The PO_2 of the dead space of a soft tissue wound varies from 20 mmHg to near-anoxia. Our data show that less than 2×10^4 macrophages cultured under conditions similar to those of a wound (2 percent O_2, 15 mmHg) and (0 percent O_2, near-anoxia) actively stimulate angiogenesis. When cultured at a PO_2 similar to that of arterial blood (10 percent O_2, 76 mmHg) and to that of tissue (5 percent O_2, 38 mmHg), macrophages did not stimulate a substantial angiogenic response. Under normal tissue culture conditions (20 percent O_2, 152 mmHg) in gas-permeable culture vessels, there was no angiogenic response at all. However, normal tissue culture O_2 concentrations are unlikely to be experienced by normal tissues other than alveoli.

The relation of angiogenesis to tissue hypoxia has been observed in other systems. The central core of a malignant neoplasm is hypoxic (*12*) owing to the metabolic hyperactivity of tumor cells and the lack of circulation in the central portion of the tumor. Macrophages constitute 10 to 30 percent of the cells in mouse (*13*) and human (*14*) tumors, but the significance of this is controversial (*15*); no correlation has been found between macrophage content of tumors and tumor vascularization. However, because the secreted products of fewer than 2×10^4 macrophages stimulate angiogenesis under hypoxic conditions, it is possible that macrophages caught in the growing tumor will experience hypoxia sufficient to increase the amount of active macrophage angiogenesis factor. The similarities, if any, between tumor angiogenesis factor (*16*) and macrophage angiogenesis factor are not known. Nevertheless, macrophages may contribute to the vascularization of certain rapidly growing hypoxic tumors.

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Cell Surface P-Glycoprotein Associated with Multidrug Resistance in Mammalian Cell Lines

Abstract. *The plasma membranes of hamster, mouse, and human tumor cell lines that display multiple resistance to drugs were examined by gel electrophoresis and immunoblotting. In every case, increased expression of a 170,000-dalton surface antigen was found to be correlated with multidrug resistance. This membrane component is of identical molecular size and shares some immunogenic homology with the previously characterized P-glycoprotein of colchicine-resistant Chinese hamster ovary cells. This finding may have application to cancer therapy.*

Selection of variants in mammalian cells that are resistant to specific drugs, such as *Vinca* alkaloids, maytansine, colchicine, anthracyclines, actinomycin D, or bleomycin, is often accompanied by expression of a complex phenotype of cross resistance to various unrelated drugs (*1–14*). This characteristic is referred to as the multidrug resistance phenotype. The generation of such variants in tumor cells may be an important mechanism by which neoplasms become resistant to treatment by combination chemotherapy.

Studies in model systems indicate that multidrug resistance results from a reduced cellular accumulation of the drugs involved (*5–19*), and changes in the plasma membrane have been observed (*17–23*). In the well-characterized colchicine-resistant (CH^R) Chinese hamster ovary (CHO) system, for example, genetic analyses involving cell-cell hybrids, drug-sensitive revertants, and DNA-mediated transformants of the CH^R phenotype indicate that multidrug resistance, colchicine resistance, and reduced drug accumulation are the result of the same genetic alteration (*21, 24, 25*). Moreover, the expression of a 170,000-dalton plasma membrane glycoprotein (P-glycoprotein) is invariably associated with this pleiotropic phenotype (*20–22, 24*). The degree of drug resistance is correlated approximately with the amount of P-glycoprotein present (*20, 22*). The objective of the present study is to determine whether or not P-glycoprotein expression is also associated with the multidrug resistance phenotype observed in other cell systems.

Each of the different mammalian cell lines examined in this study (Table 1) was originally selected for resistance to a specific drug, and in each case a multidrug resistance phenotype typified by cross resistance to unrelated compounds was observed. Such a phenotype appears to reflect a membrane-associated alteration (*6, 24, 26*). We therefore pre-

pared membranes from these cell lines for analysis of their components by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*22*). For CH^R CHO cells (lanes b to d in Fig. 1A), a protein having a molecular size of about 170,000 daltons appeared in the plasma membrane with a staining intensity proportional to the degree of resistance to the drug. As previously characterized, this band is referred to as the P-glycoprotein (*20, 22*). Similarly stained bands of approximately the same molecular size are seen in the other drug-resistant cell lines that we examined (lanes e, h, j, and l in Fig. 1A). Such a component could not be detected by protein staining in the corresponding drug-sensitive parental cells (lanes a, g, i, and k) or drug-sensitive revertant (lane f).

As a means of further characterizing the relatedness of the 170,000-dalton membrane components observed in these drug-resistant lines, an antiserum to plasma membrane vesicles of the highly colchicine-resistant CHO line CH^RC5 was prepared. The specificity of this antiserum for the P-glycoprotein was improved by cross absorption with immobilized, detergent-solubilized plasma membrane proteins of the parental drug-sensitive line (*21, 27*). The absorbed antiserum was then used to examine the membrane components of the lines shown in Fig. 1A. Each of the drug-resistant lines expressed a 170,000-dalton component that was stained by this antiserum (Fig. 1B). It is clear from the size and cross-reactivity of this component that it is similar to the P-glycoprotein of the CH^R CHO cells. Staining of other components of about 50,000 and 200,000 daltons is also observed with this antiserum. We believe that these components are not related to the P-glycoprotein or multidrug resistance because they vary with different preparations of mem-

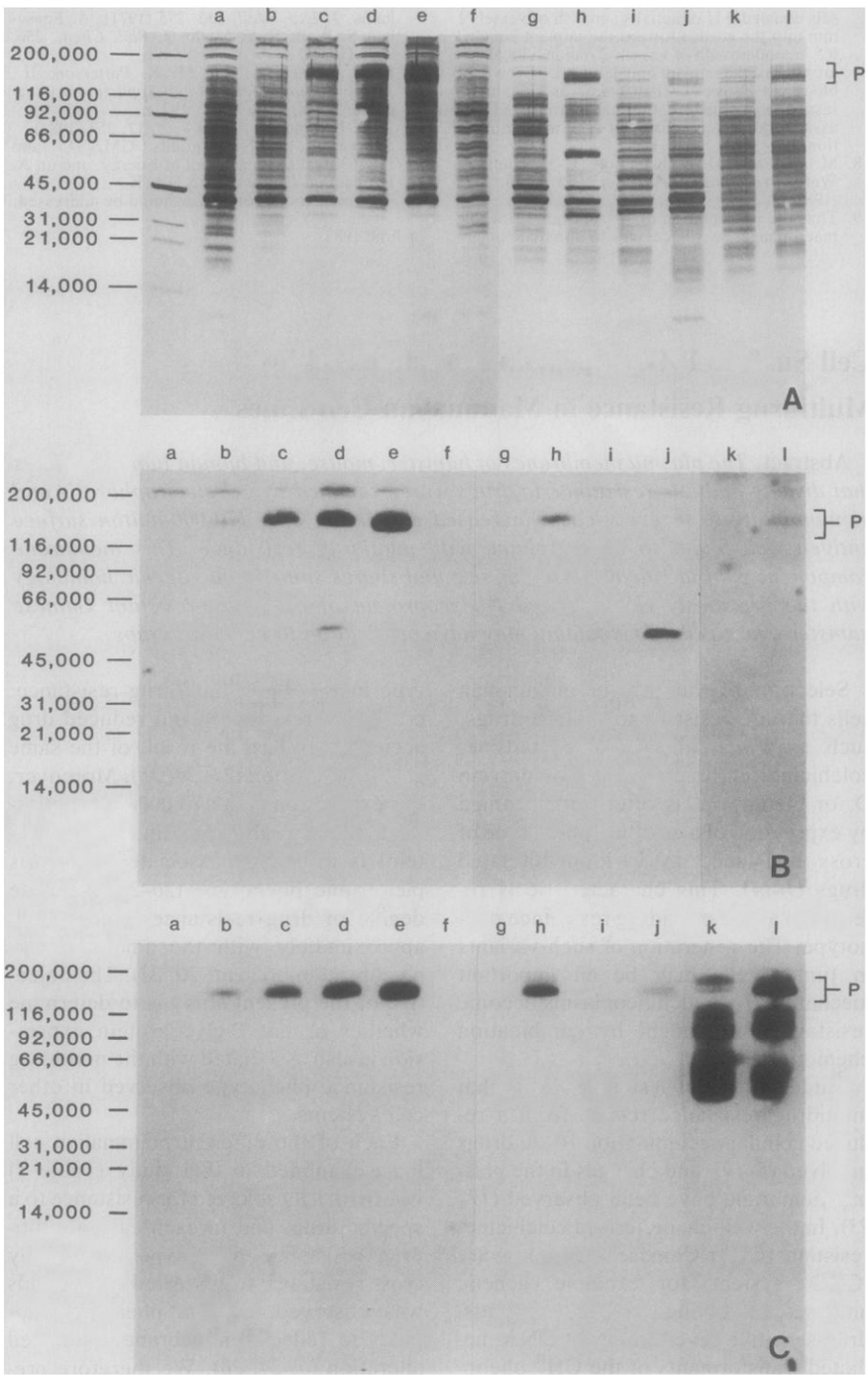

Fig. 1. Electrophoretic and immunochemical analysis of plasma membrane components. Cell culture, plasma membrane isolation, and electrophoresis were carried out as described previously (*21, 22, 27*). (A) Gels were loaded with 10 μg of membrane protein per lane. They were overstained by the silver staining method of Switzer *et al.* (*34*) and were then reduced to acceptable intensity by soaking in a solution of 0.03*M* ferric ammonium sulfate in 0.18*M* sulfuric acid diluted 1:10, followed by a final rinse in 0.3*M* sodium carbonate and thorough washing in water. Molecular size standards (Bio-Rad) are shown in the first lane. These consist of 0.1 μg each of myosin, (200,000), β-galactosidase (116,250), phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,500), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). (Lane a) Drug-sensitive parent CHO cell, AUXB1; (lane b) colchicine (0.10 μg/ml)-resistant CHO cell CH^RA3; (lane c) colchicine (3.0 μg/ml)-resistant CHO cell CH^RB3; (lane d) colchicine (10 μg/ml)-resistant CHO cell CH^RC5; (lane e) daunorubicin-resistant CHO cell DNR^R51; (lane f) colchicine-revertant CHO cell I10 (derived from CH^RC5); (lane g) sensitive parent SV40-transformed Syrian hamster cell Cl_2TSV_5S; (lane h) actinomycin D–resistant SV40-transformed Syrian hamster cell $Cl_2TSV_5R_2$; (lane i) sensitive mouse L cell $LMTK^-$; (lane j) colchicine (0.50 μg/ml)-resistant mouse L cell ECH^R; (lane k) sensitive parent human lymphoid cell CCRF-CEM; (lane l) vinblastine-resistant human lymphoid cell CEM/VLB_{100} (see Table 1 for further details). The P-glycoprotein region is indicated (*P*) at molecular size 150,000 to 170,000 daltons. Lanes a to f and g to l represent two separate gels that were run simultaneously and treated identically. (B) SDS-PAGE was performed as in (A), with the exception that 50 μg of membrane protein was loaded per lane. Western blots were overlaid with previously absorbed rabbit antiserum (diluted 1:100) against isolated plasma membranes from the colchicine-resistant CHO cells CH^RC5 and processed as described (*21, 27*). Lanes a to f and g to l are two separate blots prepared and treated identically, but lanes k and l were exposed to film four times longer than other lanes. All lanes are as described in (A). (C) SDS-PAGE was performed as in (B), but blots were overlaid with unabsorbed serum prepared against CEM/VLB_{100} plasma membranes (diluted 1:100). Lanes k and l were exposed to film for one-tenth the exposure time of other lanes.

Table 1. Description of cell lines. Cell line CH^RA3 was selected from AUXB1; CH^RB3 was selected from CH^RA3 in a second step; and CH^RC5 was selected from CH^RB3 in a third step (*6*). DNR^R51 was selected from AUXB1 in two steps (*27*). I10 is a revertant cell line, selected in a single step from CH^RC5 (*24*). Abbreviations for drugs are: ACR, acriflavine; ADR, adriamycin; AMD, actinomycin D; CCH, colchicine; CMD, colcemid; CYB, cytochalasin B; DNR, daunorubicin; EME, emetine; ERY erythromycin; ETB, ethidium bromide; GRD, gramicidin; PRO, proflavin; PUR, puromycin; VCR, vincristine; and VLB, vinblastine. Relative resistance was calculated as the ratio of drug concentration tolerated by the resistant cells to that tolerated equally by the sensitive parent cell. Tolerance was assessed by relative growth rates except for cell lines I10 and ECH^R, whose colony-forming abilities in the presence of drug were compared with the appropriate parental lines. Since cross resistance is shown only for drugs that have been tested and the results reported, absence of a drug from the column on cross resistance does not imply lack of cross resistance to that drug. The drugs are listed in order of decreasing relative cross resistance.

Species	Parent line	Resistant line	Drug of selection	Selection concentration (μg/ml)	Relative resistance	Cross resistance	References
Chinese hamster	AUXB1	CH^RA3	CCH	0.10	6	PUR ETB DNR CCH ERY	(*1, 6*)
		CH^RB3	CCH	3.0	21	PUR ETB DNR CCH EME ACR CYB ERY	(*1, 6*)
		CH^RC5	CCH	10.0	184	CCH GRD PUR DNR EME VLB ADR CMD ACR ETB CYB ERY PRO	(*1, 6*)
		DNR^R51	DNR	0.50	30	DNR PUR CCH VLB EME	(*27*)*
		I10			3	CCH CMD VLB DNR PUR	(*24*)
Syrian hamster	$C1_2TSV_5S$	$C1_2TSV_5R_2$	AMD	2.0	1750	AMD PUR PRO	(*10*)
Mouse	$LMTK^-$	ECH^R	CCH	0.50	26	CCH PUR CYB AMD EME	(*21*)
Human	CCRF-CEM	CEM/VLB_{100}	VLB	0.10	270	VCR VLB PUR CCH DNR EME	(*19*)*

*Cross resistance data were based on growth assay, as described (*1*).

branes. The relatively faint staining of the presumptive human P-glycoprotein by the antiserum is likely due to reduced cross-reactivity of this component with the antiserum against the CHO cell P-glycoprotein.

To further corroborate the above conclusions, we stained the membrane components with an antiserum to plasma membrane of the vinblastine-resistant, human cell line, CEM/VLB_{100} (Fig. 1C). In this case, the serum was not previously absorbed with membrane proteins from drug-sensitive cells, and many antigens common to both drug-resistant and drug-sensitive human cells were strongly stained (lanes k and l; note the exposure time). This crude antiserum stained only the drug resistance–associated P-glycoprotein in the rodent cell membranes (Fig. 1C). Thus, of the dozen or so different cell surface antigens that can be detected with the two antiserums, only the P-glycoprotein is consistently stained in all drug-resistant mammalian cell lines tested. These observations, and the fact that a side-by-side comparison of the different P-glycoprotein bands reveals no significant difference in molecular size, as shown in Fig. 1, strongly indicate that the P-glycoprotein is conserved relative to other mammalian membrane antigens that are detectable by Western blotting.

Our observations that P-glycoprotein is present in increased amounts in various drug-resistant lines, and that in the CH^R CHO system the amount of P-glycoprotein expressed is correlated with the degree of resistance, are consistent with a mechanism of resistance involving gene amplification. The appearance of double minute chromosomes has been correlated with unstable drug resistance in known gene-amplified systems (*28, 29*). Double minute chromosomes have also been observed in association with multidrug resistance in several mouse cell lines including colchicine-resistant lines for which the degree of resistance and P-glycoprotein expression correlate with the number of double minutes contained within the cells (*14, 30*). Double minutes have also been reported in multidrug-resistant hamster cells that were originally selected for colchicine resistance (*30, 31*). This speculation on the origin of the P-glycoprotein in drug-resistant cells suggests its preexistence, in much smaller amounts, in the drug-sensitive parent cell. In this context, we observed a barely detectable, antigenically cross-reactive band of the same molecular size as the P-glycoprotein in all the drug-sensitive cells examined in Fig. 1B when the film was exposed for a much longer period (data not shown). What specific role the P-glycoprotein might play in the maintenance of the multidrug resistance phenotype, or in the drug-sensitive cells, is unknown. It is also not yet known whether the P-glycoprotein is expressed in normal tissues.

Our findings could have important implications for cancer therapy. It is possible that clinical resistance to combination chemotherapy might result from an unchecked proliferation of tumor cell subpopulations with a multidrug resistance phenotype (*33*). The present data indicate that there is a strong correlation between the expression of multidrug resistance and surface P-glycoprotein in different species of drug-resistant cells established in vitro. It is reasonable to suppose that such a relationship might also exist in vivo. If P-glycoprotein is commonly present in tumor cells from patient biopsies, and if this antigen is expressed in increased amounts in tumors nonresponsive to treatment by combination chemotherapy, immunochemical screening for the antigen could provide a rapid diagnostic basis for planning treatment of cancer patients. Moreover, hitherto unresponsive tumors may become amenable to treatment with P-glycoprotein–targeted antibodies conjugated with toxins.

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Turnover, Membrane Insertion, and Degradation of Sodium Channels in Rabbit Urinary Bladder

Abstract. *Noise analysis of rabbit bladder revealed two components: Lorentzian noise, arising from interaction of amiloride with the Na^+ channel, and flicker noise (1/f, where f is frequency), as in other biological membranes. Hydrostatic pressure, which causes exchange between intracellular vesicular membrane and apical membrane, increases the number but not the single-channel current of the amiloride-sensitive channels. Flicker noise arises from degraded channels that have lost amiloride sensitivity and Na^+ to K^+ selectivity. The degraded channels were selectively removed by washing the mucosal surface. These results imply channel turnover by intracellular synthesis, transfer from vesicular to apical membrane, degradation, and elimination.*

Most proteins are subject to molecular turnover, which serves at least two functions: regulation of protein concentration by the balance between rates of synthesis and degradation of the protein and elimination of damaged or defective protein molecules (*1*). Among ion permeation channels, turnover has been demonstrated only for the acetylcholine receptor and gap junction (*2*). Yet turnover would seem to be essential for regulating hormonally controlled channels and for channel repair in epithelia exposed to fluids with varying composition and damaging chemicals (for example, urine). We now provide electrophysiological evidence for turnover of Na^+ channels in rabbit urinary bladder.

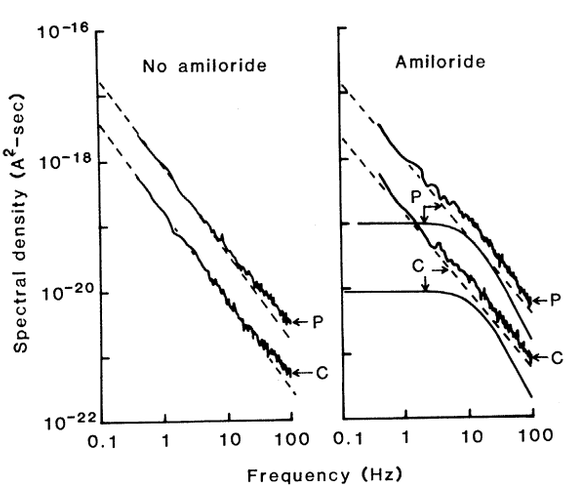

Fig. 1. Power density spectrum of a rabbit bladder, with (right) or without (left) amiloride (1.4 μM), and before (*C*) or after (*P*) punching. Data (wiggly curves) were fitted to the equation $S(f) = B/f^{\alpha}$ without amiloride, $S(f) = B/f^{\alpha} + S_o/[1 + (f/f_c)^2]$ with amiloride. The B/f^{α} component (1/*f* noise; dashed lines) is essentially the same with and without amiloride, whereas punching increases B but not α. Punching increases S_o but not f_c of the $S_o/[1 + (f/f_c)^2]$ component (Lorentzian noise; solid smooth curves).

Rabbit bladder maintains Na^+ gradients between urine and blood by active Na^+ absorption, which is regulated by aldosterone and can be inhibited partly but not entirely by the drug amiloride (*3*). The epithelial cells contain vesicles, which move between cytoplasm and the apical (lumen-facing) membrane and thereby accommodate the membrane area to fluctuations in bladder volume (*4*). Movement of vesicles depends on microfilaments and can be produced experimentally by rapid, repeated application of hydrostatic pressure to the apical surface ("punching") (*5*). This procedure increases short-circuit current (I_{sc}) and transepithelial conductance (G_t), suggesting that Na^+ entry channels are being transferred from vesicular to apical membrane.

We studied channels by the technique of noise analysis, which measures fluctuations in I_{sc} due to reversible blocking of channels by amiloride. Following the method of Lindemann and Van Driessche (*6*), we assume N statistically independent channels, each with single-channel current i. Combination of amiloride (A) and receptor (R) to form a blocked channel (AR) is described by

$$A + R \underset{K_{10}}{\overset{K_{01}}{\rightleftarrows}} AR$$

where K_{01} and K_{10} are rate constants. The residual amiloride-sensitive current (I_A') at amiloride concentration A, is

$$I_A' = iNK_{10}/(K_{01}A + K_{10}) \quad (1)$$

Fluctuations in I_A' yield a power density spectrum $S(f)$ (f, frequency in hertz) with a single time constant; that is, a Lorentzian spectrum $S(f) = S_o/[1 + (f/f_c)^2]$. The corner frequency f_c and plateau value S_o are

$$f_c = (K_{01}A + K_{10})/2\pi \quad (2)$$

$$S_o = 4iaI_A' K_{01}A/(K_{01}A + K_{10})^2$$
$$= 4Ni^2aK_{01}AK_{10}/(K_{01}A + K_{10})^3 \quad (3)$$

where a is membrane area.

We first determined S_o and f_c at five amiloride concentrations. Linear dependence of f_c on A yielded K_{01} and K_{10} by Eq. 2. Values of i and N were then determined from Eqs. 1 and 3 for each A value, and all five values were averaged (*7*).

Rabbit bladder epithelium, stripped of underlying muscle, was mounted between identical solutions at 37°C in chambers (area, 2 cm^2) designed to eliminate edge damage [see (*3*) for details of methods]. Values of I_{sc} and G_t were