Oxidative Stress and AP-1 Activity in Tamoxifen-Resistant Breast Tumors *In Vivo*

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Background: **Most breast cancers, even those that are initially responsive to tamoxifen, ultimately become resistant. The molecular basis for this resistance, which in some patients is thought to involve stimulation of tumor growth by tamoxifen, is unclear. Tamoxifen induces cellular oxidative stress, and because changes in cell redox state can activate signaling pathways leading to the activation of activating protein-1 (AP-1), we investigated whether tamoxifen-resistant growth *in vivo* is associated with oxidative stress and/or activation of AP-1 in a xenograft model system where resistance is caused by tamoxifen-stimulated growth.** ***Methods:*** **Control estrogen-treated, tamoxifen-sensitive, and tamoxifen-resistant MCF-7 xenograft tumors were assessed for oxidative stress by measuring levels of antioxidant enzyme (e.g., superoxide dismutase [SOD], glutathione *S*-transferase [GST], and hexose monophosphate shunt [HMS]) activity, glutathione, and lipid peroxidation. AP-1 protein levels, phosphorylated c-jun levels, and phosphorylated Jun NH_2-terminal kinase (JNK) levels were examined by western blot analyses, and AP-1 DNA-binding and transcriptional activities were assessed by electrophoretic mobility shift assays and a reporter gene system. All statistical tests are two-sided.** ***Results:*** **Compared with control estrogen-treated tumors, tamoxifen resistant tumors had statistically significantly increased SOD (more than threefold; $P = .004$) and GST (twofold; $P = .004$) activity and statistically significantly reduced glutathione levels (greater than twofold; $P < .001$) and HMS activity (10-fold; $P < .001$). Lipid peroxides were not significantly different between control and tamoxifen-resistant tumors. We observed no differences in AP-1 protein components or DNA-binding activity. However, AP-1-dependent transcription ($P = .04$) and phosphorylated c-Jun and JNK levels ($P < .001$) were statistically significantly increased in the tamoxifen-resistant tumors.** ***Conclusion:*** **Our results suggest that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with oxidative stress and the subsequent antioxidant response and with increased phosphorylated JNK and c-Jun levels and AP-1 activity, which together could contribute to tumor growth. [J Natl Cancer Inst 2000;92: 1926–34]**

Tamoxifen is the most prescribed drug for the prevention and treatment of breast cancer *(1,2)*. However, in breast cancer patients, the disease eventually progresses with the emergence of tamoxifen-resistant tumor cells. Tamoxifen is thought to act primarily by competitive blockade of the estrogen receptor (ER) *(3,4)*. Experimental and clinical evidence suggests that an important form of tamoxifen resistance is the acquired ability of the tumor cells to be stimulated, rather than inhibited, by the drug after prolonged treatment *(5–9)*.

We have developed an *in vivo* experimental model for tamoxifen resistance using ER-positive MCF-7 human breast cancer cells grown in athymic nude mice *(5)*. Tamoxifen treatment suppresses tumor growth for several months, but growth eventually resumes as the tumors become stimulated by tamoxifen *(5)*. The mechanisms underlying the conversion from growth suppression to growth stimulation are still unclear. However, several studies using the MCF-7 *in vivo* model have already discarded a number of potential mechanisms for growth stimulation by tamoxifen, including altered tamoxifen uptake or metabolism *(6,10)* and lost or altered ER *(11)*.

Another possible mechanism for growth stimulation by tamoxifen is an altered intracellular redox status leading to activation of downstream signaling pathways. Cellular redox status is a balance between the rate of pro-oxidant generation, either exogenous or endogenous, and the cellular enzymatic and nonenzymatic antioxidant capacities. A number of studies *(12–16)* have shown that, depending on the cellular microenvironment, tamoxifen can affect the intracellular redox status as either a pro-oxidant or an antioxidant. For example, tamoxifen has the ability to protect lipids, proteins, and DNA against oxidative damage *(13)* and can itself be activated into reactive electrophilic metabolites *(14)*. Moreover, evidence suggests that tamoxifen can induce phase I and phase II metabolizing enzymes *(15),* which may contribute to its beneficial antioxidant activity but may also be responsible for its own activation. It is also known that changes in the intracellular redox status can lead to the activation of important transcription factors, including activating protein-1 (AP-1) *(17,18)*.

AP-1 is a heterodimeric transcription factor that is composed of various members of the Jun and Fos families *(19)* and binds to DNA at specific AP-1 binding sites. AP-1 activity is determined in part by phosphorylation of these complex components. Importantly, the transcriptional activity of c-Jun is increased by phosphorylation by the Jun NH_2-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), which are preferentially activated by a variety of environmental and cellular stresses *(20)*, including oxidative stress *(21)*. AP-1 activity can also be coregulated by protein–protein interactions between AP-1 and the ER *(22)*. Furthermore, tamoxifen can function as an agonist in coactivating ER/AP-1 on promoters regulated by AP-1 sites *(22–26)*.

The observation that AP-1 is important in several mitogenic signaling pathways *(27,28)* led us to hypothesize that an increase in cellular AP-1 activity, perhaps resulting from tamoxifen-induced oxidative stress and the changes in intracellular redox status, may contribute to the devel-

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See "Notes" following References."

opment of tamoxifen-resistant tumor growth. In this study, we looked for evidence of oxidative stress and changes in AP-1 activity in the MCF-7 *in vivo* nude mouse model of tamoxifen resistance.

Materials and Methods

Breast Cancer Cells

ER-positive MCF-7 human breast cancer cells (originally obtained from Dr. H. Degani at the Weizmann Institute of Science, Rehovoth, Israel) were used for all experiments, unless otherwise stated. Tissue culture methods have been described previously *(29)*. Exponentially growing cultures were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (50 ng/mL) in the presence of serum-containing medium for the indicated times. A doxorubicin-resistant subclone of MCF-7, MCF-7 Adria (obtained from Dr. K. Cowan, National Cancer Institute, Bethesda, MD), known to express high levels of c-Jun, was used as an internal standard in the electrophoretic mobility shift assay. ER-negative human MDA-MB-435 breast cancer cells were cultured as described previously *(30)* and were used to obtain ER-negative xenograft tumors.

Athymic Nude Mouse Model of Tamoxifen-Stimulated Growth

Animal care was in accordance with institutional guidelines. Four- to 6-week-old female ovariectomized BALB/c athymic nude mice (Harlan Sprague-Dawley Inc., Madison, WI) were given a subcutaneous injection in the mammary fat pad of 5×10^6 MCF-7 cells or their transfectant derivatives (*see below*) and hormonally treated as described previously *(5,7)*. Estradiol pellets (0.25 mg; Innovative Research, Rockville, MD) were placed subcutaneously in the interscapular region of the mice to stimulate tumor growth. When tumors reached a diameter of 8–12 mm (2–4 weeks), each mouse was randomly allocated to one of the following four groups: 1) control estrogen-treated, 2) removal of the estrogen pellet (i.e., estrogen withdrawal only), 3) estrogen withdrawal and treatment with 500 μg of tamoxifen citrate (AstraZeneca, Macclesfield, U.K.) in peanut oil (subcutaneously injected daily Monday through Friday), or 4) estrogen withdrawal and treatment with 5 mg of ICI 182,780 (AstraZeneca) in castor oil (subcutaneously injected weekly). Tumor growth was assessed and tumor volumes were measured as described previously *(29)*.

Tumors were removed during estrogen treatment (control estrogen-treated tumors) and at various times after the treatment with the antiestrogen drugs tamoxifen and ICI 182,780. Antiestrogen-sensitive tumors are defined as those harvested during the first 3 months of treatment when tumor growth is inhibited by tamoxifen. Thus, these tumors are defined as tamoxifen sensitive (tamoxifen^S) and ICI 182,780 sensitive (ICI^S), respectively. Tumors were usually harvested 2 weeks after the initiation of treatment unless otherwise stated. For the DNA-binding studies, tumors were also harvested at 1, 2, and 3 months after tamoxifen treatment began. After 3–5 months of continuous treatment, growth resumes and tumor progression is evident as an increase in tumor volume. Tumors that first undergo growth inhibition and then resume growth after prolonged antiestrogen treatment are defined as tamoxifen resistant (tamoxifen^R) or ICI 182,780 resistant (ICI^R). We have shown previously that tamoxifen resistance in this model is due to tamoxifen-stimulated tumor growth *(5,7)*.

In the estrogen-withdrawal group of mice, tumors were removed after 2 weeks (estrogen withdrawal^S) or several months later after tumor growth resumed (estrogen withdrawal^R). Each tumor analyzed was from a different mouse; tumor tissues were removed from each mouse and kept at −190 °C for later analyses.

Antioxidant Enzyme Assays

Antioxidant enzyme activities were assessed in the control (estrogen-treated), tamoxifen^S, and tamoxifen^R groups (five tumors per group). Homogenates of frozen tumors (20 [wt/vol]) were prepared in a 0.25 *M* sucrose solution (0 °C) with a Potter–Elvehjem glass–Teflon homogenizer driven by an electric drill at 500 rpm with pulse homogenization six times at 20-second intervals. Homogenates were centrifuged for 10 minutes at 10 000*g* at 4 °C to remove nuclei, mitochondria, and lysosomes, and the supernatants were collected. The activities of superoxide dismutase (SOD) (Cu/Zn form) and catalase were measured in the tumor homogenates, and the activities of glutathione *S*-transferase (GST) and the hexose monophosphate shunt (HMS) were measured in the 10 000*g* supernatants. Enzyme activities were assayed with optimal incubation times and protein concentrations to ensure the linearity of the reaction velocity. SOD activity (μg/mg protein) was measured by luminometric detection of the superoxide anion produced in the xanthine–xanthine oxidase system *(31)*. Catalase activity (μg/mg protein) was determined spectrophotometrically by measuring the rate of disappearance of H_2O_2 *(32)*. GST activity (nmol · min^{-1} · mg^{-1} protein) was measured spectrophotometrically with 1-chloro-2,4-dinitrobenzene as the substrate *(33)*. HMS activity (nmol · min^{-1} · mg^{-1} protein) was assessed spectrophotometrically by the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) with the use of glucose 6-phosphate as the substrate *(34);* HMS activity represents the sum of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities.

Lipid Peroxidation and Glutathione Levels

Lipid peroxidation was assessed in tumors from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (five tumors per group) by the quantitation of the appearance of conjugated diene double bonds in lipid extracts *(35)*. Briefly, lipids were extracted from 10 000*g* supernatants with chloroform–methanol, dried under a nitrogen atmosphere, redissolved in cyclohexane, and analyzed spectrophotometrically at 233 nm to quantify diene conjugation as detected by peak absorption. Lipid peroxidation is expressed as ΔAbs/mg protein, where ΔAbs is the difference in absorbance between the sample and the cyclohexane solvent.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels (μmol/g wet wt) were determined from the control estrogen-, tamoxifen-, estrogen-withdrawal-, and ICI 182,780-treated tumors (four tumors per group). The glutathione content of tumor cytosol supernatants was estimated by high-performance liquid chromatography *(36)*. Protein content was measured by the Bradford method (Bio-Rad Laboratories, Richmond, CA).

Protein Extraction and Western Blot Analysis of Phosphorylated c-Jun and JNKs/SAPKs Forms

Phosphorylated forms of c-Jun and JNKs/SAPKs were analyzed with the use of PhosphoPlus antibody kits (Cell Signaling, Inc., Beverly, MA) according to the manufacturer's directions. Briefly, pellets of *in vitro* cultured cells or ground, frozen tumor powders from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (at least eight tumors per group) were manually homogenized in lysis buffer (Cell Signaling, Inc.). After microcentrifugation at 14 000*g* for 30 minutes at 4 °C, supernatants were collected, and the protein concentration was determined. Aliquots (25 μg) of protein from each sample were separated under denaturing conditions by electrophoresis with 10% polyacrylamide gel containing sodium dodecyl sulfate and transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The blots were stained with StainAll Dye (Alpha Diagnostic International, Inc., San Antonio, TX) to confirm uniform protein transfer. Separate membranes were then reacted with either c-Jun- or JNK/SAPK-specific PhosphoPlus antibodies that specifically recognize the phosphorylated forms. The membranes were stripped and reblotted for total c-Jun and JNK/SAPK with antibodies that recognize the respective proteins independently of their phosphorylation status. Blots were developed by chemiluminescence (Cell Signaling, Inc.). Each sample was analyzed twice on separate immunoblots. Bands were quantified by densitometric scanning of developed films with the use of the Image 1.61/ppc software program of the National Institutes of Health, Bethesda, MD.

Electrophoretic Mobility Shift Assay

Nuclear extracts from cells or individual tumors (five tumors per group) were prepared as described previously *(28)* with minor changes. Briefly, cells or ground-up, frozen tumor powders were disrupted in lysis buffer (i.e., 10 m*M* HEPES, 1 m*M* EDTA, 60 m*M* KCl, 0.5 m*M* dithiothreitol [DTT], 0.5% Nonidet P-40 [NP-40], and protease inhibitors [1 m*M* phenylmethyl sulfonyl fluoride, 0.4 μ*M* aprotinin, and 10 μ*M* leupeptin]), and nuclei were isolated by microcentrifugation at 2500*g* at 4 °C for 10 minutes. The isolated nuclei were lysed by three cycles of freezing/thawing in nuclear suspension buffer (250 m*M* Tris [pH 7.8], 400 m*M* KCl, 0.5 m*M* DTT, 20% glycerol, and protease inhibitors) and microcentrifuged at 16 000*g* at 4 °C for 10 minutes, and the supernatants (nuclear protein extracts) were collected. Protein concentrations were determined with the use of the Bradford method. Electrophoretic mobility shift assays were performed with 10 μg of nuclear protein extract in a 20-μL reaction mixture containing 20 m*M* HEPES (pH 7.9), 40 m*M* KCl, 1

m*M* EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid], 1 m*M* phenylmethyl sulfonyl fluoride, 0.5 m*M* DTT, 1% glycerol, 2 μg of poly (dI-dC), and 100 pg of [γ-^{32}P]adenosine triphosphate end-labeled, double-stranded oligonucleotide probe containing an AP-1 binding site (5′ CTAGTGATGAGTCAGCCGGATC 3′; Stratagene, La Jolla, CA; the AP-1 binding site is *underlined*). Reaction mixtures were incubated for 30 minutes at room temperature. For oligonucleotide competition experiments, the reaction mixtures were preincubated with a 100-fold excess of unlabeled, cold oligonucleotide probes containing an AP-1 or Sp-1 binding site (Stratagene) for 20 minutes before the addition of the radioactive probes. The reaction mixtures were separated on 5% nondenaturing polyacrylamide gels at 4 °C. After electrophoresis and drying, gels were autoradiographed, and shifted bands were quantified on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The AP-1 DNA-binding activity of individual tumors was normalized between gels with the use of an internal standard extract of MCF-7 Adria cells. Electrophoretic mobility shift assays of the same extracts were repeated a minimum of two times.

AP-1/CAT Reporter Constructs, Stable Transfection, and Chloramphenicol Acetyltransferase Assay

To study AP-1-dependent gene transcription, we used a chloramphenicol acetyltransferase (CAT) reporter system as described previously *(37,38)*. The reporter construct, Col-TREx5/TKCAT (TREx5), contains five copies of a consensus AP-1 binding site (5′ ATGAGTCAG 3′) upstream of the herpes simplex virus-thymidine kinase (HSV-tk) minimal promoter. The same site is also a synthetic consensus TPA responsive element (TRE). We also used a control construct, TREΔ-72/TKCAT (TREΔ-72), upstream to position −109 of the HSV-tk promoter, that contains a point mutation in the AP-1 site (5′ TGGAGTCAG 3′) that eliminates both basal and inducible AP-1 activities *(37,38)*.

To generate stable transfection clones, we plated MCF-7 cells at a density of 8×10^5/100 mm^2. After 24 hours, the cells were cotransfected with 10 μg of the TREx5 or TREΔ-72 AP-1/CAT reporter constructs and with 1 μg of the pSV2neo selection plasmid (Clontech Laboratories, Inc., Palo Alto, CA) containing the neomycin resistance gene with the use of the LipofectAMINE reagent (Life Technologies, Inc. [GIBCO BRL], Rockville, MD), according to the manufacturers directions. G418-resistant (600 μg/mL) individual clones were screened for TPA-inducible AP-1 transcriptional activity (50 ng/mL TPA for 8 hours), and CAT assays were performed as described below.

TPA-inducible TREx5 clones and noninducible TREΔ-72 clones were grown in nude mice and treated with tamoxifen as described above. Tumors were harvested from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (four to eight mice per group). Tumor samples were homogenized, and extracts were made as described previously *(39)*. Protein concentrations were determined, and the same amount of protein from each clone was analyzed for CAT activity by thin-layer chromatography and quantitated on a PhosphoImager (Ambis, San Diego, CA). We calculated the relative CAT activity by dividing the CAT activity of the tamoxifen-treated tumors by the CAT activity of the control estrogen-treated tumors of the clone. All CAT assays were repeated two times with each sample.

Statistical Methods

Differences in the mean values of tumor antioxidant enzyme activities (SOD, HMS, and GST), glutathione levels, DNA-binding activities, and AP-1-dependent CAT activities among the treatment groups were analyzed by Student's *t* test as pairwise comparisons with respect to the control estrogen-treated group or to the tamoxifen^S group, as specified. Bonferroni's correction was used to adjust for multiple comparisons. The mean values of western blot band densities of phosphorylated c-Jun and JNKs/SAPKs were compared by two-way analysis of variance. For purposes of statistical analyses, data were transformed by taking logarithms to equalize variances. All *P* values are two-sided.

RESULTS

Antioxidant Enzyme Activity

We have developed and studied an experimental *in vivo* model that mimics the clinical scenario of acquired resistance of breast cancer to tamoxifen or other endocrine therapies, such as estrogen-withdrawal or ICI 182,780 treatment *(7)*. In the nude mouse model, ER-positive MCF-7 xenograft tumors are established in the presence of estrogen (control estrogen-treated tumors), and the estrogen is withdrawn before the start of any antiestrogen treatment. Tumor growth is initially inhibited, but it eventually resumes after continued treatment. Tumors inhibited by tamoxifen are defined as tamoxifen-sensitive (tamoxifen^S) tumors, and tumors that resumed growth are defined as tamoxifen-resistant (tamoxifen^R) tumors. We have shown previously that tamoxifen stimulates tamoxifen^R tumor growth *(5,7)*. In parallel, tumors inhibited by estrogen withdrawal or ICI 182,780 are defined as estrogen-withdrawal^S or ICI^S tumors, respectively, and tumors that resumed growth are defined as estrogen-withdrawal^R or ICI^R tumors, respectively.

To investigate the relationship between tamoxifen resistance and altered cellular redox status, we first measured the activity levels of different antioxidant enzymes in control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors grown in nude mice (Table 1). SOD activity was notably increased by tamoxifen, with a greater than fourfold increase in tamoxifen^S tumors (*P*<.001) and a greater than threefold increase in tamoxifen^R tumors (*P* = .004). Catalase activity was not statistically significantly different among the tumor groups. GST activity was statistically significantly increased in the tamoxifen^R tumors (twofold; *P* = .004) relative to the control estrogen-treated or the tamoxifen^S tumors. Tamoxifen^R tumors also had increased protein levels of GST-Pi, a member of the GST enzyme complex, as measured by western blot analysis (data not shown).

The most striking effect of tamoxifen was a profound inhibition of the production of NADPH by the HMS (Table 1). In tamoxifen^S tumors, the HMS activity was statistically significantly reduced to less than half the activity detected in the control estrogen-treated tumors (*P*<.001). In tamoxifen^R tumors, the HMS activity was further statistically significantly reduced by another fourfold to 10-fold in total (*P*<.001). Thus, tamoxifen treatment and the development of tamoxifen^R by MCF-7 breast tumors *in vivo* are associated with changes in antioxidant activities, suggesting that the tumors are experiencing oxidative stress.

Oxidative Stress

Lipid peroxidation is a process generated by the effect of reactive oxygen species and occurs when the antioxidant defense mechanisms are being overwhelmed *(40)*. Glutathione, via its redox cycling, is a potent antioxidant that provides cells with a substantial degree of protection against oxidative stress *(41)*. Because decreased HMS activity and NADPH levels would be expected to greatly reduce glutathione levels, we next measured lipid peroxidation and glutathione levels (Table 1). Lipid peroxidation was statistically significantly higher in the tamoxifen^S tumors (*P* = .016) but then returned to baseline levels after resistance emerged (Table 1). In contrast, levels of both GSH and GSSG were markedly reduced in the tamoxifen^R tumors as compared with the control estrogen-treated tumors (greater than twofold; *P*<.001 and *P* = .02, respectively). GSH levels were decreased only slightly in tamoxifen^S tumors compared with those in control estrogen-treated tumors, and GSSG levels were reduced by 1.7-fold in tamoxifen^S tumors compared with those in control estrogen-treated tumors. Importantly, there were marked and statistically significant differences in the GSH levels between the tamoxifen^S and tamoxifen^R tumors (*P*<.001). These results suggest that the

Table 1. Antioxidant enzyme activity, lipid peroxidation, and glutathione levels in hormonally treated MCF-7 tumors*

Tumor group	Mean (95% confidence interval)						
	SOD,† μg/mg protein	Catalase,† μg/mg protein	GST,† nmol/min per mg protein	HMS,† nmol/min per mg protein	LPO,† ΔAbs/mg protein	GSH,‡ μmol/g protein	GSSG,‡ μmol/g protein
E_2	1.25 (0.29–2.21)	1.74 (1.33–2.15)	0.93 (0.60–1.26)	12.10 (10.34–13.86)	717 (488–946)	2.725 (2.692–2.758)	0.434 (0.279–0.589)
Tam^S	5.01 (3.76–6.26)	2.38 (1.69–3.07)	0.97 (0.77–1.17)	5.42 (4.66–6.18)	2217 (1243–3191)	2.391 (2.322–2.460)	0.248 (0.179–0.317)
Tam^R	4.34 (3.54–5.15)	2.61 (1.77–3.45)	1.83 (1.50–2.16)	1.30 (1.03–1.57)	874 (504–1244)	1.297 (1.281–1.313)	0.156 (0.103–0.209)
$-E_2^R$	ND	ND	ND	ND	ND	2.162 (2.101–2.223)	0.563 (0.357–0.769)
ICI^S	ND	ND	ND	ND	ND	2.002 (1.975–2.029)	0.301 (0.242–0.360)
ICI^R	ND	ND	ND	ND	ND	2.139 (2.053–2.225)	0.250 (0.179–0.321)

*SOD = superoxide dismutase (Cu/Zn form); GST = glutathione *S*-transferase; HMS = hexose monophosphate shunt; LPO = lipid peroxidation; GSH = reduced glutathione; GSSG = oxidized glutathione; ND = not done; E_2 = control estrogen tumors; Tam^S = tamoxifen-sensitive tumors; Tam^R = tamoxifen-resistant tumors; $-E_2^R$ = estrogen-withdrawal resistant tumors; ICI^S = ICI 182,780-sensitive tumors; ICI^R = ICI 182,780-resistant tumors.

†Tumors from five mice per treatment group. ΔAbs = difference in absorbance between the sample and the cyclohexane solvent.

‡Tumors from four mice per treatment group.

development of tamoxifen resistance in MCF-7 breast tumors *in vivo* is associated with increased susceptibility to oxidative stress and depletion of glutathione levels as the tumors attempt to respond to the oxidative stress.

To confirm that the marked decrease in total glutathione levels in resistant tumors was specific for tamoxifen, we measured glutathione levels in tumors from mice treated either with estrogen withdrawal or with the estrogen antagonist ICI 182,780. Although, compared with the levels in control estrogen-treated tumors, glutathione levels decreased slightly in tumors that had acquired resistance to estrogen withdrawal and in ICI^S or ICI^R tumors, glutathione levels in the $tamoxifen^R$ tumors were still substantially lower than those in all other tumors.

To learn whether tamoxifen-induced oxidative stress is mediated though the ER, we measured total glutathione levels in xenograft tumors of ER-negative MDA-MB-435 human breast cancer cells. Tamoxifen does not inhibit *in vivo* growth of this cell line, and long-term experiments are not possible because the tumors become too large. Tamoxifen treatment for 29 days resulted in no reduction in glutathione levels in these tumors (data not shown), suggesting that the effect of tamoxifen might be mediated through the ER.

Tamoxifen Resistance and AP-1 DNA-Binding Activity

Because oxidative stress has been shown to activate the AP-1 transcription factor, which increases cell proliferation *(27,28)*, we explored the effect of tamoxifen on AP-1 composition and activity in MCF-7 tumors *in vivo*. AP-1 is a heterodimeric transcription factor complex that is composed of proteins from the Jun and Fos families. Comparison of control estrogen-treated, $tamoxifen^S$, and $tamoxifen^R$ tumors revealed that there were no apparent changes in messenger RNA or protein levels of the Jun and Fos family members c-Jun, JunD, JunB, Fra-1, and c-Fos (data not shown).

Using electrophoretic mobility shift assays, we next compared AP-1 DNA-binding activity in nuclear extracts from control estrogen-treated, $tamoxifen^S$, and $tamoxifen^R$ tumors (Fig. 1, A). $Tamoxifen^S$ nuclear extracts were made from established tumors 2 weeks, 1 month, 2 months, and 3 months after tamoxifen treatment began. Although there was a modest reduction in AP-1 DNA-binding activity during the first 2 months of tamoxifen treatment, there were no statistically significant differences in AP-1 DNA-binding activity between control estrogen-treated tumors and tamoxifen-treated tumors at any time (Fig. 1, A). DNA-binding assays performed with AP-1 oligonucleotides containing one or five copies of the AP-1 site produced comparable results (data not shown). Using specific antibodies to various AP-1 family members (c-Jun, JunD, Fra-1, and c-Fos), we determined the composition of the AP-1 DNA-binding complexes. We found no appreciable differences in the composition of the DNA-binding complexes among any of the control or tamoxifen-treated groups (data not shown).

Although tamoxifen treatment did not appear to change AP-1 DNA-binding activity, we detected a fourfold decrease in AP-1 DNA-binding activity in $estrogen\text{-}withdrawal^S$ tumors ($P<.001$) and in $estrogen\text{-}withdrawal^R$ tumors ($P<.001$) (Fig. 1, B). We observed a 3.5-fold decrease in AP-1 DNA-binding activity in ICI^R tumors ($P<.001$). One aberrant tumor in the ICI^S treatment group did not show a decrease in AP-1 DNA-binding activity. Thus, although tamoxifen, estrogen withdrawal, and ICI 182,780 all inhibited tumor growth *in vivo,* only tamoxifen maintained AP-1 DNA-binding activity at initial levels.

Association of Increased AP-1 Transcriptional Transactivating Activity With Development of $Tamoxifen^R$ Growth

AP-1 DNA-binding activity does not necessarily reflect the transcriptional activity of this transcription factor complex *(42)*. To determine whether AP-1 DNA-binding activity is a direct reflection of its ability to promote transcription in this system, we developed stable transfectants of MCF-7 cells expressing CAT reporter gene constructs containing either five copies of a synthetic AP-1 DNA-binding site (TREx5) or a control mutated AP-1 DNA-binding site lacking basal and inducible AP-1 activities (TREΔ-72) up-

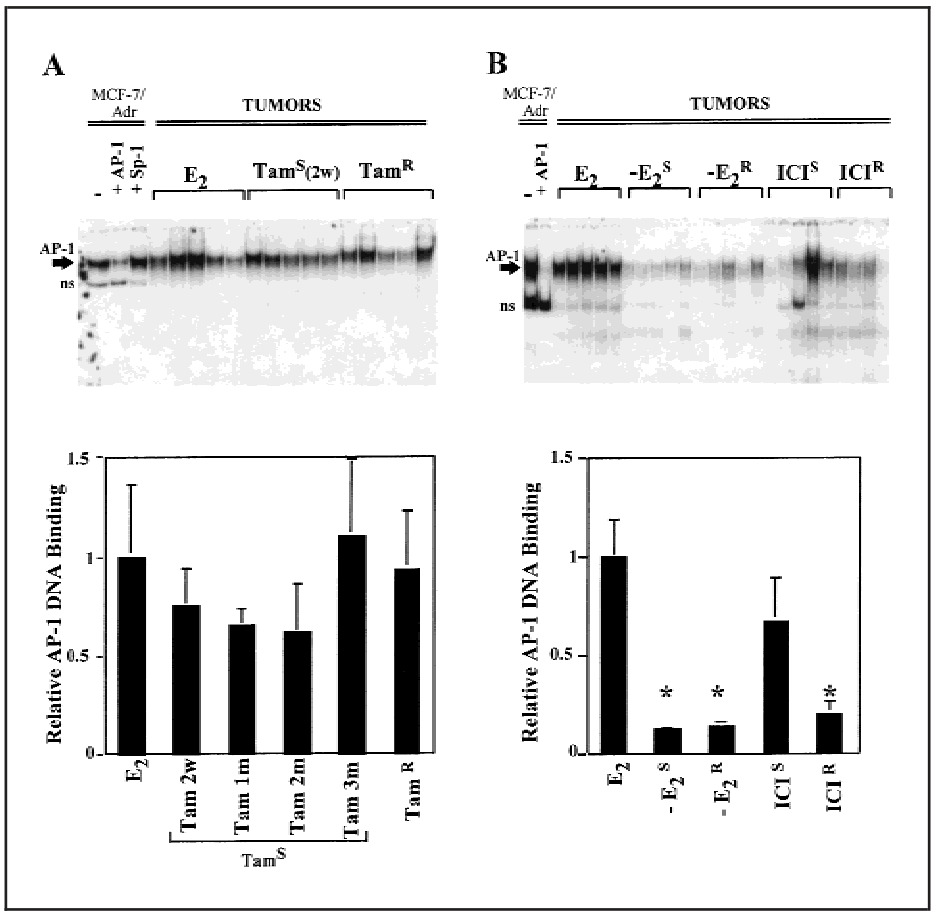

Fig. 1. Activating protein-1 (AP-1) DNA-binding activity of control estrogen-, tamoxifen-, estrogen-withdrawal-, and ICI 182,780-treated MCF-7 breast cancer xenograft tumors. Nuclear extracts from tumor groups (five mice per group) were analyzed by electrophoretic mobility shift assay with the use of an AP-1 oligonucleotide probe. **A**) Tumors from control estrogen-treated (E_2), tamoxifen-sensitive (the groups included 2 weeks [2w], 1 month [1m], 2 months [2m], and 3 months [3m] tamoxifen treatment during the growth-inhibited [Tam^S] phase), and tamoxifen-resistant (during the growth-stimulated [Tam^R] phase) xenograft mice were harvested and analyzed for AP-1 DNA-binding activity. **B**) Tumors from control estrogen-treated (E_2), estrogen-withdrawal-treated ($-E_2$), or estrogen-withdrawal-treated/ICI 182,780-treated xenograft mice at the sensitive, growth-inhibited phase (2 weeks of treatment, $-E_2^S$ or ICI^S) and at the resistant, growth-stimulated phase ($-E_2^R$ or ICI^R) were harvested and analyzed for AP-1 DNA-binding activity. **Top panels:** representative electrophoretic mobility shift assay gels of the tumor nuclear extracts. For Tam^S, only 2 weeks of treatment is shown. An internal standard extract of MCF-7 Adria cells (MCF-7/Adr) was included in each panel for normalization of the AP-1 binding reactions between gels. An excess of unlabeled AP-1 oligonucleotide (+ AP-1) was used to competitively inhibit specific AP-1 binding, and a nonspecific Sp-1 oligonucleotide (+ Sp-1) was added as a further negative control for this specificity. **Arrows** point to the specific AP-1 complexes, and the nonspecific band is designated "ns." **Bottom panels:** relative AP-1 DNA-binding activity in the treated tumors. Specific AP-1 complexes were quantitated on a PhosphoImager, and the DNA-binding activity of individual tumors was normalized between gels. AP-1 DNA-binding activity in the tumor groups was calculated relative to the control estrogen-treated group, and calculated means (±95% confidence intervals) were analyzed statistically by Student's *t* test as pairwise comparisons versus the control estrogen-treated group. * = $P<.001$. Electrophoretic mobility shift assays with the same samples were repeated a minimum of two times.

stream to a minimal promoter *(37)*. TPA induced CAT activity *in vitro* in the two TREx5 clones tested but not in the TREΔ-72 clone (Fig. 2, A). The stable transfectants were then grown as tumors in the nude mice and treated with tamoxifen. In tumors grown from the control TREΔ-72 clone, the low basal CAT activity gradually declined during tamoxifen treatment (Fig. 2, B and C). In contrast, a statistically significant increase in CAT activity was detected in tamoxifen^R tumors in both TREx5 clones (twofold to threefold; $P = .04$ and $P = .006$ for TREx5 clones 1 and 2, respectively). The finding that AP-1 transcriptional activity increased at the time of tamoxifen^R growth was reproducible in two different clones and in two independent *in vivo* experiments.

Hyperphosphorylation of c-Jun in Tamoxifen^R Tumors

AP-1 transcriptional activity is increased by phosphorylation at two specific serine residues in the c-Jun component of AP-1 *(42)*. These residues, Ser 63 and Ser 73, are phosphorylated by the JNKs *(43,44)*. Furthermore, JNK activity can be increased by various stresses *(20),* including oxidative stress *(21)* and/or glutathione depletion *(45),* conditions observed in our model with tamoxifen treatment, especially during tamoxifen^R growth.

Because we observed an induction of oxidative stress in the tumors during the emergence of tamoxifen resistance, we determined the phosphorylation status of c-Jun from control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors (Fig. 3, A). No statistically significant changes were detected between the control estrogen-treated tumors and tamoxifen^S tumors. However, the tamoxifen^R tumors (of both untransfected MCF-7 tumors and stable TREx5 transfectants) had statistically significantly higher levels of phosphorylated c-Jun (greater than twofold; $P<.001$), even after correcting for minor changes in total c-Jun levels.

Because the JNKs are the major kinases known to phosphorylate c-Jun at Ser 63 and Ser 73, we next measured phosphorylated (i.e., active) JNK family members in the control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors (Fig. 3, B). We saw that the tamoxifen^R tumors contained statistically significantly higher levels of both the 46-kd and 54-kd phospho-JNK forms compared with the tamoxifen^S tumors (>1.8-fold and 1.5-fold; $P<.001$ and $P = .008$, respectively, for 46-kd and 54-kd phospho-JNK forms, after correcting for minor changes in the level of total JNK). Thus, both increased phospho-c-Jun levels and increased JNK activity accompanied the increase in AP-1-dependent transcription in the tamoxifen^R tumors.

Discussion

The emergence of tamoxifen resistance is a major problem in the treatment of breast cancer, and understanding the mechanisms by which resistance arises could have major clinical implications for preventing or circumventing it. Our results show that the development of acquired tamoxifen resistance of xenograft MCF-7 tumors *in vivo* is associated with both increased susceptibility to oxidative

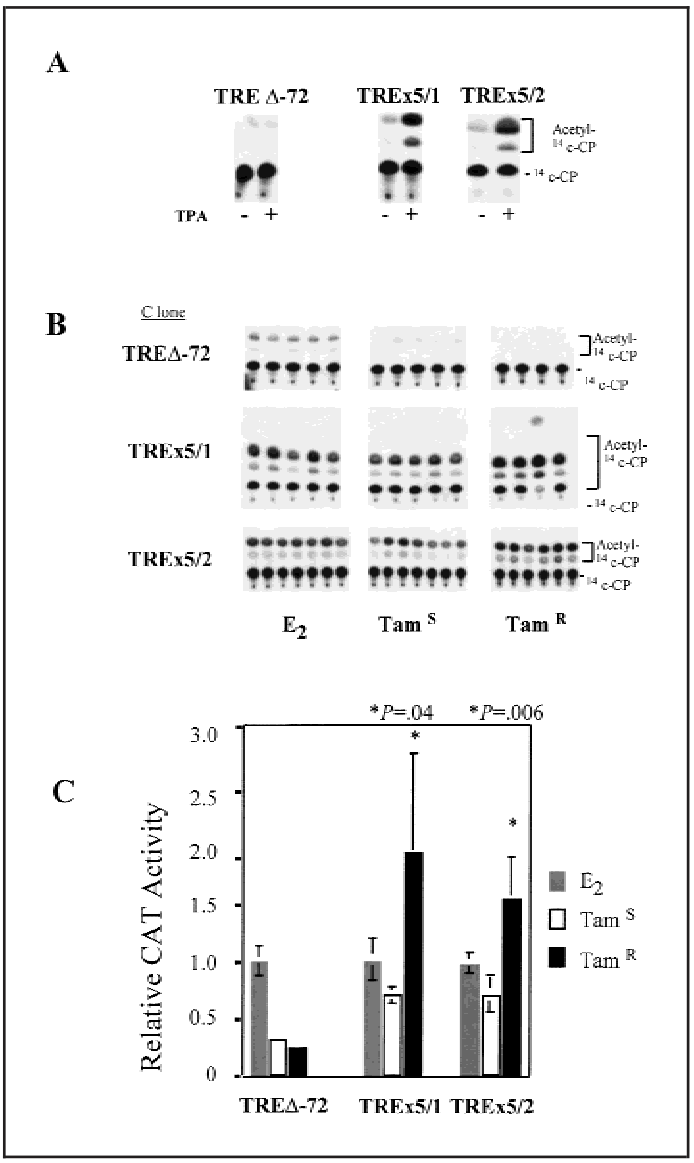

Fig. 2. Activating protein-1 (AP-1) transcriptional activity in tamoxifen-treated tumors. MCF-7 breast cancer cells were stably transfected with chloramphenicol acetyltransferase (CAT) constructs containing either five copies of a consensus AP-1 site (TREx5 clones) or a control mutated AP-1 site (TREΔ-72 construct). **A**) 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) induction of TREΔ-72 and TREx5 clones *in vitro*. Cells of two TREx5 clones (clone TREx5/1 and clone TREx5/2) and one TREΔ-72 clone were left untreated (–) or were treated with TPA (50 ng/mL) for 8 hours, and extracts were analyzed by the CAT assay, as described in the "Materials and Methods" section. In **B** and **C,** AP-1-dependent CAT activity is indicated in tamoxifen-treated tumors. The transfectant clones were injected into nude mice and treated with estrogen and tamoxifen. Tumors (four to eight mice per group) were harvested for CAT analysis after establishment in the presence of estrogen (E_2), 2 weeks after tamoxifen treatment began (during the tamoxifen-inhibited growth phase, Tam^S), and at the appearance of tamoxifen-resistant growth (Tam^R). All tumors were from individual mice. CAT assays were performed with the use of the same amount of protein extract for individual tumors of each clone. **B**) CAT assay of clones TREΔ-72 and TREx5. The TREΔ-72 clone has low basal CAT activity, so its autoradiographs were exposed longer than those of the TREx5 clones. All of the tumors analyzed from the stable transfectants are shown. CAT assays of the same samples were repeated twice. **C**) Quantitation of relative CAT activity of the TRE/CAT clones shown in **B**. The CAT assays were quantitated on a PhosphoImager, and the relative CAT activity in the tumor groups was calculated relative to that of the control estrogen-treated group of each clone. $Tamoxifen^R$ calculated means (±95% confidence intervals) were analyzed statistically by Student's *t* test compared with the $tamoxifen^S$ group. CP = chloramphenicol.

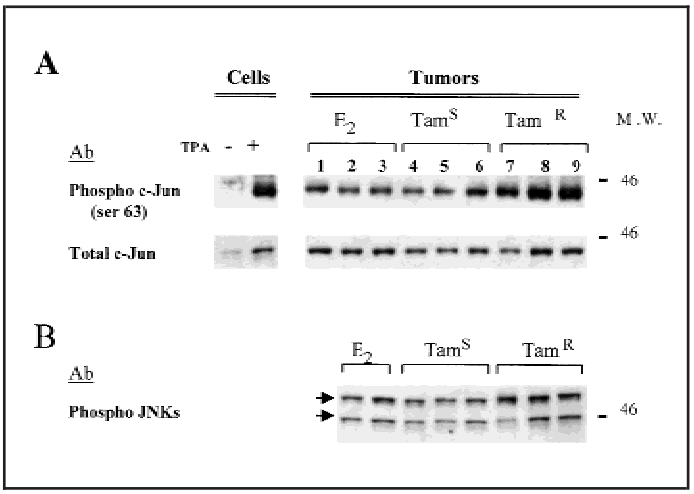

Fig. 3. Phosphorylation of c-Jun and Jun NH_2-terminal kinase (JNK) in the tamoxifen-treated tumors. Protein extracts (25 μg) of control estrogen-treated (E_2), tamoxifen-sensitive (Tam^S), and tamoxifen-resistant (Tam^R) MCF-7 breast cancer xenograft tumors were analyzed by western blot analysis with antibodies that recognize the phosphorylated forms of the proteins. **A**) Blots probed with anti-phospho c-Jun antiserum specific for Ser 63 (**top panel**) and anti-total c-Jun antiserum, which recognizes c-Jun independently of its phosphorylation status (**bottom panel**). Controls were MCF-7 cells either untreated (–) or treated *in vitro* (+) with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at a dose of 50 ng/mL for 1 hour. **B**) Blots probed with anti-phospho JNK (54 kd and 46 kd) antiserum recognizes Thr 183 and Tyr 185 phosphorylation. **Arrows** point to the 54-kd and 46-kd JNK family members. A representative gel is shown of two or three tumors per group. Mean values of western blot band densities of phosphorylated c-Jun and JNKs/stress-activated protein kinases (SAPKs) were compared by two-way analysis of variance. Ab = antibody; M.W. = molecular weight.

stress and increased AP-1 activity. Tamoxifen affects the intracellular redox status in breast tumors, increases lipid peroxidation, and induces the activity of a number of antioxidant enzymes. Prolonged tamoxifen treatment resulted in tumors that were tamoxifen resistant and growth stimulated and had a reduced antioxidant cellular capacity, as evidenced by a striking decrease in HMS activity and a marked depletion of glutathione. These oxidative changes after prolonged

treatment appear to be specific to tamoxifen because this marked reduction in glutathione levels and, consequently, increased susceptibility to oxidative stress were not found in tumors from mice treated with estrogen, with estrogen withdrawal, or with the pure antiestrogen ICI 182,780.

A remaining open question is to what extent all of the oxidative changes are ER dependent. It is known that tamoxifen's antiangiogenic effects and its potential to induce oxidative stress are, at least in part, ER independent *(46–48)*, although the role of the two ER subtypes is still unclear *(49)*. A recent study *(50)* demonstrating that the oncostatic action of melatonin on breast cancer cells is mediated by increased glutathione levels and is restricted to ER-positive cells suggests a link between cellular redox state and ER. In addition, the lack of oxidative stress in ER-negative MDA-MB-435 tumors from tamoxifen-treated mice and preliminary *in vitro* data also suggest that tamoxifen-induced oxidative stress in MCF-7 tumors may be ER mediated.

Because changes in the cellular redox status can lead to the induction of AP-1 and because AP-1 is important in a variety of mitogenic signaling pathways *(27,28),* we studied this transcription factor complex in our tamoxifen^R *in vivo* model. We found that the AP-1 transcriptional activity was increased as tumors progressed to a tamoxifen^R phenotype. It is possible that the observed increase in the antioxidant enzyme GST, whose expression is regulated by AP-1 *(51),* reflects a parallel increase in AP-1-dependent transcription. Other reports have shown that prolonged tamoxifen treatment dramatically affects the expression of a number of phase II enzymes, such as GST *(52–54),* probably through the antioxidant response element contained in the promoter of these genes *(54)* and that these genes may also be regulated by AP-1 *(51)*. In agreement with our finding, Astruc et al. *(25)* also have reported that prolonged tamoxifen treatment markedly increases the cellular response to inducers of AP-1.

Although we found increased AP-1 transcriptional activity in the tamoxifen^R tumors, neither expression of Jun and Fos family members nor AP-1 DNA-binding activity was altered. These findings are similar to those in other reports *(27,28)*. In contrast, Dumont et al. *(55)* reported that the progression of MCF-7 tumor cells (MCF-WES cells) to a tamoxifen-stimulated/tamoxifen-resistant phenotype is associated with increased AP-1 DNA-binding activity. However, MCF-WES tamoxifen^R tumors have a markedly decreased ER content, and although the tumors are still estrogen sensitive, they are globally resistant to all antiestrogens. In contrast, the tamoxifen^R tumors in this study, which have increased AP-1 transcriptional activity, express high levels of ER, remain estrogen dependent *(5),* and are growth inhibited by pure steroidal antiestrogens *(6,7)*. Thus, although both types of tamoxifen^R tumors share a common regulatory pathway, namely AP-1, they may differ in how the pathway is activated. Whether these differences in AP-1 activation account for the divergence in their cellular phenotype remains to be investigated.

Our data also demonstrate that increases in JNK activity and c-Jun phosphorylation are associated with the tamoxifen^R phenotype. Increased c-Jun phosphorylation by tamoxifen may potentiate c-Jun transcriptional activity *(42,56)* and could also enhance the agonistic effects of tamoxifen at AP-1 sites, as proposed by Webb et al. *(22)*. JNK can be activated by diverse stress stimuli *(20,42),* including oxidative stress *(21)*. Oxidative stress can alter the level of intracellular glutathione, which can be a key regulator for the induction of JNKs *(45)*. Because both oxidized and reduced glutathione levels are markedly decreased in our tamoxifen^R tumors but not in our tamoxifen^S tumors, our cumulative data suggest the possibility that chronic tamoxifen administration leads to oxidative stress and a reduction in glutathione levels followed by activation of JNK and increased AP-1 activity. The increased AP-1 activity could then provide the tumor cells with a sufficient growth stimulus to offset any growth-inhibitory effects of the antiestrogen mediated via the ER pathway. Recent data demonstrating a substantial increase in JNK activity in tamoxifen-resistant human breast tumors *(57)* further support this hypothesis.

Although our data show that prolonged tamoxifen treatment of MCF-7 tumors in nude mice results in oxidative stress and increased AP-1 activity, to conclude that these pathways mediate tamoxifen^R growth, it will be necessary to determine whether increasing or inhibiting AP-1 activity or cellular sensitivity to oxidative stress will affect the emergence of the tamoxifen^R phenotype. In this context, it is interesting that overexpression of c-Jun in MCF-7 cells results in a tamoxifen^R phenotype both *in vitro* and *in vivo (58)*. Confirmation of the role of oxidative stress and AP-1 in the development of tamoxifen^R could provide new strategies to delay or even to prevent this important clinical problem.

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NOTES

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