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13. ABSTRACT (Maximum 200 Words)

Patients with breast cancers that express estrogen receptor (ER) commonly receive antiestrogen therapy. The efficacy of this treatment depends on tight regulation of breast growth by estrogen. However, as breast cancers progress, they often become resistant to estrogens, and most patients no longer respond to anti-estrogen therapy. New antiestrogen treatment options are needed, and alternative therapies may derive from findings showing that some ER molecules occur in plasma membranes of breast cancer cells and interact with transmembrane HER-2 growth factor receptors. Expression of HER-2 receptors occurs in many breast cancers, and the protein kinase activity of HER-2 may modulate ligand-independent activation of ER. Active cross-communication between ER and HER-2 receptors occurs in breast tumors, leading to promotion of cancer growth. Thus, this axis may offer a new target for therapeutic intervention. We have partially purified a membrane-associated form of ER in breast cancer cells and have evidence that it promotes tumor growth. Using this novel signaling pathway as a target, we are assessing new treatments to prevent cancer progression in models of human breast cancer. Since HER-2 overexpression in breast cancer is associated with the failure of antiestrogen therapy, understanding the basis of interactions between ER and HER-2 receptors may help to improve patient management and survival.

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Table of Contents

INTRODUCTION

Breast cancer is the most common malignancy in women in North America and is usually a disease of post-menopausal women (1). In the clinic, endocrine therapy is an important intervention in women with breast cancers that express estrogen receptor (ER). Treatment with tamoxifen and other antiestrogens has enhanced the survival of breast cancer patients, and these agents are now used in breast cancer prevention. The success of endocrine therapy in breast cancer is dependent on tight regulation of breast cell growth by steroid receptors (1, 2). However, as cancer progresses, it usually becomes resistant to estrogens, and most patients stop responding to tamoxifen or other antiestrogens. New findings on the role of an alternate estrogen signaling pathway in breast tumors may promote design of novel and more effective antihormone treatments for breast cancers (3).

Growth factor receptor malfunction also occurs in malignant progression, with members of the HER-1 (EGF) family frequently implicated in human cancer (1-3, 4-8). The HER (erb B) receptor family includes the HER-2 (erb B2) protein, a 185-kD transmembrane tyrosine kinase encoded by HER-2 oncogene (9-11), the HER-3 protein (12) and HER-4 receptor (13,14). Overexpression of HER-2 or related growth factor receptors is estimated to occur in two-thirds of sporadic breast cancers (1), while HER-2 amplification or overexpression is found in 25-30% of breast cancers in women and 41% of breast cancers in men (15-18). Overexpression of HER-2 is a marker of poor prognosis (15-19) and is associated with failure of antiestrogen therapy (3,20-31).

Receptors for estrogen occur in a family of potentially oncogenic receptors. Sequence similarities between the erb A gene product of avian erythroblastosis virus and ER suggest that these two proteins likely evolved from a common gene (32). Erb A genes cannot induce cell transformation alone, but cooperate with viral erb B oncogenes in cell transformation (33). With this lineage of cooperativity between erb A and erb B genes, it is not surprising to find reports of significant cross-talk and interaction between erb B (HER) pathways and ER signaling (3,24,27,34-36).

It is generally held that the biologic activity of estrogen in the breast is mediated through the specific high-affinity ER located in breast cell nuclei (1,37) [see FIG.1].

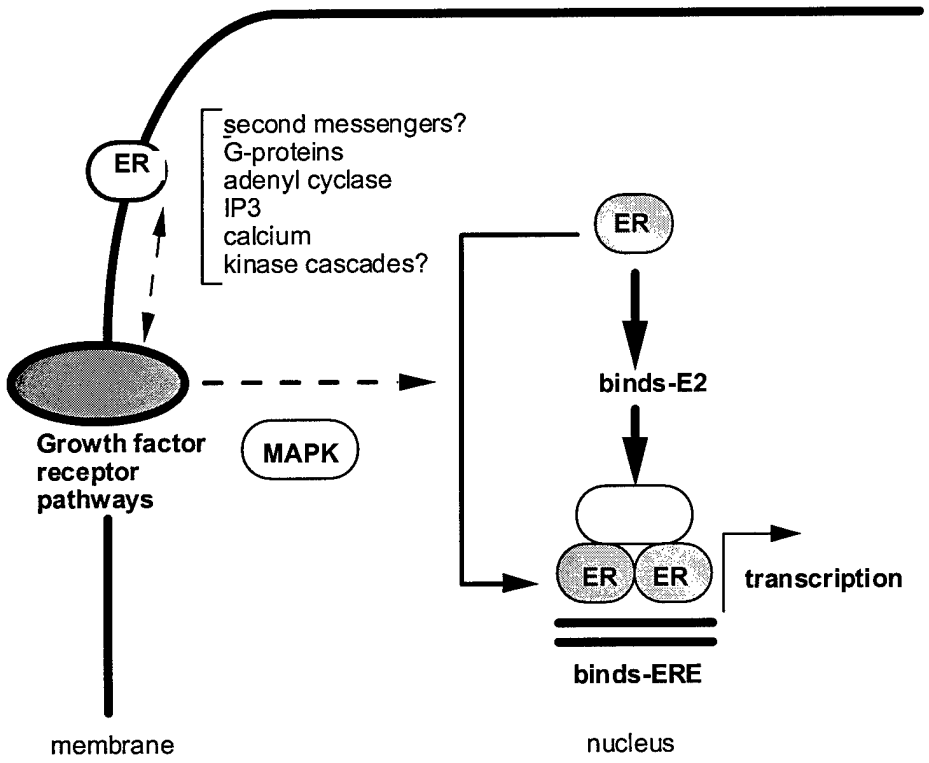

FIG. 1. Postulated cellular mechanism of action of estrogen (E2) and growth factors in breast cancers with estrogen receptor (ER). In most models of estrogen action, estrogen binding to ER in the nucleus promotes receptor dimer formation and receptor phosphorylation that enhances binding to nuclear estrogen-responsive elements (ERE) and coactivator proteins, leading, in turn, to initiation of gene transcription. However, this

model fails to account for numerous, rapid cell responses to estrogen (41-69). In the hypothesis to be tested here, estrogen may also bind to a membrane ER, with potential for stimulation of estrogenic responses via an alternate pathway. Current reports suggest that membrane-associated ER may activate one or more pathways, including interaction with growth factor membrane receptors such as HER-2 or activation of G-proteins, adenylate cyclase, inositol phosphate, calcium homeostasis and/or MAP kinase. These interactions may promote phosphorylation of ER via estrogen-induced activation of second-messengers and protein kinases or, alternatively, via ligand-independent pathways involving growth factor receptors. Growth of cells treated with estrogen may occur as a consequence of a synergistic feed-forward circuit where estrogen activates membrane signaling pathways that act, in turn, to enhance transcriptional activity of ER in the nucleus.

In the absence of estrogen, ER is considered to associate with proteins that prevent its interaction with the cell transcription apparatus. Upon estrogen binding, the receptor undergoes an activating conformational change that promotes association with target genes, thus permitting regulation of gene transcription [see FIG. 1]. In addition to the latter pathway, however, estrogen also induces rapid increases in levels of intracellular second messengers, including calcium (39,40) and cAMP (41,42), as well as activation of MAP kinase (43,44) and phospholipase (45). The timecourse of these events is similar to those elicited by peptides, lending support to the hypothesis that they do not involve genomic actions of estrogen. Both estrogens and growth factor ligands act as mitogens to promote cell growth in the breast, and the cellular effects of these agents sometimes overlap. The molecular details of this cross-talk between ER and erb B receptors are now beginning to emerge, and ER itself may be an important point of convergence (3,24,34-36).

Many of the rapid effects of estrogen are now attributed to the action of the hormone at the membrane, and these biologic actions appear to be mediated by membrane receptors that bind estrogen. The isolation and structural characterization of these native macromolecules have not yet been accomplished, and the derivation and functions of this receptor (or receptors) are largely unknown. Since activation of this alternate signaling pathway by estrogens may represent a mechanism by which estrogens regulate proliferation, we have investigated the nature and activity of this membrane response pathway in human breast cancer cells. Classical models of estrogen action that characterize this signaling pathway as solely due to the activity of an intracellular ligand-dependent transcription factor are clearly incomplete and must be modified to include estrogen receptors as significant components of other signaling pathways. As urged by others (40), "these data beg a reevaluation of the relative contributions of genomic and nongenomic activities in ER biology, an activity that is likely to support the development of pharmaceutical agents that exert differential activities in the two pathways".

RESEARCH PROGRESS

Aim 1) To assess the existence and identity of receptors for estrogen in plasma membranes of human breast cancer cells.

1.a. Enrichment of high-affinity binding-sites with specifcity for $E_2ß$ in breast cancer cell plasma membranes

To confirm earlier reports of membrane binding-sites for $E_2ß$ (52,55,61,63), we measured specific [^{3}H]$E_2ß$ binding in subcellular fractions of MCF-7 cells after controlled cell homogenization and fractionation (47,48). With recovery of more than 97% of total $E_2ß$ binding found in homogenates of MCF-7 cells, specific [^{3}H]$E_2ß$ binding was distributed among crude nuclear, microsomal, mitochondria-lysosome and cytosol fractions (see Fig. 1 in ref. 84). After purification of plasma membranes from the crude nuclear fraction by use of discontinuous-sucrose density gradient centrifugation, the PM fraction showed enhanced activity of 5'-nucleotidase, a plasma membrane marker enzyme, to about 23-times that of homogenate. Specific [^{3}H]$E_2ß$ binding in plasma membranes was enriched to 28-times homogenate activity and represented 22% of homogenate binding. This data shows that specific $E_2ß$ binding co-purifies with a plasma membrane marker protein in membrane fractions from breast cancer cells. LDH activity, highly enriched in cytosol, is not significantly detected in PM (84). In addition, cell DNA recovery was 94 $\pm$ 3 % of homogenate levels in nuclear fractions, and no DNA was detected in PM fractions (84).

Binding of [^{3}H]E_2ß by PM fractions from MCF-7 cells was analyzed further in equilibrium binding studies (see Figs. 2,3 in ref. 84). Samples of PM were exposed to [^{3}H]E_2ß concentrations ranging from 1 x 10^{-10} M to 5 x 10^{-9} M. Binding of hormone by PM is saturable, and Scatchard analyses of specific [^{3}H]E_2ß binding (cf. 48) show that the dissociation constant for the binding process is 3.6 x 10^{-10} M. Total binding sites in PM at saturation correspond to approximately 6.7 pmol E_2ß per mg membrane protein (84). In comparison with the estradiol binding properties of intact MCF-7 cells, plasma membrane estrogen-binding sites retain high affinity for specific estradiol binding and exhibit significant enrichment of ligand-binding capacity (see ref. 3). Further, ligand specificity of [^{3}H]E_2ß binding to PM was established by effective suppression by a 100-fold molar excess of unlabeled E_2ß (84). In contrast, [^{3}H]E_2ß binding by PM was essentially uninfluenced by these levels of estradiol-17α, progesterone or testosterone (84). This portion of Aim 1 is now completed.

1.b. Identification of estrogen receptor forms in subcellular fractions after gel electrophoresis

To characterize putative estrogen receptor forms associated with PM fractions, samples were subjected to Western blot analysis, and blots were probed either with anti-ER antibody Ab2 or with E_2ß-POD (84). PM purified from MCF-7 cells show significant enrichment of a primary 67-kDa protein that reacts strongly with antibody Ab2 to LBD of nuclear ER-α (see Fig. 4 in ref. 84). Similarly, breast cell nuclear fractions are enriched with this protein reactive with ER-α (84). The 67-kDa band also shows evidence of specific labeling with E_2ß-POD (84). A secondary band at 46-kDa and minor bands at 62-kDa and 97-kDa were detected in PM and other cell fractions by use of Western blot and ligand-blotting (84). Using an antibody directed to ER-ß, no significant reactivity with proteins at the expected size of 58-62 kDa was found in homogenate, nuclear or plasma membrane fractions of the MCF-7 cells (84). This portion of Aim 1 is now completed.

1.c. Purification of candidate receptors

As indicated above, work aimed at purification of candidate receptors is underway. As outlined in the original proposal, our efforts have involved the use of affinity chromatography, with recovered receptor to be used for preparation of monoclonal antibodies and for further molecular characterization and functional studies using cDNA for membrane ER. However, the yield of estrogen-binding receptor protein from purified plasma membranes has been very limited, and this strategy may require modification (85). In contrast, we have made very good progress in the isolation of membrane-associated ER from caveolae-related lipid raft subfractions of breast cancer cell plasma membranes (86), and this method may now allow further purification of membrane-associated receptor forms (87-91).

Aim 2) To assess the role of membrane estrogen receptors in promoting growth of breast cancers.

2.a. Rapid effects of E_2ß and E_2ß-BSA on activation of MAPK and Akt kinase in breast cancer cells

Post-receptor signal transduction events, such as stimulation of MAPK, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42) (43,61), may contribute to proliferative effects of E_2ß in breast cells. Thus, we assessed estrogen-induced phosphorylation of MAPK in MCF-7 cells *in vitro*. E_2ß, but not 17α-estradiol (E_2α), promotes phosphorylation of MAPK isoforms, with effects evident within 2 min (see Fig. 5 in ref. 84). To test whether activation of MAPK by E_2ß may be mediated by binding of estrogen to membrane-associated receptors, MCF-7 cells were treated with E_2ß linked to BSA, a macromolecular complex considered to be membrane-impermeant (52,61). Using E_2ß-BSA, but not control E_2α-BSA, phosphorylation of MAPK isoforms is again evident within 2 min of steroid administration. Incubation of cells with antibody against LBD of ER (Ab2) inhibited MAP kinase phosphorylation induced by E_2ß or E_2ß-BSA. Similarly, we assessed signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway after treatment of MCF-7 cells with E_2ß or E_2ß-BSA. Both ligands induced significant activation of Akt kinase (84), and inhibition of estrogen-induced effects occurred when cells were preincubated with ER antibody (Ab2), pure antiestrogen (ICI 182,780) or the PI3K inhibitor, LY 294002.

To assess the potential for MCF-7 cell activation by free estradiol liberated from E_2ß-BSA, we transfected MCF-7 cells with an ERE-CAT reporter gene as before (3). Cells were exposed *in vitro* to free estradiol-17ß or to E_2ß-BSA for only 10 minutes, then washed and incubated further. After 24 hrs, ERE-CAT reporter gene activity was measured. Short-term treatment with free estradiol-17ß stimulated a marked increase in reporter gene activity ($P<0.001$), but E_2ß-BSA elicited no significant effect (see Fig. 6 in ref. 84).

Since interaction of E_2ß-BSA with plasma membrane binding-sites may be required for intracellular signaling (52,61), we evaluated binding of fluorescein-labeled E_2ß-BSA (E_2ß-BSA-FITC) in MCF-7 cells. E_2ß-BSA-FITC binds at the surface of 77% of MCF-7 cells (see Fig. 7 in ref. 84), while only minimal background fluorescence is found among cells incubated with control ligand, BSA-FITC (84). In additional control studies, ER-positive ZR-75 breast cancer cells, as MCF-7 cells, show retention of E_2ß-BSA-FITC at the cell surface, but ER-negative MDA-MB-231 breast cancer cells or COS-7 cells do not show significant binding of E_2ß-BSA-FITC at the external membrane (84). On flow cytometric analysis (84), the E_2ß-BSA-FITC complex shows evidence of ligand specificity, with significant reduction ($P<0.01$) of E_2ß-BSA-FITC binding by competition with equi-molar amounts of free E_2ß, E_2ß-BSA, tamoxifen or ICI 182, 780, while the related steroid congener, progesterone, is not effective. Surface binding of E_2ß-BSA-FITC is significantly diminished by competition with antibody to LBD of nuclear ER, suggesting some immunologic identity of the membrane site with nuclear ER (84). As expected, after permeabilization of cells by disruption of plasma membrane with detergent, intense labeling of ER in cell nuclei is found and occurs in 96% of breast cancer cells (84). In other control studies, MDA-MB-231 cells with no ER showed no binding or retention of E_2ß-BSA-FITC label, while ZR-75 breast cancer cells with ER expression did show surface binding of the complex (84). These portions of Aim 2 are now completed.

2.b. Inhibition of cell growth in vitro by antibody to ligand-binding domain of ER-α

Since antibodies to cell surface growth factor receptors are sometimes effective in blocking tumor growth (3,77), antiproliferative activity of antibodies to ER-α was evaluated using MCF-7 cells *in vitro*. The estrogen-dependent MCF-7 cells show enhanced growth after treatment with E_2ß, but not E_2α (see Fig. 8 in ref. 84). However, prior exposure to LBD Ab1 or LBD Ab2 elicits a significant reduction ($P<0.05$) in the E_2ß growth response (84). Since some recent studies suggest that the proliferative response to E_2ß is committed within 1 min and is evoked by activation of only a small fraction (≤5%) of ER (73), we assessed the growth of breast cells after brief treatment with E_2ß-BSA. MCF-7 cells were treated with 0.5 μM E_2ß-BSA for only 10 min. Then, cells were rinsed and cultivated in estrogen-free media for an additional 72 h. The results show that E_2ß-BSA ($P < 0.001$), but not control E_2α-BSA, stimulates cell growth (84). Moreover, the proliferative effect of E_2ß-BSA is blocked by treatment of cells with ICI 182,780, a pure antiestrogen ($P < 0.001$) (84), or by prior exposure to anti-ER Ab1 ($P < 0.05$) or Ab2 ($P < 0.001$) (84). This portion of Aim 2 is now completed.

Aim 3) To investigate new treatment options to prevent breast cancer progression in human breast cancer.

3.a. Inhibition of breast tumorigenesis in vivo by antibody to ligand-binding domain of ER-α

The antitumor activity of antibodies to ER-α was evaluated further using MCF-7 tumors *in vivo*. MCF-7 cells were grown as subcutaneous xenografts in female athymic mice primed with E_2ß to promote growth of these estrogen-dependent cells (3). Antibody or control treatments were initiated when tumors grew to >30 mm^3. Anti-ER Ab2 was administered in 6 doses over a 26-day period. The results show that antibody to ER, but not control immunoglobulin, elicits a significant suppression of tumorigenesis of human MCF-7 breast cancer xenografts in female nude mice treated concomitantly with E_2ß (see Fig. 8 in ref. 84). This portion of Aim 3 is now completed.

3.b. Estrogen receptor interactions with growth factor membrane receptors

As noted above, activation of estrogen receptor-α (ER) by growth factors in the absence of estrogen is a well-documented phenomenon. To further study this process of ligand-independent receptor activation, COS-7

cells without ER were transfected with both ER and epidermal growth factor (EGF) receptor. In the absence of estrogen, EGF stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells (see ref. 87). Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGF receptors, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found (87). In confirmation of direct interactions between ER and EGF receptors, activation of affinity-purified EGF receptor tyrosine kinase *in vitro* stimulated phosphorylation of recombinant ER (87). The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways (87). However, COS-7 cells transfected with both ER and EGF receptors show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the ER. Further consideration of the cross-communication between membrane-associated ER and membrane growth factor receptors, such as EGF and HER-2 receptors, may provide new targets for intervention in the clinic (88). In addition, new findings from our laboratory suggest that proximate interactions between membrane-associated ER and growth factor receptors may occur in specialized domains of plasma membrane, the caveolae-related lipid rafts (87-91).

PERSONNEL

During the past grant period, the following personnel were engaged on this project:

Richard J. Pietras, PhD, MD (PI)
Diana Marquez, MD (Postdoctoral Fellow)
Hsiao-Wang Chen, MS (SRA)

KEY RESEARCH ACCOMPLISHMENTS

- Plasma membrane-associated binding sites with high affinity and specificity for estradiol-17ß occur in human breast cancer cells.
- Plasma membrane-associated binding sites for estradiol in human breast cancer cells may play a role in modulating cell growth and survival.
- Plasma membrane-associated estrogen receptors interact with signaling initiated by membrane growth factor receptors
- Proximate interactions between membrane-associated ER and growth factor receptors may occur in caveolae-related lipid rafts of breast cancer cells

REPORTABLE OUTCOMES

Presentations

1. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at *First International Symposium on Translational Research in Oncology*, Dublin, Ireland (2001).
2. "HER-2 receptor signaling modulates estrogen receptor in breast cancer". Presented at Medical Oncology Seminar Series, UCLA School of Medicine (2001).
3. "Steroid and growth factor receptors: Cross-talk and clinical implications". Presented at *Second International Symposium on Translational Research in Oncology*, Anaheim, California (2002).
4. "Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature". Presented at 8th Annual Multidisciplinary Symposium on Breast Disease, Amelia Island, Florida (2003).
5. "Estrogen receptor and human breast cancer therapy". Presented at Department of Pathology Seminar Series, UCLA School of Medicine (2003).
6. "HER-2 and estrogen receptor interactions in human breast cancer". Presented at Genentech BioOncology Herceptin Advisory Board Meeting, San Francisco (2003).

Abstracts

1. Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2002). Membrane-associated estrogen receptors localize to caveola-related domains and contribute to growth regulation of breast cancer cells. DOD Breast Cancer Research Program Era of Hope Meeting Proceedings.
2. Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2003). Estrogen receptor forms and HER-2/neu growth factor receptors co-localize in caveolae-related lipid rafts in human breast cancer cells. Proc. Am. Assoc. Cancer Res. 44 : 384.

Publications

1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. Endocrine **14** : 417-427.

2. Marquez, D.C. and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene 20: 5420-5430.

3. Marquez, D.C., Lee, J., Lin, T. and Pietras, R.J. (2001). Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. Endocrine 16: 73-81.

4. Marquez, D.C. and Pietras, R.J. (2003). Membrane-associated estrogen receptors and breast cancer. In: *Identities of Membrane Steroid Receptors* (Watson, C., editor), Kluwer Academic Publishers, pp. 1-10.

5. Szego, C.M., Pietras, R.J. and Nemere, I. (2003). Plasma membrane receptors for steroid hormones: Initiation site of the cellular response. Encyclopedia of Hormones (in press).

6. Pietras, R.J. (2003). Steroid and growth factor receptors: Cross-talk and clinical implications. In: *Breast Cancer Management, 2nd Edition* (Reese, D., Nabholtz, J.-M., and Slamon, D.J., editors), Lippincott, Philadelphia (in press).

7. Pietras, R.J. (2003). Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature. Breast Journal (in press).

No abstracts, patents, degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

A new approach to cancer therapy involves efforts to cut the lines of communication between hormone receptors and the cell nucleus, thus slowing or blocking cell division. Antiestrogen therapy is one well-known example of this approach, and it is often used to treat breast cancer and to prevent recurrence. Unfortunately, many patients do not respond to current therapy, and almost all treated patients eventually become resistant to antiestrogens. In addition, antiestrogens that are now available can result in abnormal uterine growth and thromboembolic events. The failure of antihormone therapy in the clinic is due to many factors, including the emergence of estrogen-independent growth that is no longer responsive to treatment with antiestrogen agonists.

New options for antiestrogen treatment are clearly needed, and alternative therapies may now derive from the current findings showing that ER molecules occur not only in the nucleus of the cell, but also in association with the surface membranes of human breast cancer cells. Moreover, these ER may interact with membrane HER-2 growth factor receptors. It is known that expression of HER-2 receptors occurs in many human breast cancers, and the enzyme activity of HER-2 may play a role in ER activation even in the absence of estrogen. If proximate interactions between ER and the HER-2 growth factor receptor occur and lead to promotion of cancer growth, this signaling axis may offer a new target for therapeutic intervention. Since overexpression of HER-2 in human breast cancers is associated with the failure of antiestrogen therapy in the clinic, understanding the biologic basis of the association between membrane ER and HER-2 receptors may help to improve decisions on patient management and to increase patient survival.

In the present work, we have made good progress in ascertaining the existence and nature of receptors for estrogen in surface membranes of human breast cancer cells. We have assessed the role of membrane ER in promoting growth of breast cancers. In challenging the dogma of estrogen action exclusively via an intracellular receptor, this work may lead to the development of previously unsuspected, less toxic antitumor therapies targeted to human breast cancer cells. A limited no-cost time extension would help us to complete all the goals of this proposal and to submit our findings for publication in peer-reviewed journals.

REFERENCES

1.) Harris J., M. Lippman, U. Veronesi & W. Willett (1992). Breast cancer. N. Engl. J. Med., 327 : 473-451.

2.) Aaronson S.A. (1991). Growth factors and cancer. Science, 254 : 1146-1152.

3.) Pietras, R.J., Arboleda, J., Wongvipat, N., Ramos, L., Parker, M.G., Sliwkowski, M.X., and Slamon, D.J. (1995). HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, 10 : 2435-2446.

4.) Carpenter G. and S. Cohen (1979). Epidermal growth factor. Ann. Rev. Biochem.,48:193-208.

5.) Bishop J.M. (1983). Cellular oncogenes and retroviruses. Ann. Rev. Biochem., 52: 301-318.

6.) Lieberman T.A., H.R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, M.D. Waterfield, A. Ullrich and J. Schlessinger (1985). Amplification, enhanced expression and possible rearrangement of the epidermal growth factor receptor gene in primary human tumors of glial origin. Nature, 313 : 144-147.

7.) Dotzlaw H., T. Miller, J. Karvelas and L. C. Murphy (1990). Epidermal growth factor gene expression in human breast cancer biopsy samples : relationship to estrogen and progesterone receptor gene expression. Cancer Res., 50 : 4204-4212.

8.) Gullick W., J. Marsden, N.Whittle, B.Ward, L.Bobrow & M.Waterfield (1986). Expression of the epidermal growth factor receptors on human cervical, ovarian and vulvar carcinomas. Cancer Res.,46: 285-293.

9.) Coussens L., T.C. Yang-Feng, Y.C. Liao, E. Chen, A. Gray, J. McGrath, P.H. Seeburg, T. A. Lieberman, J. Schlessinger et al.(1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science, 230 : 1132-1135.

10.) Schechter A.L., D. F. Stern, L. Vaidyanathan et al.(1985). The neu oncogene: an erb B related gene coding a 185,000-M tumor antigen). Nature, 312 : 513-515.

11.) Semba K., N. Kamata, K. Toyoshima and T. Yamamoto (1985). A v-erbB-related proto-oncogene, c-erb B2, is distinct from the c-erbB-1 epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA, 82 : 6479-6486.

12.) Kraus M. H., P. Fedi, V. Starks, R. Muraro and S. A. Aaronson (1993). Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constituitive activation in human breast tumor cells. Proc. Natl. Acad. Sci. USA, 90 : 2900-2905.

13.) Plowman G. D., J.-M. Culouscou, G. S. Whitney, J. M. Green, G. W. Carlton, L. Foy, M. G. Neubauer and M. Shoyab (1993). Ligand-specific activation of HER4 / p180erbB4, a fourth mamber of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA, 90 : 1746-1752.

14.) Culouscou J.-M., G. D. Plowman, G.W. Carlton, J.M. Green & M. Shoyab (1993). Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180erbB4 receptor. J. Biol. Chem.,268 : 18407-18416.

15.) Slamon D.J., G.M. Clark, S.G. Wong et al.(1987). Human breast cancer : Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, 235 : 177-181.

16.) Slamon D.J., W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, A. Ullrich and M.F. Press (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, 244 : 707-711.

17.) Slamon D., M. Press, W. Godolphin, L. Ramos, P. Haran, L. Shek, S. Stuart and A. Ullrich (1989). Studies of the HER-2/neu oncogene in human breast cancer. Cancer Cells, 7 : 371-378.

18.) Joshi M., A. Lee, M. Loda, M. Camus, C. Pedersen, G. Heatley and K. Hughes (1996). Male breast cancer : An evaluation of prognostic factors contributing to a poorer outcome. Cancer 77 : 490-498.

19.) Tiwari R., P. Borgen, G. Wong, C. Cordon-Cardo, and M. Osborne (1992). HER-2/neu amplification and overexpression in primary human breast cancer is associated with early metastasis. Anticancer Research 12 : 419-425.

20.) Wright C., B. Angus, S. Nicholson et al. (1989). Expression of c-erbB-2 oncoprotein : a prognostic indicator in human breast cancer. Cancer Res., 49 : 2087-2094.

21.) Nicholson S., C. Wright, J.R.C. Sainsbury, P. Halcrow, P. Kelly, B. Angus, J.R. Farndon and A. L. Harris (1990). Epidermal growth factor receptor as a marker for poor prognosis in node-negative breast cancer patients : neu and tamoxifen failure. J. Steroid Biochem., 37 : 811-818.

22.) Klijn J., P. Berns, P. Schmitz, J. Foekens (1992). The clinical significance of epidermal growth factor receptor in human breast cancer : review on 5232 patients. Endocr. Rev., 13 : 3-15.
23.) Wright C., S. Nicholson, B. Angus, J.R. Sainsbury, J. Farndon, J. Cairns, A. L. Harris and C. H. Horne (1992). Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br. J. Cancer, 65 : 118-124.
24.) Benz C., G. Scott, J. Sarup, R. Johnson, D. Tripathy, E. Coronado, H. Shepard and C. Osborne (1993). Estrogen-dependent, tamoxifen-resistent tumori-genic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res. Treatment, 24 : 85-92.
25.) Borg A., B. Baldetorp, M. Ferno, D. Killander, H. Olsson, S. Ryden & H. Sigurdsson (1994). ErbB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. Cancer Letters, 81:137-143.
26.) Leitzel K., Y. Teramoto, K. Konrad, V. Chinchilli, G. Volas, H. Grossberg, H. Harvey, L. Demers, and A. Lipton (1995). Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer. J. Clin. Oncol., 13 : 1129-1135.
27.) Newby J., S. Johnston, I. Smith and M. Dowsett (1997). Expression of epidermal growth factor receptor and c-erbB2 during the development of tamoxifen resistance in human breast cancer. Clin. Cancer Res., 3 : 1643-1651.
28.) De Placido S., C. Carlomagno, M. De Laurentiis and A. Bianco (1998). c-erbB2 expression predicts tamoxifen efficacy in breast cancer patients. Brst. Cancer Res. Trtmt., 52: 55-64.
29.) Nass, S., H. Hahm and N. Davidson (1998). Breast cancer biology blossoms in the clinic. Nature Medicine, 4: 761-762.
30.) Pegram, M., G. Pauletti and D. Slamon (1998). Her-2/neu as a predictive marker of response to breast cancer therapy. Brst. Cancer Res. Trtmt., 52: 65-77.
31.) Houston S., Plunkett T., Barnes D., Smith P., Rubens R., and Miles D (1999). Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. British Journal of Cancer, 79:1220-1226.
32.) Green S. and P.Chambon (1986). A superfamily of potentially oncogenic hormone receptors. Nature, 324 : 615-618.
33.) Beug H. and T. Graf (1989). Cooperation between viral oncogenes in avian erythroid and myeloid leukaemia. Eur. J. Clin. Invest., 19 : 491-501.
34.) Read L., D. Keith, D. Slamon and B.Katzenellenbogen (1990). Hormonal modulation of HER-2/neu protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. Cancer Res., 50 : 3947-3955.
35.) Russell, K. and M.-C. Hung (1992). Transcriptional repression of the neu protooncogene by estrogen stimulated estrogen receptor. Cancer Res., 52 : 6624-6632.
36.) Tang C.K., C. Perez, T. Grunt, C. Waibel, C. Cho and R. Lupu (1996). Involvement of heregulin-ß2 in the acquisition of the hormone-independent phenotype of breast cancer cells. Cancer Research, 56: 3350-3358.
37.) Green, S. and P. Chambon (1988). Nuclear receptors enhance our understanding of transcription regulation. Trends Genet., 4 : 309-314.
38.) Karin, M. (1998). New twists in gene regulation by glucocorticoid receptor : Is DNA binding dispensable? Cell, 93 : 487-490.
39.) Pietras, R. and Szego, C. (1975). Endometrial cell calcium and oestrogen action. Nature, 253: 357-359.
40.) Improta-Brears, T., A. Whorton, F. Codazzi, J. York, T. Meyer and D. McDonnell (1999). Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium. Proc. Natl. Acad. Sci. USA, 96: 4686-4691.
41.) Szego, C. and Davis, J. (1969). Adenosine 3',5'-monophosphate in rat uterus : acute elevation by estrogen. Proc. Natl. Acad. Sci. USA, 58 : 1711-1715.
42.) Aronica, S., Kraus, W. and Katzenellenbogen, B. (1994). Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc. Natl. Acad. Sci. USA, 91:8517-8521.

43.) Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E. and Auricchio, F. (1996). Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO Journal, 15:1292-300.
44.) Endoh, H., Sasaki, H., Maruyama, K., Takeyama, K., Waga, I., Shimizu, T., Kato, S. and Kawashima, H. (1997). Rapid activation of MAP kinase by estrogen in the bone cell line. Biochem. Biophys. Res. Commun., 235:99-102.
45.) Le Mellay, V., B. Grosse and M. Lieberherr (1997). Phospholipase C ß and membrane action of calcitriol and estradiol. J. Biol. Chem., 272: 11902-11907.
46.) Pietras, R. and Szego C. (1977). Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature, 265:69-72.
47.) Pietras R. Szego C. (1979). Metabolic and proliferative responses to estrogen by hepatocytes selected for plasma membrane binding-sites specific for estradiol-17ß. J. Cellular Physiology, 98:145-159.
48.) Pietras, R. and C. Szego (1980). Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. Biochem. J., 191 : 743-760.
49.) Nenci, I., Fabris, G., Marchetti, E. and Marzola, A. (1980). Cytochemical evidence for steroid binding sites in the plasma membrane of target cells. In *Perspectives in Steroid Receptor Research* (Ed. by F. Bresciani), Raven Press, New York: pp.61-69.
50.) Pietras, R., Szego, C. and Seeler, B. (1981). Immunologic inhibition of estrogen binding and action in preputial gland cells and their subcellular fractions. J. Steroid Biochem., 14: 679-691.
51.) Pietras R.J. and C.M. Szego (1984). Specific internalization of estrogen and binding to nuclear matrix in isolated uterine cells. Biochem. Biophys. Res. Commun.,123 : 84-90.
52.) Berthois, Y., N. Pourreau-Schneider, P. Gandilhon, H. Mittre, N. Tubiana and P. Martin (1986). Estradiol membrane binding sites on human breast cancer cell lines. Use of a fluorescentn estradiol conjugate to demonstrate plasma membrane binding systems. J. Steroid Biochem., 25: 963-972.
53.) Lieberherr, M., Grosse, B., Kachkache, M and Balsan, S. (1993). Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional non-nuclear receptors. J. Bone Mineral Res., 8: 1365-1376.
54.) Matsuda,S., Y. Kadowaki, M. Ichino, T. Akiyama, K. Toyoshima and T. Yamamoto (1993). 17ß-Estradiol mimics ligand activity of the c-erb B2 protooncogene product. Proc. Natl. Acad. Sci. USA, 90 : 10803-10808.
55.) Pappas, T., B. Gametchu and C. Watson (1995). Membrane estrogen receptors identified by multiple antibody labeling and impeded ligand binding. FASEB J., 9 : 404-410.
56.) Pappas, T., B. Gametchu and C. Watson (1995). Membrane estrogen receptor-enriched GH3/B6 cells have an enhanced non-genomic response to estrogen. Endocrine, 3 : 743-749.
57.) Tesarik, J. and C. Mendoza (1995). Nongenomic effects of 17ß-estradiol on maturing human oocytes. J. Clin. Endocrinol. Metabolism, 80 : 1438-1443.
58.) Fiorelli, G., Gori, F., Frediani, U., Franceschelli, F., Tanini, A., Tosti-Guerra, C., Benvenuti, S., Gennari, L., Becherini, L. and Brandi, M.(1996). Membrane binding sites and non-genomic effects of estrogen in cultured human pre-osteoclastic cells. J. Steroid Biochem. Mol. Biol., 59:233-40.
59.) Watters J., Campbell J., Cunningham M., Krebs E. and Dorsa D. (1997). Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology, 138 : 4030-3.
60.) Zheng, J. and Ramirez, V. (1997). Demonstration of membrane estrogen binding proteins in rat brain by ligand blotting using a 17ß-estradiol-[125I]bovine serum albumin conjugate. J. Steroid Biochem. Molec. Biol., 62 : 327-336.
61.) Razandi, M., Pedram, A., Greene, G. and Levin, E. (1999). Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and Erß expressed in Chinese Hamster Ovary cells. Mol. Endocrinol., 13 : 307-319.
62.) Szego, C.M. and R.J. Pietras (1981). Membrane recognition and effector sites in steriod hormone action. In: *Biochemical Actions of Hormones*, Vol. VIII (G. Litwack, editor), Academic Press, NY, pp.307-464.

63.) Szego, C.M. and R.J. Pietras (1984). Lysosomal function in cellular activation : Propagation of the actions of hormones and other effectors. Int. Review of Cytology, 88 : 1-246.
64.) Wehling, M. (1997). Specific, nongenomic actions of steroid hormones. Ann. Rev. Physiol., 59 : 365-393.
65.) Nemere, I. and M. Farach-Carson (1998). Membrane receptors for steroid hormones. Biochem. Biophys. Res. Commun., 248 : 443-449.
66.) Zhang, Q.-X., A. Borg and S.A. Fuqua (1993). An exon 5 deletion variant of the estrogen receptor frequently coexpressed with wild-type estrogen receptor in human breast cancer. Cancer Res., 53 : 5882-5892.
67.) Chen, Z., Yu, L. and Chang, C. (1998). Stimulation of membrane-bound guanylate cyclase activity by 17-beta estradiol. Biochem. Biophys. Res. Commun., 252:639-42.
68.) Gu, Q., Korach, K. and Moss, R. (1999). Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. Endocrinology, 140 :660-666.
69.) Kushner, P., Hort, E., Shine, J., Baxter, J. and Greene, G. (1990). Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. Mol. Endocrinol., 4: 1465-1473.
70.) Levenson, A. and Jordan, V. (1994). Transfection of human estrogen receptor (ER) cDNA into ER-negative mammalian cell lines. J. Steroid Biochem. Molec. Biol., 51: 229-239.
71.) Pietras, R.J. and C.M. Szego (1979). Estrogen receptors in uterine plasma membrane. J. Steroid Biochem. 11: 1471-1483.
72.) Otto, A. (1995). A one minute pulse of estradiol to MCF-7 breast cancer cells changes estrogen receptor binding properties and commits cells to induce estrogenic responses. J. Steroid Biochem. Molec. Biol., 54 :39-46.
73.) Chun, T.-Y., Gregg, D., Sarkar, D. and Gorski, J. (1998). Differential regulation by estrogens of growth and prolactin synthesis in pituitary cells suggests that only a small pool of estrogen receptors is required for growth. Proc. Natl. Acad. Sci. USA, 95 : 2325-2330.
74.) Welshons WV; Grady LH; Judy BM; Jordan VC; Preziosi DE. (1993). Subcellular compartmentalization of MCF-7 estrogen receptor synthesis and degradation. Mol. Cell. Endocrinol., 94 :183-194.
75.) Pasic R; Djulbegovic B; Wittliff JL. (1990). Comparison of sex steroid receptor determinations in human breast cancer by enzyme immunoassay and radioligand binding. J. Clin. Lab. Anal.,4 : 430-436.
76.) Paech, K., Webb, P., Kuiper, G., Nilsson, S., Gustafsson, J-A., Kushner, P. and Scanlan, T. (1997). Differential ligand activation of estrogen receptors ERα and ERß at AP1 sites. Science, 277 : 1508-1510.
77.) Lewis G., Figari, I., Fendly, B., Wong, W., Carter, P., Gorman, C. and Shepard, H. (1993). Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. Cancer Immunol. Immunother., 37: 255-263.
78.) Meyer, C., Schmid, R., Scriba, P. and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding sites(s) from porcine liver membranes. Eur. J. Biochem., 239: 726-731.
79.) Greene, G., Nolan, C., Engler, J. and Jensen, E. (1980). Monoclonal antibodies to human estrogen receptor. Proc. Natl. Acad. Sci. USA, 77: 5115-5119.
80.) Puca, G., Medici, N., Molinari, A., Moncharmont, B., Nola, E. and Sica, V. (1980). Estrogen receptor of calf uterus : An easy and fast purification procedure. J. Steroid Biochem., 12: 105-113.
81.) Holmes W., M. Sliwkowski, R. Akita, W. Henzel, J. Lee, J. Park, D. Yansura, N. Abadi, H. Raab, G. Lewis, H. Shepard, W.-J. Kuang, W. Wood, D. Goeddel and R. Vandlen (1992). Identification of heregulin, a specific activator of p185erbB2. Science, 256 : 1205-1209.
82.) Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M. and Chambon, P. (1986). Cloning of the human oestrogen receptor cDNA. J. Steroid Biochem., 24: 77-83.
83.) Walter, P., Green, S., Greene, G., Krust, A., Bornert, J.-M., Jeltsch, J.-M., Staub, A., Jensen, E., Scrace, G., Waterfield, M. and Chambon, P. (1985). Cloning of the human estrogen receptor cDNA. Proc. Natl. Acad. Sci. USA, 82: 7889-7893.
84.) Marquez, D.C. and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene 20 : 5420-5430.
85.) Thomas, P., Zhu, Y. and Pace, M. (2002). Progestin membrane receptors involved in the meiotic maturation of teleost oocytes: A review with some new findings. Steroids 67 : 511-517.

86.) Marquez, D.C., Lee, J., Lin, T. and Pietras, R.J. (2001). Epidermal growth factor receptor and tyrosine phosphorylation of estrogen receptor. Endocrine 16 : 73-81.
87.) Marquez, D.C. and Pietras, R.J. (2003). Membrane-associated estrogen receptors and breast cancer. In: *Identities of Membrane Steroid Receptors* (Watson, C., editor), Kluwer Academic Publishers, pp. 1-10.
88.) Szego, C.M., Pietras, R.J. and Nemere, I. (2003). Plasma membrane receptors for steroid hormones: Initiation site of the cellular response. Encyclopedia of Hormones (in press).
89.) Pietras, R.J. (2003). Steroid and growth factor receptors: Cross-talk and clinical implications. In: *Breast Cancer Management, 2nd Edition* (Reese, D., Nabholtz, J.-M., and Slamon, D.J., editors), Lippincott, Philadelphia (in press).
90.) Pietras, R.J. (2003). Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature. Breast Journal (in press).
91.) Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2003). Estrogen receptor forms and HER-2/neu growth factor receptors co-localize in caveolae-related lipid rafts in human breast cancer cells. Proc. Am. Assoc. Cancer Res. 44 : 384.