*Biochimica et Biophysica Acta* 814 (1985) 77–84
Elsevier



BBA 72495

# Characterization of cellular and extracellular plasma membrane vesicles from a non-metastasizing lymphoma (Eb) and its metastasizing variant (ESb)

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(Received October 16th, 1984)

Key words: Plasma membrane isolation; Lipid composition; Membrane shedding; Metastatic tumor; (Murine lymphoma)

**Plasma membrane fragments from two variants of a murine lymphoma, Eb and ESb, with different metastatic capacity were investigated. Plasma membranes were isolated from tumor cells recovered from the peritoneal cavity. They differed in their lipid composition, indicating a more fluid state of the plasma membranes derived from the highly metastatic tumor line ESb. Extracellular membrane vesicles could be isolated from the ascites of the tumor-bearing mice. The shedding capacity of ESb cells was much higher than that of Eb cells. The extracellular membranes by chemical analysis and the measurement of marker enzymes proved to be derived from the plasma membranes. However, they differed from the plasma membranes from which they were derived in several aspects: (i) the lipid to protein ratio was diminished; (ii) the activities of some plasma membrane-associated enzymes were lower while others were identical in plasma membranes and extracellular membranes; (iii) the content of saturated fatty acids in phopholipids was enhanced in extracellular membranes. These effects were more pronounced in the highly metastasizing tumor line ESb. It is thus concluded that shedding of extracellular membranes is not a random process. The biochemical differences found in the plasma membranes and the extracellular membranes of the two tumor lines are discussed with respect to the different metastatic capacity of the tumors.**

## Introduction

The spontaneous release of complete membrane vesicles from the cell surface has been described for normal and for tumor cells in vivo and in vitro [1–6]. Membrane shedding seems to be a selective rather than a random process. The extracellular membranes from leukemic cell lines have been shown to contain a specific protein and lipid composition as compared to the plasma membrane; thus, in extracellular membranes from YAC cells, a receptor for lectin was found with increased activity as compared to that of the intact cells [2]. Furthermore, the extracellular membranes from GRSL cells have been shown to be enriched in cholesterol and tumor antigens [7]. It is not clear whether the selectivity of the shedding process reflects a preexisting asymmetry or a segregation of membrane components during the exfoliation of membrane fragments.

Extracellular membranes from leukemic cells can be used for an active immunization of mice against viable lymphoma cells [2]. Based on these observations of antigenic recognition of vesicles by the immune system it was suggested that the formation of plasma membrane vesicles by mouse

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

leukemic cells may be a mechanism of escape of tumor cells from immune destruction. In accordance with this assumption, it has been found that high-metastatic tumor cells show a higher shedding of vesicles than low-metastatic ones [8].

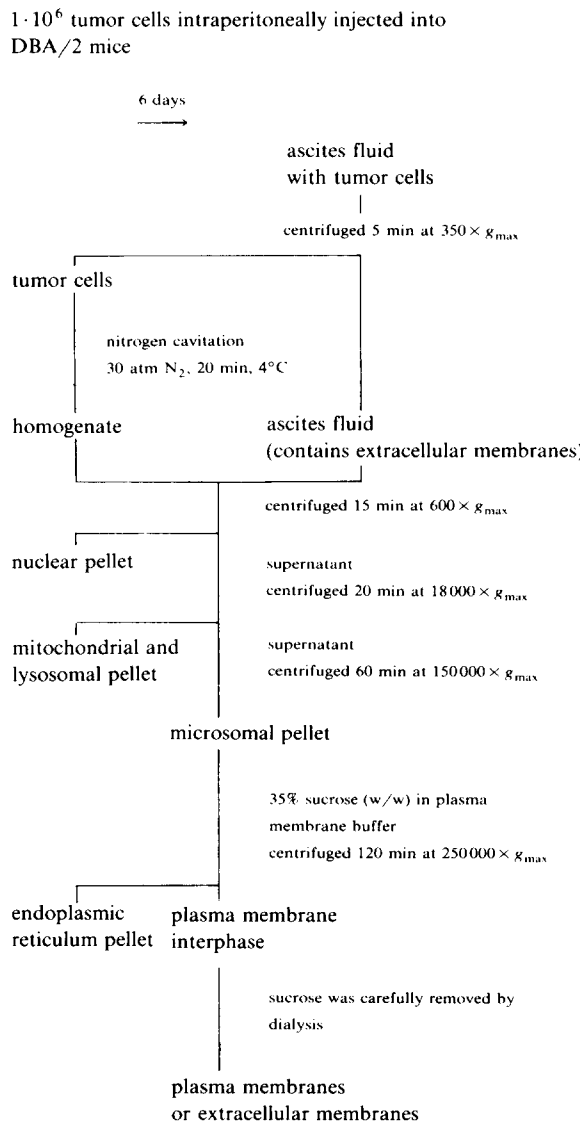
The model system we used are the two syngeneic tumor lines Eb with a low metastatic capacity and ESb with a high metastatic capacity. They are derived from the methylcholanthrene A induced T cell lymphoma L5178Y [9]. The etiology of the tumors and other characteristics like their invasion capacity [10], their immunogenicity and their tumor-associated transplantation antigens have been described [11]. Recently, the close genetic relationship between the two lines has been confirmed [12,13].

In the present study, the biochemical characteristics of the purified plasma membrane and extracellular membrane of the two tumor lines were compared. Differences were found between the plasma membrane of the two tumors as well as between the extracellular membrane and the plasma membrane of the respective tumors.

## Materials and Methods

*Preparation of plasma membrane vesicles and extracellular membrane vesicles.* Plasma membrane vesicles were prepared from Eb and ESb ascites cells and from the ascites fluid. The isolation procedure was adapted from a protocol for the isolation of calf thymocyte membranes [14] and is summarized in Scheme I.

Ascites tumor cells and ascites fluid were harvested from the peritoneal cavity of DBA/2 mice 6 days after intraperitoneal inoculation with $1 \cdot 10^6$ tumor cells. The ascites fluid was separated from the cells by low-speed centrifugation and the cellular sediment treated with hypotonic buffer to lyse the erythrocytes. Cell disruption was achieved by the nitrogen cavitation method with $5 \cdot 10^7$ cells/ml buffer (10 mM Hepes (pH 7.0)/140 mM KCl/0.15 mM $MgCl_2$/0.02% $NaN_3$) in a pressure homogenizer (Fike, Blue Springs, U.S.A.). Both the cell homogenate and the ascites fluid were made 1 mM with EDTA and fractionated in parallel (Scheme I). Membranes were stored at −80°C in 10 mM Hepes (pH 7)/140 mM KCl/0.02% $NaN_3$ (= plasma membrane buffer).




Scheme I. Isolation of purified plasma membrane vesicles from tumor cells and from the ascites fluid.

*Analytical procedures.* Protein was determined by the procedure of Lowry et al. [15]. DNA was analyzed by the diphenylamine reaction according to Dische [16]. Cholesterol was determined enzymatically by the method of Röschlau et al. [17] using a commercial test combination (Boehringer, Ingelheim, F.R.G.). Total phospholipid was analyzed according to Zilversmith and Davis [18] with a commercial test combination (Boehringer, Ingelheim, F.R.G.). Phospholipids were extracted from the plasma membranes as described earlier

[19] and separated by thin-layer chromatography on precoated silica gel glass plates (Schleicher and Schüll, F 1500) using the solvent system chloroform/methanol/acetic acid/0.9% NaCl (50:25:8:2, v/v). Individual phospholipid spots were scraped out and the fatty acids transesterified by sodium methylate. Fatty acid analysis was done by gas-liquid chromatography on a Fractovap 4160 (Carlo Erba Strumentazione) using a FFAP glass capillary column [20].

*Enzyme assays.* β-*N*-Acetylglucosaminidase (EC 3.2.1.2.0) was assayed according to Kornfeld and Siemers [21]; succinate dehydrogenase (EC 1.3.99.1) according to Earl and Korner [22]; lactate dehydrogenase (EC 1.1.1.27) according to Capaud and Wrobleski [23] with a commercial test combination (Sigma); alkaline *p*-nitrophenylphosphatase (EC 3.1.3.1) and acyl-CoA : lysolecithin acyltransferase (EC 2.3.1.23) according to Ferber et al. [24]; ATPases (EC 3.6.13)) according to Averdunk and Lauf [25] modified by Szamel et al. [19]; γ-glutamyltransferase (EC 2.3.2.2) according to Szasz [26] with a commercial test combination; glucose-6-phosphatase (EC 3.1.3.9) according to Swanson [27]; 5′-nucleotidase (EC 3.1.3.5) according to Weaver and Boyle [28]. Inorganic phosphate liberated in enzyme assays was analyzed as described by Anner and Moosmeyer [29].

*Electron microscopy.* Fractions were fixed with 2% glutaraldehyde for 30 min at room temperature. After washing, the samples were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 30 min on ice. Samples were stained with 5% uranyl acetate in 70% acetone for 20 min on ice. After dehydration and embedding, thin sections were cut and poststained with lead citrate for 10 min. Samples were examined in a Siemens 102 electron microscope.

## Results

Plasma membranes were prepared from the low and high metastatic tumor lines Eb and ESb as described under Materials and Methods. They were biochemically characterized and compared to the purified extracellular membrane vesicles which had been shed from the respective tumor cells into the ascites fluid.

*Shedding capacity of Eb and ESb tumor cells*

The amount of cells that could be harvested from the ascites of one mouse at the sixth day after inoculation of $1 \cdot 10^6$ tumor cells was about $1 \cdot 10^8$ cells in Eb-tumor-bearing mice and $(1.5–2) \cdot 10^8$ cells in ESb-tumor-bearing mice. The yield of protein in the plasma membranes and extracellular membranes derived from $1 \cdot 10^{10}$ cells of Eb and ESb is shown in Table I. The total amount of protein in the cell homogenates and the yield of plasma membrane protein derived thereof were similar for both cell types, in spite of the marked morphologic differences of the cells [9]. However, the amount of protein in the ascites fluid from Eb and ESb differed greatly. It corresponded with a 3-times higher yield of the extracellular membrane protein shed from $1 \cdot 10^{10}$ ESb cells as compared to that of $1 \cdot 10^{10}$ Eb cells.

*Characterization of the membrane preparations*

*Morphology of plasma membrane and extracellular membrane preparations.* Electron micrographs obtained from thin sections of the membrane pre-

TABLE I

THE YIELD OF PROTEIN IN THE CELLULAR AND EXTRACELLULAR PLASMA MEMBRANES DERIVED FROM $1 \cdot 10^{10}$ TUMOR CELLS

Data are means of ten experiments. Values are expressed as mg protein/$10^{10}$ tumor cells. PM, plasma membrane; ECM, extracellular membrane.

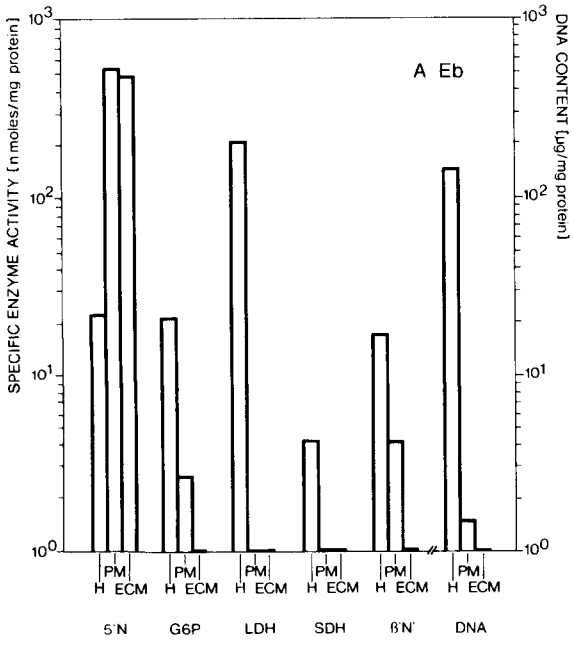
| Tumor | Cell homogenate [a] | PM | Ascites fluid [b] | ECM |
|---|---|---|---|---|
| Eb | 742 ± 212 (100%) | 27 ± 6.3 (3.6%) | 321 ± 81 | 6 ± 1.2 |
| ESb | 793 ± 253 (100%) | 26 ± 4.2 (3.3%) | 945 ± 212 | 20 ± 2.4 |

[a] Prepared by nitrogen cavitation.

[b] After separation from $1 \cdot 10^{10}$ tumor cells.

parations showed that plasma membranes as well as extracellular membranes from Eb and ESb tumor cells contained mostly membrane vesicles devoid of contamination by cytoplasmic organelles such as mitochondria, lysosomes or rough endoplasmic reticulum. No differences were observed between the plasma membranes or extracellular membranes from the two tumor sublines. Micrographs are not shown because they don't provide additional information.

*Purity of the plasma membrane and extracellular membrane fractions.* The purity of the membrane preparations was determined by measuring DNA and marker enzyme activities. In Fig. 1, the data found in the homogenate and the membrane fractions are depicted. Mitochondrial, lysosomal or cytoplasmatic marker enzyme activities (succinate dehydrogenase, $\beta$-*N*-acetylglucosaminidase and lactate dehydrogenase), respectively, were not detectable or measurable only in trace amounts in the plasma membrane fractions as well as in the extracellular membrane fractions. Glucose-6-phosphatase, a marker enzyme of the endoplasmic reticulum, was highly enriched in the microsomal fraction, but hardly measurable in the plasma membrane and extracellular membrane fractions after purification by sucrose gradient centrifuga-



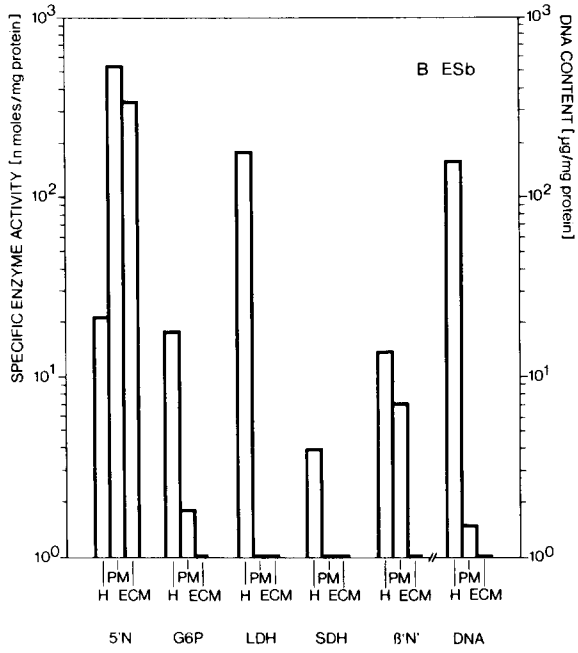




Fig. 1. DNA and subcellular marker enzyme distribution in the homogenate (H) and the membrane fractions (plasma membrane and extracellular membrane) from (A) Eb and (B) ESb tumor cells. PM, plasma membrane; ECM, extracellular membrane; 5'N, 5'-nucleotidase (marker enzyme for membrane fractions); G6 P, glucose-6-phosphatase (peak activity in microsomal fraction: (A) 61.8 nmol/mg, (B) 73.4 nmol/mg); LDH, lactate dehydrogenase (peak activity in cytosol fraction: (A) 436.8 nmol/mg, (B) 457.3 nmol/mg); SDH, succinate dehydrogenase (peak activity in mitochondrial and lysosomal fraction: (A) 29.3 nmol/mg, (B) 30.4 nmol/mg); $\beta$'N', $\beta$'-*N*-acetylglucosaminidase (peak activity in mitochondrial and lysosomal fraction: (A) 66.2 nmol/mg, (B) 78.4 nmol/mg); DNA, desoxyribonucleic acid (peak activity in nuclear fraction: (A) 358.3 $\mu$g/mg, (B) 375.5 $\mu$g/mg).

TABLE II

PHOSPHOLIPID AND CHOLESTEROL CONTENT OF PLASMA MEMBRANES AND EXTRACELLULAR MEMBRANES FROM Eb AND ESb TUMOR CELLS

Data are means of three independent determinations. PM, plasma membrane; ECM, extracellular membranes

| | Eb | | ESb | |
|---|---|---|---|---|
| | PM | ECM | PM | ECM |
| Cholesterol ($\mu$mol/mg protein) | 1.75 | 0.065 | 0.92 | 0.04 |
| Phospholipid ($\mu$mol/mg protein) | 2.9 | 0.1 | 2.2 | 0.09 |
| Cholesterol/ phospholipid | 0.61 | 0.65 | 0.42 | 0.44 |

TABLE III

MOLAR RATIO OF SATURATED VERSUS UNSATURATED FATTY ACIDS IN THE PLASMA MEMBRANES AND EXTRACELLULAR MEMBRANES FROM Eb AND ESb TUMOR CELLS

Data are means of four independent determinations. PM, plasma membrane; ECM, extracellular membrane.

| C-16+C-18 | Eb | | ESb | |
|---|---|---|---|---|
| 18:1+18:2+20:4 | PM | ECM | PM | ECM |
| Phosphatidylcholine | 1.6 | 1.45 | 1.0 | 4.5 |
| Phosphatidylinositol | 2.2 | 3.7 | 0.9 | 4.6 |
| Phosphatidylserine | 6.4 | 3.7 | 2.7 | 4.15 |
| Phosphatidyl-ethanolamine | s1.3 | 1.7 | 1.0 | 3.45 |

tion. The highest specific activities of 5′-nucleotidase, a typical plasma membrane marker enzyme, were found in the microsomal fraction and were further enriched by a factor of 4–6 in plasma membranes and extracellular membranes.

*Lipid composition of plasma membranes and extracellular membranes.* One of the most obvious differences between extracellular membranes and plasma membranes in both cell types was the highly reduced phospholipid and cholesterol to protein ratio (Table II). The extracellular membrane contained much more protein while the ratio cholesterol to phospholipid remained stable. With respect to the latter parameter, the two tumor lines were found to differ with ESb cells showing a lower cholesterol/phospholipid ratio than Eb cells. The fatty acid distribution of the individual plasma membrane phospholipids was measured as described under Materials and Methods. As an indicator of the fluidity of the different membranes, the ratio of the most prominent saturated and unsaturated fatty acids was determined. As can be seen from Table III, there were some differences between the plasma membrane from Eb and ESb. ESb plasma membranes contained more unsaturated fatty acids and were thus more fluid. While plasma membranes and extracellular membranes from Eb cells were rather similar, extracellular membranes from ESb cells differed markedly from the original plasma membrane: the degree of saturation was significantly increased.

*Membrane enzymes.* The specific activities of plasma membrane-bound enzymes were similar in the membranes from Eb and ESb cells (Table IV). In contrast, the pattern of enzymatic activities were changed in the shed extracellular membrane: some enzyme activities such as alkaline phosphatase, γ-glutamyltransferase and total ATPase were reduced, whereas others such as 5′-nucleotidase and lysolecithin acyltransferase were unaltered. The decrease in enzyme activity was more pronounced in the extracellular membranes from the ESb than from the Eb tumor.

TABLE IV

MEMBRANE-BOUND ENZYME ACTIVITIES IN PLASMA MEMBRANES AND EXTRACELLULAR MEMBRANES FROM Eb AND ESb TUMOR CELLS

Data are means of five preparations and enzyme activities are expressed in nmol/mg protein. PM, plasma membrane; ECM, extracellular membrane.

| | Eb | | ESB | |
|---|---|---|---|---|
| | PM | ECM | PM | ECM |
| Alkaline phosphatase | 1079 | 400 | 1077 | 128 |
| γ-Glutamyltransferase | 8.1± 1.21 | 2.3± 0.71 | 10.8± 1.37 | 1.7± 0.28 |
| ATPase (total) | 139 ± 16.4 | 22 ± 3.2 | 114 ±18.9 | 25 ± 3.7 |
| 5′-Nucleotidase | 555 ±136.2 | 518 ±76.5 | 597 ±89.8 | 373 ±53.2 |
| Oleoyl-CoA: lysolecithin acyltransferase | 1.2 | 0.9 | 0.9 | 0.9 |
| Arachidonoyl-CoA: lysolecithin acyltransferase | 2.9 | 3.0 | 2.8 | 2.8 |

## Discussion

We could show previously that the two tumor sublines Eb and ESb, which have low and high metastatic capacity, respectively, differ markedly in morphological, antigenic and functional properties [9,11,30–32]. The major biological difference of the two tumor variants consists in their in vivo growth pattern: while ESb cells metastasize easily from virtually every site, Eb cells do not metastasize from a local site [33]. Since it is known that the metastatic capacity is much dependent on cell surface characteristics [34], the cellular and extracellular plasma membranes of both cell types were compared with biochemical methods.

As can be seen from marker activities, the plasma membrane was highly purified and free of contaminating intracellular membranes. The electron micrographs showed similar size distributions for the plasma membrane vesicles of Eb and ESb cells. The lipid matrix of the plasma membrane was altered in the metastasizing tumor variant ESb as compared to the parental line Eb: the cholesterol to phospholipid ratio was diminished, indicating a higher fluidity of the membrane. This was further supported by the higher degree of unsaturation of the phospholipid fatty acids of the ESb plasma membrane. These findings are consistant with previous results that the plasma membranes of leukemic cells are more fluid than the plasma membranes of normal lymphocytes [1,35].

Extracellular membrane vesicles could be purified from the ascites fluid of both tumors by the same procedure as used for the isolation of plasma membrane vesicles. In electron micrographs, these extracellular membrane vesicles were similar to the plasma membrane vesicles but somewhat smaller in size. Several lines of evidence indicate that the extracellular membranes were indeed derived from the plasma membranes exclusively: (1) some plasma membrane marker enzymes including 5′-nucleotidase and lysolecithin acyltransferase were present in extracellular membranes and plasma membranes with similar specific activity; (2) the cholesterol to phospholipid ratio was identical in plasma membranes and extracellular membranes of the corresponding tumor variants; (3) the content of the terminal sugar sialic acid in extracellular membranes was similar to that of the tumor cell microsomal membranes, which consist of about 60% plasma membrane (data not shown).

While the cholesterol to phospholipid ratio in plasma membranes and extracellular membranes derived thereof was identical, the protein content was much higher in extracellular membranes. Moreover, the relative enrichment of proteins in extracellular membranes appears to be selective since some plasma membrane-associated enzymes such as alkaline phosphatase, γ-glutamyltransferase or total ATPase appeared in extracellular membranes at much lower specific activities than in plasma membranes. Moreover, the phospholipid fatty acid composition of the extracellular membrane was not identical with that of the parental membrane. This was most evident in ESb cells, where all phospholipids of the extracellular membrane had a higher content of saturated fatty acids compared to the plasma membrane. These differences found between plasma membranes and extracellular membranes indicate that extracellular membranes do not consist of random plasma membrane debris from living or dead cells but may result from an active shedding process of specific regions of the plasma membrane. It cannot be excluded completely that a small subpopulation of cells with relatively rigid membranes are preferentially killed during the tumor growth nor that rigid plasma membrane fragments of a lysed cell would be more stable in the ascites fluid. However, since the viability of the tumor cells from the ascites was 98% and since no cytoplasmic enzyme activity was found in the ascites fluid it is more likely that special rigid plasma membrane regions are predominantly released from living cells.

The two associated events – the fluidization of the plasma membrane via a decrease in the ratio of cholesterol to phospholipids and the formation of the extracellular membrane are quantitatively more prominent in ESb. Both might have significant implications for the metastatic capacity and the invasion potential of the tumor. Membrane fluidity changes might be implicated in the loss of normal growth control. This is suggested from observations in other systems, where differences were seen in membrane fluidity of tumor cells compared to their normal counter parts [35–38]. Thus, the shedding process could be regarded as

one mechanism by which tumor cells can change their membrane fluidity.

Shedding of membrane vesicles by tumor cells could be of functional significance with respect to tumor immune escape. It has been shown earlier that both Eb and ESb cells evoke specific host defense mechanisms directed against tumor-cell-associated antigens [12]. Furthermore, we were able to show that Eb- and ESb-derived extracellular membrane vesicles could block specifically anti-Eb or anti-ESb cytotoxic T lymphocytes [41]. Since the tumor variant ESb sheds 3-fold more extracellular membranes than Eb cells, this increased release of tumor-antigen-carrying vesicles could potentially facilitate blocking of specific host defense mechanisms in vivo.

The shed membrane vesicles could also be of functional significance for cancer invasion into surrounding tissues. We have recently characterized two tumor-derived degradative enzymes, a proteinase [39] and a heparan sulfate degrading endoglycosidase [40], both of which can degrade components of the basement membrane. Preliminary findings indicate that both enzymatic activities are displayed by ESb-derived membrane vesicles (Kramer, M., et al., unpublished data). It could thus be anticipated that shedding of extracellular membranes carrying degradative enzymes into surrounding tissue could lead to partial tissue degradation and may thus facilitate subsequent tumor cell penetration.

## Acknowledgements

We would like to thank Dr. H.-P. Zimmermann, Deutsches Krebsforschungszentrum, Heidelberg, for help with the electron micrographs. The skillful technical assistance of Ms. A. Stifter and the expert typing assistance of Mrs. H. Zimmermann and Mrs. M. Aschoff is also gratefully acknowledged.

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