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Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats

(hyperplastic liver nodules/pleiotropic drug resistance/aryl hydrocarbon hydroxylase/cytochrome P_1-450/glutathione transferase)

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ABSTRACT **MCF7 human breast cancer cells selected for resistance to doxorubicin (adriamycin; Dox^R) have developed the phenotype of multidrug resistance. Multidrug resistance in Dox^R MCF7 cells (called Adr^R MCF7 cell line in previous publications) is associated with biochemical changes similar to those induced by carcinogens in rat hyperplastic liver nodules (HNs) and associated with resistance to xenobiotics in that system. In HNs and Dox^R cells, exposure to a single agent results in the selection of cells that are cross-resistant to a wide variety of structurally dissimilar toxic agents. Resistance in both systems is associated with decreases in intracellular accumulation of toxins and changes in phase I (decreased cytochrome P_1-450) and phase II (increased glutathione transferase and glucuronyltransferase) drug-metabolizing activities. In HNs and Dox^R cells, resistance is associated with the induction of relatively stable levels of an immunologically related anionic glutathione transferase isozyme (EC 2.5.1.18). The finding of similar biochemical changes associated with the development of resistance to various xenobiotics in HNs and to many naturally occurring antineoplastic agents and at least one carcinogen (benzo[*a*]pyrene) in Dox^R MCF7 cells suggests that the mechanisms of resistance in these two models may be similar.**

One of the interesting problems in cancer therapy has been the finding that cells selected for resistance to a specific class of anticancer drugs often develop cross-resistance to structurally dissimilar agents (1). Multidrug-resistant cells often possess defects in drug accumulation (2) and frequently contain increased levels of membrane glycoproteins of high molecular mass (130–170 kDa) (3–5) and cytosolic proteins of low molecular mass (19–30 kDa) (6, 7). However, the precise mechanisms whereby cells can develop simultaneous resistance to multiple agents that differ markedly in both structures and mechanisms of action are as yet unclear.

We have isolated a doxorubicin (adriamycin)-resistant human breast cancer cell line (Dox^R MCF7, previously called Adr^R MCF7) that has developed the phenotype of multidrug resistance (MDR). Preliminary studies have demonstrated that resistance in these cells is associated with decreased hydroxyl radical formation in the Dox^R MCF7 cells exposed to doxorubicin compared with wild-type (WT) cells (8). This change is associated with an increase in glutathione peroxidase activity and an increase in an anionic glutathione S-transferase (GSHTase) isozyme that possesses high levels of intrinsic peroxidase activity.

The induction of increased levels of a similar anionic GSHTase isozyme is also noted in rat hyperplastic liver nodules (HNs), a model of carcinogenesis (9, 10). Numerous studies have shown that HNs with similar morphologic and biochemical properties are produced following exposure of rats to any of a variety of different carcinogens (10–16). Moreover, these carcinogen-induced changes in HNs are associated with the development of resistance to many structurally diverse hepatotoxins (10–16), prompting Farber to use the term "resistant hepatocyte model" in describing this system (16). The induction of stable levels of an anionic GSHTase isoenzyme in HNs resistant to various hepatotoxins, and in Dox^R cells resistant to many naturally occurring antineoplastic agents, prompted us to compare the biochemical changes in these two models in more detail. The results presented in this report suggest that similar mechanisms may be involved in the development of resistance in these two systems.

MATERIALS AND METHODS

Cell Culture. The conditions for the growth of WT and Dox^R MCF7 cells and the determination of drug sensitivity were described earlier (17). Drug transport studies were done using [^{14}C]daunomycin (Drug Development Branch, National Cancer Institute) as described previously (18).

Enzyme Assays. GSHTase activity was assayed using dichloronitrobenzene as the substrate (19), glutathione peroxidase was assayed using H_2O_2 or cumene hydroperoxide (20), and UDP-glucuronyltransferase (21) was assayed using 4-nitrophenol according to assays previously described. Aryl hydrocarbon hydroxylase (AHHase) activity was measured using a fluorometric assay (22), and sulfotransferase activities (I/II and III/IV) were measured as described previously (23). Immunoprecipitation analysis of GSHTase was performed as described (17). Antibody directed against the anionic GSHTase purified from the Dox^R MCF7 cells was prepared from goats (17) while antibody against GSHTase purified from rat hyperplastic liver nodules was kindly provided by K. Sato and K. Satoh (24).

Nucleic Acid Studies. The preparation of DNA of high molecular weight, the isolation of RNA using guanidine isothiocyanate and cesium chloride density-gradient centrifugation, and the separation of polyadenylylated RNA by oligo(dT)-cellulose chromatography were done using stan-

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Abbreviations: HN, rat hyperplastic liver nodule; MDR, multidrug resistance; AHHase, aryl hydrocarbon hydroxylase (EC 1.14.14.1); GSHTase, glutathione *S*-transferase (EC 2.5.1.18); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WT, wild-type cell line; Dox^R, doxorubicin (adriamycin)-resistant cells; cytochrome P_1-450, form of cytochrome P-450 monooxygenase that is inducible in mouse in "c57BL"/6N inbred mice by TCDD and is most closely associated with aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity.

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dard techniques (25). Hybridization conditions using radiolabeled probes prepared by nick-translation were described previously (18, 25, 50). A cDNA clone, pGP5, that codes for the anionic GSHTase present in rat HNs, was generously provided by A. Muramatsu (26), while a clone containing the 3′ portion of the human cytochrome P_1-450 cDNA was provided by D. Nebert (27).

RESULTS

Table 1 shows the relative resistance of Dox^R MCF7 cells to various antineoplastic agents. Although selected by exposure to doxorubicin, Dox^R MCF7 cells are markedly cross-resistant to vinca alkaloids (vincristine and vinblastine), an epipodophyllotoxin (VP-16), and the antitumor antibiotic actinomycin D. Thus these Dox^R MCF7 cells possess the characteristic phenotype of MDR.

Although the mechanisms associated with MDR and with carcinogen resistance in rat HNs are not well understood, defects in intracellular toxin accumulation have been noted in both systems (2, 15). Farber and coworkers (15) reported a decrease in intracellular accumulation of the carcinogen aminoacylfluorene, as well as a decrease in the binding of this carcinogen to the DNA of rat HNs compared with normal hepatocytes. Similarly, there was a 2- to 3-fold decrease in the rate of uptake and in the intracellular accumulation of radiolabeled daunomycin into Dox^R MCF7 cells compared with WT MCF7 cells (Fig. 1). At the time indicated by the arrow, radiolabeled drug was removed from the medium, and the efflux of intracellular drug was examined. As has been reported in other doxorubicin-resistant cell lines (51), less intracellular drug remained in the Dox^R cells compared with the WT cells suggesting that enhanced efflux may be involved in this defect. Similar differences were found when radiolabeled doxorubicin was used in the transport studies. This 2- to 3-fold defect in intracellular drug accumulation, however, seems insufficient to account for the overall level of drug resistance of these cells.

Alterations in Phase I Drug-Metabolizing Enzymes. Since many carcinogens, including benzo[*a*]pyrene, are converted to more active species intracellularly by a variety of enzyme activities referred to as phase I drug-metabolizing enzymes, the role of these enzyme activities in the development of resistance to carcinogens in HNs has been examined (29–32). Indeed, HNs contain lower levels of cytochrome P-450 and decreased activities of several cytochrome P-450-dependent enzymes, including aryl hydrocarbon hydroxylase (AHHase), aminopyrene *N*-demethylase, and NADPH-cytochrome c_2 reductase relative to that present in normal hepatocytes (29–32). Because AHHase is markedly inducible by polycyclic hydrocarbons in WT MCF7 cells (27), the activity and the regulation of this enzyme activity were compared in both cell lines. As shown in Table 2, WT and Dox^R MCF7 cells have low basal levels of AHHase activity (<2 pmol/mg per min). While exposure of WT MCF7 cells to 100 nM TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) for 24 hr markedly induced AHHase activity, similar treatment of Dox^R MCF7 cells produced no detectable increase in AHHase activity.

Table 1. Relative resistance of Dox^R MCF7 cells

Drug	Resistance, fold
Doxorubicin	192
VP-16*	100
Vincristine	>250
Vinblastine	375
Actinomycin D	175

Resistance is measured as IC_{50} (Dox^R)/IC_{50} (WT) as described (17) for each drug.
*An epipodophyllotoxin.

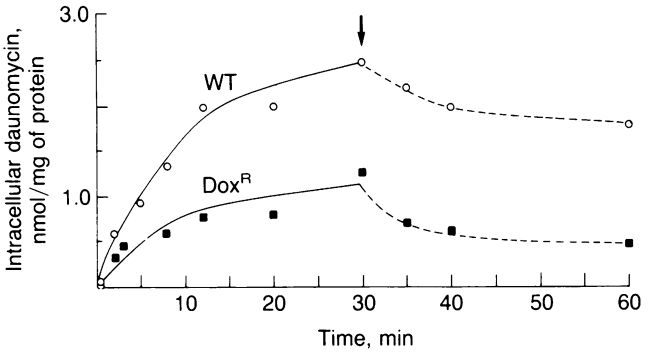

FIG. 1 [^{14}C]Daunomycin uptake into WT and Dox^R MCF7 cells. Cells were incubated in 2 μM [^{14}C]daunomycin at 37°C for various periods; at indicated times the amount of intracellular drug was determined as described (18). At the time indicated by the arrow, the medium was removed, the cells were washed twice with drug-free medium, and the amount of drug remaining in cells was measured after incubation for additional time at 37°C.

To determine the level of the defect in AHHase induction in the Dox^R MCF7 cells, mRNA levels for the gene encoding this activity (cytochrome P_1-450) in these cells were compared in WT and Dox^R MCF7 cells (Fig. 2*A*). While the induction of AHHase activity by TCDD in WT MCF7 cells was associated with a marked increase in cytochrome P_1-450 mRNA, there was no change in the level of this mRNA in Dox^R MCF7 cells treated with TCDD. Thus, the altered regulation of cytochrome P_1-450 gene expression in Dox^R MCF7 cells apparently involves a defect at the level of transcription.

At present there is no evidence that implicates this alteration in the regulation of AHHase directly with the development of resistance to doxorubicin or any of the other agents listed in Table 1. However, since AHHase is involved in the intracellular metabolism of the carcinogen benzo[*a*]pyrene to an active metabolite 3-OH-benzo[*a*]pyrene (33), this change should alter sensitivity of the Dox^R MCF7 cells to the cytotoxic effects of this carcinogen. Indeed, the IC_{50} of the Dox^R MCF7 cells to benzo[*a*]pyrene was 30-fold higher than that for WT MCF7 cells (23 vs. 0.74 μM).

Alterations in Phase II Drug-Metabolizing Enzymes. One of the most consistent changes produced in HNs is an increase in an anionic isozyme of GSHTase that is immunologically related to the anionic isozyme present in rat placenta (24, 26). The Dox^R MCF7 cell line also contains a marked increase (45 fold) in GSHTase activity of which more than 90% of the increased activity is present in the form of an anionic species not found in WT MCF7 cells (17). Using a polyclonal antibody generated from animals immunized with the anionic GSHTase enzyme purified from Dox^R MCF7 cells, we compared the homology of this enzyme with other GSHTases. As shown in Fig. 3*A*, this antibody inhibited GSHTase activity in Dox^R MCF7 cells but does not cross-react with the activity present in WT MCF7 cells. Thus, the GSHTase basic isozyme (pI 9.5) present in WT MCF7 cells

Table 2. AHHase activity in WT and Dox^R MCF7 cells

	Activity, pmol/min per mg
WT control	<2.0
WT + TCDD	80.0
Dox^R control	<2.0
Dox^R + TCDD	<2.0

AHHase activity was determined in cells incubated for 24 hr with or without 100 nM TCDD. Results represent the mean of two experiments, each done in duplicate; variation between samples was <10%.

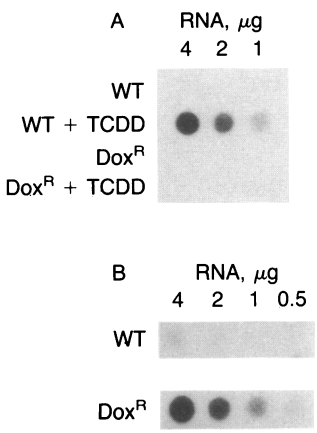

FIG. 2. Dot blot analysis of WT and Dox^R RNA. (*A*) RNA was prepared from WT and Dox^R MCF7 cells after 24 hrs incubation at 37°C with or without 100 nM TCDD. Poly(A)$^+$ RNA was hybridized with ^{32}P-labeled DNA sequences containing the 3′ end of the human cytochrome P_1-450 gene (26) as described in *Materials and Methods*. (*B*) Poly(A)$^+$ RNA from WT and Dox^R MCF7 cells was bound to nylon membranes and hybridized with radiolabeled pGP5, a cDNA coding for anionic GSHTase from rat HNs (26).

(17) is immunologically unrelated to the anionic species present in the resistant subline. However, the anionic isozyme in the Dox^R MCF7 cells was immunologically related to the enzyme present in human placenta, a tissue which contains an anionic GSHTase (34) (Fig. 3*A*).

Since the GSHTase isozyme produced in rat HNs is also an anionic isozyme that is similar to that in rat placenta, we used a polyclonal antibody directed against the anionic GSHTase from Dox^R MCF7 cells to probe for homology with the GSHTase in rat HNs (17). As shown in Fig. 3*B*, this antibody did not cross-react with the GSHTase activity in normal rat liver, a tissue that contains predominantly basic GSHTase isozymes, but the antibody did cross-react with the GSHTase enzyme present in HNs. Moreover, antibody prepared against GSHTase purified from rat HNs (24) cross-reacted with enzyme present in Dox^R MCF7 cells but not with the enzyme in WT MCF7 cells (Fig. 3*C*). Thus, the GSHTase isozyme induced in the Dox^R MCF7 cells is biochemically (anionic) and immunologically related to the isozyme present both in human placenta and the enzyme induced in rat HNs. However, this GSHTase isozyme differs from other human anionic isozymes in that it possesses a high level of intrinsic organic peroxidase activity (17), a property generally associated with basic transferases and not anionic GSHTases (28).

The results of an RNA dot blot analysis in which poly(A)$^+$ RNA from WT and Dox^R MCF7 cells was hybridized with radiolabeled probe for the rat anionic GSHTase from HNs (26) is shown in Fig. 2*B*. There was a marked increase in the concentration of RNA homologous to this anionic GSHTase cDNA probe in the Dox^R compared with the WT MCF7 cells. Thus, the induction of this anionic GSHTase is due to the transcriptional activation of this gene in the resistant subline.

Previous studies have shown that the phase II drug-conjugating enzyme glucuronyltransferase is also induced in rat HNs (35, 36). As shown in Table 3, this enzyme activity was increased 2.5-fold in Dox^R MCF7 cells. Moreover, a similar isozyme of glucuronyltransferase, one capable of conjugating 4-nitrophenol, was induced in both rat HNs (12, 35, 36) and in Dox^R MCF7 cells. There was also a 2.5-fold increase in sulfotransferase, another phase II drug-metabolizing enzyme, in Dox^R MCF7 cells (Table 3). This differs from that reported in rat HNs in which a decrease in sulfotransferase activity apparently occurs (37).

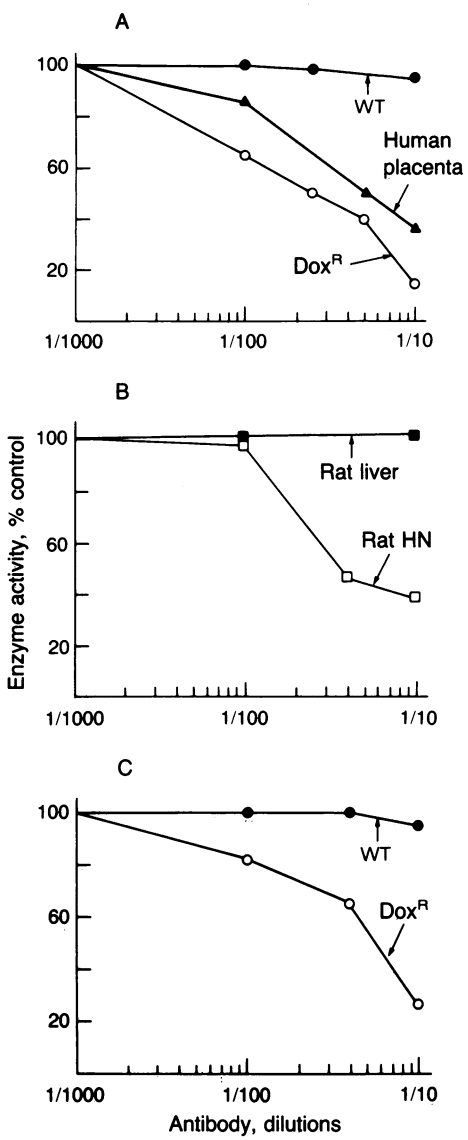

FIG. 3. Immunoprecipitation studies of GSHTase. The activity of GSHTase was measured in cell cytosol following incubation with various concentrations of antibodies as described in text. Results are expressed as % control, that is, activity of extracts incubated with preimmune serum. (*A*) Polyclonal antibody prepared against the anionic GSHTase in Dox^R MCF7 cells was incubated with cytosol from WT MCF7 cells (●), Dox^R MCF7 cells (○), and human placenta (▲). (*B*) The same antibody as in *A* was incubated with cytosol from normal rat liver (□) and from rat hepatic nodules (■). (*C*) Antibody directed against the GSHTase in rat hepatic nodules was incubated with cytosol from WT (●) and Dox^R MCF7 (○) cells.

DISCUSSION

These studies indicate that remarkably similar biochemical changes occur in human breast cancer cells selected for

Table 3. Drug-conjugating enzyme activities in WT and Dox^R MCF7 cells

Enzyme	Activity, nmol/mg per min		Increase, fold
	WT	Dox^R	
GSHTase	3.6 ± 0.2	161.0 ± 6.1	44.7
UDP-glucuronyltransferase	5.1 ± 0.5	11.8 ± 1.0	2.3
Sulfotransferase			
I/II	15.1 ± 1.0	36.8 ± 1.1	2.4
III/IV	14.1 ± 2.0	39.2 ± 0.7	2.6

resistance to multiple antineoplastic agents and in rat HNs that develop resistance to a wide variety of hepatotoxins (Table 4). In both model systems, selection by chronic exposure to a single agent, a naturally occurring antineoplastic in one case and a carcinogen in the other, leads to the development of resistance to the selecting agent and to cross-resistance to a wide variety of structurally dissimilar agents. In both systems, cellular resistance is associated with a decrease in intracellular accumulation of cytotoxic agents. In many MDR cell lines, increased levels of membrane glycoproteins of high molecular weight (P170) have been associated with decreased drug accumulation (3–5). Moreover, amplified, overexpressed P170 or homologous genes have been isolated from several different multidrug resistant cell lines (38–42) including these Dox^R MCF7 cells (43). Whether overexpression of a similar gene is involved in the development of decreased carcinogen uptake and resistance in rat HNs remains to be determined.

In Dox^R MCF7 cells and rat HNs, resistance is associated with similar changes in phase I and phase II drug-metabolizing enzyme activities. In rat HNs, both decreases in the overall intracellular content of cytochrome P-450 and decreases in AHHase activity and other cytochrome P-450-dependent enzyme activities have been reported (29–32). Similarly, we have found a marked decrease in TCDD-inducible AHHase activity and cytochrome P_1-450 gene transcription in Dox^R MCF7 cells.

The role of this defect in the regulation of cytochrome P_1-450 gene expression in the development of MDR in these cells is not clear. Previous studies have suggested that metabolism of anthracyclines may involve cytochrome P-450-dependent mixed-function oxidases (44) and that the drug VP-16, an epipodophyllotoxin, may be converted to reactive quinones by microsomal enzymes (45, 46). However, there is no evidence that AHHase activity is directly involved in the metabolism of any of the antineoplastic agents listed in Table 1. Since this enzyme does metabolize benzo[*a*]pyrene to more toxic intermediates, the loss of this enzyme activity in Dox^R MCF7 cells and in HNs is associated with the development of cross-resistance to this carcinogen in both systems. Furthermore, in addition to AHHase, several other enzymes are induced by polycyclic hydrocarbons such as TCDD (47). It is possible that the loss of AHHase regulation in the Dox^R MCF7 cells represents not a structural mutation in the gene encoding cytochrome P_1-450, but rather a defect in a regulatory locus involved in the coordinated regulation of other genes whose products may be involved in the molecular mechanisms of antineoplastic drugs.

Resistance in Dox^R MCF7 cells is also associated with the induction of an anionic GSHTase that displays biochemical (anionic), immunological, and nucleic acid sequence homology to the isozyme induced in HNs. GSHTases are a family of isozymes with several properties well suited to play a potential role in protecting cells from toxic agents (48), including the ability to bind toxins either covalently or noncovalently, the ability to inactivate toxins through conjugation with glutathione, and the ability to protect cells from organic peroxides that may be formed. Peroxidase activity of GSHTases may be particularly important in the development of resistance to doxorubicin, an agent whose toxicity involves the generation of intracellular free radicals (49). Thus, the observation that the GSHTase in Dox^R cells differs from other anionic isozymes in having a high level of intrinsic organic peroxidase activity may have particular relevance for these cells (17, 28).

Table 4. Properties of Dox^R MCF7 cells and rat HNs

Dox^R MCF7	Rat HNs	Ref(s).*
Resistance to cytotoxic drugs and benzo[*a*]pyrene	Resistance to carcinogens and hepatotoxins	10–16
Decreased accumulation of drug	Decreased accumulation of carcinogen	16
Decreased regulation of phase I enzyme: AHHase	Decreased activity of phase I enzyme: AHHase	24, 29–31
Increased activities of phase II enzymes: anionic GSHTase	Increased activities of phase II enzymes: anionic GSHTase	9, 16, 26
glucuronyltransferase	glucuronyltransferase	31, 32
Immunologic similarity to anionic GSHTase of rat HNs	Immunologic similarity to anionic GSHTase of Dox^R MCF7	

*Previous references for HNs.

The studies presented in this report demonstrate a marked similarity in the biochemical changes associated with the development of resistance to xenobiotics in carcinogen-induced HNs, and with the development of resistance to many naturally occurring antineoplastic agents in human breast cancer cells exhibiting the phenotype of MDR. Farber (16) has previously suggested that carcinogenesis may involve the selection of an altered clone of cells which has developed biochemical changes that render them "resistant" to the cytotoxic effects of carcinogens to which they are exposed and that have a higher probability of evolving into a cancer. The finding that MCF7 cells selected for multidrug resistance develop changes similar to those found in HNs suggests that these changes may also render the transformed cell insensitive to the cytotoxic effects of antineoplastic agents. GSHTase and glutathione peroxidase activities are reportedly higher in colon and breast cancers than in their respective normal tissues (52, 53). Thus, it may be that the mechanisms of *de novo* resistance to therapy in tumors associated with increased carcinogen exposure, such as colon cancer and lung cancer, and the mechanisms associated with resistance to antineoplastic agents acquired during treatment may involve similar mechanisms.

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