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Alterations in Cell Surface Membranes in Chinese Hamster Lung Cells Resistant to Adriamycin

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Summary: Studies have been carried out to analyze plasma membrane proteins in chinese hamster lung cells resistant to the action of adriamycin. The cell surface was labeled using the lactoperoxidase catalyzed iodination procedure and the proteins were analyzed after gel electrophoresis. The results suggest that the plasma membranes of resistant cells are chemically altered. These alterations include a marked reduction in the labeling of a 100,000 molecular weight protein which is a major iodinated component of sensitive cells. In addition, a protein having a molecular weight of about 180,000 is iodinated in resistant cells but is not detected in cells sensitive to adriamycin. A protein with this same molecular weight is also highly labeled in resistant cells grown in the presence of [^{14}C]glucosamine. In sensitive cells this protein is not detected. The finding that these same surface changes are observed in several different isolates of drug resistant cells suggest they may be involved in the molecular basis of adriamycin resistance.

Introduction: Adriamycin is an anthracycline antibiotic which is currently being used as an effective chemotherapeutic agent in the treatment of patients with certain leukemias or solid tumors (1). One major problem, however, with adriamycin chemotherapy is that in some instances patients acquire a resistance to the drug and thus do not respond to its cytostatic action. The basis of this resistance is unknown.

Adriamycin or the closely related analog daunomycin can induce resistance in vitro when cells are grown in the presence of drug (2-5). In many systems examined these cells show a cross resistance to various chemicals which are structurally unrelated to adriamycin (6, 7). Biochemical analysis suggests that resistance is primarily due to low levels of drug transport into cells (2, 4, 5). Other factors such as an enhanced outward transport of drug and lower affinity for intracellular binding sites may also contribute to the resistant phenotype (5, 8). Since membrane restriction to drug may play an important role in adriamycin resistance we have analyzed cell surface proteins

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in sensitive and resistant cells. The results indicate that plasma membranes in several isolates of resistant cells are structurally altered in a consistent manner.

Materials and Methods

Materials. Adriamycin was provided by the Developmental Therapeutics Program of the Division of Cancer Treatment, NCI.

Cell culture. Chinese hamster lung cells (HT-1) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Isolation of Cells Resistant to Adriamycin. HT-1 cells were grown in the presence of 0.1 μg/ml of adriamycin and survivors were thereafter passed and continuously grown in the presence of increasing concentrations of drug. Cells capable of growing in adriamycin at a concentration of 10 μg/ml were maintained in drug for at least three weeks and thereafter cultured for three weeks in the absence of drug prior to use in the experiments described below. Cells isolated in this manner have exhibited a stable resistant phenotype for at least 6 months in culture.

Measurement of Drug Uptake. Adriamycin was added to sensitive and resistant cells in 4 ml of standard medium at a final concentration of 10 μg/ml. At various times after the addition of the drug, the media was removed and cells were scraped into 5 ml of 0.01 M sodium phosphate (pH 7.4) - 0.15 M NaCl and thereafter centrifuged. The drug contained in the cell pellet was extracted with 0.3 N HCl - 50% ethanol (11) and the fluorescence of the supernate was determined (excitation, 470 nm; fluorescence 585 nm).

Isotopic Labeling of the Cell Surface. HT-1 sensitive or resistant cells were grown in 60 mm dishes for 24 hours and the cell surface was labeled with ^{125}I as described by Hynes (9). Incubations with lactoperoxidase and glucose oxidase were carried out for 10 min at room temperature. At the end of the incubation period the cells were scraped from the dish and washed 3 times with 0.01 M sodium phosphate (pH 7.2) - 0.15 M NaI. The final cell pellet was suspended in 0.01 M Tris-HCl (pH 7.6) - 5mM 2-mercaptoethanol. Aliquots in duplicate were thereafter taken for determination of acid-insoluble radioactivity. The iodinated proteins were analyzed in sodium dodecyl sulfate polyacrylamide slab gels as described by Laemmli (10). Proteins were detected by autoradiography. In some instances the autoradiograms were traced with a Joyce-Loebl densitometer.

Results and Discussion: HT-1 cells treated with drug and isolated as described in Methods were analyzed further in order to characterize the adriamycin resistant phenotype. In our initial studies we examined the effect of adriamycin on DNA and RNA synthesis in sensitive and resistant cells since previous studies have shown that the drug brings about an extensive inhibition of nucleic acid synthesis in cells growing in culture (12, 13). In these studies cells were incubated with adriamycin (5 μg/ml) for periods from 2-4 hours and thereafter pulse labeled for 20 min with either [^3H]thymidine or [^3H]uridine. The results demonstrated that after a 4 hr incubation period with drug both

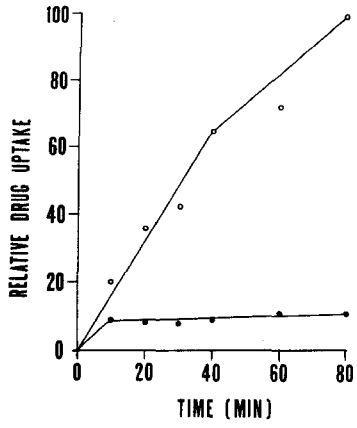

Figure 1: Adriamycin uptake in sensitive and resistant HT-1 cells. Drug uptake in sensitive and HT-1 (R6) resistant cells was determined as described in Methods. Drug uptake is relative to the amount of adriamycin contained in sensitive cells after an 80 min. incubation period. 0——0, sensitive cells; ●——●, resistant cells.

DNA and RNA synthesis were inhibited 75% in sensitive cells whereas in resistant cells nucleic synthesis was essentially unaffected (not shown).

Additional studies have been carried out to measure cellular uptake of adriamycin in sensitive and resistant cells. In sensitive cells adriamycin uptake (plus binding) proceeds for about 40 min and thereafter begins to plateau (Figure 1). In resistant cultures however, only very low levels of drug either binds to or is taken up by the cells (Figure 1).

Previous studies have shown that in adriamycin resistant Ehrlich ascites (5) or P388 leukemia cells (8) there is an energy dependent efflux mechanism which considerably reduces intracellular drug levels. Attempts to demonstrate such an efflux mechanism in HT-1 resistant cells have thus far been unsuccessful. Thus the results of the uptake experiments are in agreement with other workers (2, 4, 5) and suggest that a major contributing factor to resistance is a membrane defect which prevents drug from binding to or entering the cells.

In consideration of the above findings studies were carried out to determine if HT-1 resistant cells contain alterations in plasma membrane proteins. Specific labeling of these proteins was performed by using the lactoperoxidase catalyzed iodination procedure as described in Methods. In studies carried out thus far the iodination pattern of four different isolates of adriamycin resistant cells have been examined. The results obtained with

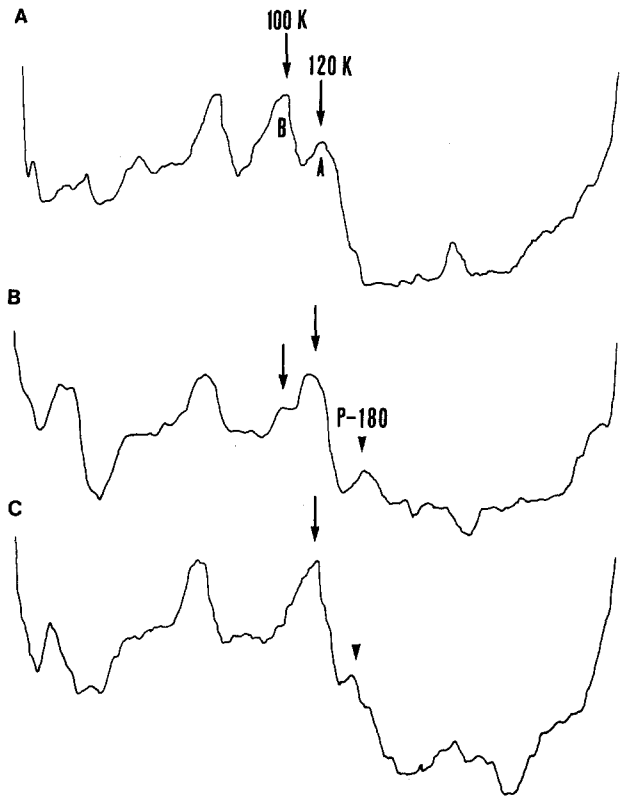

Figure 2: Polyacrylamide gel electrophoresis of iodinated proteins in sensitive and resistant cells. Labeling of HT-1 sensitive and resistant cells was as described in Methods. Labeled proteins were electrophoresed in a 5% polyacrylamide gel and the autoradiogram was traced with a Joyce Loebl densitometer. A, sensitive HT-1 cells; B, resistant cells, isolates R6; C, resistant cells, isolates R24.

two of these isolates are shown in Figure 2. In sensitive and resistant cells several proteins are labeled in the lactoperoxidase procedure. Two of the major components of sensitive cells are those designated A and B which have molecular weights of 120,000 and 100,000 respectively. In resistant cells we have consistently observed a significant alteration in the labeling pattern of protein B. In the two drug resistant isolates examined protein B is either not labeled (Figure 2C) or represents about 20% of that present in sensitive cells (Figure 2B). Studies with two additional drug resistant isolates also show that the iodination of protein B is 20% of that obtained with sensitive cells. It has also been observed that after iodination of resistant cells a minor high molecular weight protein can be detected which is not present in cells sensitive to adriamycin (Figure 2B and C). The molecular

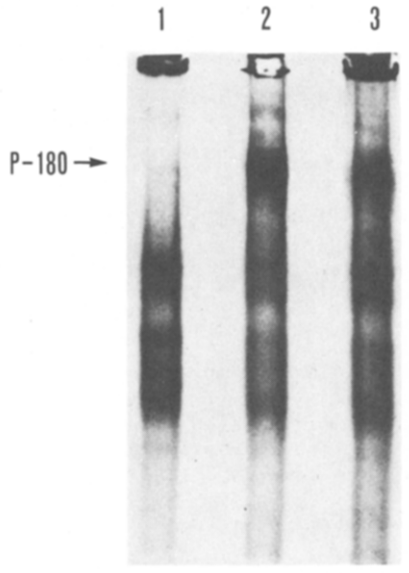

Figure 3: Polyacrylamide gel electrophoresis of [^{14}C]glucosamine labeled proteins in HT-1 sensitive and resistant cells. Growing cells in standard medium were labeled for 24 hours with [^{14}C]-D-glucosamine (0.25 μCi/ml). After the labeling period media was removed and the cells were washed twice in 0.01 M sodium phosphate (pH 7.2) - 0.15 M NaCl. The labeled proteins were electrophoresed in a 7% polyacrylamide gel as described in Methods. Lane 1, sensitive cells; lanes 2 and 3 resistant cells, isolates R6 and R24 respectively.

weight of this protein in polyacrylamide gels is somewhat variable and ranges from 160,000-180,000. In the majority of experiments the protein has a molecular weight of 180,000 and has been designated P-180. Although P-180 represents a minor component of the iodination pattern this protein has been consistently observed in four different isolates of drug resistant cells.

To determine if the iodinated proteins are actually membrane associated cells were labeled in the lactoperoxidase reaction and the plasma membranes were thereafter isolated (14, 15). The labeled proteins were analyzed after gel electrophoresis. The results of these experiments demonstrated that the labeling pattern and protein composition of sensitive and resistant cells was essentially the same as that shown in Figure 2A and B respectively. The protein, P-180 was present as a distinct component and was not detected in the membrane preparation from sensitive cells. These studies thus suggest that protein B and P-180 are integral components of plasma membranes.

Additional studies have been carried out in which sensitive and resistant cells were grown in the presence of [^{14}C]glucosamine and the labeled proteins

analyzed after polyacrylamide gel electrophoresis. Analysis of proteins from resistant cells reveals the presence of a major glycosylated component having a molecular weight of 180,000 (Figure 3, lanes 2 and 3). This 180,000 molecular weight glycoprotein has also been identified in two additional drug resistant isolates. However as shown in Figure 3 a protein with this molecular weight is not detected in sensitive cells grown in the presence of [^{14}C]-glucosamine.

The labeled proteins of plasma membranes isolated from cells grown in the presence of [^{35}S]methionine have also been examined. The results of these experiements demonstrated that membranes from resistant cells contain a minor protein having a molecular weight of 180,000. This protein was absent from membranes isolated from resistant cells.

In conclusion, two distinct protein changes have been found to occur in plasma membranes of adriamycin resistant cells. The finding that four different drug resistant isolates all contain the same membrane changes suggests that these cell surface alterations may play an important role in the resistant phenotype. We also find that the resistant isolates restrict the action of the antitumor agent 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione (16). Thus the cells are not specific for adriamycin and may be similar to other isolates which restrict the activity of certain anthracyclines and other compounds as well (6,7). It may thus be possible to relate the observed cell surface protein changes to a membrane defect which results in impaired transport of several different biologically active compounds.

It is also of interest to note that in cells sensitive to vinblastine there is also a new 180,000 molecular weight glycoprotein which appears on the cell surface (17). Whether this protein is identical to that which appears in adriamycin resistant cells remains to be determined.

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