Advances in Brief

Increased Activator Protein-1 DNA Binding and c-Jun NH$_2$-Terminal Kinase Activity in Human Breast Tumors with Acquired Tamoxifen Resistance


Abstract

Human breast tumors that are initially responsive to tamoxifen (TAM) eventually relapse during treatment. Estrogen receptor (ER) expression and function are often preserved in these tumors, and clinical evidence suggests that this relapse may be related to TAM’s known agonistic properties. ER can interact with the activator protein-1 (AP-1) transcription factor complex through protein-protein interactions that are independent of ER DNA binding and, in certain ER-positive cells, this may allow TAM to exert an agonist response on AP-1-regulated genes. We, therefore, assessed both AP-1 DNA binding and the known AP-1 activating enzyme, c-Jun NH$_2$-terminal kinase (JNK), in a panel of 30 ER-positive primary human breast tumors with acquired TAM resistance, as compared to a matched panel of 27 untreated control ER-positive breast tumors and a separate control set of 14 primary tumors, which included 7 ER-positive tumors that were growth-arrested by 3 months of preoperative TAM. AP-1 DNA binding activity was measured from cryopreserved tumor extracts using a labeled oligonucleotide probe containing a consensus AP-1 response element by electrophoretic mobility shift assay. JNK was first extracted from the tumor lysates by incubation over a Sepharose-bound c-Jun(1–89) fusion protein, and its activity was then measured by chemiluminescent Western blot by detection of the phosphorylated product using a phospho-Jun(Ser-63)-specific primary antibody. The set of control ER-positive breast tumors growth arrested by TAM showed no significant difference from untreated control tumors in their AP-1 DNA binding and JNK activities. In contrast, there was a significant ($P < 0.001$) increase in mean AP-1 DNA binding activity for the panel of ER-positive TAM-resistant (TAM-R) tumors as compared to its matched control panel of untreated tumors. Mean JNK activity in the TAM-R tumors was also significantly higher than that found in the untreated tumors ($P = 0.038$). Overall, there was no significant correlation between JNK activity and AP-1 DNA binding; however, regression analysis showed that, for any given level of JNK activity, the TAM-R tumors possessed a 3.5-fold increase in AP-1 DNA binding activity as compared to the untreated tumors. These findings indicate that, when compared to untreated ER-positive primary breast tumors, TAM-R tumors demonstrate significantly increased levels of AP-1 DNA binding and JNK activity, consistent with experimental models suggesting that TAM-stimulated ER-positive tumor growth may be mediated by enhanced AP-1 transcriptional activity. These observations support the need for further evaluation of these markers in breast tumors as predictors of TAM resistance.

Introduction

Human breast tumors that are hormone-dependent and initially responsive to antiestrogen therapy with TAM eventually relapse with acquired resistance. An animal model of TAM-R MCF-7 breast cancer suggested that such tumors may develop a TAM-dependent phenotype in vivo, with growth becoming stimulated by the partial agonist properties of TAM (1). In clinical practice, patients with TAM-R breast cancer can respond to second-line endocrine therapy, including aromatase inhibitors, indicating that the TAM-R phenotype does not necessarily signify resistance to all ER-mediated endocrine agents (2). We have previously shown that, in TAM-R breast tumors, ER expression and ER DNA binding capacity are preserved (3, 4). The pharmacological and molecular bases for the TAM-R phenotype remain unknown, despite several recent clinical studies that examined changes in TAM metabolism and the expression of variant/mutant forms of ER (5).

It is possible that TAM-R development involves an enhanced cellular response to various extracellular signals mediated through signal transduction pathways from the cell membrane through the

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3 The abbreviations used are: TAM, tamoxifen; TAM-R, TAM-resistant; ER, estrogen receptor; AP-1, activator protein-1; SAPK, stress-activated protein kinase; JNK, c-Jun NH$_2$-terminal kinase; EMSA, electrophoretic mobility shift assay; TPA, 12-O-tetradecanoylphorbol-13-acetate.
cytoplasm to the nucleus. One complex of proteins that responds to extracellular signals and regulates gene transcription is AP-1, composed of Jun and Fos family members that dimerize and bind DNA at specific AP-1 response elements within a given gene’s promoter region (6, 7). These Jun (c-Jun, Jun B, and Jun D) and Fos (c-Fos, Fos B, Fra 1, and Fra 2) family members individually respond to a variety of extracellular signals (growth factors, hormones, and cytokines) and physical agents (UV and stress) as immediate-early genes and are also transcriptionally regulated by various mitogen-activated protein kinases, MAPKs. The collective AP-1 transcriptional activity in a cell may be increased by changes in the relative abundance of these Jun or Fos family components or by specific phosphorylation of c-Jun at its Ser-63 and Ser-73 residues from the SAPK isozymes encoded by any of the three known JNK genes (7, 8).

ER can coactivate genes along with AP-1; this coactivation occurs independently of its ER DNA-binding activity and via protein-protein interactions with c-Jun. Furthermore, in some ER-positive cells, TAM functions as an agonist in coactivating ER/AP-1 (9). ER-positive MCF-7 cells selected for TAM resistance show increased AP-1 DNA-binding (10), and more recently, it has been shown that MCF-7 cells transfected with c-Jun develop TAM activity and exhibit increased invasiveness (11). Together, these findings suggest that enhanced AP-1 activity may bypass hormone-dependence and/or associate with an agonistic TAM response in ER-positive TAM-R breast cancers. We, therefore, compared both AP-1 DNA binding activity and JNK activity in a panel of TAM-R human breast tumors along with a matched panel of untreated control tumors and a set of tumors that were growth-arrested on TAM therapy.

Materials and Methods

Human Tumors and ER Analysis. The effect of primary treatment by TAM on AP-1 DNA binding and JNK activity was first examined in ER-positive primary breast carcinomas from 14 consecutive postmenopausal patients (median age, 71 years) who attended the Breast Unit at The Royal Marsden Hospital. These patients had been randomized in a neoadjuvant endocrine study (Protocol No. 932) to receive either primary TAM at 20 mg/day for 3 months followed by surgery (n = 7) or initial surgery followed by adjuvant TAM (n = 7). In addition, these assays were performed on TAM-R tumors (n = 30) obtained at relapse from postmenopausal patients (median age, 73 years) whose resistance developed after an initial objective (complete or partial) response of their metastatic breast cancer (median age, 69 years).

Tumor extracts were prepared from 100–200 mg of cryopreserved (−80°C) samples of tumor which were pulverized in a tissue dismembrator (Braun Medical Ltd.). Frozen tumor powder was added to 1.5 ml of ice-cold extraction buffer containing appropriate protease inhibitors [20 μM Tris (pH 7.5), 10 mM EDTA, 20% glycerol, 0.4 mM KCl, 5 μg/ml leupeptin, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml pepstatin] and solubilized by Polytron homogenization. The resulting homogenate was centrifuged at 10,000 rpm for 15 min at 4°C, and the clear supernatant was removed and stored at −80°C until assayed. Total immunoreactive ER was assayed in the cytosol by ER-EIA (Abbott Laboratories), and protein concentration was quantified by the Bradford method as described previously (3).

AP-1 DNA Binding Assay (EMSA). Tumor extracts (5 μg total protein) were preincubated with 2 μg of poly(dIdU) (Boehringer Mannheim) in 100 mM KCl, 10 mM Tris (pH 7.5), 2 mM DTT, and 5% (v/v) glycerol at room temperature for 10 min. DNA binding was initiated by incubation with 32P-end-labeled AP-1 oligonucleotide containing a single AP-1 consensus binding site (underlined; 5'-CGCTTTGATGACTCAGCCGGA-3') at room temperature for 30 min. Bromphenol blue dye in concentrated gel loading buffer was added to the samples, which were then loaded onto a 4.2% loosely cross-linked native polyacrylamide gel to separate protein-bound DNA from free oligonucleotide, as described previously (4). The gels were vacuum-dried, and the gel-shifted bands were analyzed by autoradiography or PhosphorImager (Molecular Dynamics). Positive controls included in each experimental run consisted of MCF-7 extracts from untreated or phorbol ester (TPA)-pretreated cells. Gel-shifted AP-1 complexes were confirmed by their disappearance in the presence of excess unlabeled competing AP-1 oligonucleotide probe, as compared to a competing nonsense probe sequence. Tumor AP-1 DNA-binding activity was measured by densitometry of the labeled AP-1 complex, compared to a standard curve of AP-1 DNA binding activity from a control MCF-7 extract (showing r = 0.993 linear correlation coefficient of AP-1 DNA binding over a 200-fold range of control extract protein concentration).

JNK Assay. A solid-phase SAPK/JNK assay (New England BioLabs, Beverley, MA), which measures the activity of all known JNK isozymes, was used to assay the cryopreserved tumor extracts. Glutathione-S-transferase-c-Jun fusion protein bound to glutathione Sepharose beads (2 μg) was added to a solution containing 150 μg of extract protein in 300 μl of lysis buffer and incubated for 18 h at 4°C. The beads were then washed twice in 500 μl of lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium diphosphate, 1 mM β-glycerolphosphate, 1 mM Na2VO4, and 1 mg/ml leupeptin], followed by two washes in 500 μl of kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na2VO4, and 10 mM MgCl2]. The kinase reaction was performed by adding 100 μM cold ATP and incubating at 30°C for 30 min. The reaction was terminated by adding 25 μl of 3× SDS buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue] and boiling the sample for 5 min. Samples were run on an SDS-polyacrylamide gel and analyzed by Western blotting using a phospho-c-Jun(Ser-63)-specific primary antibody (1:1000 dilution). Detection was by incubation of the membrane with conjugated antirabbit secondary antibody (1:2000) followed by chemiluminescent horseradish peroxidase detection, quantified by PhosphorImager.

Statistical Methods. For both AP-1 and JNK assays, tumor samples were analyzed by investigators who were blind to their control or prior treatment status. Because the data for AP-1 were normally distributed in each panel, these were analyzed by unpaired Student’s t test. Due to batching of samples, the JNK assays were performed in four separate experiments, although each one contained an equal number of control and treated samples. ANOVA (Kruskal-Wallis test) was performed within each panel of control or treated tumors across the four JNK experiments, prior to analysis of the data from each experiment.
nonparametric comparisons made by Mann-Whitney test between the tumor panels. Regression analyses were performed to compare AP-1 DNA binding activity with ER content and JNK activity.

Results

ER Content of Tumor Groups. All tumors that were growth-arrested on TAM treatment were ER positive at the time of surgery, although their ER content was somewhat lower (mean = 57 fmol ER/mg protein; n = 7) than that of their randomized untreated control set (mean = 106 fmol ER/mg protein; n = 7). Among the set of growth-arrested tumors, 43% demonstrated partial clinical responses (≥50% reduction in tumor volume), and none demonstrated clinical progression during the 3 months of preoperative (neoadjuvant) TAM treatment. The ER content of the panel of TAM-R tumors (mean = 95 fmol ER/mg protein; n = 30) did not significantly differ from that of the panel of age-matched untreated control tumors (mean = 80 fmol ER/mg protein, n = 27). As reported previously, PgR and pS2 were also often coexpressed in these tumors (3).

Increased AP-1 DNA Binding in TAM-R Tumors. There was no significant difference in AP-1 DNA binding activity between the ER-positive tumors growth-arrested after 3 months of TAM treatment and the untreated control tumors, although a trend favoring lower AP-1 DNA binding was observed in the former set (Fig. 1A). In contrast, several ER-positive TAM-R tumors demonstrated higher AP-1 DNA binding comparable to that found in TPA-treated MCF-7 cell extracts, which were routinely included as internal positive experimental controls with each assay (Fig. 2). Overall, there was a significant increase in AP-1 DNA binding activity for the TAM-R tumors as compared to the matched panel of untreated control tumors (P < 0.001, unpaired Student’s t test; Fig. 3). No significant correlation was observed between tumor ER level and AP-1 DNA binding activity in either the control (r = 0.301) or TAM-R (r = 0.101) panels.

Increased JNK Activity in TAM-R Tumors. JNK activity was not significantly different between the ER-positive tumors growth-arrested after 3 months of TAM treatment and the untreated control tumors, with a trend favoring lower JNK activity in the former set (Fig. 1B). Sufficient extracts were available to perform the JNK assay in 22 of 27 control and 23 of 30 TAM-R tumors. Several TAM-R tumors demonstrated increased JNK activity, as illustrated on the Western blot in Fig. 4. An ANOVA showed no difference in JNK values obtained for either control tumors or TAM-R tumors between the four experiments. Overall JNK activity was significantly higher in the panel of TAM-R tumors as compared with the control panel (P = 0.038, Mann-Whitney test).

Independent Relationship between AP-1 DNA Binding and JNK Activity. The EMSA determined AP-1 DNA binding activities showed no significant correlation with JNK activities across the entire tumor collective (TAM-R plus control panels), confirming that these mechanisms can be up-regulated independently. In contrast, when AP-1 DNA binding and JNK activities were analyzed by linear regression for each of the two tumor panels and the two regression curves then compared by ANOVA, significantly different AP-1 (but not JNK) intercept values were observed in association with similar regression slopes (Fig. 5). That is, for any given level of JNK activity, the TAM-R tumors possessed a 3.5-fold higher level of AP-1 DNA binding activity, consistent with the measured univariate difference in mean AP-1 DNA binding activities between the two tumor panels.

Discussion

Activation of the AP-1 transcription factor complex has been shown to mediate growth factor-induced proliferation of breast cancer cells in vitro (12). In MCF-7 cells, peptide growth factors and the tumor promoter TPA induce expression of Jun and Fos family members and enhance both AP-1 DNA-binding and transcriptional activity. Furthermore, a dominant-negative form of c-Jun can inhibit AP-1 transactivation resulting in suppression of cell proliferation (12). Genes that are known to be transcriptionally up-regulated by AP-1 include collagenase, cathepsin D, and the P-glycoprotein multidrug resistance gene; therefore, enhanced AP-1 activity may be associated with cellular growth deregulation and transformation to a more malignant and invasive phenotype (6).

Recent evidence indicates that growth-promoting AP-1 transcriptional activity may be coregulated by ligand-bound ER. Several studies have now shown that estrogen treatment of ER-positive cells results in enhanced AP-1 transcriptional activity and gene expression that is mediated by DNA-bound Jun-Jun or Fos-Jun dimeric complexes (9, 13–16). This coactivation appears to require protein-protein contacts between Jun and ER as well as a structurally intact ER amino (NH2)–transactivator domain, but it is independent of ER DNA-binding to its cognate ERE (9). Of interest, increased expression of the Fos family member, Fra-1, to levels present in some breast cancer cells represses this AP-1 coactivation by estrogen-bound ER, indicating that ER/AP-1 coactivation is also dependent on the Jun dimerization partner within the AP-1 complex (16). In some ER-positive cell lines, such as MCF-7, the antiestrogen TAM antagonizes ER/AP-1 coactivation, whereas in others, such as Ishikawa cells, TAM-ligated ER is a potent agonist of the AP-1 pathway (9, 14). With the clinical development of acquired TAM resistance, the TAM-R tumors in this study may have converted their phenotype from ER-positive cells such as...
MCF-7 to those with TAM agonistic properties similar to Ishikawa cells, independent of any change in AP-1 DNA-binding activity. Alternatively, the prolonged clinical exposure to TAM may have induced the observed increase in AP-1 DNA binding, which may have been associated with sufficient AP-1 transcriptional activity to bypass any potential coregulatory effects by TAM-liganded ER.

There is evidence that, in ER-positive cells, continuous exposure to TAM may induce agonistic activity at AP-1-regulated promoter sites (17). Long-term culture treatment of ER-positive breast cancer cells with low (nanomolar) TAM concentrations was found to enhance AP-1 transcriptional activity by 4-fold. This stimulation was mediated by ER and was not observed in ER-negative cells, leading the authors to conclude that continuous TAM treatment known to induce TAM-R may result in TAM stimulated tumor growth by increasing AP-1 transcriptional activity. Another group has shown that AP-1 DNA binding activity is significantly elevated in a subline of hormone-independent MCF-7 tumors stimulated in vivo by TAM (10). This MCF-WES subline, derived after prolonged estrogen withdrawal or TAM therapy, demonstrates a hormone-independent phenotype, in that it remains responsive to estradiol but is also growth-stimulated by TAM and the pure steroidal antiestrogen ICI 182,780. Despite the lower level of nuclear ER expressed by this subline, Dumont et al. (10) suggested that enhanced AP-1 DNA binding activity represented the most likely mechanism underlying the observed antiestrogen stimulated growth pattern.

This study provides the first clinical evidence that human breast tumors with acquired resistance to TAM possess enhanced AP-1 DNA binding. In a set of ER-positive tumors that were growth-arrested following a short (3-month) course of neoadjuvant TAM treatment, mean AP-1 DNA binding activity is nonsignificantly reduced relative to untreated control tumors (Fig. 1), indicating that exposure to TAM is not sufficient in itself to increase AP-1 DNA binding. This would be supportive of the in vitro observations in wild-type MCF-7 cells that TAM antagonized ER/AP-1 co-activation (9, 14). In contrast, the group of TAM-R tumors that acquired their resistance to TAM after a median of 28 months of therapy showed a significantly higher AP-1 DNA binding activity over untreated control tumors (Fig. 3). We have reported previously that ER remains expressed in these TAM-R tumors and that this ER is fully capable of binding to its cognate estrogen response element (3, 4). Thus, this clinical finding of enhanced AP-1 DNA binding activity in primary ER-positive TAM-R breast tumors supports experi-
mural models, suggesting that the development of TAM resistance may be causally related to increased AP-1 DNA binding activity (10, 17).

It remains unclear which components of the AP-1 transcription factor complex are responsible for the enhanced DNA binding activity observed in TAM-R tumors. There is a preliminary report indicating that overexpression of c-Jun in MCF-7 breast cancer cells results in increased AP-1 DNA binding activity, also in the TAM-R phenotype accompanied by altered cell morphology and increased cell invasiveness (11). Increased c-Fos expression has been detected in some TAM-R human breast tumors. More recently, investigators studying MCF-7 xenografts carrying an AP-1-driven reporter gene have demonstrated increased AP-1 transcriptional activity occurring with in vivo acquisition of the TAM-R phenotype, although they were unable to detect significant changes in tumor AP-1 DNA binding activity or expression of Fos or Jun family members (18, 19).

Independently of altered levels in any of the Fos or Jun family members constituting the AP-1 complex, AP-1 transcriptional activity can be increased by protein kinase signals, which may not necessarily increase AP-1 DNA binding activity. Isozymes of the stress-activated JNK family bind to the NH2-terminal transactivator domain of c-Jun and phosphorylate Ser-63 and Ser-73 residues, enhancing AP-1 transcriptional activity (20, 21). This SAPK/JNK signaling pathway may become activated in cells in response to physical stress (UV light, ionizing radiation, and osmotic shock) as well as extracellular signals from inflammatory cytokines such as tumor necrosis factor-α (22). Of note, our assay specifically detected activated JNK within the tumor lysates and not tumor content of the activated c-Jun product, which can be similarly phosphorylated by a number of MAPK-related pathways (22, 23). Recently, it has been shown that activated forms of Ha-ras as well as G protein-coupled receptors and receptor tyrosine kinases all signal through the SAPK/JNK pathway (21, 24), thus providing many convergent transduction mechanisms leading to c-Jun phosphorylation and up-regulation of AP-1. Because JNK activation does not by itself increase AP-1 DNA binding and given the many different signaling pathways convergent on AP-1, it is not surprising that we found no significant association between increased JNK activity and increased AP-1 DNA binding in either our TAM-R or control tumors. It is also recognized that other transcription factors may be substrates of JNK (8), including ATF2 (25) and Elk-1 (26). Equally, our work does not determine whether increased AP-1/JNK activity represents increased upstream signaling, although we have previously reported no significant increase in epidermal growth factor receptor or c-erbB2 expression during the development of TAM resistance in human breast cancer (27).

The mechanism by which increased AP-1 transcriptional activity is associated with TAM resistance may well depend on the nature of the liganded ER isof orm interacting with the DNA-bound AP-1 complex at critical AP-1 target genes. With the recent cloning of a second ER isof orm, ERβ (28), consideration must now be given to the relative abundance of the two known ER isof orms in TAM-R breast tumors as TAM-ligated ERα and ERβ have been shown to have strikingly different effects on AP-1 target genes (29). In breast cancer cells such as MCF-7, TAM-ligated ERα exerts antagonistic effects on both ER and AP-1 target genes; in contrast, TAM-ligated ERβ activates AP-1 target genes in all cell types tested to date (29). Because TAM-ligated ERβ has the same stimulatory effect on AP-1 target genes as estrogen-ligated ERα, the development of TAM resistance in breast tumors accompanied by increased expression of ERβ would result in the stimulation of AP-1 target genes critical for tumor cell proliferation and invasion.

In conclusion, our present findings suggest that up-regulation

Fig. 4 Representative Western blot of phosphorylated c-Jun product resulting from solid phase kinase assay of activated JNK present in tumor lysates. As described in "Materials and Methods," the bands represent phosphorylated c-Jun(63) fusion product (M, 33,000–35,000) with greater band intensities seen in two of the TAM-R tumor samples as compared with the untreated tumor lysates. The slower migrating bands represent c-Jun product phosphorylated at only Ser-63. MCF-7 cell lysate was included in each experiment as an internal positive control.

Fig. 5 Relationship between AP-1 DNA binding activity and JNK activity for both untreated control tumors (○) and TAM-R tumors (●). Linear regression analysis showed no correlation between JNK and AP-1 in each tumor panel. Although the slopes of the regression lines were not significantly different (Control, y = 0.101x + 11.42; TAM-R, y = −0.019x + 40.48), for any given JNK activity, the AP-1 DNA binding appeared 3.5-fold greater in TAM-R tumors.

of both AP-1 DNA binding and JNK activity occur in ER-positive human breast tumors showing acquired resistance to TAM. These clinical observations confirm experimental models (10, 11, 17) and lend support to the hypothesis that up-regulated AP-1 transcriptional activity contributes in part to the phenotype of acquired antiestrogen resistance, which includes TAM-stimulated breast tumor growth. Studies are underway to determine the origin of the cell signals resulting in the increased JNK and AP-1 DNA binding activities observed in TAM-R tumors and to investigate further the nature of TAM-ligated ER interactions with AP-1. As well, additional clinical evaluation is warranted to assess the potential value of JNK activity and AP-1 DNA binding as predictive markers of TAM-R ER-positive breast tumors.

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References


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