Biochimica et Biophysica Acta, 1029 (1990) 191–195
Elsevier

BBAMEM 70663

BBA Report

Ultrastructural alterations in plasma membranes from drug-resistant P388 murine leukemia cells

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(Received 4 May 1990)

Key words: Freeze-fracture; Cellular multidrug resistance; Intramembrane particle; Membrane protein; Chemotherapy drug; Anthracycline; Daunomycin

Freeze-fracture studies of daunomycin-sensitive and daunomycin-resistant P388 cell lines, reveal a significant increase in the numerical density of intramembrane particles at both, the protoplasmic and the exoplasmic leaflets of the plasma membrane from the drug-resistant cells. Such change in plasma membrane architecture is not accompanied by overexpression of P-glycoproteins. Furthermore, drug-sensitive cells exhibited an increased number of exo-endocytotic images when compared to drug-resistant cells. Our observations suggest that there are global changes in the structural organization of the plasma membrane, which are related to the acquisition of the cellular drug-resistant phenotype.

The plasma membrane of tumour cells is receiving increasing attention in regard to cellular multidrug resistance (MDR) [1,2]. MDR cell lines exhibit a reduced net accumulation of drug relative to the parental drug-sensitive cell lines and most authors have explained these observations in terms of differences in membrane transport of the drug in and out of the cells [3,4]. Moreover, the degree of drug resistance in several cell lines and tumors, has been correlated with the overexpression of P-glycoproteins, a well known family of plasma membrane, high molecular weight proteins (reviewed in Ref. 5). These proteins predictably have the characteristics of pore-forming proteins and act as ATP-dependent pumps to actively eliminate drug and other hydrophobic substances from cells [2,5,6].

The above paragraph emphasizes the importance of alterations in plasma membrane phenomena and/or plasma membrane components in MDR. In spite of this, information on how the pleitropic changes found in MDR cells are reflected on the ultrastructural organization of the plasma membrane, is scarce. Arsenault and co-workers [7], reported freeze-fracture studies in colchicine-resistant Chinese Hamster Ovary (CHO) cell lines and in a vinblastine-resistant human leukemia cell line, and concluded that an increase in the numerical density of intramembrane particles (IMPs) in the protoplasmic leaflet of the plasma membrane, accompanies resistance. The drug-resistant CHO and human cell lines used in those studies overexpressed P-glycoproteins and in fact, the authors explain their results based on a correlation between the number of IMPs and P-glycoprotein overexpression.

We report here freeze-fracture studies of plasma membranes from daunomycin-sensitive P388 murine leukemia cells (P388/S) and from a stable, daunomycin-resistant subline (P388/R) (approx. 50-fold resistance in in vitro cytotoxicity assays) (see legend to Fig. 1 for details and experimental conditions). Examples of the appearance of fracture faces of plasma membranes from P388/S and P388/R cells, are given in Fig. 1. Plasma membranes from P388/S and P388/R cells differ in the frequency of appearance of exo-endocytotic images (Fig. 1), a finding which was not apparent in the CHO cells study [7]. These images, pits in the protoplasmic face and domes in the exoplasmic face, were quantitated in both fracture faces. Plasma membranes from P388/S cells exhibit more than a 2-fold increase in the number of exo-endocytotic images, as compared to those from P388/R cells (Table I). Skovsgaard and co-workers have proposed that drug resistance results from an increased exocytosis of drug previously trapped in endosomal/lysosomal complexes and in fact, they reported an increased plasma membrane traffic in drug-resistant P388 [8] and Ehrlich ascites cells [9]. Although these are measurements of membrane recycling dynamic processes, which can not be formally related to our observations, one would expect that differences in plasma membrane traffic should be reflected somehow in the morphology of the plasma membrane. Therefore, it is tempting to speculate that our observa-

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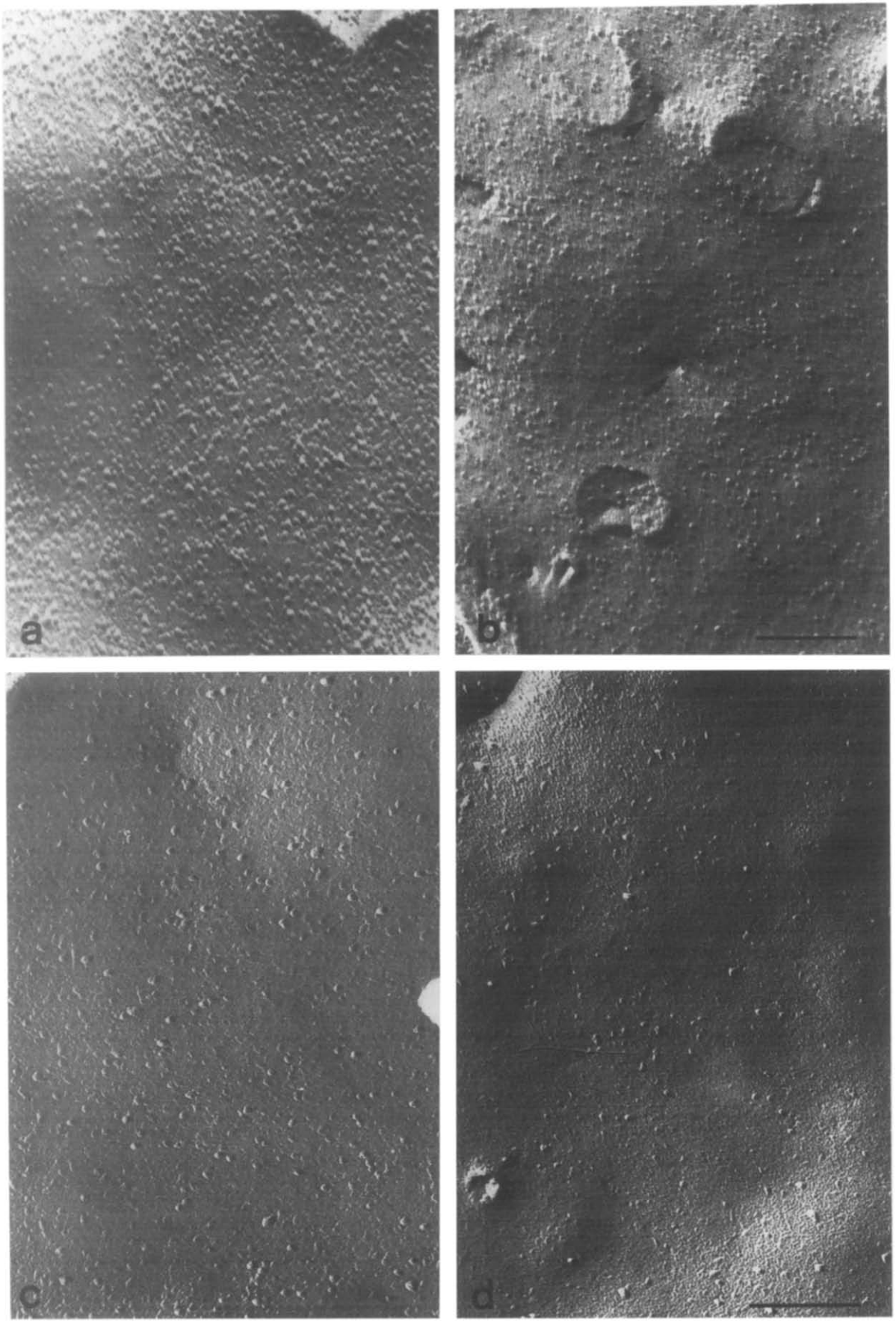
a
b
c
d

TABLE I

Number [a] and size [a,b] of intramembrane particles and frequency of appearance of exo-endocytotic images [a] in plasma membranes from P388 cells [c]

	P388/S cells	P388/R cells
Exo-endocytotic images per 100 μm^2		
Protoplasmic face	232 ± 31 *	94 ± 19
Exoplasmic face	212 ± 23 *	89 ± 18
Intramembrane particles per μm^2		
Protoplasmic face	1095 ± 118 **	1579 ± 132
diameter ⩽10 nm	108 ± 12	157 ± 29
diameter 10–14.9 nm	723 ± 78 *	1200 ± 90
diameter ⩾15 nm	264 ± 28	222 ± 13
Exoplasmic face	128 ± 34 *	342 ± 35
diameter ⩽10 nm	13 ± 3	16 ± 2
diameter 10–14.9 nm	78 ± 21 *	296 ± 30
diameter ⩾15 nm	37 ± 10	30 ± 3

*,** Value significantly different (*, $P < 0.001$; ** $P < 0.02$), from that of the resistant cells.

[a] Each value is the mean ± S.E. from 20 cells. For each cell and each membrane face, at least 1 μm^2 of membrane area were evaluated.

[b] For IMP size measurements, 500 IMPs were evaluated from each cell type.

[c] The number of IMPs and exo-endocytotic images were recorded within a calibrated test square grid, superimposed on photographic prints of the freeze-fracture membrane, as described in Ref. 17. The size of all the IMPs inside the test square was measured using a calibrated eyepiece. The diameter recorded was the length of the base of the triangular shadow projected by the particle, perpendicular to the direction of the shadow. Since the angle of platinum shadowing with respect to the variable curvature of fracture faces may introduce changes in the apparent size of the particles, only flat regions of the membrane, with similar shadowing, were selected for quantitative analysis.

tions on the number of exo-endocytotic images in P388 cells, are partly the result of a different membrane traffic activity.

Fig. 1 also shows that globular IMPs are observed homogeneously distributed in the protoplasmic and exoplasmic fracture faces of plasma membranes from both cell lines, without IMP patches or clusters. Quantitative assessment of IMPs, as indicated in the legend to Table I, does not reveal any differences in IMP size or number, between cells of the same type. On the contrary, comparison between the two cell types demonstrates an increased number of IMPs in the plasma membrane of the drug-resistant cells (Table I). In contrast with the CHO cells report [7], we observe an increased number of IMPs at both, the protoplasmic and the exoplasmic faces of the plasma membrane from the drug-resistant P388 cells. Nevertheless, the absolute number of plasma membrane IMPs in the protoplasmic face of our P388/S and P388/R cells (1095 and 1579 IMP/μm^2, respectively), have a remarkable similarity with those found in that of drug-sensitive CHO cells and of a subline with intermediate resistance of colchicine (970 and 1590 IMP/μm^2, respectively), while highly resistant CHO cells, had an even higher density of particles [7]. Since IMPs represent protein containing structures embedded in the membrane matrix, it should be expected that these observations are the morphological counterpart of differences in protein contents and/or in lipid–protein interactions within the membranes. As indicated previously, Arsenault and co-workers [7], correlated their observations with overexpression of P-glycoproteins in their drug-resistant cell lines. Nevertheless, there are MDR cell lines and tumors which seem not to overexpressed P-glycoproteins [10–13] and therefore, such correlation may not be valid in all cases. In drug-resistant P388 cells, increased expression of P-glycoproteins was initially reported in sublines selected for an extremely high level of resistance [14]. Other authors [15], reported that moderately resistant P388 cells also overexpressed P-glycoprotein, but to a much lower extent than the drug-resistant CHO cells. To determine whether our moderately resistant P388/R cells overexpressed P-glycoprotein, we use the fluorescein-labelled C219 mouse IgG_{2a} monoclonal antibody (P-GlycoCHEK, Centocor Inc., Malvern, PA) in a flow cytometry assay (Fig. 2), using an Epics Profile I instrument equipped with a 0.75 Watts argon laser (488 nm emission line). Monoclonal antibody C219 recognizes a highly conserved aminoacid sequence found in cytoplasmic domains of all the P-glycoprotein isoforms characterized to date [16]. Fig. 2 shows the distribution of each cell population analyzed in terms of the fluorescence due to their reactivity against the C219 antibody. Mean fluorescence channel numbers indicated within the Figure, are proportional to the mean fluorescence intensity exhibited by each cell line. The flow cytometry data indicate, therefore, that there are no significant differences in immunoreactivity against C219 between the moderately resistant P388/R cells and their parental drug-sensitive P388/S cell line (panels A and B). On the contrary, mouse L1210

Fig. 1. Freeze-fracture replicas of the plasma membranes from P388/R (panels a, c) and P388/S (panels b, d) cells. a, b: examples of protoplasmic faces, 90000×. c, d: examples of exoplasmic faces, 100000×. Arrowheads indicate exoendocytotic images. Scale bars, 0.2 μm. P388/S and P388/R cells were continuously propagated as ascitic tumors by serial passage into the abdominal cavity of DBA-2J mice [18]. The LD_{50} values for daunomycin in in vitro proliferation assays were of 0.008 and 0.4 μM, respectively, in the P388/S and P388/R cells. For freeze fracturing freshly isolated cells, previously separated from erythrocytes by centrifugation in Ficoll-Hystopaque and washed in phosphate-buffered saline, were centrifuged in Eppendorf tubes and the resulting pellets were fixed in 1% glutaraldehyde and 1% paraformaldehyde in phosphate buffer at 22°C, for 2 h. Fixed samples were washed several times with buffer, soaked for 2 h in buffer containing 20% glycerol, coated with polyvynyl alcohol [19], frozen in Freon 22 cooled with liquid nitrogen, fractured at −110°C and shadowed with platinum/carbon in a Balzers 400 D apparatus. A total of 3–6 replicas were prepared from each cell pellet. Fracture faces were photographed in a Jeol 100B electron microscope.

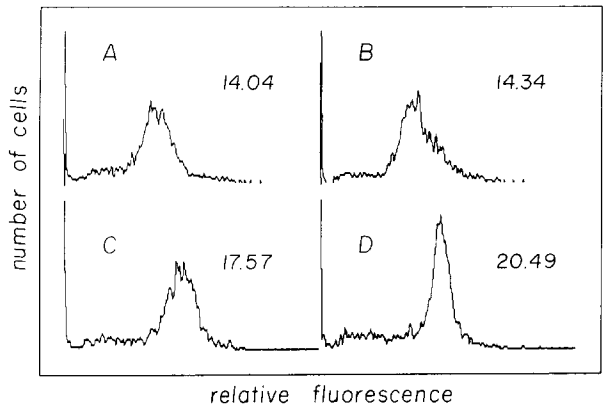

Fig. 2. Detection of P-glycoprotein by flow cytometry using fluorescein-labelled C219 monoclonal antibody. Panels correspond to flow cytometric analysis of Daunomycin-sensitive P388/S (A), approx. 50-fold resistant P388/R (B), approx. 100-fold resistant P388 (C) and 50–60-fold resistant L1210 (D) cells. Numbers within the figure are the mean fluorescence channels at which the different cell populations have been detected. A non-reactive, fluorescein-labelled IgG_{2a} monoclonal antibody was also used to evaluate the fluorescence due to non-specific antibody binding. Use of this non-reactive antibody resulted in detection of all cell populations analyzed at fluorescence channels ranging 11 to 12. For the flow cytometry assay, approx. 10^6 viable cells of each cell line, were washed in PBS several times, resuspended in 70% methanol and incubated at $-20\,^{\circ}C$ during 5–10 min to permeabilize the cell membrane, centrifuged and resuspended again in PBS. Then, 100 μl aliquots containing $2 \cdot 10^5$ permeabilized cells were incubated for 1 h, in a cold chamber, with 0.5 μg of either the C219 or the non-reactive antibodies, in the presence of 1% bovine serum albumin, and submitted to flow cytometry within 2–4 h. A minimum of 5000 cells of each cell line were analyzed per assay.

leukemia cells (panel D), with a similar degree of resistance to daunomycin as the P388/R cells (about 50 – 60-fold resistance) (panel C), and a P388 cell line selected for a higher resistance to daunomycin (approx. 100-fold resistance), reacted positively against antibody C219, indicating the occurrence of P-glycoprotein overexpression only in the latter cell lines. Therefore, regardless of the similarities found in the number of IMPs at the protoplasmic faces of plasma membranes from similarly resistant P388 and CHO cells, it is unlikely that P-glycoproteins would be responsible for the observed increase in IMP number reported here in the moderately resistant P388 cells. This suggests the possibility that there might be common features in the MDR phenotype of different cell lines, such as the increased number in plasma membrane IMPs, which are even more general resistance 'markers' than P-glycoprotein overexpression.

Analysis of IMP size (Table I) indicated that the observed differences in IMP number in the plasma membranes, are due to a population of IMPs 10 to 14.9 nm in diameter, which are present at a much greater density in membranes from P388/R cells. Particles smaller than 10 nm and those equal or larger than 15 nm, are distributed at similar numerical densities in both P388/S and P388/R cells. The observed size distribution of IMPs in P388 cell membranes does not resemble that reported in CHO cells [7]. This could partly be due to differences in the criteria used to measure IMP size (see footnote C in Table I), but it could also indicate that IMPs in P388 and CHO cells, result from the presence of different proteins at their plasma membranes. In fact, there are proteins other than P-glycoproteins, which undergo changes associated to MDR [1–3,12] and it is possible that they could be related to the observed morphology.

In summary, our results indicate that the expression of the daunomycin-resistant phenotype in P388 cells is associated to marked morphological alterations in the plasma membrane organization, which include differences in the number of exo-endocytotic images and IMPs. These results further suggest that modifications at the plasma membrane may be related to the mechanisms by which tumor cells become resistant to drugs.

This work has been supported by grants PM89-0004, PB87-0790 and PB87-0791 from the DGICYT of Spain. A.V.F.M. and P.V.E. have been recipients of predoctoral fellowships from 'Fondo de Investigaciones Sanitarias de la Seguridad Social' and 'Program de Formacion de Personal Investigador', respectively.

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