in part, are carried by these structures. Vesicles shed *in vivo* were frequently associated with a network of fibrin fibers. The observed association between vesicles and fibrin fibers raises the possibility that vesicles contain some procoagulant activities that account for the fibrin deposition associated with many types of malignancy. A similar association between vesicles and fibrin fibers has been reported for ascitic fluid of ovarian carcinomas [27], which we have also recently observed (unpublished). Moreover, procoagulant activities have been found in vesicles released by hepatocarcinomas and murine breast carcinoma cells [31].

Vesicles shed *in vitro*, as well as vesicles found in the pleural effusion of a carcinoma patient, were HLA class I positive and rich in tumor-associated antigens. The expression of HLA class I antigens on vesicles points to the possibility that these structures present antigenic molecules to cells of the immune system. On the other hand, the presence of TGF-β (unpublished observation) would not only prevent the stimulation of a specific lymphocyte population, but actually selectively inhibit it.

In summary, these results confirm that membrane vesicles are shed by breast carcinoma cells from the plasma membranes and suggest that tumor markers detected in the blood of carcinoma patients are, at least in part, carried by shed vesicles. They also point to a potential role for vesicles in immunological escape and the acquisition of metastatic behavior.

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