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Altered Gene Expression in Drug-resistant Human Breast Cancer Cells

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ABSTRACT

It is increasingly recognized that drug-resistant cells undergo transitions not directly linked to "classical" drug resistance. We examined the expression of growth factors, growth factor receptors, and the estrogen receptor in 17 drug-resistant and 2 revertant human breast cancer sublines to provide an understanding of the phenotypic changes that occur and how these changes could affect the biology of the cell. These sublines were derived from five parental human breast cancer cell lines (MCF-7, ZR75B, T47D, MDA-MB-231, and MDA-MB-453). The expression of estrogen receptor was absent or decreased in 6 of the 15 resistant MCF-7, ZR75B, and T47D sublines. Increases of as much as 49-fold compared to parental levels were observed in transforming growth factor α, epidermal growth factor receptor, c-erbB2, and/or c-erbB3 mRNA expression in 14 of the 17 resistant sublines. Altered amphiregulin and insulin-like growth factor-I receptor expression was observed in nine and four drug-resistant sublines, respectively. No major alterations were observed in epidermal growth factor and c-erbB4 expression. Few alterations were observed in two sublines derived from estrogen receptor-negative cells. Higher levels of phosphotyrosine residues were detected in a subset of the resistant sublines, indicating an increased tyrosine kinase activity in these cells. Interestingly, decreased growth rates were observed in all of the sublines, despite up-regulated growth factor-related gene expression. Taken together, these data suggest that loss of estrogen receptor, increased expression of growth factor pathway genes, and decreased growth rate regularly occur in drug-resistant breast cancer cells.

Although we do not know whether the altered expression of growth factor pathway genes is linked as a cause or a consequence of the reduced growth rate, it is well established that decreased growth rate confers drug resistance. These phenotypic changes in drug-resistant human breast cancer cells could serve to initiate, support, or extend the drug resistance.

INTRODUCTION

Multiple mechanisms of drug resistance exist, including altered drug uptake, efflux, metabolism, or target (1). However, several reports have suggested that cells that have developed a drug-resistant phenotype *in vitro* express a wide variety of additional phenotypic alterations, including protein kinase C overexpression, loss of epithelial markers, increased accumulation of p53 protein, altered expression of hormone or growth factor receptors, or changes in growth rate (2–9). In addition, it is increasingly recognized that proteins unrelated to "classical" drug-resistant phenotypes may contribute to drug resistance. For example, in transfection studies, it has been shown that overexpression of EGF[4] receptor or c-erbB2 in breast cancer cells, c-ras in NIH 3T3 cells, and c-bcl 2 in lymphoid cells leads to an increase in cellular resistance to chemotherapeutic drugs (10–13). Also, absence of p53 expression in mouse embryonic fibroblasts results in resistance (14), and a ribozyme that cleaves c-fos mRNA was shown to reverse drug resistance in an ovarian carcinoma cell line (15). In addition, mitogen activation of the *MDR*-1 promoter, mediated through the Raf-1 kinase, has been demonstrated (16), suggesting a role of signal transduction, apoptosis, or cell cycle components in the drug-resistant phenotype.

Both clinical and laboratory data have suggested that growth factor signaling may play a role in drug resistance in breast cancer. Vickers *et al.* (17) showed, in an unrelated MCF-7 subline (MCF-7/Adr[R]), that doxorubicin resistance and P-glycoprotein overexpression were accompanied by an increased expression of EGF receptor and loss of estrogen receptor. Previously, we demonstrated increased levels of EGF receptor and TGF-α, as well as decreased levels of the estrogen receptor in a doxorubicin-resistant human breast cancer MCF-7 subline (MCF-7 AdVp; Ref. 7). In patients with breast cancer, loss of estrogen receptor and an increased level of EGF receptor expression in tumor samples are correlated with a poor prognosis (18–21).

Growth factors interact with the extracellular binding domain of their specific, transmembrane spanning receptors, activating the tyrosine kinase function of the intracellular domain,

initiating a signaling pathway that leads to gene expression and increased growth rate (22). Numerous growth factor receptors have been shown to initiate the growth factor signaling cascade in human breast cancer cells, including the EGF receptor, c-erbB2, c-erbB3, c-erbB4, and IGF-I receptor. The EGF receptor is activated by at least three ligands: TGF-α and EGF, which stimulate growth; and amphiregulin, which stimulates or inhibits growth, depending upon the model system (23). The proto-oncogenes c-erbB2, c-erbB3, and c-erbB4 encode receptor tyrosine kinases with extensive homology to the EGF receptor (24–26). Recent data indicate that ErbB2 interacts with the EGF receptor, ErbB3, and ErbB4, in a ligand-dependent manner (27). The ligands for ErbB3 and ErbB4, termed neu differentiation factor or heregulins, either stimulate mitogenesis or promote differentiation, depending upon the breast cell line used (28). Amplification of the c-erbB2 gene has been described in human mammary carcinomas and is correlated with a poor disease prognosis (29–31).

In the present study, we evaluated the generality of changes in the expression of estrogen receptor and growth factor-related genes in human breast cancer cells that developed drug resistance. Expression of *MDR*-1 and topoisomerase II were evaluated as mechanisms of drug resistance. In addition, we examined the growth rate of the drug-resistant cells to determine whether the studied phenotypic alterations had an effect on the proliferative activity of the cells.

MATERIALS AND METHODS

Cell Culture Methods and Cell Lines. All cells were grown as monolayer cultures at 37°C with 5% CO_2 and maintained by regular passage in IMEM supplemented with 10% FCS, 2 mM glutamine, 15 mM HEPES, and 25 μg/ml gentamicin. MCF-7 TH and MCF-7 AdP500 cells are drug-resistant sublines isolated from parental MCF-7 cells by intermittent exposure to 1 μM and 500 nM doxorubicin, respectively. The MCF-7 TH cells were a gift from Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The remaining resistant sublines were generated by stepwise increases in the concentration of the selecting agent and referred to by selecting agent and by drug concentration. The sublines were maintained in the peak selecting concentration. Cells were consistently cultured for 5–7 days out of drug before the start of the experiments.

Cytotoxicity Assay and Growth Curve Experiments. Cytotoxicity studies were performed in 96-well plates in the presence of increasing concentrations of the cytotoxic compound in IMEM + 10% FCS. Between 1000 and 4000 cells/well were plated and treated 24 h later. After 4 days in culture, the cells were fixed in 10% trichloroacetic acid and then stained with 0.4% sulforhodamine B (Sigma Chemical Co., St. Louis, MO) in 1% acetic acid (32). The bound dye was solubilized in 200 μl of 10 mM unbuffered Tris solution, and the absorbance (*A*) was determined at a wavelength of 540 nm in an Elisa microplate reader (Bio-Rad) in quadruplicates. Untreated control wells were assigned a value of 100%, and the IC_{50} was defined as the dose required to inhibit *A* measured at 540 nm to 50% of the control value. Results are representative of two to three separate experiments. The growth curve experiments were performed in similar conditions. Sixteen h and 3, 5, and 7 days

after plating, cells were fixed and analyzed as described above. A growth curve was generated, and from there the doubling time was calculated.

Riboprobes Used in RNA Analysis. The 141-bp fragment of EGF receptor, 603-bp fragment of TGF-α, and 220-bp fragment of *36B4* were described previously (7, 33) and used to generate radiolabeled antisense riboprobes for the RNase protection experiments. A 289-bp *SphI-StyI* fragment of EGF, subcloned in pGEM 3, a 470-bp fragment of c-erbB2 also subcloned in pGEM3, and a 700-bp *EcoRI* fragment of IGF-I receptor subcloned in pGEM3Z were used to detect EGF, c-erbB2, and IGF-I receptor mRNA, respectively (34, 35). The 300-bp *EcoRV* fragment of amphiregulin, the 466-bp *EcoRV* fragment of c-erbB3, and the 725-bp *EcoRV-NsiI* fragment of c-erbB4, all subcloned in pBluescript-SKII+, were generously donated by Greg Plowman (Sugen, Redwood City, CA) for use in making riboprobes for detection of amphiregulin, c-erbB3, and c-erbB4 mRNA (26, 36, 37). The 300-bp fragment of estrogen receptor was described previously and used to detect estrogen receptor mRNA (7). A 572-bp PCR fragment (10B) subcloned in pGEM3Z was kindly provided by Tito Fojo (NIH, Bethesda, MD) to detect topoisomerase IIα expression. A 1.4-kb cDNA containing sequences from the middle third of *MDR*-1 subcloned in a pGEM vector was used for riboprobe synthesis and Northern blot analysis of the *MDR*-1 message (38).

RNA Extraction, Northern Analysis, and RNase Protection Assay. Total RNA was extracted from cells by homogenizing in guanidine isothiocyanate buffer followed by centrifugation over a CsCl cushion (39). For Northern analysis, 8 μg of total RNA were electrophoretically separated in a 1% agarose, 6% formaldehyde gel and transferred onto a nylon membrane (Gene Screen Plus; DuPont NEN, Boston, MA). Equal loading, blotting, and quality of RNA samples were verified by staining the membrane with methylene blue (40). Subsequently, the blots were hybridized with ^{32}P-labeled *MDR*-1 riboprobe and exposed to a film.

For RNase protection assays, 30 or 60 μg of total RNA were hybridized with 3×10^5 cpm labeled riboprobe and then digested for 30 min at 25°C with 40 μg/ml RNase A and 28 μg/ml RNase T1. Following extraction, samples were separated on a 6% polyacrylamide gel, and autoradiography was performed. ^{32}P-Labeled riboprobes were prepared by SP6, T3, or T7 polymerase transcription (Promega, Madison, WI) as appropriate for each probe in the presence of [α-^{32}P]UTP (specific activity, 3000 Ci/mmol; DuPont NEN; Ref. 41). The *36B4* gene has been used as a control by investigators in studies of mRNA regulation in breast cancer cells because its expression is not affected by estrogen or other mitogens (33, 42). The intensity of the autoradiographic signal was determined with a Fotoeclipse densitometer (Fotodyne, Inc., Hartland, MI). Pearson correlation coefficients were calculated using the Desa-Plot program written by Dr. M. Schliak (Klinge Pharma, Munich, Germany).

Immunoblot Analysis. Cells were plated in 100-mm Petri dishes in serum-containing medium. ZR75B and T47D cells and sublines were cultured 48 h prior to cell lysis. MCF-7 cells and sublines were placed in serum-free medium for 24 h prior to stimulation with 10^{-8} M EGF for 30 min, lysis in TNESV [50 mM Tris (pH 7.6), 1% NP40, 2 mM EDTA, 100 mM NaCl, and 1 mM vanadate] with 20 μg/ml aprotinin, 20 μg/ml leupeptin,

Table 1 Resistance phenotype in selected sublines

Cell line	Selecting cytotoxic drug[a]	IC_{50} (ng/ml)	IC_{50} + Vp[b] (ng/ml)	Relative resistance[c]	Cell doubling time (h)	Relative *Topo* II expression[d]
MCF-7						
Parent	Ad	21.5	18.3		34.6	1
TH	Ad	3224	207	150	50.6	0.87
TH Rev	Ad	74	41	3.4	36.6	1.03
AdVp	Ad	3888	4097	181	58.1	1.44
AdVp RevA	Ad	1221	854	57	39.5	1.22
AdVp1000	Ad	15×10^3	ND	698	ND	1.39
Ad75	Ad	622	175	29	77.3	0.19
AdP500	Ad	ND	ND	ND	ND	0.14
Parent	Tx	1.4	1.9		34.6	1
Tx8	Tx	9.8	1.8	7.0	54.7	0.40
Tx200	Tx	344	52	246	79.3	0.96
Parent	Ida	1.7	ND		34.6	1
Ida6	Ida	40	ND	24	ND	ND
ZR75B						
Parent	Ad	17.3	16		30.5	1
Ad24	Ad	215	15.4	12.4	41.3	2.14
Ad120	Ad	517	35	30	47.8	0.95
Parent	Tx	2.8	2.8		30.5	1
Tx10	Tx	60	3.9	21	34.1	1.07
Tx40	Tx	77	7.3	27.5	43.5	0.34
Parent	Vb	0.7	0.5		30.5	1
Vb10	Vb	100	1.5	141	38.2	0.54
T47D						
Parent	Ad	33.4	40.7		49.2	1
Ad12	Ad	240	173	7.2	68.2	1.73
Ad60	Ad	2154	1287	65	86.0	1.63
MDA-MB-453						
Parent	Ad	18.7	ND		68.8	1
Ad15	Ad	111.5	ND	6.0	120.5	0.46
MDA-MB-231						
Parent	Vb	0.7	0.5		26.7	1
Vb100	Vb	150	1.5	214	33.5	1.72

[a] Ad, doxorubicin; Ida, idarubicin; Tx, paclitaxel; Vb, vinblastine.

[b] IC_{50} as determined in the presence of the P-glycoprotein blocker verapamil.

[c] Relative resistance refers to the fold increase in IC_{50} of the drug-resistant subline compared to the IC_{50} of the parental cell line.

[d] Topoisomerase II (*Topo*II) expression was determined by RNase protection assay. Levels of expression were normalized to *36B4* levels and depicted as ratios compared to the parental cells. ND, not determined.

and 1 μM phenylmethylsulfonyl fluoride, and centrifugation for 30 min at 4°C at 14,000 rpm in an Eppendorf centrifuge. For analysis of total protein, 70–80 μg protein extracts were fractionated by SDS-PAGE, transferred to Immobilon (Millipore, Bedford, MA), and probed with the primary antibodies to detect phosphotyrosine (clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY; Ref. 43). Proteins were visualized using horseradish peroxidase-labeled secondary (Amersham Corp., Arlington Heights, IL) antibody and light emitting nonradioactive enhanced chemiluminescence (ECL, Renaissance; DuPont NEN) followed by exposure to an autoradiographic film.

RESULTS

Resistance to Cytotoxic Drugs in the Drug-selected Human Breast Cancer Cells. The resistance of 17 drug-selected sublines to the selecting agent is shown in Table 1. Of the MCF-7 sublines, the relative resistance was highest in the MCF-7 TH, AdVp, and Tx200 sublines, 150-, 181-, and 246-fold, respectively. The resistance to drug was 30-fold for ZR75B Ad120, 28-fold for ZR75B Tx40, and 141-fold for the ZR75B

Vb10 sublines. The T47D Ad60, MDA-MB-453 Ad15, and MDA-MB-231 Vb100 sublines were 65, 6, and 214 times more resistant than the parental cells, respectively. Treatment with 5 μg/ml verapamil, a P-glycoprotein antagonist, reversed resistance in MCF-7 TH, Tx 8, and Tx200 cells and in the resistant ZR75B and MDA-MB-231 sublines. The reversal of resistance by verapamil implicates P-glycoprotein as a major mechanism of drug resistance in these cells. Incomplete reversal of resistance by verapamil may be explained by very high levels of P-glycoprotein in the MCF-7 TH subline and by the existence of other mechanisms of resistance in the MCF-7 Ad75 subline.

Expression of *MDR*-1 in Drug-resistant Breast Cancer Sublines. To confirm the presence of P-glycoprotein, we analyzed the level of *MDR*-1 mRNA, as depicted in Fig. 1. Expression of *MDR*-1 mRNA was detected in 10 of the 18 resistant sublines examined and correlated with reversal of verapamil (Table 1), suggesting that the main mechanism of drug resistance in the 10 sublines expressing *MDR*-1 is P-glycoprotein mediated. The highly resistant subline MCF-7 AdVp showed no *MDR*-1 expression and has previously been

MCF-7

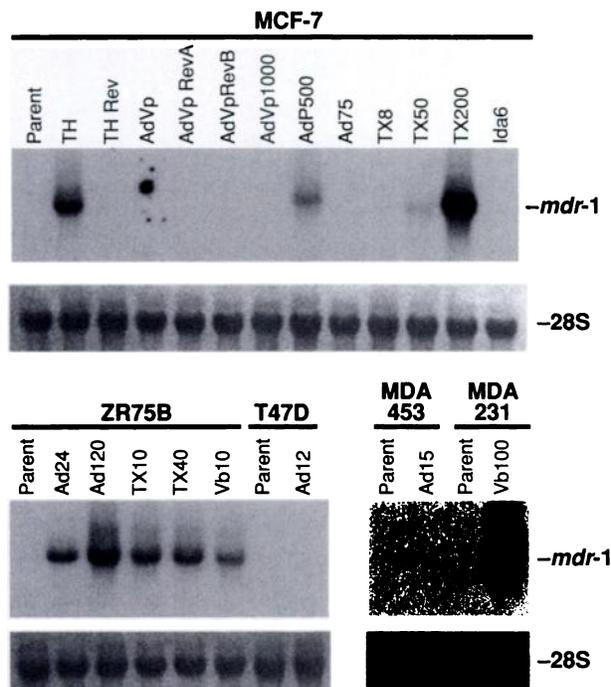**ZR75B**   **T47D**   **MDA 453**   **MDA 231**

Fig. 1 Expression of *MDR*-1 mRNA in parental and drug-resistant cells. The *MDR*-1 expression was analyzed by Northern blot technique using 8 μg of total RNA, and expression in parental cells was compared with the drug-resistant sublines. Methylene blue staining of the membrane shows the integrity and loading of the RNA samples. The sublines are referred to as in Table 1 by the selecting drug and maintenance concentration in ng/ml. The Tx50 cells were an intermediate selection between the Tx8 and Tx200 cells and were not included in the remaining studies.

shown to display a non-P-glycoprotein-mediated resistant phenotype (44). The MCF-7 Ad75 subline and the T47D Ad12 and Ad60 sublines demonstrated only a small degree of reversal with verapamil and did not express *MDR*-1, suggesting that resistance in these cells is also not mediated by P-glycoprotein.

Decreased Estrogen Receptor Expression in Doxorubicin-resistant Sublines. It was reported previously in two human breast cancer sublines that loss of estrogen receptor occurs with the development of drug resistance (7, 17). We, therefore, determined the estrogen receptor mRNA expression in the parental and drug-resistant sublines by RNase protection. As shown in Fig. 2, expression of estrogen receptor was reduced or absent in 6 of 15 resistant MCF-7, ZR75B, and T47D sublines. Decreased estrogen receptor mRNA expression was observed in MCF-7 AdVp and AdVp 1000 cells and in T47D Ad60 cells at 57, 17, and 10% of parental levels, respectively. Estrogen receptor mRNA was undetectable in the MCF-7 TH subline and the ZR 75B Ad24 and Ad120 sublines and, as expected, was absent in the two MDA-MB-453 and MDA-MB-231 Vb100 sublines derived from the estrogen receptor-negative breast cancer cell lines.

Altered Expression of Growth Factor Pathway-related Genes in Drug-resistant Breast Cancer Cells. The results of RNase protection assays for TGF-α, EGF, amphiregulin, EGF receptor, c-*erb*B2, c-*erb*B3, c-*erb*B4, and IGF-I receptor are

displayed in Fig. 3. TGF-α expression increased in the MCF-7 AdVp (30-fold), Tx200 (17-fold), ZR75B Vb10 (3-fold), and T47D Ad60 (2-fold) sublines. No changes in EGF expression were observed in the drug-resistant sublines compared to the parental cells. Amphiregulin mRNA expression was affected particularly in the MCF-7 sublines; the level was increased in the MCF-7 AdVp (2.5-fold) and AdVp1000 (2-fold) sublines but reduced in the MCF-7 TH, AdP500, Ad75, Tx8, Tx200, and Ida6 sublines. Increased EGF receptor mRNA expression was observed in MCF-7 TH (49-fold), AdVp (18-fold), AdVp1000 (10-fold), ZR75B Ad120 (3-fold), and Tx10 (3-fold) cells. Expression of c-*erb*B2 mRNA was increased in the MCF-7 Tx200 (2-fold), Ida6 (3-fold), ZR75B Ad24 (3-fold), Ad120 (5-fold), and T47D Ad12 (2-fold) sublines. Expression of c-*erb*B3 was increased in MCF-7 AdP500 (4-fold), Tx8 (6-fold), Tx200 (4-fold), Ida6 (5-fold), and ZR75B Ad120 (10-fold) cells. Less than 2-fold alterations in c-*erb*B4 expression were observed for most sublines. The IGF-I receptor mRNA expression is reduced in MCF-7 TH and ZR75B Ad24 sublines but increased in the MCF-7 Ad75 (3-fold) and Tx200 (2-fold) sublines.

The MCF-7 TH Rev and AdVp RevA sublines displayed a phenotype similar to the drug-resistant sublines from which they were derived (MCF-7 TH and AdVp, respectively) rather than to the MCF-7 parental cells. In the revertant MCF-7 TH subline (MCF-7 TH Rev), only the TGF-α expression was similar to the MCF-7 parental level, whereas in the AdVp subline, amphiregulin and c-*erb*B3 expression were similar to parental expression.

Pearson correlation coefficients were obtained for the resistant sublines testing the correlation between growth factor pathway gene expression and relative resistance. In the whole set, no correlations were observed. Among the nine sublines with a relative resistance less than or equal to 30, EGF receptor expression was significantly correlated with $r = 0.840$, $P = 0.01$. There was a suggestion that c-*erb*B2, c-*erb*B3, and IGF-1 expression correlated with relative resistance, $r = 0.447, 0.436$, and 0.521, respectively, but these correlations did not reach statistical significance ($P = 0.2, 0.2$, and 0.1, respectively).

Acute Exposure to Doxorubicin, Vinblastine, and Paclitaxel Does Not Affect the Expression of Growth Factor-related Genes. To exclude the possibility that the alterations observed in Fig. 4 were reversible changes induced by drugs, we examined the effect of a 24-h exposure to doxorubicin, vinblastine, and paclitaxel on gene expression. As shown in Fig. 4, no consistent acute effects of the chemotherapeutic agents on gene expression were observed. Parental cells were also exposed to these agents for 4 weeks prior to RNA harvest. Again, no consistent effects of the chemotherapeutic agents on gene expression were observed (data not shown).

Increased Level of Cellular Tyrosine-phosphorylated Proteins in Drug-resistant Cells. The binding of growth factor to its receptor activates the receptor tyrosine kinase activity to initiate signal transduction, which in turn increases cellular tyrosine phosphorylation. We evaluated the basal cellular phosphorylation of tyrosine residues by anti-phosphotyrosine immunoblotting in parental and drug-resistant sublines (Fig. 5). The overall cellular tyrosine phosphorylation was increased to a variable extent in the drug-resistant sublines. The most dramatic increase was observed in the MCF-7 TH subline, the subline with the greatest increase in EGF receptor levels. Four of the
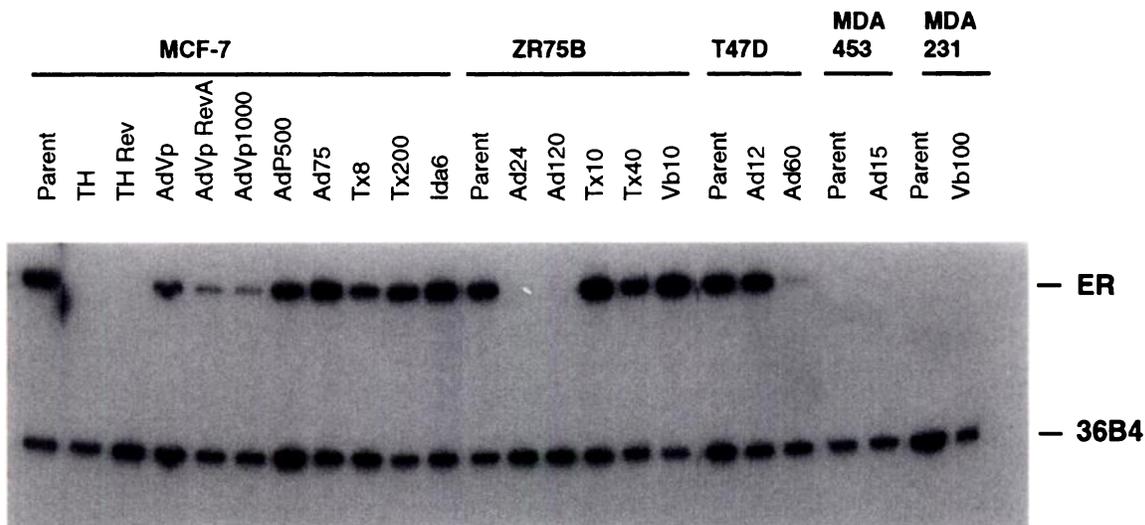
Fig. 2 Estrogen receptor expression in parental and drug-resistant breast cancer cells. The expression levels were determined by RNase protection analysis of 60 μg of total RNA. The *36B4* signal was used to confirm regular loading of the RNA samples in the RNase protection assay.

MCF-7 sublines were examined with and without stimulation by EGF. Increased tyrosine phosphorylation was observed in two of the four sublines, demonstrating that in these sublines the EGF receptor could be functionally activated.

Decreased Growth Rate in Drug-resistant Breast Cancer Cells. Because the expression of growth-related genes is altered in cells that developed drug resistance, we compared the growth rate of the drug-resistant sublines to that of the parental cells. Table 1 shows that the proliferation rate of all of the resistant sublines grown in the absence of drug was reduced compared to the parental cells. The growth rate of the revertants MCF-7TH Rev and AdVp RevA was increased compared to that of the resistant MCF-7TH and AdVp sublines from which they were derived. This result suggests that overexpression of growth factor or growth factor receptors does not necessarily lead to increased proliferation, and that a selective advantage may exist for cells with reduced growth rates.

Most Drug-resistant Breast Cancer Sublines Demonstrate Little Variation in Topoisomerase IIα Expression. Reduced topoisomerase II expression can result in reduced growth rate and has been proposed as a mechanism of drug resistance, through a reduction in available drug target for binding of such anticancer agents as doxorubicin, VP-16, and *m*-amsacrine (1, 45–47). Because our sublines both are drug-resistant and display decreased growth rates, one possible explanation would be reduced topoisomerase II expression. Table 1 presents the results, measured by RNase protection assay, as ratios to the levels in the parental cells. Only the MCF-7 AdP500 and Ad75 sublines demonstrated markedly reduced topoisomerase IIα expression: 0.19- and 0.14-fold expression compared to the parental cells, respectively. For the other sublines, topoisomerase IIα expression ranged between one-half and two times the level of the parental cells. The undetectable *MDR*-1 level but decreased topoisomerase IIα level in the MCF-7 Ad75 cells suggests that the reduced topoisomerase IIα

expression contributes to the drug resistance phenotype in this subline.

DISCUSSION

In the study presented here, we evaluated phenotypic changes occurring in drug-resistant breast cancer cells. We examined 17 drug-resistant and 2 revertant sublines derived from MCF-7, ZR75B, T47D, MDA-MB-453, and MDA-MB-231 cell lines by exposure to various cytotoxic drugs. We determined the expression of nine genes involved in mitogenesis, including estrogen receptor, EGF, TGF-α, amphiregulin, EGF receptor, c-*erb*B2, c-*erb*B3, c-*erb*B4, and IGF-I receptor. The results presented demonstrate the following phenotypic changes in the drug-resistant sublines: decreased estrogen receptor expression in 6 out of 15 resistant MCF-7, ZR75B, and T47D sublines and increases in TGF-α, amphiregulin, EGF receptor, c-*erb*B2, c-*erb*B3, and/or IGF-I receptor mRNA expression in 15 of 17 resistant sublines. Expression of EGF and c-*erb*B4 was essentially unchanged, which implies that these genes are unlikely to be of importance in drug resistance. Significant up-regulation of growth factor and growth factor receptor was most commonly observed in the MCF-7 sublines. A correlation between EGF receptor expression and drug resistance was observed among those drug-resistant sublines with a relative resistance <30, whereas no apparent correlations were observed if all of the resistant sublines were included in the analysis. In addition, we demonstrated that the basal level of tyrosine phosphorylation in drug-resistant cell lines was increased. Interestingly, this increase in growth factor gene expression level or tyrosine phosphorylation is not translated into faster growth because all drug-resistant sublines display slower growth rates. Although overexpression of P-glycoprotein is the major mechanism of resistance in at least 10 of the sublines, the frequent occurrence of altered expression of growth factor genes suggests that these alterations have a role in drug resistance.
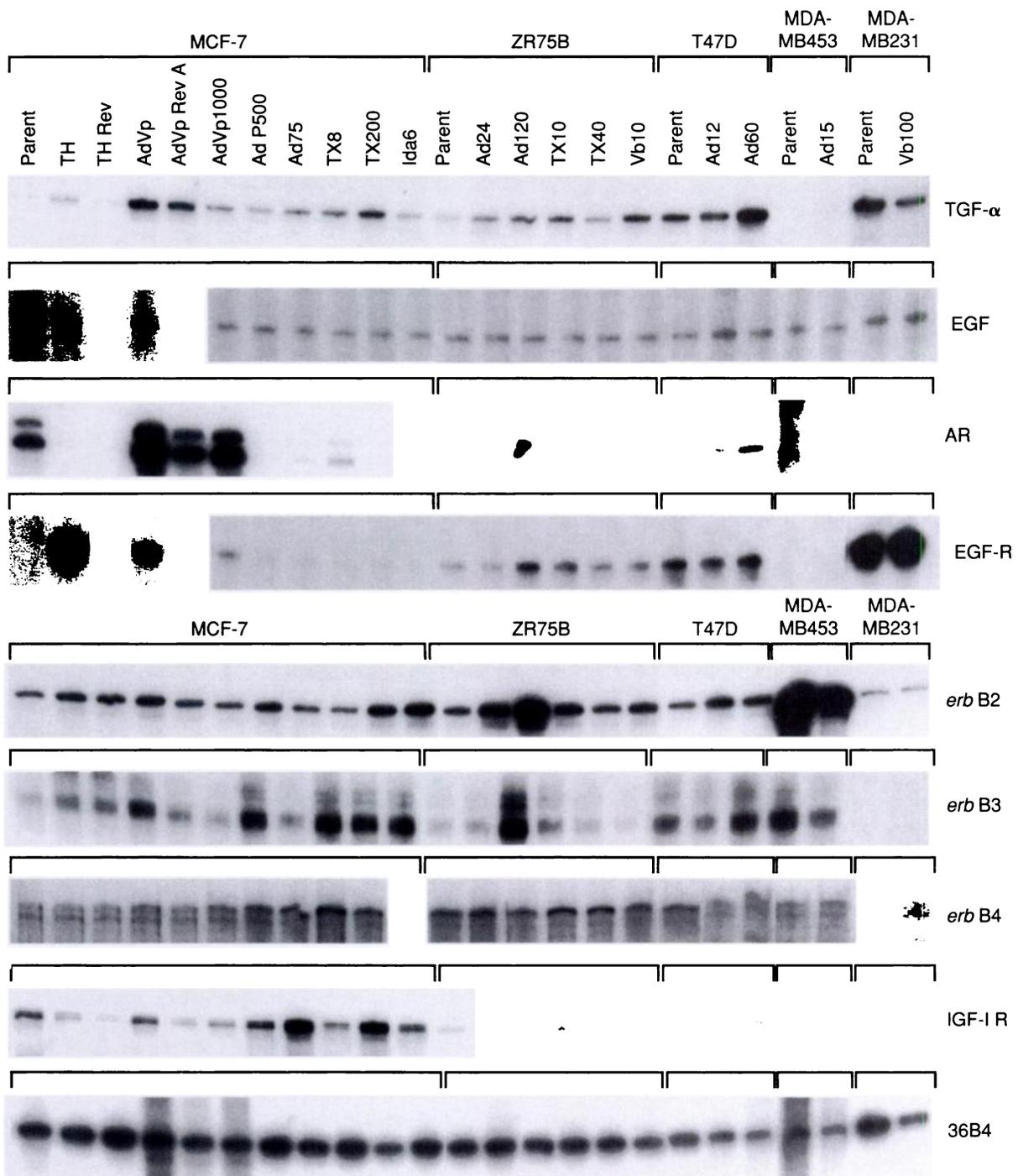
Fig. 3 Expression of growth factor signaling pathway components in parental and drug-resistant sublines. TGF-α, EGF, amphiregulin, EGF receptor, c-erbB2, c-erbB3, c-erbB4, IGF-I receptor, and 36B4 mRNA expression were determined by RNase protection assay using 30 μg of total RNA from the cell lines shown in the figure. The 36B4 mRNA level was determined to confirm regular loading of the RNA samples.

This altered expression could be an epiphenomenon, could provide a selective growth advantage, or could participate in a type of kinetic resistance in the drug-selected cells.

Estrogen receptor mRNA levels were decreased or lost in at least one of the resistant sublines derived from all three estrogen receptor-positive parental lines. It has been proposed that estrogen receptor-negative breast cancer cells derive from hormone-responsive cells and that loss of estrogen receptor is the result of cells progressing toward a more aggressive phenotype. Our results suggest that in some instances, exposure to cytotoxic drugs pushes the
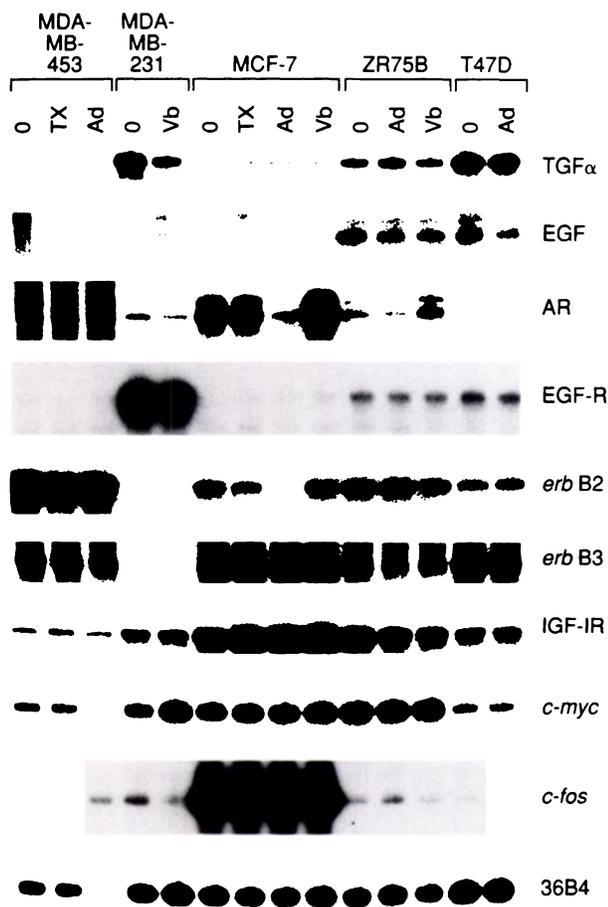
Fig. 4 Acute effect of the cytotoxic agents on gene expression. The MDA-MB-453, MDA-MB-231, MCF-7, ZR75B, and T47D cell lines were treated with 100 ng/ml paclitaxel, doxorubicin, or vinblastine. After 24 h, cells were harvested, and total RNA was prepared and analyzed by RNase protection assay to protect the fragments of the genes shown in the figure. Thirty μg of total RNA were used for each experiment.
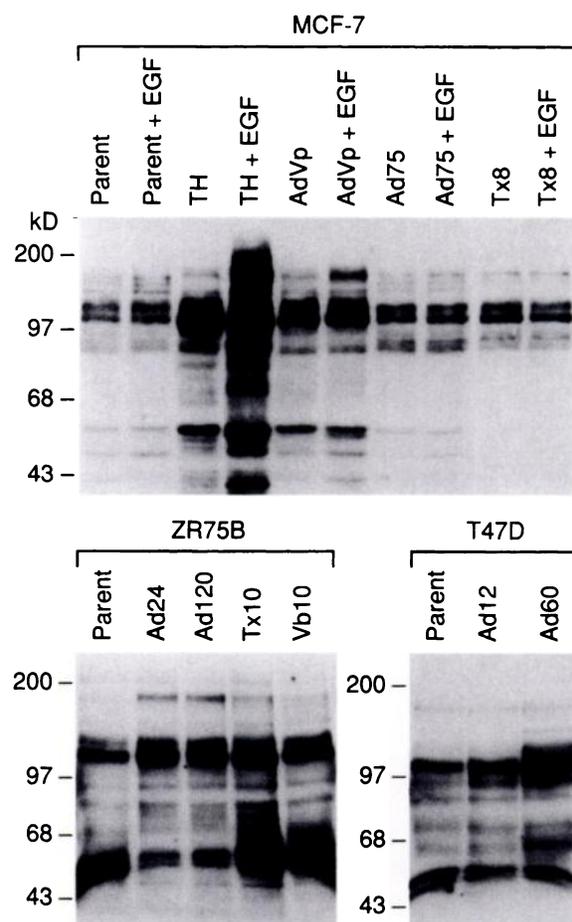

Fig. 5 Immunoblot assay of protein tyrosine phosphorylation in parental and resistant sublines. All parental and drug-resistant sublines were plated in IMEM containing 10% FCS. ZR75B and T47D cells and sublines were cultured for 48 h prior to harvest. MCF-7 parental and four resistant sublines were cultured 24 h in serum-free medium and then treated with 10^{-8} M EGF for 30 min prior to harvest. Cells were lysed, and 70 μg of protein extracts were examined by immunoblot using antiphosphotyrosine antibody. The electrophoretic mobility of molecular size standards is shown in kilodaltons.

cells toward an estrogen receptor-negative phenotype. Interestingly, all of the sublines with decreased estrogen receptor expression were selected in doxorubicin.

Altered expression of EGF receptor has been observed previously in drug-resistant sublines derived from an epidermoid cancer cell line, a large cell lung cancer cell line, neuroblastoma cells, and breast cancer cells (4, 6, 7, 17, 48). Both increases and decreases in EGF receptor levels were reported. Increased EGF receptor expression was observed in our drug-resistant human breast cancer cells. EGF receptor expression in the MCF-7 TH subline increased 49-fold to a level comparable to that in the MDA-MB-231 cell line. In addition, these cells lost their estrogen receptor expression, resulting in an estrogen receptor-negative, EGF receptor-positive phenotype. The most attractive and logical explanation for the overexpression of the EGF receptor is that increased expression allows a cell to adapt to the growth inhibition resulting from drug exposure. Increased growth factor signaling would serve as a cell survival mecha-

nism, as implicated for reduced apoptotic signaling. Such cells would have a proliferative advantage and would select more rapidly from a population exposed to drug. For this hypothesis to be correct, one would have to postulate that the proliferative advantage was present only while the cells were exposed to drug to reconcile the results of growth studies that showed reduced growth rates in the absence of drug.

One possible explanation for the reduced proliferation and for drug resistance would be a reduction in expression of topoisomerase II, which is required for DNA replication, and has been correlated with the proliferative state of cells (49). Reduced expression of topoisomerase IIα has been implicated as a mechanism of resistance to topoisomerase II inhibitors such as doxorubicin, m-amsacrine, and the epipodophyllotoxins (1). Markedly reduced topoisomerase IIα levels were observed in only two sublines, MCF-7 AdP500 and Ad75. No correlation between topoisomerase IIα expression and proliferative activity

was present ($r = -0.04$), suggesting that decreased topoisomerase IIα is not a major mechanism of resistance in most of these sublines and cannot explain the decreased growth rate.

It is well established that tumor responsiveness to chemotherapy is significantly related to proliferative activity of the tumor cells *in vitro* and *in vivo* (9, 50–53). It has been proposed that cells that are growth stimulated are more sensitive to drug (54) because they traverse the G_0 and G_1 phase of the cell cycle more rapidly, allowing less time for DNA repair. Thus, a higher proliferation rate in drug-resistant cells would be detrimental, and a decreased proliferative rate would be advantageous. We hypothesize that the reduced proliferation observed in the drug-resistant breast cancer cells is a cellular response to drug selection that allows the cells to become less susceptible to cell death. This form of drug resistance, presumably by providing the opportunity to repair or prevent damage, may also allow more potent mechanisms of resistance to emerge over time. That proliferation is important for resistance is supported by the observation that the two revertant sublines had a faster doubling time, closer to that of the parental cells. Some of these selected cell lines have levels of drug resistance higher than that which could be found clinically, and the limitations of such experimental models must be understood. However, the level of resistance that could be conferred by reduced proliferation is certainly within the range of clinical drug resistance. Consistent with this hypothesis is a clinical study showing that the proliferative activity of tumor cells decreased after treatment with chemotherapy (55).

Increased EGF receptor without concomitant mitogenic activity has been observed previously in at least two model systems. Both MDA-MB-468 and MDA-MB-231 have high levels of EGF receptor but are growth inhibited following treatment with 10^{-8} M EGF (56, 57). In related studies, Warri *et al.* (58) reported that enhanced expression of c-*erb*B2 is associated with hormone deprivation and growth arrest of the estrogen-dependent ZR75–1 breast cancer cell line (58). They postulated that nonamplified c-*erb*B2 participates in the inhibitory pathways of growth regulation. Interestingly, the ZR75B Ad120 and to a lesser extent the T47D Ad60 sublines displayed c-*erb*B2 overexpression and loss of estrogen receptor expression, presumably resulting in estrogen unresponsiveness and decreased growth rate, similar to the observations made by Warri *et al.*

The relationship between altered expression of growth factors and their receptors and the reduced proliferation observed in the drug-resistant breast cancer cells is unknown. Drug resistance in cancer is undoubtedly multifactorial, and a slower growth rate represents one component of drug resistance. What is not clear is how reduced proliferation occurs in the presence of up-regulated growth factor or receptor expression. We found impaired growth factor signaling at different levels in each of four drug-resistant MCF-7 sublines (MCF-7 TH, AdVp, Ad75, and Tx200) and postulated that these interruptions lead to the reduced growth rate.[5] Reduced growth rate in parental MCF-7 cells increased resistance to doxorubicin and paclitaxel, con-

firming that decreased proliferative activity contributes to drug resistance in these cells. Whatever the mechanism, the recurrent alterations in growth factor and receptor expression suggests that they are important in the biology of drug resistance. It is our hypothesis that perturbed EGF receptor signaling mediates the reduced growth rate in the drug-resistant cells and contributes to the onset or maintenance of the drug-resistant phenotype. The growth factor and growth factor receptor overexpression may be a cause or a consequence of the impaired signaling. The refractory nature of tumors with low growth rates has been long recognized, and clinical approaches aimed at circumventing resistance due to decreased proliferation are needed.

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