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13. ABSTRACT (Maximum 200 Words)

Tamoxifen is the hormonal therapy of choice for patients whose tumors are classified estrogen receptor (ER)- positive. However, in advanced breast cancer, ER-positive tumors that may be initially responsive to tamoxifen become resistant. ER can interact with the activator protein-1 (AP-1) transcription factor complex through protein-protein interactions and tamoxifen functions as an agonist in coactivating ER/AP-1 in some ER positive cells. Tamoxifen has been shown to induce oxidative stress and tamoxifen resistant ER positive cell lines are associated with increased AP-1 binding, suggesting that enhanced AP-1 activity can account for tamoxifen-stimulated growth. In this study, I focus on the functional effect of oxidant stress on the zinc finger structure of ER. In order to accurately study the effect of the oxidant stress on full-length recombinant ER and on ER purified from cell lines and tumor samples, I have developed an alkylation and in gel-digestion protocol. This procedure is described in detail and will allow me to circumvent the unexpected pitfalls discussed in this report. Additionally, an analysis of 70 ER-positive breast tumors extracts for AP-1 and Sp1 DNA-binding and phosphorylated extracellular signal-regulated protein kinase 5 (P-Erk5) content has been completed.

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Breast Cancer, Tamoxifen, Estrogen Receptor, AP-1, Sp1

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INTRODUCTION

The hormonal therapy of choice for the treatment of ER-positive breast cancer has been the antiestrogen, tamoxifen. However, either from the onset or after prior treatment with tamoxifen, many ER-positive tumors demonstrate a tamoxifen-resistant or tamoxifen-stimulated phenotype. Studies of the ER in these cases have shown the persistent expression of immunoreactive 67 kDa ER, suggesting that ER function has somehow become altered permitting tumor growth in the presence of tamoxifen (1). As well, for nearly a third of primary ER positive breast tumors the extracted ER is unable to bind to its cognate DNA estrogen response element (ERE), an effect that we have found may be due to oxidation of one or both of the zinc fingers in the ER DNA binding domain (2). This defect may prevent ligand (or antiestrogen) bound ER from binding to its ERE but not prevent it from co-activating genes through other DNA-binding transcription factors such as AP-1 and Sp1. ER is known to interact with both these transcription factors when they are DNA-bound and through protein-protein interactions independent of ERE-mediated ER DNA binding. Likewise, the zinc finger protein Sp1 may similarly loose its capacity to bind an Sp1 response element yet retain its activity to co-activate specific genes containing an ERE bound to ER. Genes that are known to be transcriptionally upregulated 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 resistant deregulation and transformation to a more malignant and invasive phenotype. Genes that are known to be dependent on a cooperative ER/Sp1 promoter interaction include progesterone receptor (PR), cathepsin D, pS2 (trefoil factor), Bcl2 and cyclin D. Oxidant stress is associated with increase in AP-1 activity and DNA-binding as well as loss of Sp1 and ER DNA-binding function.; in turn, tamoxifen has been shown to induce oxidative stress (3) and tamoxifen-resistant breast tumors have been shown to possess increased AP-1 binding (4). This project continues to evaluate the functional effect of oxidant stress on the zinc-finger structure of ER, compares cellular oxidant stress effects on ER, AP-1 and Sp1 DNA-binding function, and will also measure the intracellular impact of these stresses on ER/AP-1 and ER/Sp1 regulated gene expression. As described in the first and subsequently amended Progress Report on our two original Technical Objectives, first year attempts to develop a cell-free AP-1 transcript forming assay to supplement our standard AP-1 DNA-binding assay (Technical Objective 1) were deferred due to methodological difficulties, and in favor of our more exciting progress toward a new objective (Technical Objective 3) involving mass spectrometric identification of the cysteine (Cys) residue defects observed within the DNA-binding domain (DBD) zinc-fingers of oxidant stressed ER-- progress that we expect will ultimately provide a new analytical tool and assay for analysis of ER-positive breast cancer. As well, analysis of 70 ER-positive cryopreserved primary breast tumor extracts for AP-1 and Sp1 DNA-binding, as well as content of the recently described oxidant stress parameter, phosphorylated (P)-Erk5, has now been completed and this work has been submitted as a new manuscript (5) reporting on the oxidant stress of aging and its clinically significant impact on ER-inducible gene expression in primary human breast tumors.

BODY

Progress Overview:

Progress since year 01 according to the technical objectives as originally proposed and subsequently amended (11/30/00).

Technical Objective 1: Develop a new assay to measure AP-1 transcriptional activity as a correlate of increased AP-1 DNA-binding and JNK activity in human tumor samples.

Unchange since original Progress Report and with reprioritization and addition of new objective (Technical Objective 3).

The new assay is based on a two-step process: The first step is to use a luciferase reporter construct in vitro transcription. The second step is to measure luciferase activity after in vitro translation. As proposed, a (AP-1)4-TK-CA plasmid (and one mutated to inactivate the tandem AP-1 response elements) obtained from H. Rochefort was modified into an (AP-1)-TK-LUC plasmid. The luciferase transcripts were then be purified (proteinase-K digestion, phenol/chloroform extraction followed by ethanol precipitation) and used to prime a rabbit reticulocyte in vitro translation system (Promega) that generates the luciferase product. The amount of product was quantified by luminometer in a standard luciferase assay.

To develop the assay, AP-1 containing nuclear and whole cell extracts from MCF-7 cell lines were used. The levels of AP-1 activity were compared in control vs. 12-O-tetradecanoylphorbol-13-acetate (TPA)-pretreated MCF-7 cells. TPA is a tumor promoter which induces expression of Jun and Fos family members, and enhances both AP-1 DNA binding and transcriptional activity (1); therefore the cells pretreated with TPA should have higher activity level. Transient transfections using the WT and MUT 4AP1/TK/Luc plasmids showed that the plasmids were able to induce Ap-1 activity in whole cell extracts from uninduced and TPA-induced MCF7 cells. TPA caused a 23-fold increase in Ap-1 activity from the WT plasmid (4527 $\pm$ 3910 vs 195 $\pm$ 63 relative luminosity units) but did not increase activity and in fact appeared to inhibit activity from the MUT plasmid (181 $\pm$ 113 vs. 35 $\pm$ 29). Based on this data, we attempted to measure luciferase activity after the in vitro transcription/translation protocol. Using the 4AP1/TK/Luc constructs, no difference in luciferase activity was detectable in TPA-treated MCF7 cell extracts (Mutant: 9.1 $\pm$ 14.8 vs. Wild-type: 5.3 $\pm$ 8.8). Therefore, although all of the components of the transcription/translation pathway are present in the MCF7 cells as demonstrated by the transient transfection experiments, we were unable to measure an increase in AP-1 activity by TPA in the in vitro assay.

Because the TK promoter has binding sites for other transcription factors that could be masking the effects of TPA, we made a new plasmid construct by replacing the TK

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promoter for the viral major late promoter E1b that does not contain additional transcription factor binding sites. Using this construct for transient transfection of MCF7 cells treated with and without TPA, we found that the E1b-containing promoter did have lower baseline luciferase activity compared to the TK-containing promoter. Furthermore, similar to the TK construct, TPA induced a major increase in Ap-1 activity from the wild-type, but not mutant, construct. Unfortunately, no difference in AP-1 activity was seen between TPA-stimulated WT and MUT 4AP1/E1b/Luc (Mutant: 29.0 ± 12.9 vs. WT: 28.0 ± 9.1).

While increased luciferase activity with TPA stimulation of both the 4AP1/TK/Luc and 4AP1/E1b/Luc plasmids is measurable in transient transfections, no increase was detected in the in vitro assay. Chromatinization has now been shown to be required for proper transcription and translation in several in vitro systems using a variety of promoters. In particular chromatin structure is required to set low-level baseline promoter activity. One reason our in vitro assay is not performing as expected is that despite the decrease in baseline activity of the E1b vs. TK construct, chromatinization is probably required. One unsuccessful attempt to form chromatin structures from the 4AP1/E1b/Luc construct has been made in the Tjian laboratory at UC Berkeley, where the process was developed. The protocols for chromatinization have been published but require 6-12 months to develop optimally performing reagents. Thus, we will continue to monitor progress in and consult with the Tjian lab who have been collaborating in our assay development effort. Thus, our own effort to develop an in vitro AP-1 transcription assay is now entirely dependent on significant progress and resources from other labs, and our efforts toward Technical Objective 1 will be deferred until such progress in the field of plasmid chromatinization has been convincingly and reproducibly demonstrated.

Summary:
The in vitro transcription assay remains challenging and is no longer under active development in this project to allow for our reprioritization of effort toward new Technical Objective 3, which shows substantial progress and promise.

Technical Objective 2: Compare zinc finger (ER, Sp1) DNA-binding with ER content, AP-1 DNA-binding, and associated mitogen activated protein kinase (Jun N-terminal kinase (JNK) or Erk5) activity from primary breast tumors.

Methods: We correlated age at diagnosis with expression of breast cancer markers ER, PR, pS2, Bcl2 and cathepsin D, quantitated by enzyme immunoassays from a European collective of ~3000 cryobanked primary breast cancers and ~300 adjacent non-malignant breast tissues. Results were compared with ER and PR data reported to the SEER registry for 83,541 US cancers diagnosed during 1992-1997. A homogeneous subset of 70 ER-positive tumors preselected from the European collective was blindly analyzed for age-specific changes in the DNA-binding content of the redox-sensitive transcription factors, AP1 and Sp1, and the oxidant stress-activated protein kinase, phosphorylated (P)-Erk5.

Results: Increases in breast tumor ER from patients aged <30 to >80 years mirrored 10-fold lower increases in non-malignant breast tissue ER up to age 60, rising faster thereafter and reaching a near 25-fold differential between malignant and non-malignant breast tissue by age 80. ER-inducible markers PR, pS2, Bcl2 and cathepsin D were overexpressed in tumors relative to non-malignant breast tissue but appeared to be independent of age in both tissue types. All ethnic groups showed an increasing proportion of ER-positive/PR-negative breast cancers with age. Loss of Sp1 DNA-binding, increased P-Erk5 and lower PR content correlated significantly with age in the subset of ER-positive breast tumors. Almost all extracts showed detectable AP-1 DNA-binding; no age-related changes in AP-1 DNA-binding were detected, however a borderline significant increase in AP-1 DNA-binding was observed in tumors extracts associated with earlier metastatic recurrence.

Conclusions: These findings support two hypotheses: i) dysregulated ER expression underlies the age-specific increase in breast cancer incidence especially after age 50; and ii) oxidative stress and loss of Sp1 DNA-binding likely contribute to the increase in higher risk ER-positive/PR-negative breast cancers seen with aging. Future studies including larger sample sizes and statistically powered to show changes associated with patient outcome (e.g. metastatic recurrence after tamoxifen therapy) are needed to conclusively demonstrate that increased AP-1 DNA-binding is associated with tamoxifen resistance.

Technical Objective 3: Extend recently developed mass spectrometric approach to identify differentially oxidized cysteine (Cys) residues within the ER DBD zinc-fingers and characterize the oxidative damage detected in purified full-length human breast tumor ER.

Mass spectrometric (MS) analysis of oxidant stressed recombinant estrogen receptor- DNA-binding domain (ER-DBD) has shown that its two zinc fingers are susceptible to oxidation, particularly the second zinc finger that engages in ER dimerization (2). Our goal is to carry these studies a step further and look at the affects of oxidative stress on the ER-DBD in the context of the full-length (67 kDa) protein. Our initial studies focused on the full-length recombinant ER (purchased from PanVera) and involved the digestion of the protein (Lys C endoprotenaise) followed by the isolation and identification of the relevant ER-DBD fragments by liquid chromatography (reverse phase) and mass spectrometry (LC/MS). In the process of such studies we found several technical challenges not encountered in the studies of the isolated recombinant ER-DBD. For example, we immediately recognized that during the analysis of the protein, the ER-DBD fragments readily oxidized. This spontaneous oxidation of Cys residues via thiol-disulphide exchange reactions is a common phenomenon and most likely occurs after digestion and during the LC/MS analysis of the ER-DBD peptides, making it difficult to accurately asses the susceptibility of the different cysteine (Cys) residues to oxidants in the context of the folded protein. Additionally, treatment of full-length recombinant ER with oxidizing agents (H_2O_2, diamide) followed by digestion and LC/MS analysis caused

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a significant loss in absolute signal of each ER-DBD fragment (up to 90%). This loss in signal is likely due to the various inter- and intra-molecular disulphide bond formations that occur within the oxidized protein. In turn, these interactions likely cause much of the protein to fall out of solution resulting in the low signals registered by the mass spectrometer.

Thus, in an effort to efficiently study the effect of oxidative stress on the full-length recombinant protein as well as on ER purified from cell lines and tumor samples we have developed a two-stage Cys alkylation and two-protease in-gel digestion protocol (See Fig.1) that will allow us to circumvent the problems mentioned above. This involves the initial selective carboxymethylation of non-oxidized Cys residues in ER with iodoacetic acid, an in-gel reduction and a second alkylation step with doubly labeled ^{13}C bromoacetic acid to identify Cys residues that were originally oxidized, followed by in-gel proteolytic digestion and peptide analysis by LC/MS. Endoproteinase Lys-C and Asp-N enzymes have been selected giving peptides that each contain two Cys residues, showing mass differences of 2 or 4 Da for single or double incorporation of ^{13}C labeled carboxymethyl groups.

This method will allow us to: 1) circumvent the problem of spontaneous oxidation of cysteines by alkylating these reactive residues; 2) circumvent the loss of peptide signal by reducing the protein prior to the second alkylation and after oxidant treatment; 3) more precisely quantitate the amount of reduced versus oxidized zinc finger peptides by using chemically identical but isotopically tagged alkylating agents of different molecular weights to selectively label free cysteines with one agent and previously oxidized cysteines with the other. The in-gel digestion and extraction are necessary as part of ER's purification procedure from cell lines and tumors sample.

Employing this two-step alkylation protocol on recombinant ER that has been treated with various concentrations of oxidizing agents will establish an oxidant sensitivity profile of Cys residues within full-length protein. Additionally, it will allow for the analysis of *in vivo*-induced Cys oxidation in ER purified from cultured breast cancer cells and freshly resected breast tumors to correlate these oxidant stress-induced structural changes with the measurable loss of ER DNA-binding function. A goal of these studies is to develop a clinical test that will identify breast tumors possessing oxidatively damaged ER and less likely to respond to endocrine agents like the antiestrogen tamoxifen.
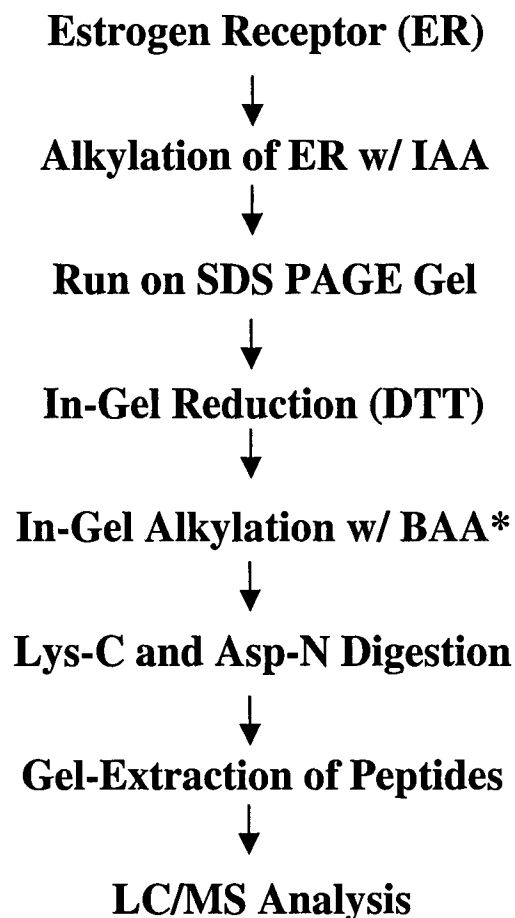
Estrogen Receptor (ER)

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Alkylation of ER w/ IAA

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Run on SDS PAGE Gel

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In-Gel Reduction (DTT)

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In-Gel Alkylation w/ BAA*

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Lys-C and Asp-N Digestion

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Gel-Extraction of Peptides

↓

LC/MS Analysis

Figure 1: The two-stage alkylation and two-protease in gel-digestion and extraction procedure used in our studies. The alkylation steps circumvent the spontaneous oxidation of Cys residues that occurs during the analysis of ER. Alkylation with iodoacetic acid (IAA) adds 58 Da to the molecular weight per reactive cysteine, while doubly ^{13}C-labeled bromoacetic acid (BAA*) adds 60 Da.

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5. Quong, J.; Eppenberger-Castori, S.; Moore, D.; Scott, G.K.; Birrer, M.J.; Kueng, W.; Eppenberger, U.; and Benz, C.C. Age-dependent changes in breast cancer hormone receptors and oxidant stress markers. J. Natl.Cancer Inst., (Submitted; Aug. 2001).

APPENDIX

Key Research Accomplishments (according to funding year):

1. (year 1) Two different 4AP1/Luc constructs were made during the first year and tested for the early feasibility assessment of a potential in vitro transcription assay; collaborations with the UCB Tjian lab were put into place for assistance in reagent production and assay development.
2. (years 1-2) Completed analysis of ER content, AP-1 DNA-binding, Sp1 DNA-binding, Sp1 protein content, and Erk5 activation in 70 outcome-linked and biomarker-characterized breast tumors. Significant statistical associations with patient age at diagnosis were discovered, potentially explaining the increasing incidence of ER-positive/PR-negative breast cancers occurring with age >50 years, as confirmed by review of SEER database information on >80,000 US breast cancer cases diagnosed between 1992-1997.
3. (year 2) Developed a two-step Cys alkylation and two-protease in-gel digestion and extraction protocol that has enabled us to successfully isolate and identify fragments comprising the ER-DBD from full-length recombinant ER by mass spectrometry. Our preliminary data suggest that this method will allow us to circumvent the unforeseen problems described above (i.e. spontaneous oxidation of Cys residues and loss of peptide signals) and enable us to efficiently study the effect of oxidative stress on the ER-DBD within full-length recombinant ER and on ER purified from cell lines and tumor samples.

Reportable Outcomes:

1. Quong, J.N.; Eppenberger-Castori, S.; Birrer, M.; Eppenberger, U., and Benz, C.C. Redox-sensitive kinase (Erk5) activation and altered transcription factors (Ap-1, SP1) DNA binding in untreated primary breast tumors. American Association of Cancer Research 2000 Meeting, April 1-5, San Francisco, CA.
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