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Characterization of cellular and extracellular plasmamembrane vesicles from a low metastatic lymphoma (Eb) and its high metastatic variant (ESb): inhibitory capacity in cell–cell interaction systems

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Spontaneously shed extracellular plasma membrane vesicles (ECM) of a highly metastatic murine tumor line (ESb) were compared (i) with plasma membrane vesicles (PM) of the same cells prepared by the nitrogen cavitation method and (ii) with ECM and PM preparations of the related low metastatic tumor line Eb. From a previous biochemical analysis it was concluded that the exfoliation of ECM vesicles, which is very pronounced in metastatic ESb cells, is not a random process. This conclusion is further corroborated by the present functional analysis. Compared to ESb PM, ESb-derived ECM were selectively enriched for Fc receptors and depleted in glycoproteins with affinity for hepatocytes. Tumor-derived ECM carried the same tumor antigen as the corresponding tumor line and showed in comparison to PM material an increased inhibitor capacity in a T-cell-mediated tumor-specific cytotoxicity test.

Introduction

The spontaneous release of plasma membrane vesicles has been described for normal and tumor cells in vivo and in vitro [1–7]. Plasma membrane vesicles shed from tumor cells were found to express tumor antigens [3,8] and it was therefore suggested that they might play a role in the escape of tumor cells from immune control [6,8,9]. In accordance with this hypothesis, it was observed that metastasizing mammary carcinomas shed more membrane vesicles than related non-metastatic lines [10]. It was furthermore suggested that plasma membrane vesiculation might represent a cellular response to injury [11], because it could be artificially induced by aldehydes, reducing agents and detergents [11–13].

In order to understand more about the shedding of membrane vesicles and its potential significance in cancer metastasis we have performed a systematic analysis of cellular plasma membranes and shed vesicles in a tumor system consisting of a low metastatic parental line (Eb) and a spontaneous high metastatic variant (ESb) [14]. Cellular plasma membranes (PM) and extracellular membrane vesicles (ECM) of these two tumor lines were prepared in large quantities. Interestingly, the metastatic variant was found to produce about 3-times as much ECM material as the low metastatic line [15]. By chemical analysis and measurement of marker enzymes the ECM proved to be derived from the plasma membranes [15]. However, the ECM differed from PM in lipid composition, rigidity and in the activities of some membrane-associated enzymes [15].

Here we report on our functional analysis of the tumor-derived plasma membranes. We decided to test the inhibitory capacity of the membrane material in three different cell–cell interaction systems which had been studied with these

tumor lines before: (i) tumor cell binding to hepatocytes [16], (ii) binding of antibody-coated erythrocytes (EA) to tumor cells [17], and (iii) recognition and lysis of tumor cells by tumor-specific cytotoxic T lymphocytes [18]. These assays allow testing for expression of (i) hepatocyte-binding cell surface glycoproteins, (ii) immunoglobulin-binding Fc receptors and (iii) tumor-associated antigens. The differences observed between ECM- and PM-derived material as well as those seen between low (Eb) and high (ESb) metastatic tumor lines will be discussed with regard to potential functional significance.

Materials and Methods

Tumor cell lines. The two tumor cell lines Eb and ESb are sublines of the methylcholanthrene-induced DBA/2 mouse lymphoma L5178Y. Eb has low and ESb high metastatic capacity [14]. The etiology of the cell lines, their characteristics and their maintenance in tissue culture have been described in detail elsewhere [18,19].

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 as described in detail [15].

Analytical procedures. Protein content was determined by the procedure of Lowry et al. [20]; phospholipids and fatty acids were determined as described [15].

Hepatocyte tumor cell rosette assay. Hepatocytes were isolated by in situ collagenase perfusion as described [16]. Washed hepatocytes were cultured in Williams medium E (Flow Laboratories No. 12-502-54) in the presence of 15% fetal calf serum and insulin (Sigma No. I-5500) for 2–3 h at 37°C in flat-bottom microtiter plates (Multiwell Nr. 3008). For the rosette assay, $2 \cdot 10^4$ hepatocytes per well were co-cultured with $2 \cdot 10^6$ ESb tumor cells in the presence or absence of the indicated amounts of plasma membranes. The percentages of hepatocyte rosettes (hepatocytes with 3 or more tumor cells bound) were counted under an inverted microscope.

Detection of Fc receptors by the EA rosette assay. 100 µl of a 50% suspension of washed sheep erythrocytes (SRBC) in 0.9% NaCl were incubated for 30 min at room temperature with 100 µl of a mouse anti-SRBC antiserum. The antibody-coated erythrocytes (EA) were then washed two times and diluted to a 1% suspension. In the rosette assay 50 µl containing $3 \cdot 10^5$ test cells (e.g. ESb cells) were mixed in small plastic tubes (Greiner Nr. 100101) with 50 µl of a 1% EA suspension and 50 µl of RPMI 1640 medium with or without plasma membrane material. The mixture was centrifuged for 3 min at low speed (1000 rpm) in a WIFUG table centrifuge. The mixture of cells was then incubated for 15 min on ice and the percentage of rosettes determined under the microscope. Rosette-forming cells were those which had 3 or more erythrocytes bound to their cell surface. Details have been described by Schirrmacher and Jacobs [17].

T-cell-mediated tumor-specific cytotoxicity test. Tumor-specific cytotoxic T cells were induced by immunization in vivo and restimulation of immunized spleen cells in vitro for 4 days as described [16]. In the cytotoxicity test $5 \cdot 10^3$ ^{51}Cr-labelled tumor target cells in 100 µl medium were mixed with 100 µl medium containing cytotoxic T cells and centrifuged for 3 min to mediate contact between effector and target cells. The assay was performed in microtiter plates in triplicate and different ratios of effector to target cells were tested in each experiment. After 4 h of incubation the extent of target cell lysis was determined by ^{51}Cr release. Details have been described [18]. In the inhibition assay effector cells were preincubated with different amounts of plasma membranes for 30 min at 37°C in microtiter plates before the labelled target cells were added. Cytotoxic activity was then determined as described above.

Results

Inhibitory capacity of tumor-derived plasma membranes in a hepatocyte tumor cell rosette assay

Plasma membranes (PM) and extracellular membranes (ECM) obtained from Eb or ESb tumor cells were tested for their capacity to inhibit the interaction between normal hepatocytes and metastatic ESb tumor cells. The results are illustrated in Fig. 1. In the absence of inhibitors, 68% of the hepatocytes formed spontaneous

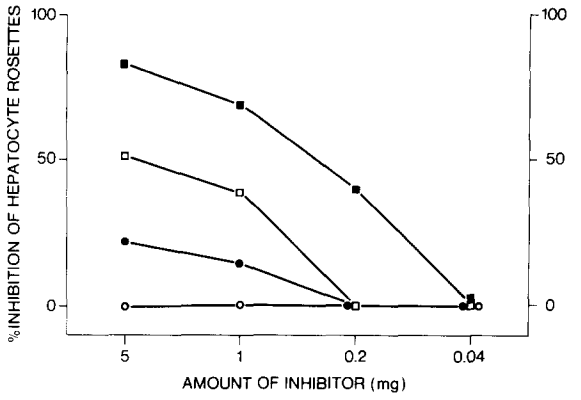

Fig. 1. Inhibitory capacity of tumor-derived plasma membranes in the ESb hepatocyte rosette assay. $2 \cdot 10^4$ hepatocytes per well were co-cultured with $2 \cdot 10^6$ ESb tumor cells in the presence or absence of the indicated amounts of plasma membranes. The percentages of hepatocyte tumor cell rosettes were counted under an inverted microscope. ○, Eb-derived ECM; ●, Eb-derived PM; □, ESb ECM; ■, ESb PM. The results are expressed as percent inhibition of rosette formation in dependence on the amount of membranes used.

rosettes with the ESb tumor cells. The strongest inhibition of rosette formation was obtained with plasma membranes from ESb tumor cells, where 84% inhibition was observed at the highest concentration. ECM of ESb cells were less effective at all concentrations tested. Plasma membranes of Eb tumor cells inhibited only 20% at the highest concentration and ECM obtained from Eb tumor cells did not inhibit the rosettes at all.

These results corroborate our previous findings that ESb cells carry a number of cell surface glycoproteins which can directly bind to hepatocytes, while Eb cells do not [19]. The differences observed with ECM compared to PM of ESb cells suggest that ECM express far fewer binding glycoproteins than PM.

Inhibitory capacity of tumor derived plasma membranes in an EA rosette assay

When Eb and ESb tumor cells were tested for expression of Fc receptors on their cell surface, about 58–70% of ESb cells, but only 2–12% of Eb cells were found to form rosettes with IgG-coated sheep erythrocytes (EA). To test for the presence of functional Fc receptors on isolated cellular and extracellular plasma membranes we performed rosette inhibition assays. For this purpose antibody-coated sheep erythrocytes were preincubated for 45 min at room temperature with the indicated amounts of plasma membranes, washed two times and then added to ESb tumor cells as indicator cells in the EA rosette assay. The results are illustrated in Fig. 2. In contrast to the hepatocyte rosette assay, the ECM of ESb tumor cells had in this assay a much higher inhibitory capacity than the PM preparation. ECM of Eb cells were inhibitory only at concentrations of greater than 100 μg/ml, while the CM of Eb cells did not inhibit at all.

The following points should be noted when comparing the results of the two different assays.

1. To obtain 50% inhibition with PM from ESb cells about 80 μg/ml was required in the EA rosette assay and about 500 μg/ml in the hepatocyte rosette assay (a factor of > 6 difference).

2. The corresponding concentrations for 50% inhibition obtained with ECM from ESb cells were only 10 μg/ml in the EA rosette assay and as much as 5 mg/ml in the hepatocyte assay (a factor 500 difference).

3. Membrane material from Eb cells was much less inhibitor (25% maximal inhibition) and here EA rosette inhibition was observed only with ECM, while hepatocyte inhibition was observed only with PM-derived material.

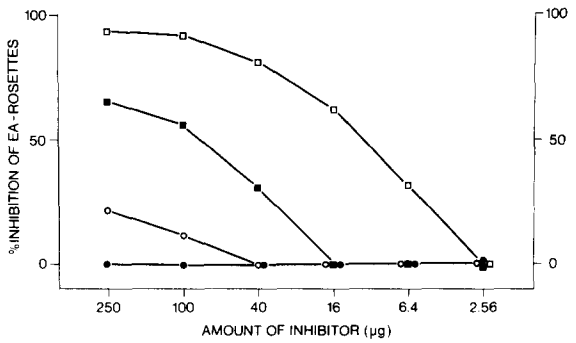

Fig. 2. Inhibitory capacity of tumor-derived plasma membranes in the EA rosette assay. Plasma membranes (PM) of either Eb (●) or ESb cells (■) or ECM of either Eb (○) or ESb cells (□) were tested for capacity to inhibit the binding of antibody-coated erythrocytes (EA) to ESb tumor cells.

Taken together, it can be stated that there is a difference in the functional characteristics of cellular and extracellular tumor-derived plasma membrane vesicles. In the EA rosette assay ESb derived ECM had a much stronger inhibitory capacity then PM, while in the hepatocyte rosette assay ESb derived ECM was much less inhibitory than respective PM. Compared to PM, the ECM material was thus enriched for Fc receptor expression while it was depleted for expression of membrane glycoproteins with affinity for lectin receptors on hepatocytes [21]. In both assays Eb-derived plasma membrane material was much less inhibitory than ESb membrane material, confirming our previous findings with intact cells [16,21].

Inhibitory capacity of tumor-derived plasma membranes in a tumor-specific T-cell-mediated cytotoxicity test

We have reported previously that Eb and ESb cell types express tumor-associated transplantation antigens (TATA) which induce protective immunity in vivo [22] and specific cytotoxic T cells in vitro [18]. The TATA expressed by Eb and ESb cells were distinct for each line so that there was cross-reactivity neither in protective immunity in vivo nor on the level of cytotoxic T cells in vitro. To test whether shed ECM might play a role in tumor immune escape it was important to know (i) whether the vesicles expressed TATAs and (ii) wheter they could inhibit TATA-specific cytotoxic T lymphocytes (CTL).

We therefore tested the tumor-derived plasma membranes for their inhibitor capacity in specific T-cell-mediated cytotoxicity. The results of these competition experiments performed in vitro are illustrated in Figs. 3 and 4. In Fig. 3 Eb-specific CTL were preincubated in microtiter plates for 30 min at 37°C with tumor-derived plasma membranes at the indicated concentrations. Then, ^{51}Cr-labeled Eb tumor target cells were added, the killer and target cells were co-incubated for 4 hours and the specific ^{51}Cr release was determined. The results in 3A illustrate that in the absence of inhibitory membranes Eb tumor target cells were lysed in a dose-dependent fashion, whereas ESb target cells (specificity control) were not lysed. B, C and D show the results when either 100, 10 or 1 μg of tumor-derived plasma mem-

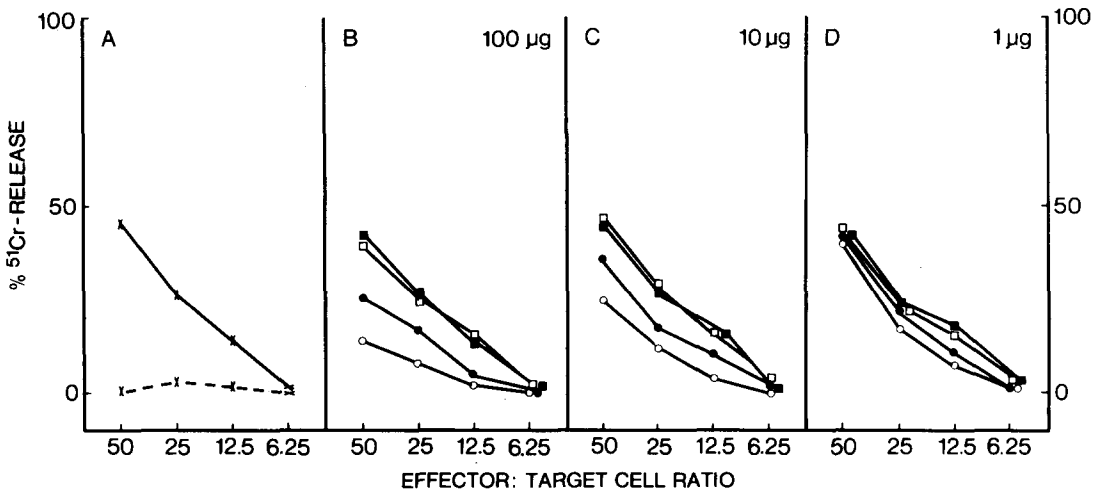

Fig. 3. Inhibitory capacity of tumor-derived plasma membranes in a tumor-specific T-cell-mediated cytotoxicity test with anti-Eb cytotoxic T lymphocytes (CTL). Anti-Eb CTL were induced by immunization in vivo and 4 day restimulation in vitro of immunized spleen cells as described [16]. Solid lines indicate the cytotoxicity measured with ^{1}Cr-labeled Eb target cells, while the striped line in A (specificity control) indicates the cytotoxicity against ESb tumor target cells. B, C and D illustrate the cytotoxicity against Eb cells obtained after competitive inhibition with either Eb ECM (○), Eb PM (●), ESb ECM (□) or ESb PM (■). Inhibition was seen only with Eb-derived plasma membranes, where ECM material was more strongly inhibitory than PM material.

brane material had been added for competitive inhibition. No inhibition of cytotoxicity was observed with ESb-derived material, whereas Eb-derived membranes could partially inhibit the Eb-specific CTL when using either 10 or 100 μg. It can be seen furthermore that Eb-derived ECM material had a higher inhibitory capacity than PM at every effector-to-target-cell ratio tested.

Fig. 4 shows the results of the corresponding analysis performed with ESb-specific CTL. Now the inhibitory capacity of Eb versus ESb-derived membranes was just reversed. Eb-derived membranes did not inhibit ESb-specific killer cells, while ESb-derived membranes showed partial competitive inhibition. Similar to the previous analysis, ECM had a higher specific inhibitory capacity than PM. These results show that tumor-derived plasma membranes have a specific inhibitory capacity in T-cell-mediated cytotoxicity and thus express tumor antigens. Spontaneously shed vesicles also express these antigens and have even higher inhibitory capacity than PM material. It is thus possible that in a local microenvironment shed ECM material could interfere with T-cell-mediated anti-tumor activity.

Discussion

In a previous study [15] we compared cellular (PM) and extracellular (ECM) plasma membrane vesicles from the low metastatic lymphoma Eb and its high metastatic spontaneous variant ESb by means of biochemical procedures. It was concluded that spontaneous membrane shedding (i) was more pronounced in the high than in the low metastatic line and (ii) was a selective rather than a random process. Here we report on functional differences of PM and ECM preparations from Eb and ESb cells and discuss their possible implications for cancer metastasis and immune escape.

First we investigated the inhibitory capacity of the tumor-derived membrane preparations in a hepatocyte tumor cell rosette assay. ESb cells which metastasize predominantly to the liver have the capacity to form spontaneous rosettes with liver parenchymal cells [16]. This binding, which was not observed with Eb cells, could be shown to be due to the recognition via hepatocyte lectin-like receptors of ESb cell surface glycoproteins with free Gal and GalNac residues [21,25]. We also reported evidence to suggest that this selective

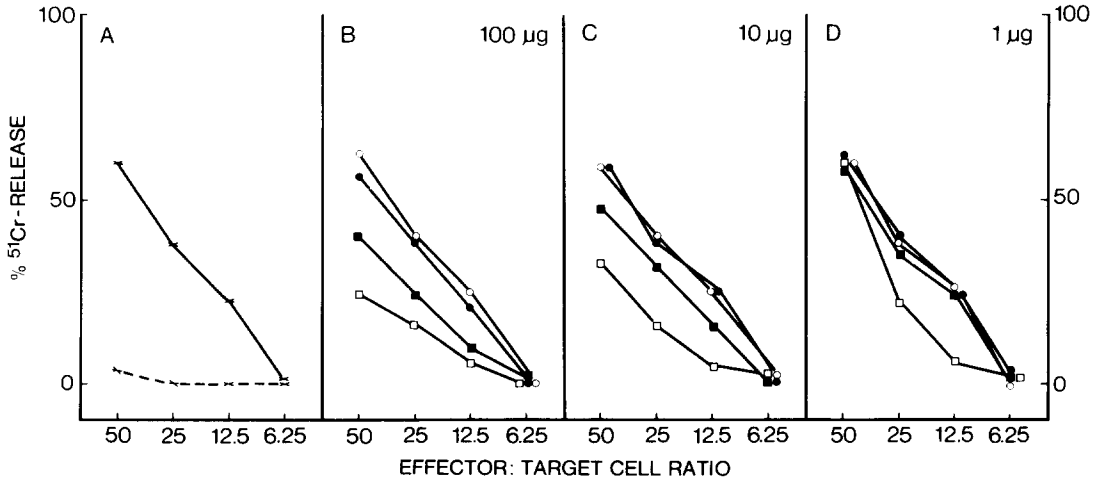

Fig. 4. Inhibitory capacity of tumor-derived plasma membranes in a tumor-specific T-cell mediated cytotoxicity test with anti-ESb CTL. Anti-ESb CTL were induced by immunization in vivo and 4 day restimulation in vitro of immunized spleen cells as described [16]. Solid lines indicate the cytotoxicity tested against ^{51}Cr-labeled ESb target cells either in the absence (A) or in the presence (B, C and D) of different amounts of plasma membranes. The striped line in A (specificity control) shows the cytotoxicity against Eb tumor target cells. The symbols for the results observed in the presence of inhibitory membranes are as in Fig. 3. Inhibition of ESb-specific cytotoxicity was detected only with ESb-derived plasma membranes, and ESB ECM material was superior to ESb PM-derived material.

interaction of a metastatic tumor variant with a normal organ-derived parenchymal cell could lead to tumor cell trapping and enhanced organ colonization [21,23,24]. It was now found that ESb-derived membranes could inhibit the rosettes while those from Eb cells were very poorly or not inhibitory, thus corroborating our previous finding with intact cells. When comparing ESb-derived ECM and PM material we found that the shed vesicles inhibited about 10-times less well than the PM material. It would thus be difficult to imagine that shed vesicles could reach a local concentration sufficiently high for competitive inhibition of ESb-hepatocyte binding, i.e. about 5 mg/ml. In comparison to PM the ECM of ESb cells seem to be depleted of hepatocyte-binding glycoproteins.

Quite different results were obtained when analyzing the membranes for expression of Fc receptors. From the EA rosette inhibition curves it appeared that ESb-derived ECM carried significantly more Fc receptors than the corresponding PM membranes, while those of Eb cells were only very poorly or not inhibitory. The different inhibitory capacity of ESb ECM in the two rosette assays is very remarkable and amounts to a 500-fold difference when comparing concentrations required for 50% inhibition. Compared to ESb PM the shed ECM vesicles were selectively enriched for Fc receptors and selectively depleted for hepatocyte-binding sites. It can now be stated that there is a continuous line of increase in Fc receptor expression from Eb PM via Eb ECM to ESb PM and from there to ESb ECM (see Fig. 2). In concordance with this we previously described cell-free Fc receptors in serum or ascites fluid from tumor-bearing animals [17]. The release of Fc receptors into body fluids might have implications for the regulation of host immune responses. An immunosuppressive activity has indeed been ascribed to an immunoglobulin-binding factor (IBF) produced by L5178Y lymphoma cells [26] from which Eb and ESb cells are derived.

Since in both functional tests discussed so far ECM material behaved differently from PM material it may be questioned whether the ECM was really derived from the tumor cells or whether it could not have been derived from host cells within the ascites fluid. Very specific markers for tumor cells are their tumor antigens, which can be recognized by syngeneic tumor-specific cytotoxic T lymphocytes. Using such cells for typing we have previously shown that both tumor lines, Eb and ESb, carry distinct non cross reactive tumor antigens [18]. These antigens were not detectable on syngeneic normal cells and were recognized on the tumor cells surface in association with H-2 antigens [27]. As shown in Figs. 3 and 4, it was possible to inhibit tumor-specific CTL activity selectively with the respective tumor-derived PM preparations, which shows that PM carry tumor antigens as well as H-2 antigens. When testing ECM material, it was found that these inhibited also specifically the corresponding cytotoxic T cells and thus also expressed the tumor antigens. ECM-mediated inhibition was even more pronounced than that by PM preparations, which is similar to the GRSL leukemia [10] and suggests (i) that ECM was definitely tumor-derived and (ii) that shed ECM vesicles might play a role in specific immune escape mechanisms. To our knowledge this is the first demonstration of specific inhibition of anti-tumor CTL activity by purified spontaneously shed ECM vesicles.

When considering the results from our biochemical and functional analysis together, it seems that the exfoliation of membrane vesicles by metastatic tumor cells is a highly selective process. Both in qualitative and quantitative aspects, plasma membrane vesiculation was more pronounced in metastatic ESb cells. This suggests that this process is of selective advantage for cells to become metastatic. This could be achieved (i) by changing the membrane fluidity [15], (ii) by facilitating tumor cell invasion into surrounding tissues via release of degradative enzymes [28,29] and (iii) by evading host anti-tumor immune responses [30,31].

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