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P-glycoprotein expression and modulation of cell-membrane morphology in adriamycin-resistant P388 leukemia cells*

S. Radel[1], W. Fredericks[2], E. Mayhew[2, 3], and R. Baker[3]

[1] Department of Oral Biology, State University of New York at Buffalo, Buffalo, NY, 14214, USA
[2] Department of Experimental Pathology, and
[3] Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, NY 14263, USA

Summary. Electron microscopy of cultured P388 leukemia cells revealed morphological differences between the Adriamycin-resistant (P388/ADR) and -sensitive (P388/0) cell lines. P388/ADR cells showed longer villus-like protrusions and large foldings of the plasma cell membrane, whereas P388/0 cells had only short membrane protrusions. Western blot analysis of cells revealed the increased expression of a glycoprotein of 170–180 kDa (P-glycoprotein) in P388/ADR cells as compared with P388/0 cells, which is consistent with the findings of other studies. A modulation of the membrane morphology of in vitro P388/ADR cells was evident from one i.p. in vivo passage for 10 days, by a reversion to the non-ruffled sensitive-cell morphology. Long-term P388/ADR culture cells showed a reduction in membrane folding with as little as a 4-h exposure to the peritoneal ascitic fluid obtained when the tumor was harvested. However, there was no alteration in the expression of P-glycoprotein or in the sensitivity to Adriamycin in either of the P388 cell lines when grown in vivo or when exposed to ascites fluid from an i.p. tumor. Thus, the modulation of membrane morphology was related to the tumor cell environment rather than the abundance of P-glycoprotein in the plasma membrane.

Introduction

The effective treatment of malignant disease is often complicated by the presence or development of resistance to the chemotherapeutic drugs used. Tumors usually consist of heterogeneous cell populations displaying a broad response to anticancer agents [18, 21]. The administration of antineoplastic drugs can eliminate the sensitive cell population, whereas the resistant tumor cells may remain unaffected. Tumor cells resistant to many drugs have been shown to accumulate chemotherapeutic agents at a lower rate than the sensitive parental cell lines [6, 11]. Anthracycline resistance in the P388 Adriamycin-resistant murine leukemia cell line is associated with an energy-dependent, outward drug-transport system that results in sublethal intracellular concentrations of drug [4, 7]. The expression of a 170- to 180-kDa cell-surface glycoprotein (P-glycoprotein) at much higher levels in drug-resistant cells as compared with sensitive cells is correlated with chemotherapeutic resistance [8].

The present investigation was conducted to determine whether any alterations in membrane morphology between P388 Adriamycin-resistant (P388/ADR) and -sensitive (P388/0) leukemia cells exist and, if so, whether this modulation in morphology might be correlated to the degree of drug resistance. Some of the results have previously been reported in an abstract [15].

Materials and methods

Tumor cells. P388/ADR and P388/0 murine leukemia cells were supplied by the National Cancer Institute's Frederick Cancer Research Facility (Frederick, Md). These cells (determined to be mycoplasma-free) were grown in vitro in antibiotic-free media and have been maintained in our laboratory for 2 years, with routine drug-resistance checks (once every 2 months). P388/ADR cells have remained 93-100 times more resistant than P388/0 cells throughout this period (ID_{50}: P388/ADR, 100–140 n*M*; P388/0, 1.0–1.5 n*M*).

Cell culture. Tumor cells were maintained in plastic 75-cm^2 T-flasks (Becton-Dickinson Labware, Oxnard, Calif) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY) and a 10 μ*M* concentration of 2-mercaptoethanol (Fisher Scientific Co., Fair Lawn, NJ). Cell cultures were grown in a 37° C humidified atmosphere of 5% CO_2 in air. Each cell line was resupplied with fresh media every 24–28 h, maintaining a concentration of $4.0–6.0 \times 10^5$ cells/ml, which resulted in exponential growth with a cell-doubling time of approximately 24 h.

Electron microscopy. Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out on P388/ADR and P388/0 tumor cells. For both TEM and SEM, the P388 cell samples were fixed for 90 min in 3% glutaraldehyde in Sorensen's phosphate buffer (pH 7.3). The cell samples for TEM were then rinsed in Sorensen's buffer and postfixed in 1% OsO_4 (in Sorensen's buffer) for 90 min. The samples were dehydrated in graded alcohol solutions, embedded in epon-araldite, and sectioned on an LKB Ultratome III.

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Offprint requests to: S. Radel

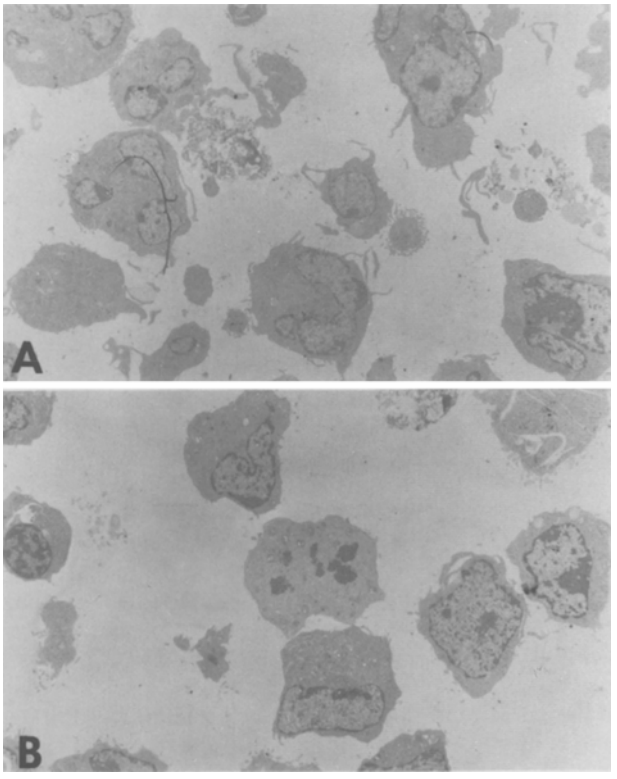

Fig. 1. Transmission electron micrographs of **A** P388/ADR cells and **B** P388/0 cells, ×6,000

The sections were then stained with uranyl acetate, counterstained with lead citrate, and examined on a Siemen's 101 electron microscope.

After fixation, the cell samples for SEM were filtered onto a 0.45 μm Nucleopore filter (Nucleopore Corporation, Pleasanton, Calif), rinsed in Sorensen's buffer, and postfixed in 1% OsO_4 for 90 min. The samples were dehydrated in graded alcohol solutions and critical-point-dried. The temperature was raised to 31° C under 1,080 psi pressure for 5 min, and then both pressure and temperature were slowly reduced, leaving a dry sample. The filter paper containing the cells was then attached to a mounting stub by painting with silver. The samples, under a vacuum, were coated with gold by heating a gold wire to its evaporation point in an Edwards E306 evaporator. The samples were then examined and micrographed in an ETEC Autoscan electron microscope.

P388 ascites cells. Established P388 culture cells (>200 passages) were injected at 2.5×10^6 cells/mouse (6- to 8-week-old female DBA/2J; Jackson Labs, Bar Harbor, Me). After 10 days, cells and ascitic fluid were harvested aseptically from the peritoneal cavity. Both the normal culture cells and the in vivo passaged culture cells were then plated into microtest plates (Falcon Labware, Oxnard, Calif) at 50,000 cells/well. The cells were incubated in either culture media or the harvested ascitic fluid. Samples were then removed at various times over a 24-h period and photographed under oil immersion (1,000) on

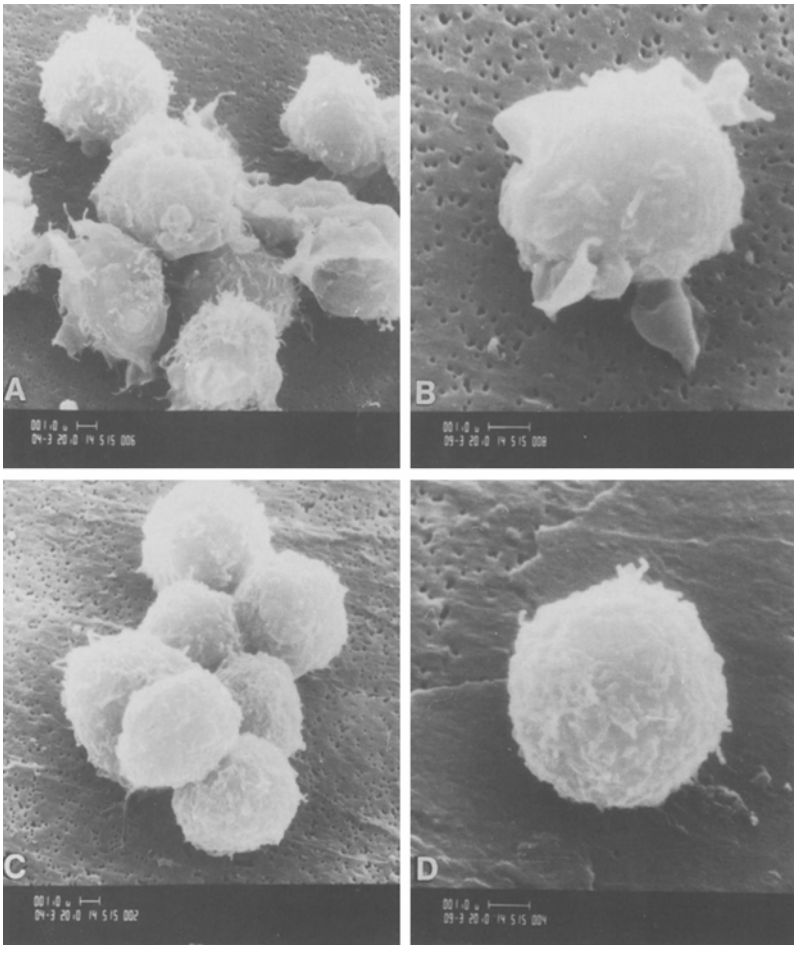

Fig. 2. Scanning electron microscopic photographs of (**A**, **B**) P388/ADR cells and (**C**, **D**) P388/0 cells. **A**, **C**, ×4,000; **B**, **D**, ×9,000

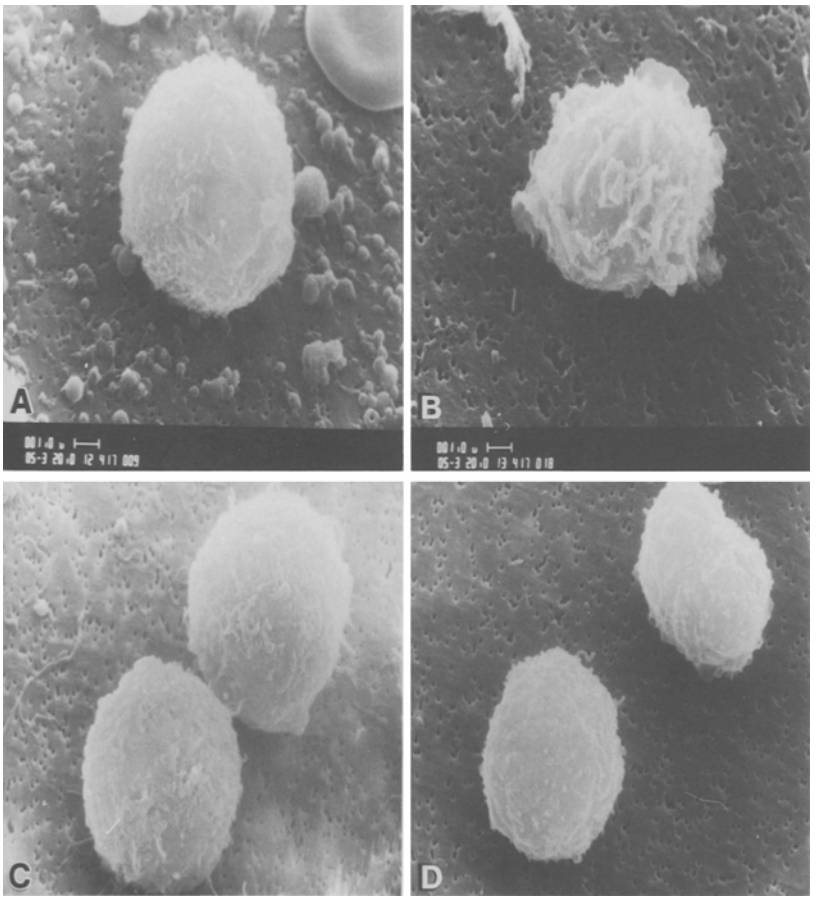

Fig. 3. Scanning electron micrographs of **A** P388/ADR cells and **C** P388/0 cells 10 days after implantation in the peritoneal cavity of a 7 week old DBA/2J mouse. After an additional 7 days of growth in culture. **B** P388/ADR cells revert to their original ruffled morphology indicative of drug resistance, whereas **D** P388/0 cells remain morphologically unchanged

an Olympus automatic photomicrographic system (Model PM-10ADS).

Membrane preparation. For each cell line assayed, a crude membrane fraction of microsomes and plasma membranes were prepared from frozen suspensions as described by Riordan and Ling [16], with slight modifications. After thawing briefly in a 37° C water bath, cells were placed on ice and maintained at 4° C throughout the subsequent steps. Cells were washed three times with ice-cold PBS by centrifugation at 400 *g* for 15 min and then resuspended to $1.0-5.0\times10^7$ cells/ml in a hypotonic lysis buffer [10 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* TRIS-HCl (pH 7.4)] to which a cocktail of protease inhibitors had been added [2 m*M* phenyl-methyl sulfonyl fluoride (PMSF), 1 m*M* *N*-*p*-tosyl-*l*-arginine methyl ester (TAME), 1% aprotinin]. After 15–30 min, the swollen cells were ruptured using a Stansted cell disrupter and the crude subcellular fractions were removed by sequential centrifugations. Nuclei and cell fragments were pelleted by centrifugation at 400 *g* for 10 min and the mitochondrial fraction was removed by pelleting at 4,000 *g* for 10 min. A membrane fraction was collected by ultracentifugation at 100,000 *g* for 1 h and was resuspended in 8.6% sucrose in 5 m*M* TRIS (pH 7.4) and frozen at −70° C. Protein content from this membrane fraction was determined by Peterson's modification of the Lowry procedure [14].

Electrophoresis and Western blotting procedures. Membranes were solubilized in a buffer consisting of a final concentration of 2% SDS, 50 m*M* dithiothreitol, 1 m*M* EDTA, 10% sucrose, and 10 m*M* TRIS-HCl (pH 8.0) by boiling in a water bath for 5 min, after which urea was added to a concentration of 4.5 *M*. A modification of the Fairbanks procedure [5] that includes the addition of 1% SDS and 9 *M* urea to the SDS-PAGE of the solubilized membrane fraction was used. Electrophoretic fractionation was done at 5 W constant power for 3–4 h using pyronin-Y as a tracking dye. Gel protein profiles were transferred onto nitrocellulose by the Towbin Western electroblotting procedure [20] for 4 h in a Hoeffer transfer apparatus. The nitrocellulose filter was then incubated in 5% BSA in PBS for 24 h at 37° C to block remaining protein-binding sites and, again incubated overnight at 4° C in ^{125}I-labeled P-glycoprotein-specific monoclonal antibody C219 [9] at $2.0-5.0\times10^5$ cpm/ml; 5–10 mCi/mg in blocking solution. Blots were washed 4 times in PBS with gentle shaking. The air dried blots were then autoradiographed onto Kodak X-Omat R film in cassettes fitted with Dupont Cronex Lightening-Plus intensifying screens at −90° C for 24–48 h. In addition, the amount of bound label was quantitated by excising relevant portions from the blots and counted directly. From the slope ratios of plots of the amount of bound label per microgram of membrane protein loaded, different cell lines' P-glycoprotein levels were compared.

Results

Electron microscopy of P388 culture cells

P388 cells were examined using both Transmission electron microscopy (TEM) and Scanning electron

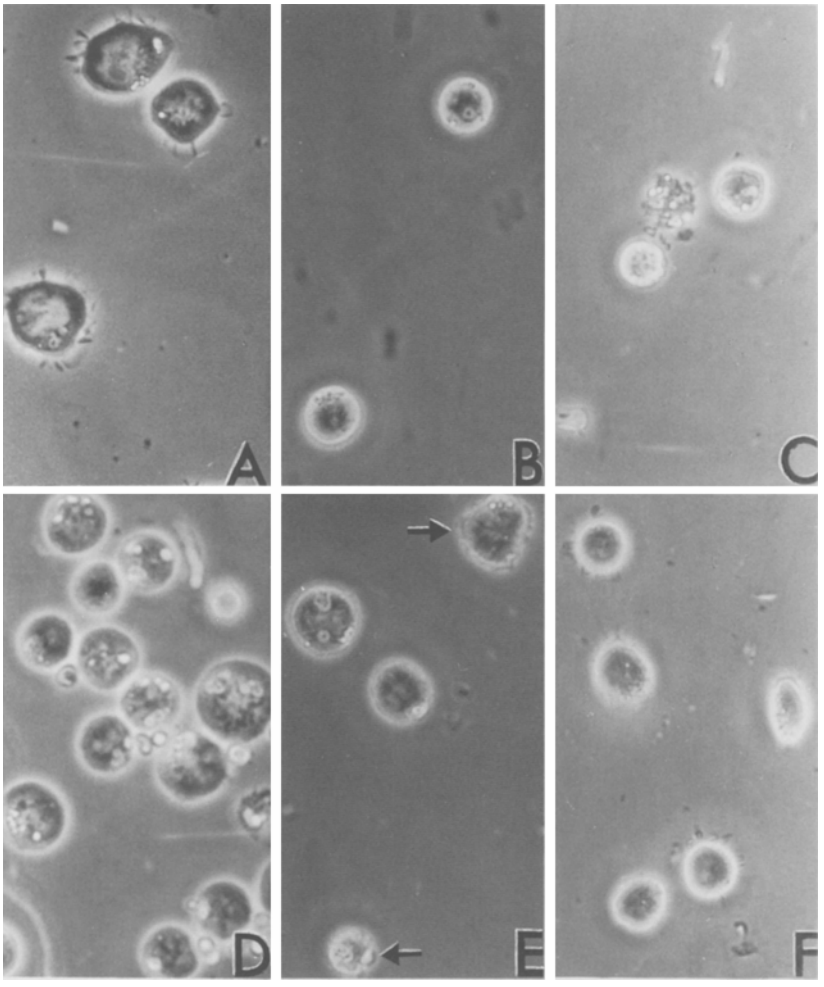

Fig. 4. Photographs of P388/ADR cells exposed to tumor ascitic fluid or normal culture media (ascitic fluid harvested after 10 days from the peritoneal cavity of a mouse bearing an i.p. tumor). **A** Culture cells exposed to media (morphology did not change over the treatment period); **B, C** Culture cells exposed to undiluted ascitic fluid for 4 and 24 h, respectively; **D, E, F** in vivo passaged cells exposed to media for 0, 4 and 24 h, respectively

microscopy (SEM). TEM revealed that P388/ADR cells had long, villus-like processes of the plasma membrane, which P388/0 cells lacked (Fig. 1). SEM examination revealed more clearly the cell-membrane differences between the two cell lines (Fig. 2). P388/ADR cells (Fig. 2 A, B) exhibited not only the longer villus-like processes seen in TEM but also large foldings of the cell membrane that were absent in P388/0 cells (Fig. 2 C, D). These membranous folds could greatly increase the surface area of P388/ADR as compared with P388/0 cells.

Effects of in vivo passage on P388 culture cells

Established P388/ADR and P388/0 leukemia culture cells were passed once in vivo (i.p. for 10 days) in DBA/2J mice. The effect on cell morphology is shown in the electron micrographs of harvested cells (Fig. 3). After 10 days in vivo, the P388 Adriamycin-resistant cells (Fig. 3 A) demonstrated an alteration in morphology to the smoother, non-ruffled appearance of the drug-sensitive cells (Fig. 3 C). However, after growth in culture for 7 days, a reversion to the "drug-resistant morphology" was evident in the P388/ADR cells (Fig. 3 B). No change in the morphology of the Adriamycin-sensitive cells (Fig. 3 D) resulted from this treatment.

Possible alterations in P388/0 and P388/ADR leukemia cell morphology was also examined using ascitic fluid or normal growth media. Both normal culture cells and in vivo passaged culture cells were plated in microtest plates containing undiluted tumor ascitic fluid (obtained when cells were harvested from the peritoneal cavity) or normal cell culture media and then monitored for 24 h (tumor cells incubated in ascites fluid were in a static growth phase). The morphology of P388/0 cells did not change under any of the conditions to which the cells were subjected. Figure 4 A illustrates the membrane folding seen in the continuously cultured P388/ADR cells. However, exposure of P388/ADR culture cells to ascitic fluid resulted in a modulation in the surface morphology to a less ruffled "drug-sensitive morphology" after only 4 h (Fig. 4 B) that was maintained after 24 h (Fig. 4 C). Conversely, development of the cellular "drug-sensitive morphology" in P388/ADR cells after one in vivo passage (Fig. 4 D) showed a partial reversion to the original, ruffled "drug-resistant morphology" after a 4-h incubation with media (Fig. 4 E; *arrows* indicate membrane ruffling), which progressed further by 24 h (Fig. 4 F). After 7 days in culture media, the in vivo passaged P388/ADR cells returned to their original "drug-resistant morphology". This apparently indicates that either a tumor-cell-associated factor(s) or a product released by other cells in vivo can modulate the surface-membrane morphology of Adriamycin-resistant P388 cells.

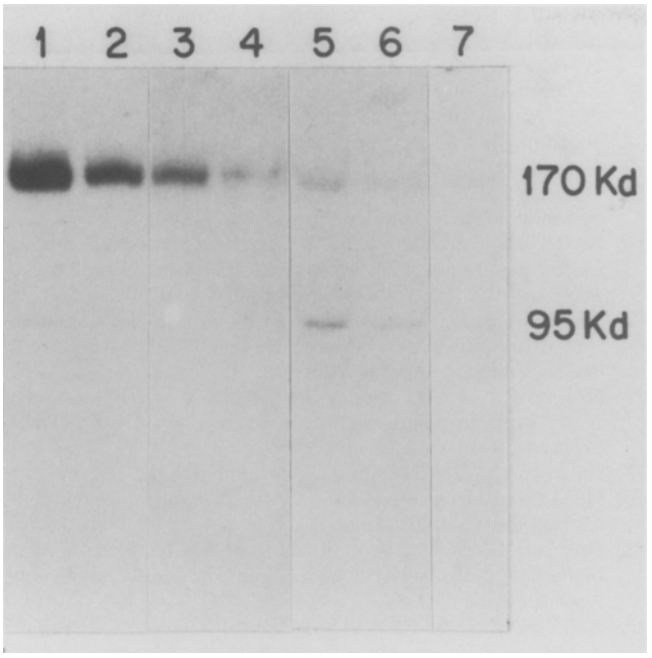

Fig. 5. Comparison of P-glycoprotein expression in P388/ADR and P388/0 cells. Plasma membranes were examined through western blot analysis using ^{125}I labeled monoclonal antibody C219 specific for P-glycoprotein. *Lanes 1–4:* P388/ADR culture cells loaded at 50, 25, 12.5 and 6.25 µg, respectively. *Lanes 5–7:* P388/0 culture cells loaded at 100, 50, and 25 µg, respectively

P-glycoprotein content of P388 cells

All cell lines (both the normal culture cells and the in vivo passaged cells) were examined through western blot analysis as to membrane P-glycoprotein content. Chinese hamster ovary (CHO) cell line $AuxB_1$ and its multidrug-resistant clones A_3 and C_5 were used as controls. The colchicine sensitivity of the cell lines was determined as follows: B_1 was sensitive, A_3 was 2-fold resistant, and C_5 was 184-fold resistant [1]. The drug-resistant cell lines also displayed the typical pleiotropic pattern of cross-resistance to other chemotherapeutic agents. Figure 5 shows a much higher expression of P-glycoprotein in P388/ADR cells than in P388/0 cells, as has previously been reported [10]. No modulation in the expression of P-glycoprotein was evident for either the sensitive or resistant lines when normal culture cells were compared with in vivo passaged cells (Fig. 6). The only difference observed was an increase in a 95 kDa component from the in vivo passage, which could be a modulation in the expression of a different protein or a degradation product of the P-glycoprotein. This lack of an effect on the expression of membrane P-glycoprotein correlated with the lack of modulation in the degree of Adriamycin sensitivity when cultured cell lines were compared with in vivo passaged cell lines.

Discussion

The emergence of a drug-resistant tumor-cell population is one of the major causes of the failure of cancer chemotherapy [17, 18]. One of the mechanisms of drug resistance in many tumors is believed to be an increased outward transport of drug [3, 19]. Studies have demonstrated the enhanced expression of a 170,000- to 180,000-dalton cell-surface glycoprotein (P-glycoprotein) in many multidrug-resistant tumor cells that was detected at low levels in the sensitive parental line [13]. The increased expression of P-glycoprotein has been shown to be connected to an active drug-efflux process [2, 7]. P388/ADR culture cells in our system also expressed a much higher level of surface P-glycoprotein than did P388/0 cells.

Electron microscopic investigation revealed membrane morphological differences between P388/ADR and P388/0 cells. SEM photographs of P388/ADR cells showed long villus-like protrusions and large foldings of the cell membrane, whereas P388/0 cells had only short membrane protrusions. The large, ruffled foldings of the cell membrane in P388/ADR cells would produce a greater cell surface area, than P388/0 cells, which could better facilitate the outward transport of Adriamycin through an increased number of drug-efflux units. However, a recent study reported that an Adriamycin-sensitive murine mammary adenocarcinoma cell line had a rougher membrane surface, with more ruffles than its 3-fold-resistant counterpart, as seen by SEM [22]. This is exactly the opposite of the morphological variation in the plasma membranes of the sensitive P388 cell line. Changes in cell-membrane morphology could be related to the magnitude of drug resistance and/or the type of tumor, or even to chance variation in clonal characteristics.

A study recently reported that, in addition to the decreased accumulation of intracellular drug attributed to the P-glycoprotein, another mechanism of increased drug resistance in P388/ADR cells involved an altered glutathione redox cycle that detoxifies reactive oxygen

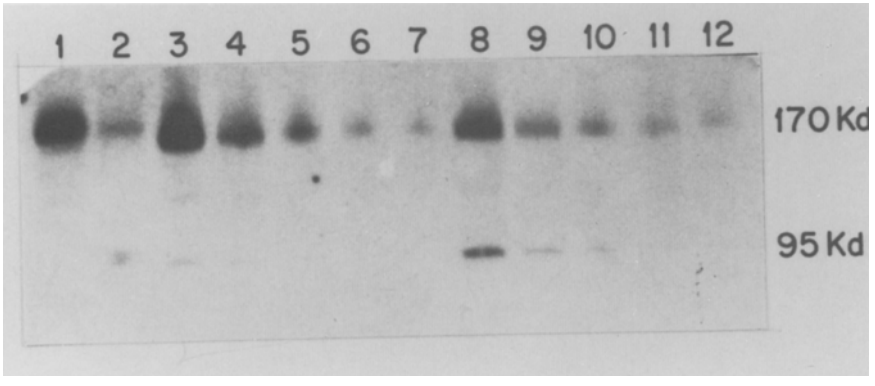

Fig. 6. Western blot analysis of P388/ADR cells: Quantitation of P-glycoprotein expression. Plasma membranes, isolated from resistant cells were examined using ^{125}I labeled monoclonal antibody C219 specific for P-glycoprotein. *Lane 1:* the colchicine resistant A_3 cell line; *Lane 2:* the colchicine sensitive B_1 cell line (each loaded at 50 µg). *Lanes 3–7:* P388/ADR culture cells loaded at 50, 25, 12.5, 6.25 and 3.12 µg, respectively. *Lanes 8–12:* P388/ADR culture cells passed once in vivo (10 days i. p.) loaded at 50, 25, 12.5, 6.25, and 3.12 µg, respectively

species [12]. It is not known whether plasma-membrane morphology could be related to the activity of other such mechanisms of drug resistance.

The modulation of membrane morphology seemed to be related to the tumor-cell environment. One passage in vivo of P388/ADR cells altered their morphology to that of the sensitive cells (more rounded in appearance). However, this alteration did not result in a concomitant down-modulation in the expression of surface P-glycoprotein; it could be due to a change from an in vitro environment, where conditions are kept constant, to an in vivo environment, where many other factors would affect the tumor cells. This morphological change seemed to be related to factor(s) present in the ascitic fluid, since P388/ADR culture cells incubated with ascitic fluid removed from the periotoneal cavity of a mouse bearing an i. p. tumor demonstrated a reversion to the less-ruffled, sensitive-cell morphology after as little as a 4-h exposure. Conversely, P388/ADR cells passed in vivo and then incubated in regular media in vitro displayed a modulation from their rounded morphology to a more ruffled morphology within 24 h of exposure. Furthermore, in vitro Adriamycin toxicity studies demonstrated no alteration in the degree of resistance in these morphologically altered P388/ADR cells. This modulation in the membrane morphology of tumor cells in vivo is likely to be the result of tumor-cell or host factors.

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