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BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION OF MCF-7 DRUG-SENSITIVE AND Adr^R MULTIDRUG-RESISTANT HUMAN BREAST TUMOR XENOGRAFTS IN ATHYMIC NUDE MICE

EDWARD G. MIMNAUGH,* CRAIG R. FAIRCHILD, JOHN P. FRUEHAUF and BIRANDRA K. SINHA

Biochemical and Molecular Pharmacology Section, Clinical Pharmacology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD 20892, U.S.A.

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Abstract—The phenotypic expression of multidrug resistance by the doxorubicin-selected Adr^R human breast tumor cell line is associated with overexpression of plasma membrane P-170 glycoprotein and increased cytosolic selenium-dependent GSH-peroxidase activity relative to the parental MCF-7 wild-type line (WT). To determine whether doxorubicin resistance by Adr^R cells persists *in vivo*, and to further investigate the possibility of biochemical differences between WT and Adr^R solid tumors, both tumor cell lines were grown as subcutaneous xenografts in athymic nude mice. Tumorigenicity depended upon cell inoculation burden, and tumor incidence was similar for both cell lines (> 80% tumor takes at 10^7 cells/mouse) at 14 days, provided 17β-estradiol was supplied to the animals bearing the WT tumors. However, the growth rate for the Adr^R xenografts was only about half that of WT xenografts. Doxorubicin (2–8 mg/kg, i.p., injected weekly) significantly diminished the growth of the WT tumors, but Adr^R solid tumors failed to respond to doxorubicin. The accumulation of ^{14}C-labeled doxorubicin was 2-fold greater in WT xenografts that in Adr^R, although there were no differences in host organ drug levels in mice bearing either type of tumors. Membrane P-170 glycoprotein mRNA was detected by slot-blot analysis in the Adr^R tumors, but not in WT. Electron spin resonance 5,5-dimethylpyrroline-*N*-oxide-spin-trapping experiments with microsomes and mitochondria from WT and Adr^R xenographs demonstrated a 2-fold greater oxygen radical (superoxide and hydroxyl) formation from activated doxorubicin with WT xenographs compared to Adr^R. Selenium-dependent glutathione (GSH)-peroxidase, superoxide dismutase and GSH-*S*-aryltransferase activities in Adr^R xenografts were elevated relative to WT. Although the activities of the latter two enzymes were similar to those measured in both tumor cell lines, GSH-peroxidase activities were elevated 70-fold (WT) and 10-fold (Adr^R) in xenografts compared to tumor cells. In contrast, in both WT and Adr^R solid tumors *in vivo*, catalase, NAD(P)H-oxidoreductases, and glutathione disulfide (GSSG)-reductase activities, and GSH and GSSG levels were not markedly different, and were essentially the same as in cells *in vitro*. Like the MDR cells in culture, Adr^R tumor xenografts were extremely resistant to doxorubicin and retained most of the characteristics of the altered phenotype. These results suggest that WT and Adr^R breast tumor xenografts provide a useful model for the study of biochemical and pharmacological mechanisms of drug resistance by solid tumors *in vivo*.

A major barrier to the effective treatment of human malignancies is the acquisition of broad-based anticancer drug resistance by tumor cells. The development of "pleiotropic" or multidrug resistance (MDR†) is particularly insidious because this phenotype is usually cross-resistant to several of the clinically most effective antitumor agents, including the naturally occurring antibiotics doxorubicin and daunorubicin; the vinca alkaloids, vincristine and vinblastine; and the epipodophilotoxins, VP-16 and VM-26 [1–3].

Mechanistic investigations of acquired MDR in tumor cell lines *in vitro* have identified differences in the biochemical and pharmacological characteristics of MDR tumor cells compared to parental, drug-sensitive counterparts [4], and it is likely that several mechanisms contribute cooperatively to the diminished drug sensitivity of MDR tumor cells. In our laboratory we have studied extensively the Adr^R cell line, an acquired drug-resistant variant selected for resistance *in vitro* from the MCF-7 human breast tumor parental line by escalating doxorubicin exposures [5]. Adr^R cells display many of the hallmarks of typical MDR. Compared to the parental, drug-sensitive, MCF-7 wild-type line (WT), Adr^R tumor cells are highly resistant to doxorubicin, significantly cross-resistant to other cytotoxic agents derived from natural products, show decreased intracellular accumulation of several drugs, overexpress the *mdr1* P-glycoprotein drug-efflux pump, and have greatly elevated anionic glutathione (GSH)-transferase (π isozyme) (EC 2.5.2.12) and

* Address requests for reprints to: Dr. E. G. Mimnaugh, National Cancer Institute, NIH, Building 10, Room 6N119, Bethesda, MD 20892.

† Abbreviations: MDR, multidrug-resistant or multidrug resistance; WT, wild-type MCF-7 human breast tumor cell line; Adr^R, doxorubicin-resistant MCF-7 cell line; ESR, electron spin resonance; IMEM, improved minimal essential medium; DMPO, 5,5-dimethylpyrroline-*N*-oxide; GSH, reduced glutathione; GSSG, oxidized glutathione; and DETPAC, diethylenetriaminepentaacetic acid.

selenium-dependent GSH-peroxidase (EC 1.11.1.9) enzyme activities [5–9]. In addition, compared to the WT line, the Adr^R subline generates several-fold less hydroxyl free radical derived from doxorubicin oxidation–reduction cycling [10], contains diminished activities of drug-activating enzymes, including NADPH–cytochrome P450 monooxygenases [11], is inherently less sensitive to extracellular and intracellular oxygen free radicals and hydrogen peroxide [7], and can be effectively sensitized to doxorubicin cytotoxicity following GSH depletion with buthionine sulfoximine [12].

While MDR has been studied extensively in tumor cell lines *in vitro*, there is a paucity of information about the biological determinants of MDR in human cancers *in situ* and in animal solid tumor models. Although P-glycoprotein has been detected in several types of human tumors and in normal tissues [13, 14], and increased GSH-transferase activity has been associated with drug-resistant human tumors [14, 15], the biochemistry of MDR *in vivo* has not been investigated thoroughly. To address this question we have established both WT and Adr^R human tumor cell lines as solid tumor xenografts in athymic nude mice. Our main objectives were to determine whether the well characterized Adr^R resistance to doxorubicin persisted *in vivo* and to investigate whether the enzymology of the WT and Adr^R tumor cell lines changed as a consequence of the shift from monolayer growth *in vitro* to solid tumor aggregate growth *in vivo*. We have focused primarily on the tumor enzymes that are capable of activating doxorubicin to a free radical semiquinone intermediate and reactive oxygen-scavenging enzymes that might influence the response to doxorubicin by either WT or Adr^R tumor xenografts.

MATERIALS AND METHODS

Chemicals and drugs. Xanthine, xanthine oxidase (50 units/mL) superoxide dismutase (3050 units/mg protein), catalase (10,000 units/mg protein), NADPH, NADH, reduced glutathione, glutathione reductase (500 units/mg protein), fatty acid-free bovine serum albumin, EDTA, DETPAC, cytochrome *c*, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide, 5,5′-dithiobis-nitrobenzoic acid, and acetic anhydride were from the Sigma Chemical Co. (St. Louis, MO); hydrogen peroxide, heparin sodium and sulfosalicylic acid were from the Fisher Chemical Co. (Fair Lawn, NJ); and ultrapure Tris was purchased from Bethesda Research Laboratories (Gaithersburg, MD). 5,5-Dimethylpyrroline-*N*-oxide (DMPO) was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was purified by passage over activated charcoal. All other chemicals were of the highest purity. Doxorubicin hydrochloride (NSC 123127) was provided by the Drug Development Branch, National Cancer Institute, NIH, Bethesda, MD. 17β-Estradiol 60-day release pellets (0.72 mg/pellet) were from Innovative Research of America, Toledo, OH. [^{14}C]Doxorubicin (55 mCi/mmol) was from the Amersham Corp., Arlington Heights, IL; TS-1 tissue solubilizer from Research Products International Corp., Mount Prospect, IL; and Aquasol from New England Nuclear, Boston, MA. The water used in this study was deionized and glass-distilled.

Cell culture and xenografts. Wild-type MCF-7 human breast tumor cells and the MDR Adr^R variant were grown in improved minimal essential medium supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin and 5% fetal bovine serum (IMEM) (GIBCO, Grand Island, NY) in 5% carbon dioxide in air in a humidified incubator at 37°. The Adr^R cell line was derived from the parental line by selection in increasing concentrations of doxorubicin and is capable of slowly proliferating in 10 μM drug [5]. MDR of the Adr^R variant line is reasonably stable without further doxorubicin exposure for nearly a year in continuous culture [5]. Both tumor cell lines were examined for, and found to be free from, contaminating viruses and mycoplasma prior to inoculation into mice.

Adr^R cells were grown for at least 8 but not more than 25 passages in drug-free IMEM medium before they were harvested by trypsinization and injected into mice. Both WT and Adr^R tumor cells (up to 10^7 in 0.25 mL of IMEM medium containing 10% fetal bovine serum) were inoculated s.c. into the intrascapular region of female inbred athymic BALB/c (nu^+nu^+ genotype) mice weighing 20–25 g (10–20/group) (Harlan Sprague–Dawley, Madison, WI). Mice were kept under sterile conditions and were given sterilized food and water *ad lib*. The WT tumor cells require exogenous estrogen for efficient tumorigenicity [16], and mice intended for WT xenografts were therefore aseptically implanted s.c. in the flank area with 0.72-mg 60-day release 17β-estradiol pellets 2–3 days prior to injection of tumor cells. Ard^R tumor cells are estrogen-independent and do not respond to estradiol stimulation of proliferation *in vitro* or *in vivo* [17]. The growth of WT and Adr^R xenografts was monitored every 7–14 days by measuring the greatest diameters of the tumors with calipers in two and three dimensions. While it is difficult to accurately measure the thickness of incipient tumors (< 2 mm) through mouse skin, measurement of tumor diameters in three dimensions are possible as the xenografts grow larger. Therefore, tumor size is expressed as length × width^2/2, and the volume calculations were validated in the larger tumors by the spheroid volume formula: length × width × thickness × π/6, as described for murine solid tumors [18]. Tumor weights were determined when the animals were killed.

Histopathology. WT and Adr^R intact tumors (60–75 days growth) were removed from the mice, rinsed in phosphate-buffered saline (PBS), dissected free of skin and connective tissue, and fixed for several days in 10% neutral-buffered formalin. Tissue thin sections were cut from embedded paraffin blocks and stained with hematoxylin and eosin.

Doxorubicin sensitivity. Doxorubicin in sterile normal saline (0.9%) was administered at 2, 4 or 8 mg/kg, i.p., weekly, starting 7 days after inoculation of the tumor cells, a time when the solid tumors were just palpable, and progressed for a total of six to eight treatments until body weight losses exceeded 25%, or until the mice began to die. Tumor response to doxorubicin was assessed by comparing tumor volumes measured during the course of drug

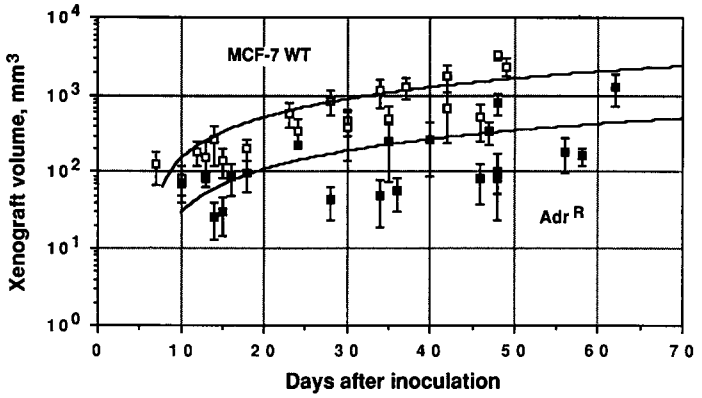

Fig. 1. Growth of WT and Adr^R xenografts in nude mice after inoculation of 10^7 tumor cells per mouse. Data are expressed as the mean ± SD for the calculation (length × width^2)/2 from three separate experiments combined in these plots. The curves were computer-fitted (Macintosh) by regression analysis using second-degree polynomial equations.

treatments, and in some cases several weeks after the last dose of doxorubicin.

Doxorubicin distribution. To determine the relative concentrations of doxorubicin in the two tumor types, and to contrast tumor drug levels with host tissues, [^{14}C]doxorubicin (approximately 2 μCi/mouse) at 8 mg/kg was administered i.p. to mice bearing xenografts weighing approximately 0.5 to 1.0 g. Two hours later the animals were killed by cervical dislocation, and tumors, livers, kidneys, and hearts were removed, washed in PBS, weighed and frozen for later processing. Blood was collected from the severed axillary vessels into heparinized pipets, transferred to tubes, and centrifuged; the plasma was separated from the cells before storage. Samples were thawed within 7 days, the tumors were quartered and trimmed of obvious grossly necrotic area, and tissues (100- to 200-mg samples of tumors and livers, one entire kidney and the intact hearts) and plasma (100–200 μL) were digested in TS-1 solubilizer for 48 hr at 50°. Following neutralization with HCl, Aquasol scintillant was added and the radioactivity was determined with a Packard model 2000 CA liquid scintillation analyzer using both quench and chemiluminescence corrections. Drug levels are expressed as nanomoles of doxorubicin equivalents per gram of tissue wet weight.

ESR experiments with tumor subcellular fractions. To compare the relative capabilities of the WT and Adr^R xenografts to enzymatically active doxorubicin to a semiquinone intermediate that generates oxygen free radicals, both microsomal and mitochondrial fractions were isolated from solid tumor homogenates by differential centrifugation techniques described previously in detail [19, 20]. The purified microsomal and mitochondrial fractions were suspended in 150 mM KCl–50 mM Tris buffer, pH 7.4, and diluted appropriately for incubations with doxorubicin following assay for protein.

ESR spectra were recorded on an ER 200-D SRC IBM-Brucker spectrometer operating at 9.5 GHz, equipped with a TM cavity and an ER 035 nuclear magnetic resonance gaussmeter. Freshly-isolated solid tumor subcellular fractions (2 mg/mL) were mixed with doxorubicin (200 μM), DMPO (50 mM) and NADPH or NADH (1 mM), all final concentrations, and then transferred to the ESR flat cell; the spectra were recorded between 15 and 20 min of the reactions. The production of superoxide and of hydroxyl radical was estimated from their respective characteristic DMPO-OOH and DMPO-OH spin-adduct spectra as described [21]. Verification of direct spin-trapping of hydroxyl radical was done by adding either ethanol or dimethyl sulfoxide and identifying the respective DMPO-carbon-centered spin-trap spectra as previously described [10] (data not shown).

Biochemical and enzyme assays. Solid tumor samples were homogenized in appropriate buffers for each of the assays prior to the isolation of the microsomal, mitochondrial or cytosolic fractions. The two principle doxorubicin-activating enzymes, microsomal NADPH–cytochrome P450 reductase (EC 1.6.2.4) [22] and mitochondrial NADH-dehydrogenase (EC 1.6.99.3) [23], were assayed spectrophotometrically by measuring the rate of reduction of cytochrome *c* as the electron acceptor [24], and the activities are expressed as nanomoles of ferricytochrome *c* reduced per milligram of protein per minute. The cytosolic antioxidant and GSH-related enzymes were assayed in supernatant fractions following centrifugation of tumor homogenates at 30,000 *g*. Glutathione peroxidase activity was measured by the method of Paglia and Valentine [25] using 73 μM H_2O_2 as substrate; glutathione *S*-aryltransferase (EC 2.5.1.18) activity was assayed by monitoring the catalyzed combination of glutathione with 1-chloro-2,4-dinitrobenzene [26], and glutathione disulfide reductase (EC 1.6.4.2) by measuring the rate of NADPH oxidation [27]. Superoxide dismutase (EC 1.15.1.1) activities were measured by the method of McCord and Fridovich [28] using acetylcytochrome *c* prepared as described by Azzi *et al.* [29], and catalase (EC 1.11.1.6) activity was measured by following the rate of H_2O_2 decomposition at 240 nm [30]. Protein was measured by the bicinchoninic acid method as described by Smith *et al.* [31] with bovine serum albumin as the standard. GSH levels were assayed by the kinetic assay of Tietze [32] in sulfosalicylic acid-deproteinized

samples, and oxidized glutathione (GSSG) concentrations were measured following treatment of sample aliquots with excess vinylpyridine to complex GSH [33].

Analysis for P-170 glycoprotein mRNA. Frozen WT and Adr^R tumor samples (−80°) were pulverized with a mortar and pestle on dry ice to a fine powder just prior to total RNA extraction and isolation by guanidine isothiocyanate-cesium chloride gradient centrifugation [34]. For RNA slot-blot analysis, aliquots of tumor RNA were placed on a nitrocellulose filter and the blots were hybridized with a ^{32}P-labeled pAdr-1 cDNA fragment isolated from the 3′ end of the P-glycoprotein gene that had been cloned from a cDNA library made from mRNA isolated from Adr^R cells [9, 35]. Blots were also separately probed with ^{32}P-labeled actin cDNA to control for the amount of RNA assayed in each sample.

Statistics. Data were analyzed by Student's *t*-test [36], and differences between mean values at $P < 0.05$ were considered to be significant.

RESULTS

Growth characteristics of MCF-7 WT and Adr^R xenografts in nude mice. It has been shown previously that supplemental estradiol stimulates the proliferation of the MCF-7 WT breast tumor line in culture [37] as well as MCF-7 WT xenografts in nude mice [16, 17]. Without estradiol treatment, only 4 of 62 mice (intact ovaries) that were inoculated with 10^7 MCF-7 WT tumor cells developed any palpable tumors ($> 2 \times 2$ mm) within 3–4 weeks, but 89 of 103 mice (86%) surgically implanted s.c. with 17β-estradiol 60-day timed-release pellets prior to tumor cell inoculation developed solid tumors. In contrast, solid tumors arising from the inoculation of 10^7 Adr^R cells s.c. averaged 82% without estradiol supplementation, and estradiol did not enhance the percent of takes or growth rates of this drug-resistant tumor. In subsequent experiments, all mice to be injected with WT tumor cells were pretreated with 17β-estradiol pellets.

The tumorigenicity of MCF-7 WT and Adr^r tumor cells in athymic nude mice also depended upon the number of tumor cells inoculated. The incidence of solid tumors palpable at 2 weeks increased dramatically as the inoculation of tumor cells was escalated from 1 to 5 to 10 million cells/mouse, yielding $< 20\%$, 40–60%, and $> 80\%$ takes, respectively (data not shown). Based upon these observations, for all other experiments animals were routinely treated with 17β-estradiol pellets and injected with 10^7 WT or Adr^R tumor cells isolated freshly from culture.

Although the overall efficiency of tumor takes at 10^7 cells/mouse was similar for both the WT and resistant Adr^R tumor cells, the relative rates of growth differed markedly as shown in Fig. 1, where data are combined from several separate groups of mice treated with tumor cells. The growth of both types of tumors varied slightly from experiment to experiment for unknown reasons, but the WT xenografts consistently grew faster than matched Adr^R xenografts. As early as 3–4 weeks after inoculation, the average volume of the WT tumors was more than twice the volume of their Adr^R counterparts. This observation was verified by tumor spheroid volume calculations from the greatest diameter measurements in three dimensions (data not shown).

It has been reported that MCF-7 WT breast tumors in mice metastasize from orthotopic mammary fat pad primary sites to host lungs, liver and spleen [38]. Nevertheless, at no time did we find distant organ tumor metastasis in the animals bearing either WT or Adr^R tumors s.c., although a histopathological examination of host tissues was not done. In spite of the presence of tumors as large as 4 g that were allowed to grow for 4 months, the mice otherwise appeared healthy and active, and no obvious tumor-related deaths were noted.

Histopathology. Hematoxylin and eosin-stained thin sections of the two types of xenografts were typical of human breast adenocarcinomas. Both WT and Adr^R tumors contained central area caseous coagulative necrosis interspersed with many regions of viable tumor cells near small blood vessels and a margin of actively proliferating cells several millimeters in depth at the tumor periphery (Fig. 2). The overall vascularity of the two tumor types was similar and, in general, larger tumors (> 0.5 g) had more extensive central ischemic necrosis. At high magnification, basophilic pyknotic nuclei were observed bordering the regions of viable cells in the transitions leading into eosinophilic necrotic areas. Compared to WT, Adr^R xenografts tended to have more anaplastic cells. Both types of solid tumors contained minimal regions of lymphocytic cell infiltrations localized predominantly near the tumor surface, and the areas of concentrated lymphocytes typically were less than 5% of the tumor cross-section areas. Thus, there were no major differences in the way the xenografts grew that could be related to doxorubicin resistance.

Response of WT and Adr^R xenografts to doxorubicin. Tumor-bearing mice were treated with doxorubicin on a schedule of weekly injections rather than a single acute drug administration to more closely mimic the clinical situation. We also waited 7 days after tumor inoculations before beginning doxorubicin treatments to allow the tumors to coalesce and to develop a peritumor vascular system. Doxorubicin at 2 mg/kg every 7 days diminished the growth of the WT tumors by approximately 50% (Fig. 3A). In contrast, doxorubicin was not very effective against the Adr^R xenografts (Fig. 3B). Mice tolerated this regime reasonably well, and it offered the best therapeutic index. This experiment was repeated twice with independent groups of mice with essentially the same outcome. The 4 mg/kg dose of doxorubicin given weekly was not significantly better than the 2 mg/kg dose in preventing tumor growth, and it caused 100% lethality following only five doses. Doxorubicin at 8 mg/kg, given twice at 7 and 14 days, actually caused WT tumor regression at 3 weeks, but body weight loss by this time was considerable, and it was apparent the mice could not tolerate a third treatment. The Adr^R tumors failed to respond to the higher doses of doxorubicin

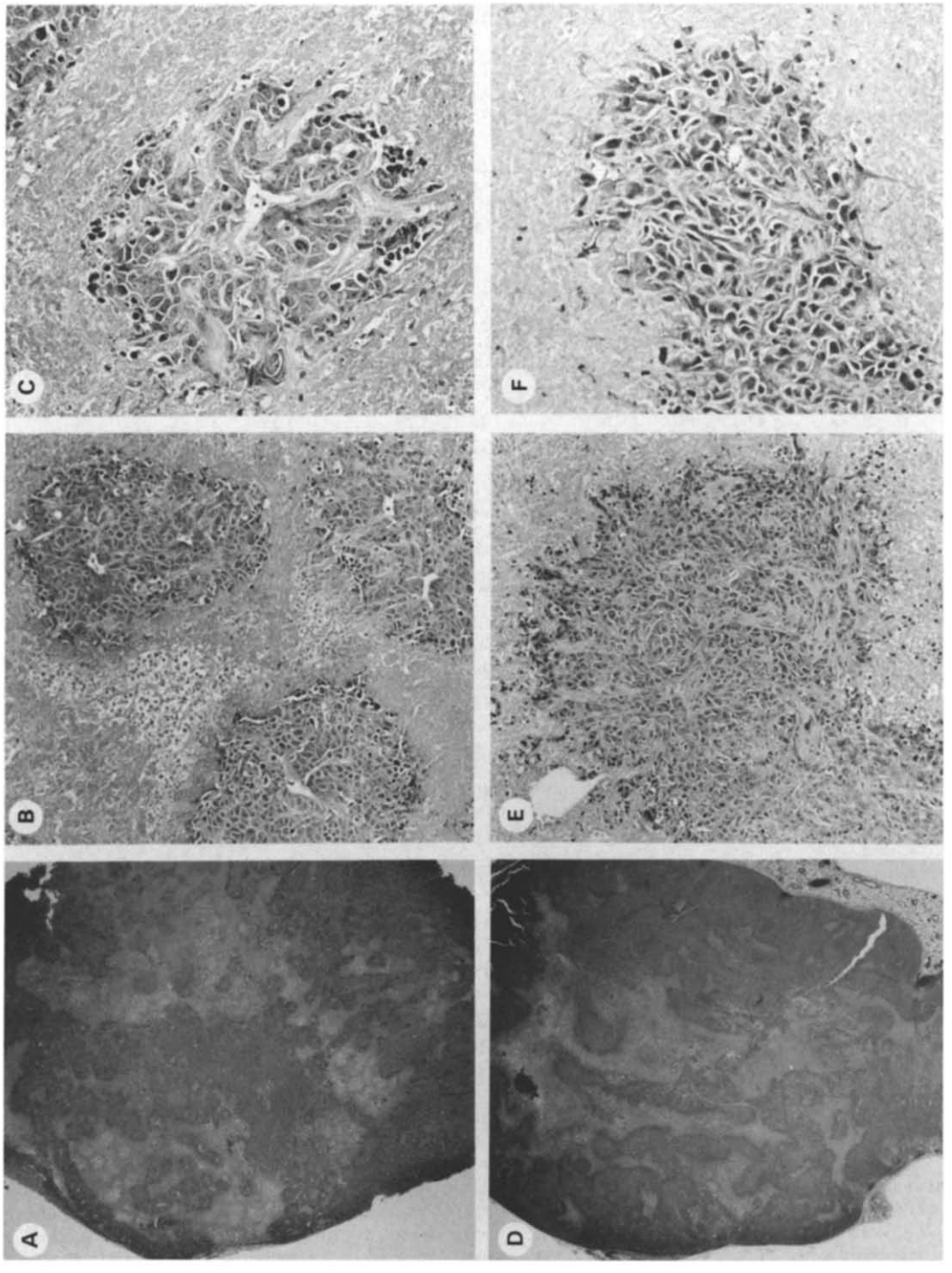

Fig. 2. Photomicrographs of hematoxylin and eosin-stained thin sections of the WT and Adr^R xenografts. Note the similar overall patterns of necrotic areas interspersed with regions of viable, proliferating tumor cells localized near blood vessels in both the mostly necrotic tumor interior and near the tumor periphery. Key: (A) WT 12×, (B) WT 32×, (C) WT 62×, (D) Adr^R 12×, (E) Adr^R 32×, and (F) Adr^R 62× magnification.

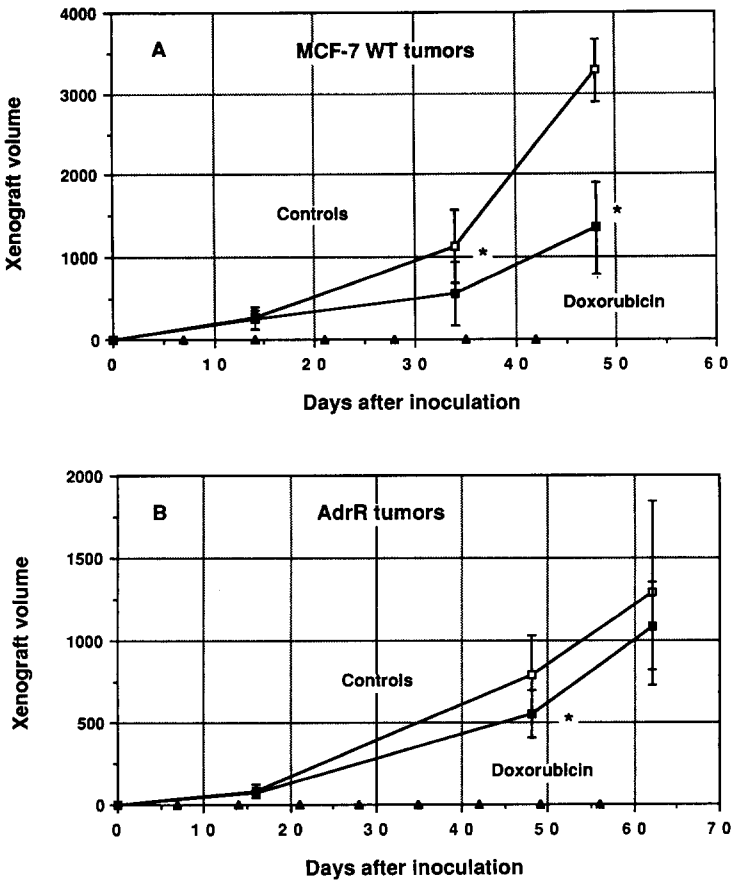

Fig. 3. Response of the WT (A) and Adr^R (B) xenografts to weekly i.p. injections of doxorubicin at 2 mg/kg (indicated by the ▲ symbols). This dose and schedule yielded the best balance between antitumor effectiveness and host toxicity. Tumor volumes (mean ± SD) have been calculated from greatest diameter measurements in two directions by the use of the formula: volume = (length × width^2)/2 for control and doxorubicin-treated mice. The total cumulative dose of doxorubicin was 12 mg/kg for mice with WT tumors and 16 mg/kg for the Adr^R tumor-bearing mice. The WT tumor volumes in the group treated with doxorubicin were significantly smaller than those in the WT control group (Student's t-test, at $P < 0.05$, denoted by an asterisk).

given weekly until cumulative drug toxicity to the mice became lethal (data not shown).

Comparing cumulative doxorubicin doses in the range of 10–16 mg/kg revealed that WT xenograft volumes were restricted to 33, 41 and 51% of controls in three independent experiments. When 16 mg/kg of drug was given in two 8 mg/kg injections 7 days apart, WT tumor volumes were inhibited 77% within 2 weeks; however, nearly half the mice died of drug toxicity within 5 weeks. At all doses tested, doxorubicin failed to slow significantly the growth of Adr^R xenografts with the exception of the single value at 48 days in the experiment shown in Fig. 3B. In other experiments not shown, Adr^R tumors from doxorubicin-treated mice were actually larger than those in parallel saline-treated animals (117, 104 and 148% of control volumes) at cumulative doses within the range of 10–14 mg. This enhancement of Adr^R xenograft growth by doxorubicin was never observed with WT xenografts. In the various experiments with both types of tumors, all doxorubicin cumulative doses greater than 16 mg/kg caused unacceptable lethality that approached 100% within several weeks.

In vitro, the sensitivity of the Adr^R tumor cell line is 250- to 500-fold less for doxorubicin in continuous exposure compared to the WT line, depending upon the choice of cytotoxicity assay [5, 7, 8]. It was not possible, however, to algebraically estimate the relative doxorubicin resistance by the Adr^R tumors *in vivo* because of the lack of any drug response by these tumors.

Tumor concentrations of doxorubicin. We have observed previously that the Adr^R cell line accumulates one-third as much [^{14}C]daunorubicin as the WT line when exposed to 2 μM drug for 30 min [6], and following 90 min of exposure to 10 μM [^{14}C]daunorubicin, the steady-state drug level in WT cells is more than ten times that of Adr^R cells [39]. The decreased intracellular daunorubicin steady-state levels in Adr^R relative to WT are consistent with the MDR phenotype and most likely contribute to both daunorubicin and doxorubicin resistance

Table 1. Comparison of doxorubicin concentrations in WT and Adr^R tumors and host mouse tissues

Tissue	Tissue doxorubicin concentration levels*	
	WT-bearing mice	Adr^R-bearing mice
Tumor	1.34 ± 0.14†	0.59 ± 0.20‡
Hearts	3.67 ± 2.29	3.34 ± 1.15
Kidney	11.1 ± 1.6	9.07 ± 1.19
Liver	16.6 ± 4.9	18.3 ± 1.8
Plasma	0.32 ± 0.06	0.35 ± 0.03

Tumors were grown in athymic nude mice for 40–60 days prior to i.p. injections with [^{14}C]doxorubicin; 2 hr later the animals were killed, and tissues were removed and processed for liquid scintillation counting of doxorubicin-derived radioactivity.

* Expressed as doxorubicin equivalents, nmol/g tissue wet weight or nmol/mL of plasma.

† Values are means ± SD for 4–8 separate samples.

‡ Statistically significant difference ($P < 0.05$) from WT value.

[39–41]. Therefore, it was important to measure the doxorubicin concentrations in WT and Adr^R xenografts to determine whether similar differences in drug levels existed *in vivo* in solid tumors. The data in Table 1 show that Adr^R xenografts, on average, contained only about half as much doxorubicin as the WT tumors at 2 hr after injection (approximately 1–2 μM). It should be pointed out that the Adr^R cell line *in vitro* shows no cytotoxic response to doxorubicin at 2 μM following a 1-hr exposure [8]. Mouse host tissues accumulated much more doxorubicin than the xenografts (6- to 12-fold), most likely because of better vascular perfusion. The tissue to plasma doxorubicin ratios at 2 hr were: WT tumor, 2.6; Adr^R tumor, 1.5; liver, 52; kidney, 35; and hearts, 11 for 5–9 samples. When doxorubicin levels in livers, kidneys and hearts from mice bearing either the WT or Adr^R xenografts were compared, there were no significant differences between the two groups. The relative WT and Adr^R tumor and host organ doxorubicin levels were similar to those in Table 1 when both tumor types were grown contralaterally and simultaneously in mice. These patterns were also observed 4 hr after doxorubicin injection, although the organ drug levels, in general, were lower. We have not yet investigated the metabolism of doxorubicin by the breast tumor xenografts, and therefore drug levels are expressed as doxorubicin equivalents.

P-170 Glycoprotein mRNA expression. The autoradiogram of tumor RNA slot blots probed with a cDNA for P-170 glycoprotein (pAdr-1) shows that the *mdr1* gene product was detected only in the Adr^R xenograft samples (Fig. 4). The cDNA probe hybridized with as little as 1 μg mRNA from the two Adr^R samples, but no hybridization was seen with 10 μg mRNA from the two separate WT extracts.

Doxorubicin-activating enzymes. Cytochrome P-450 reductase activity in microsomes from Adr^R xenografts was 60% of the activity in microsomes from WT xenografts (Table 2), suggesting only a slightly diminished potential for doxorubicin activation. In contrast, mitochondrial NADH-dehydrogenase activity was higher in Adr^R tumors relative to the WT (Table 2). The activities of both of these enzymes are similar to those previously measured in microsomes and mitochondria isolated from the WT and Adr^R tumor cells grown in culture [7, 21]. The yields of microsomes and mitochondria from the two solid tumors were nearly the same (microsomes: 10.0 ± 2.2 and 9.6 ± 1.3 mg protein/g and mitochondria: 4.5 ± 0.4 and 5.8 ± 1.3 mg protein/g wet weight for WT and Adr^R tumors, respectively).

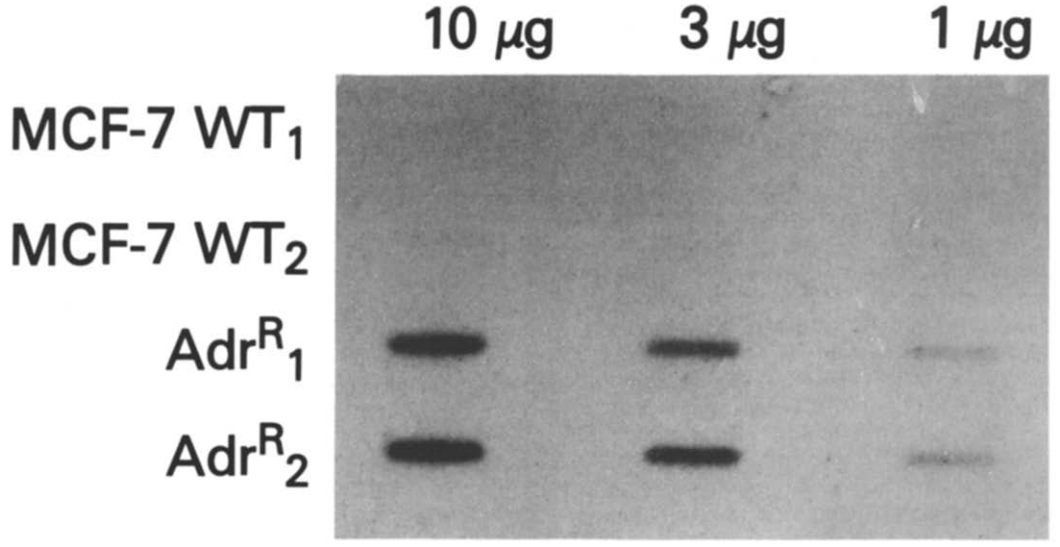

Fig. 4. P-Glycoprotein mRNA expression in MCF-7 WT and Adr^R xenografts. RNA was isolated from two WT and two Adr^R solid tumors grown in separate mice, aliquotted onto a nitrocellulose filter, and probed with a radiolabeled *mdr1* cDNA probe for P-glycoprotein, as described in the text.

Table 2. Activities of the major doxorubicin-activating enzymes and cytosolic antioxidant enzymes in WT and Adr^R human breast tumor xenografts

Enzyme	WT tumors	Adr^R tumors
Microsomal NADPH–cytochrome P450 reductase*	52 ± 9†	32 ± 3‡
Mitochondrial NADH-dehydrogenase*	43 ± 2	49 ± 3
Glutathione peroxidase§	66 ± 10	117 ± 12‡
Copper, zinc-superoxide dismutase‖	3.9 ± 0.3	5.9 ± 0.5‡
Catalase¶	7.9 ± 1.5	6.0 ± 0.5

Freshly excised tumors were homogenized in appropriate buffers, and microsomes, mitochondria and cytosolic fractions were isolated by differential contrifugation. Each enzyme was assayed in the fractions where the specific activities are known to be the highest.

* Expressed as nmol ferricytochrome *c* reduced/mg protein/min at 37°.

† Values are means ± SD for 3–12 determinations.

‡ Statistically significant difference ($P < 0.05$) from WT tumor values.

§ Expressed as the rate of NADPH oxidation (nmol/mg protein/min) in the glutathione reductase-coupled assay using 73 μM H_2O_2 as cosubstrate.

‖ Expressed in units/mg protein where one unit is defined as the amount of enzyme necessary to inhibit by half the superoxide-dependent reduction of acetylcytochrome *c*.

¶ Expressed as μmol hydrogen peroxide decomposed/mg protein/min.

Antioxidant enzymes in xenografts. Catalase activities in the WT and Adr^R xenograft cytosols were similar, but copper, zinc-superoxide dismutase activity was 50% higher in Adr^R compared to WT tumors (Table 2). Adr^R tumor glutathione peroxidase activity was nearly 2-fold greater than the WT, and, interestingly, both WT and Adr^R xenografts have much higher peroxidase activities than the tumor cell lines in culture (70- and 10-fold, respectively [5, 7]. Overall, the Adr^R xenografts had significantly greater antioxidant enzyme protection than the WT solid tumors.

Levels of GSH, GSSG and glutathione-related enzymes. Glutathione is the obligatory cofactor for both glutathione peroxidase and the glutathione transferases, and it also functions independently as an electrophile-detoxifying agent [42]. The constitutive levels of GSH (nmol/mg total protein) and GSSG-reductase specific activities were both marginally higher in WT than in Adr^R xenografts (20%) (Table 3). GSH levels normalized to tissue wet weights were 2.60 ± 0.25 and 1.59 ± 0.27 μmol/g tumor, respectively, for WT and Adr^R ($P < 0.05$). In contrast, slightly more (50%) glutathione disulfide was measured in the cytosols from the Adr^R tumors. Collectively, these results indicate that there are no major differences in glutathione regulation in the two types of breast tumors grown as xenografts in mice.

Of note is the nearly 10-fold increase in GSH-*S*-aryltransferase activity in the Adr^R xenografts compared to the WT (Table 3). A similar magnitude of difference in total GSH-transferase activities between WT and Adr^R tumor cells in culture has been reported [5, 7], and the increased GSH-transferase activity in Adr^R tumor cells is almost exclusively due to the overexpression without amplification of the gene coding for the anionic π GSH-transferase isozyme [43]. Interestingly, the GSH-transferase activities were almost the same *in vivo* and *in vitro* for both WT and Adr^R, suggesting considerable stability of the regulation of the overexpression of glutathione *S*-aryltransferase in the resistant tumors.

The treatment of mice with 17β-estradiol pellets did not influence GSH-peroxidase, or GSH-transferase activities or P-170 glycoprotein mRNA levels in the Adr^R xenografts. The doxorubicin-activating oxidoreductases were measured in microsomes and mitochondria isolated from WT and Adr^R tumors grown in 17β-estradiol-treated mice.

Superoxide and hydroxyl radical production. Negligible amounts of superoxide and hydroxyl radicals were detected by ESR DMPO-spin-trapping when microsomes or mitochondria from either WT or Adr^R solid tumors were incubated without doxorubicin. However, significant generation of oxygen free radicals occurred after doxorubicin and either NADPH or NADH were added to reaction mixtures. Figure 5 shows examples of the DMPO-oxygen radical adduct ESR spectra obtained from microsomes and mitochondria from WT and Adr^R xenografts incubated aerobically with doxorubicin and appropriate cofactors. With tumor microsomes, superoxide radical was the predominant species combined with a small amount of hydroxyl radical that could be identified by the DMPO-OOH/DMPO-OH adduct spectra. In contrast, hydroxyl radical DMPO-OH spectra were observed exclusively with mitochondria from WT and Adr^R tumors (Fig. 5). Quantitatively, with doxorubicin at 200 μM, approximately 2-fold more superoxide/hydroxyl radical-DMPO adducts were formed with WT microsomes than with Adr^R microsomes, and Adr^R mitochondria generated only half as much hydroxyl radical as mitochondria from WT solid tumors.

DISCUSSION

Anticancer drug sensitivity, growth characteristics, biochemistry and the hormone-responsiveness of the

Table 3. Glutathione levels and glutathione-related enzyme activities in WT and Adr^R breast tumor xenografts

Parameter*	WT xenografts	Adr^R xenografts
Reduced glutathione	26 ± 4†	20 ± 3‡
Oxidized glutathione	2.7 ± 1.2	4.1 ± 0.4
GSSG-reductase activity	93 ± 9	70 ± 11‡
GSH-*S*-transferase activity	17 ± 3	160 ± 15‡
Protein	86 ± 9	83 ± 12

Tumor homogenates in 0.1 M phosphate buffer, pH 7.5, were deproteinized with sulfosalicylic acid for the glutathione measurements. Solid tumor samples assayed for GSSG-reductase and GSH-transferase activities were centrifuged at 15,000 *g* to remove membraneous material following homogenization and freeze-thawing to disrupt organelles.

* Glutathione levels are expressed as nmol/mg total protein, GSSG-reductase activity as nmol NADPH oxidized/mg cytosolic protein/min, GSH-*S*-transferase activity as nmol GSH-adduct formed/mg cytosolic protein/min, and protein as mg/g wet weight.

† Values are means ± SD for 3–9 determinations.

‡ Statistically significant difference ($P < 0.05$) from WT values.

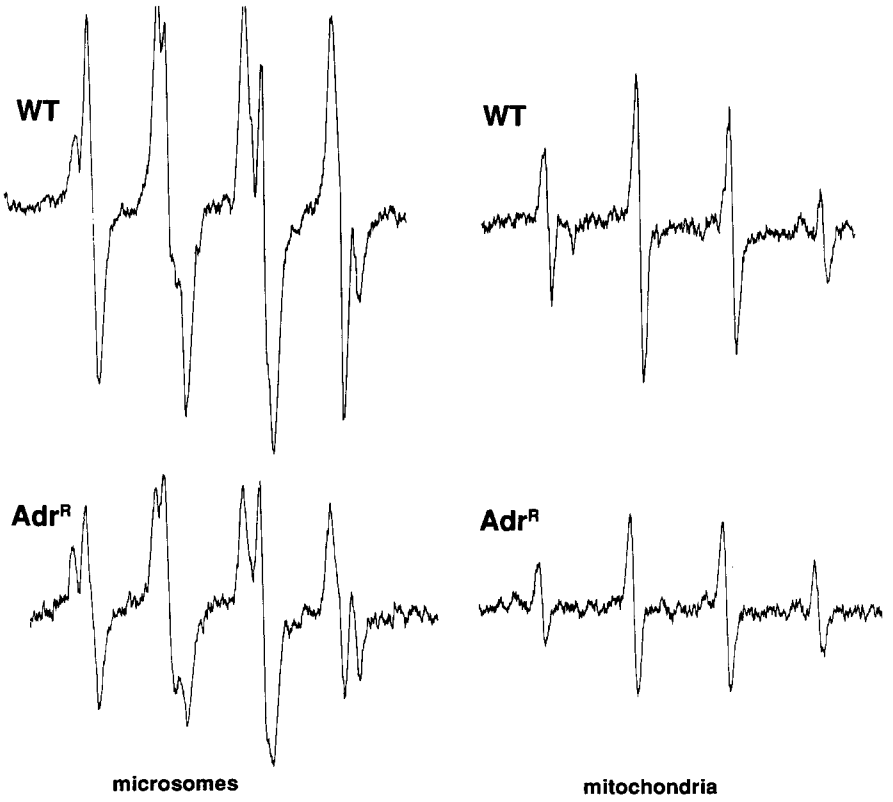

Fig. 5. Electron spin resonance DMPO spin-trapping of oxygen radicals generated by microsomes and mitochondria (2 mg protein/mL) isolated from WT and Adr^R xenografts and incubated *in vitro* at 25° with DMPO (50 mM) doxorubicin (200 μM) and NADPH or NADH (1 mM). With microsomes (spectra on left), mostly superoxide radical (DMPO-OOH) with a small amount of hydroxyl free radical (DMPO-OH) were spin-trapped, and with mitochondria, the ESR spectra were due almost exclusively to DMPO-hydroxyl radical adducts (spectra on the right). The instrument settings were: center field, 3480 G; scan range, 100 G; scan time, 500 sec; frequency, 9.79 GHz; microwave power, 10 mW; field intensity modulation, 2 G; gain, 2×10^6 and time constant, 2.5 sec for microsomes and gain 1×10^6 and time constant 1.25 for mitochondria. The splitting constants are: $a^N = a^H = 15.0$ G for DMPO-OH; $a^N = 14.7$ G, $a_\beta^H = 11.7$ G, and $a\gamma^H = 1.2$ G for DMPO-OOH. To quantitatively compare the microsomal spectra with the mitochondrial spectra, the mitochondrial spectra must be doubled. Oxygen radical spin-trapping is shown with doxorubicin at 200 μM for the purpose of showing the details of the ESR spectral signatures, although with WT tumor cells, DMPO-oxygen free radical spin-adducts have been detected at lower doxorubicin concentrations [10].

MCF-7 WT and the Adr^R MDR variant cell lines *in vitro* have been characterized extensively, and multiple factors have been identified that collectively contribute to the decreased drug sensitivity of the Adr^R cell line [6–12]. Resistance to doxorubicin by Adr^R cells is clearly associated with the amplification and overexpression of the *mdr1* gene that codes for the P-170 glycoprotein multidrug-transporter [9] that decreases doxorubicin accumulation relative to the sensitive WT cells [6, 39]. Decreased sensitivity to doxorubicin by Adr^R cells has also been linked to a 12-fold increased selenium-dependent GSH-peroxidase activity that detoxifies doxorubicin-generated H_2O_2 and prevents the formation of the extremely reactive and toxic hydroxyl free radical [10, 12, 17, 21]. Because of the extensive biochemical and pharmacological characterization that has been done on Adr^R cells, we chose this tumor cell line to establish xenografts in mice to examine the doxorubicin sensititivity and biochemistry of MDR *in vivo*.

Overall, the tumorigenicities of the WT and Adr^R xenografts were similar, except for the estradiol independence and slower growth of the Adr^R MDR tumors, characteristics also observed in cell culture. Neither tumor type metastasized from the primary sites or extensively invaded nearby tissues within 2 months, although larger tumors occasionally eroded through the skin or grew into the muscle layers. A microscopic examination of stained thin sections from WT and Adr^R xenografts failed to show any gross differences in morphology. Tumor vascularization, desmoplasia, the proportion of central region necrosis, and the extent of lymphocytic cell infiltration in the two types of xenografts were similar. Histologically, both tumors could be clearly identified as adenocarcinomas.

Doxorubicin levels in the WT tumors were 2-fold greater in Adr^R tumors 2 hr after drug injection, a time when tissue doxorubicin levels in nude mice have been reported to be near maximum following the i.p. route of administration [44]. The diminished doxorubicin accumulation by the Adr^R xenografts relative to the WT reflects the difference in the comparative doxorubicin levels seen in the two cell lines *in vitro* [39]. These results suggest that the P-glycoprotein drug pump, which was found exclusively in the Adr^R xenografts by RNA slot-blot analysis, may have diminished the Adr^R solid tumor doxorubicin levels, and thus contributed to doxorubicin resistance. Compared to host tissues, doxorubicin accumulation by tumors was relatively poor. Doxorubicin was only twice the plasma concentration in Adr^R and four times plasma levels in WT tumors at 2 hr, while the liver/plasma ratio was more than 50. The low tumor doxorubicin concentrations most likely resulted from poor vascularization and limited drug delivery. Perhaps because of these restrictions, the WT xenografts only partially responded to doxorubicin treatment, and because Adr^R xenografts accumulated even less drug, they failed to show any objective response. In fact, the doxorubicin levels in Adr^R xenografts following a dose of 8 mg/kg were far below the doxorubicin concentrations necessary for cytotoxicity *in vitro* (400 μM IC_{50} for a 1-hr exposure). In this way, the xenografts may resemble the human situation where clinically attainable tumor drug levels are frequently insufficient to kill or even slow the growth of MDR tumors.

While many of the previously measured enzymatic activities of the WT and Adr^R cell lines *in vitro* were similar in the xenografts derived from those lines, several important biochemical differences were noted. Most conspicuous among these was the marked elevation of selenium-dependent GSH-peroxidase activities in both types of xenografts. Relative to tumor cells in culture [7], the activity of this enzyme was increased >70-fold in WT xenografts, and 10-fold in Adr^R xenografts, to levels found in normal mouse organs including breast tissue [45]. Additionally, the GSH-peroxidase activity of Adr^R xenografts was nearly twice that of WT xenografts. These elevated tumor GSH-peroxidase levels did not result from contamination of the tumor tissue by erythrocytes. Hemoglobin in clear supernatants from both types of solid tumors was $< 0.5\%$ of mouse whole blood hemoglobin levels, indicating minimal erythrocyte content (data not shown). Moreover, both catalase and superoxide dismutase levels are known to be very high in erythrocytes, yet the activities of these two enzymes in xenografts were comparable to or less than those of tumor cells in culture [7], further indicating minimal erythrocyte contamination. The increase in tumor GSH-peroxidase *in vivo* probably results from the greater availability of selenomethionine, selenocysteine and selenite in the rodent diet, while the only source of selenium *in vitro* was the fetal bovine serum. Selenium is essential for the activity of GSH-peroxidase [42], and it also appears to up-regulate GSH-peroxidase mRNA expression [46]. Chu *et al.* [47] have shown recently that the addition of 30 nM selenite to MCF-7H6 tumor cell transfectants expressing a GSH-peroxidase cDNA increases this enzyme activity more than 20-fold compared to that of cells grown in selenium-deficient medium, and elevated the mRNA for this enzyme 3- to 4-fold.

The important role for GSH-peroxidase activity in doxorubicin resistance by Adr^R tumor cells *in vitro* has been well documented experimentally [48]. The activity of this enzyme is elevated 12- to 14-fold in Adr^R cells compared to WT [5, 7, 10]. Adr^R tumor cells are 4-fold more resistant than WT cells to exogenous H_2O_2 generated from glucose oxidase, although catalase, the alternative H_2O_2-scavenging enzyme, has equivalent activity in both cell types [7, 10]. Treatment of Adr^R tumor cells with bithionine sulfoximine depletes cytosolic GSH and limits the ability of GSH-peroxidase to scavenge H_2O_2, enhances hydroxyl radical generation in tumor cells incubated with doxorubicin, and potentiates doxorubicin cytotoxicity 5-fold [12]. We have shown that either purified GSH-peroxidase or Adr^R cell lysate added to sonicated WT cells incubated with doxorubicin blocked hydroxyl radical DMPO-spin-trapping [21], and "scrape-loading" of this enzyme into WT MCF-7 breast tumor cells has been reported to ameliorate doxorubicin cytotoxicity [49]. Collectively, this evidence supports the concept that oxygen radicals and hydrogen peroxide-mediated

mechanisms contribute to the cytotoxicity of doxorubicin toward tumor cells in culture.

The DMPO-spin-trapping experiments with microsomes and mitochondria from WT and AdrR xenografts indicate that both types of solid tumors have the ability to enzymatically activate doxorubicin to a semiquinone free radical intermediate that reacts with dioxygen to form superoxide and hydroxyl free radicals. It is reasonable, then, to hypothesize that in addition to P-170 glycoprotein, the greater GSH-peroxidase and superoxide dismutase activities in Adr^R compared to WT xenografts played a role in the lack of doxorubicin sensitivity by the Adr^R tumors by scavenging drug-generated superoxide radical, H_2O_2 and possibly secondary organic peroxides, preventing oxidative damage to critical macromolecules. Additionally, the high glutathione peroxidase activity in the WT xenografts compared to WT tumor cells in culture may explain why doxorubicin treatments *in vivo*, even at the high dose of 8 mg/kg, only slowed the growth of these tumors when tumor drug levels were several-fold higher than the cytotoxic IC_{50} concentration for doxorubicin toward WT cells determined in culture (0.3 to 0.5 μM for a 1-hr exposure). It would be extremely interesting to examine human breast tumor samples for GSH-peroxidase activity, and to attempt to correlate prospectively enzyme levels with doxorubicin response.

It should be pointed out that the GSH content and GSSG-reductase activity in Adr^R xenografts were 80% of WT xenograft values, suggesting only a slightly diminished ability of Adr^R tumors to maintain glutathione in its reduced form. Although reduced GSH is important as the obligatory cofactor for GSH-peroxidase, the GSH concentration in Adr^R tumors would be sufficient ($\approx$ 2 mM) to supply adequate reducing equivalents to this enzyme for peroxide removal. Overall, the coupling of GSSG-reductase, GSH and especially the high GSH-peroxidase activity would permit extensive detoxification of doxorubicin-generated H_2O_2 in the millimolar range.

In summary, Adr^R and WT xenografts in nude mice were tumorigenically and morphologically similar, but only the WT solid tumors responded to doxorubicin treatment, indicating that the doxorubicin resistance of Adr^R tumor cells, and by extension the MDR phenotype, persists *in vivo*. Adr^R xenografts accumulated less doxorubicin than WT, and this observation can be attributed to the expression of membrane P-170 glycoprotein by Adr^R. Both WT and Adr^R xenografts enzymatically activated doxorubicin and generated oxygen free radicals, but WT tumor subcellular fractions produced about twice as much superoxide and hydroxyl radicals as the corresponding Adr^R fractions. Additionally, the Adr^R xenografts had a greater potential to detoxify H_2O_2 by the action of selenium-dependent GSH-peroxidase and superoxide dismutase. While there were some important changes in the biochemistry of both WT and Adr^R tumor cell lines when they were transferred from the well-defined conditions of cell culture to the extremely complex environment of a living animal, in general, the phenotypes of the two cell lines were retained when they were grown as xenografts. Further work may establish human breast tumor xenografts as useful *in vivo* models to study biochemical mechanisms and regulation of MDR.

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