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TITLE: Membrane Estrogen Receptors - Improving Predictions of
Responsiveness of Breast Cancers to Anti-estrogenic
Therapies

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13. ABSTRACT (Maximum 200 Words) An alternative mechanism of action of steroids B action via a membrane form of steroid receptors B is not well studied. The pathway for steroid action is likely to be just as important as the well-studied genomic pathway for predicting responsiveness of cells for a variety of functions. There are basic responses to steroids that have not been fully explained; the most clinically important of these is how a steroid causes a cancer cell to divide. The significance of the membrane form of the estrogen receptor-α (mER) in estrogen-induced cell proliferation is unexplored. If the mER is involved, its measurement should contribute to the accuracy of clinical tests for predicting if breast cancer patients will respond to estrogen-antagonist therapies. We used immunocytochemical strategies to distinguish mER from the nuclear receptor to determine the expression levels and appearance of mER in mER+ and mER- breast cancer cells. We adapted an enzyme-linked detection and quantitation system, nuclear and membrane forms of ERα, and made these measurements in mER+, mER-, and wild type MCF-7 cells. We used determinations of cell number to assess cell proliferation and apoptosis responses of mER+, mER-, and wild-type cells to a wide range of concentrations of estrogens.				
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INTRODUCTION

Understanding the mechanisms by which breast tumor cells grow in response to estrogen has long been the goal of clinicians trying to devise prevention and treatment strategies. Exposure to estrogenic compounds can both elicit and exacerbate breast tumor growth. We have previously identified a sub-population of GH₃/B6 pituitary tumor cells which bear a novel form of the estrogen receptor- α (mER α) on their plasma membranes, in addition to the classical intracellular receptor. These membrane estrogen receptors (mERs) mediate rapid nongenomic responses to estrogen. Further proof of action through membrane receptors is the initiation of responses to the impeded ligand, BSA-conjugated estradiol (which cannot enter cells rapidly). We recently found preliminary evidence that this same type of mER α could be visualized on MCF-7 breast cancer cells via immunocytochemistry. Our task, using this funding, was to further characterize these receptors via immunocytochemical technique optimization for this antigen, and through development of quantitative antibody-based measurement techniques. Our hypothesis is that the membrane receptor form of estrogen receptor- α is responsible for the initiation of estrogenic responses leading to tumor cell proliferation. To test this hypothesis we used ER α -specific antibodies to isolate mER-bearing MCF-7 cells and then asked if these selected cells were more sensitive to estrogen-induced cell proliferation than were cells which lack or have a reduced quantity of mER α . Such a correlation will provide a functional link between these tumor growth-inducing estrogenic effects and the membrane form of the estrogen receptor- α .

Our experiments in the following report did support the hypothesis that mER levels can predict estrogenic proliferative responses. Accurate measurement of receptor subtypes responsible for initiating tumor cell growth should improve the selection of patients who will benefit from hormone-targeted therapies. Targeting the initial (rapid) receptor response may have greater therapeutic efficacy than targeting later genomic responses. As we understand more about these novel forms of the ER, new ideas for therapeutic interventions in the estrogenic influences on breast cancer initiation and growth will become possible.

BODY

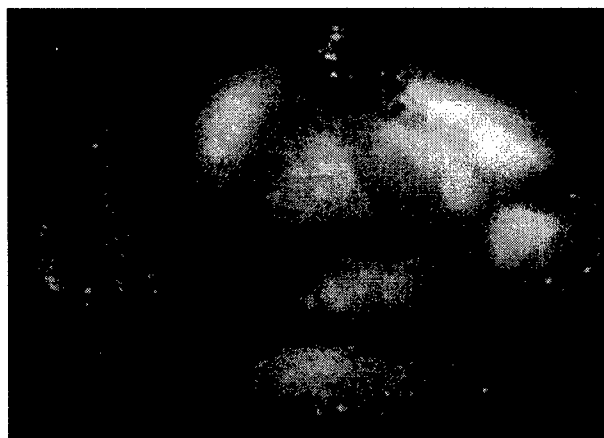
Although this work was funded in May of 2001, the recruitment of a postdoctoral fellow (Dr. Zivadinovic) was delayed due to visa complications. Therefore she could not begin work on the mER quantitative measurement aspects of this proposal until around August of 2001. A technical assistant (Bridget Hawkins) also participated in these studies to accomplish the proliferation assays within the time frame of this grant.

Objective 1. Develop immunocytochemical strategies to distinguish mER from the nuclear receptor in MCF-7 breast cancer cells. Determine the qualitative expression and appearance of mER in mER⁺ and mER⁻ selected breast cancer cells.

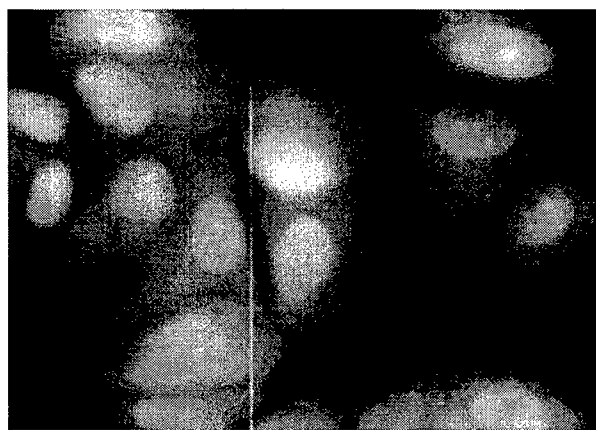
Various aldehyde fixation and background blocking techniques were optimized for our selected MCF-7 cells. We found conditions that would prevent cell permeabilization,

and thus prevent entry into the cell, of antibodies for ER α . Such techniques are necessary to measure mER α without the interfering signal from the nuclear form of the receptor. In separate preparations, controlled permeabilization with detergents was optimized for antibody entry into cells to allow comparison to the nuclear receptor labeling. Thus we established fixation parameters for the observation of mER and nuclear ER in the same cells. The signal in these assays is provided by secondary antibody conjugated to alkaline phosphatase which generates a colored product from the Vector Red substrate (Vector Laboratories).

A - MCF7⁺⁺ nonpermeabilized



B - MCF7⁺⁺ permeabilized



C - MCF7⁻ nonpermeabilized

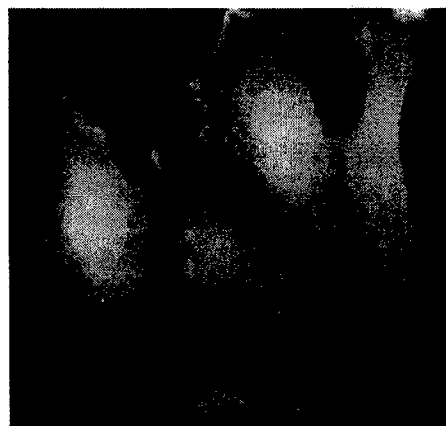


Figure 1: ER α staining of different subpopulations of MCF-7 cells enriched or depleted for mER α by immunopanning together with fluorescence-activated cell sorting (FACS).

A: MCF-7 cells highly selected for mER α (immunopanning + FACS): Using mild fixation conditions that did not permeabilize the cells, high levels of punctuate staining (red) is seen on the membrane.

B: Cells permeabilized with detergent (NP-40) show large amounts of intracellular staining with greatest intensity in the nucleus (orange-yellow). Some punctuate staining is observed as well, possibly from the cell surface. For this reason in our subsequent quantitative analysis we use the value for permeabilized cells to represent nuclear plus membrane receptor.

C: Nonpermeabilized mER⁻ cells. Cells were selected by immunpanning to not bind to ER α antibody and therefore are enriched for a population of cells which has no or low levels of mER α . These cells mostly show absence of the punctate membrane staining seen on mER⁺ cells. Occasionally, some of these cells show some membrane staining. Subsequent quantitative analysis confirms that there is a small amount of membrane receptor staining remaining in this population, which are obviously not devoid of mER, but have much reduced quantities of it.

Very recently we have acquired software (digital deconvolution) and microscope adaptations to demonstrate that this punctate staining is indeed on the membrane of these unpermeabilized cells. Such a demonstration will accompany our future publications to support a membrane localization for ER α in these cells.

Objective 2. Adapt an enzyme-linked detection and quantitation system for the measurement of mER α .

We based this optimization on our recently developed assay for nuclear and membrane forms of ER α in pituitary tumor cells (Campbell and Watson, Steroids 2001). Specifically, cells in 96-well plates were fixed according to our membrane-preserving fixation techniques optimized for breast cancer cells for immunocytochemistry (see objective 1). Then cell surface ER α was detected via a biotinylated secondary antibody linked to alkaline phosphatase which was subsequently used to convert a colorless substrate (paranitrophenylphosphate) to a soluble yellow-colored product (paranitrophenol). This product was then read in a plate reader. A subsequent crystal violet assay was used to estimate cell number so that receptor values could be normalized to cell numbers in each well.

First we verified that measuring cell numbers via the crystal violet assay was valid for our system. For this assay cells are fixed in glutaraldehyde, stained in 0.1% crystal violet, the dye is extracted with 10% acetic acid and the absorbance of the extracted dye is read in plate reader at 590 nm, directly in the well. Figure 2 shows that for our MCF-7 cell lines, this assay does a very good job of predicting cell number.

Fig. 2

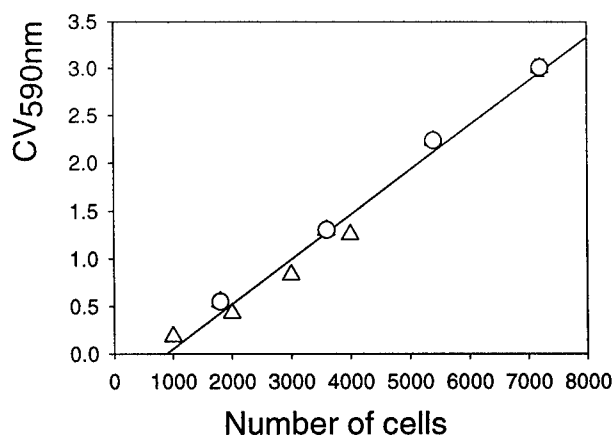
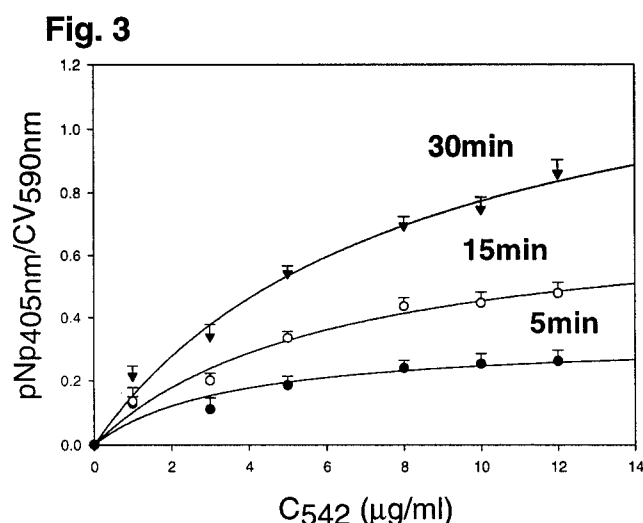


Figure 2: Correlation between crystal violet assay and the number of cells estimated by counting them in hemocytometer at the day of their plating. These values were compared 5 days after plating the cells (2 days grown in regular growing medium and three additional days in medium supplemented with 4X charcoal-stripped serum. There is a linear correlation between CV and cell number in the case of MCF-7 mER⁺⁺ (triangles) and MCF-7 mER⁻ (circles) cells. The line does not go through zero because there is a higher cell number due to cell proliferation since plating 5 days prior.

Since the crystal violet assay is a far more convenient way of estimating cell number for normalization of receptor values and also for assessing cell numbers in a proliferation assay (see objective 3), we adopted it for the rest of our studies.

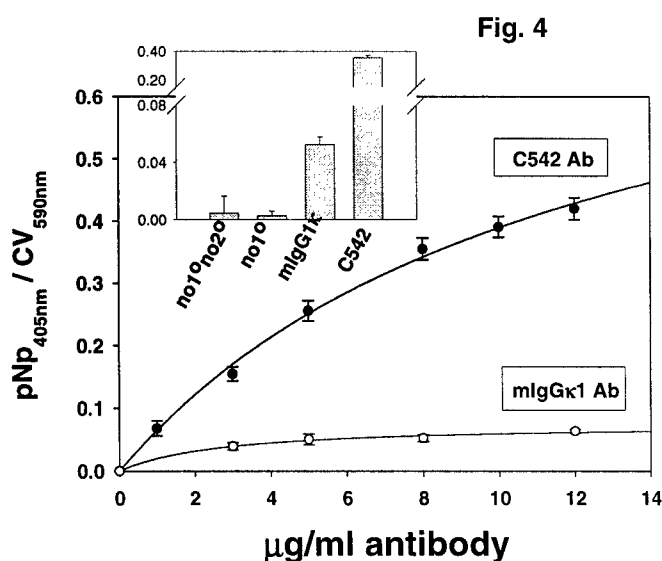


Now we began to develop, optimize, and verify our antibody-based quantitative assay for the measurement of mERα. Figure 3 shows the saturation of ERα-specific antibody binding to its antigen in unpermeabilized cells. The reaction generating the signal was allowed to proceed at 37° for 5-30 minutes to determine the conditions yielding a measurable result with low errors and within the measurement range of absorbance for the plate reader. We tested different concentrations of C542 antibody in the range from 1 to 12

μg/ml. All pNp absorbance measurements were normalized against cell number in each well.

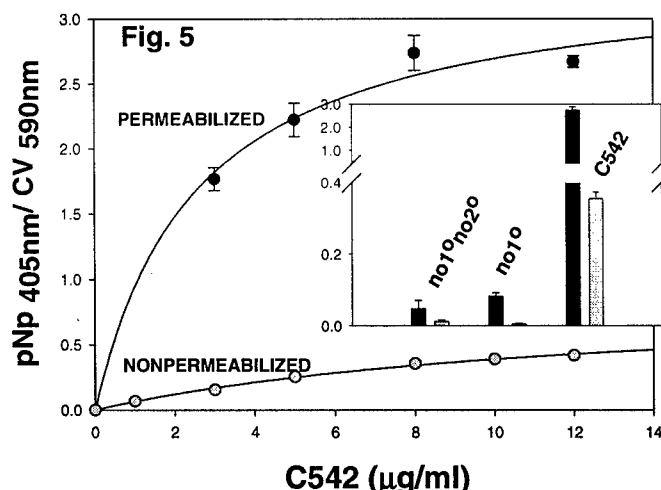
As can be seen in Figure 3 (and in Figure 4) the binding was saturable and 8 μg/ml of C542 antibody was enough to reliably saturate the antigen for measurement under these conditions. We chose 15 min of incubation at 37°C as the optimal time since sufficient product is developed, the values were within the detection level of the instrument, and sample to sample variation was minimal.

Next we compared this type of binding to several controls for nonspecific binding of the antibody to cells: IgG subclass binding, no primary antibody (1°), and no 1° or secondary (2°) antibody. The later control was used to show absence of endogenous alkaline phosphatase in MCF-7 breast cancer cells which might interference with the assay (give false positive results). As can be seen from Figure 4, these assays were very specific for ERα, based on these multiple specificity criteria. The specificity of C542 antibody as compared to other antibodies was checked by comparing its binding to the binding of mIgG1k, both being of the same immunoglobulin isotype. In a wide range of tested concentrations mIgG1k displayed very low binding. At 8ug/ml it



measures only about 10% of the level of C542 binding. Values for the other measurements of nonspecific binding are shown in the inset. That the signal from endogenous alkaline phosphatase did not interfere with our assay was confirmed by signal generated in the absence of 1° and 2° antibody. Therefore the levamasole concentration used in our assays to inhibit endogenous phosphatases was adequate. Nonspecific binding of the secondary antibody by itself was also extremely low.

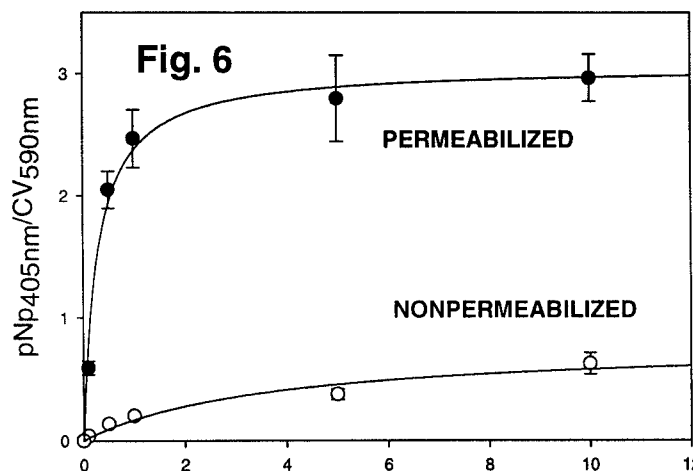
We also required a procedure for measuring the quantity of nuclear ER α



for comparison. To quantitate nuclear receptors in parallel preparations, the cells were carefully optimized for permeabilization with detergent (NP-40) present during the fixation process (optimized separately, data not shown). Thus we measured a value for total ER α , and subtracted the quantity of the membrane fraction to quantify a value for the nuclear fraction. Figure 5 shows that 8

μg/ml of C542 antibody can also be used for the estimation of total ER α since the plateau level is reached with this concentration in permeabilized cells. The inset in this figure compares again several measures of non-specific signal (no 1°, no 2°; no 1°) to values obtained with C542 at saturating concentrations. The black bars represent total receptor and the gray bars represent membrane receptor.

To prove that we were indeed looking at membrane antigen under nonpermeabilizing conditions and nuclear antigen under permeabilizing conditions, we verified our assessments of these compartments by looking at another protein which is abundant in the cell and resides just under the plasma membrane. Thus, if the membrane is still



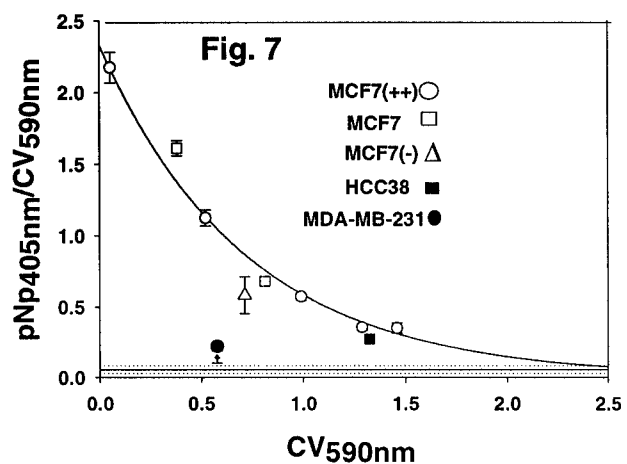
intact after fixation, this would be a sensitive measure of this integrity. For this purpose, clathrin was detected with anti-clathrin antibody in non permeabilised and permeabilised cells in parallel. As can be seen from Figure 6, nonpermeabilised cells have low anti-clathrin antibody binding, while permeabilised cells

display extremely high binding. Therefore, we confirmed that our fixation conditions promote measurement of the correct subpopulations of estrogen receptors, much as we showed previously for pituitary tumor cells.

Figure 6: Antibody to clathrin confirms the permeabilization status of MCF-7 mER⁺⁺ cells.

We then went on to confirm an important regulatory parameter for mER in MCF7 cells that we had previously observed in our GH3 pituitary tumor cell line studies -- cell density dramatically influences the number of cell surface ERs. As can be seen in Figure 7, receptor signal normalized to cell number exponentially decreases as cell number increases over a range of cell densities typically used in many studies.

Figure 7: mER declines with increasing cell density. Open symbols represent different MCF-7 cell subtypes. Circles represent MCF-7 mER⁺⁺ cells selected by immunopanning and fluorescence-activated cell sorting. The triangle represents MCF-7 cells selected for membrane receptor depletion. The empty squares are the values for receptor positive, estrogen-



responsive MCF-7 cells obtained from collaborator Dr. Barbara Boyan (Univ. of Texas San Antonio and Georgia Institute of Technology). Two ER negative cell lines were tested as well. MDA-MB-231 cells (filled diamond) have a very low signal in our assay, even when they are plated at low density. These cells have been reported to be receptor negative, but most of these measurements previously have been made on nuclear receptor. They are also non-responsive to estrogens in cell proliferation assays. Our results suggest that they may also have very low levels of mER. HCC38 cells (filled square) in our assays, in retrospect, were obviously plated at too high of a cell density to predict mER levels, and thus they will have to be re-assayed at lower densities before we can report on their mER status. However, their values were below the curve seen with estrogen-responsive cell lines. These results show that cell density is a very important parameter for use of this assay clinically – cells must be plated at low densities in order for this assay to be useful in measuring levels of membrane receptor and thus diagnosing estrogen sensitivity. (Our previous studies in pituitary tumor cells suggest that this is not as important

a parameter when measuring nuclear receptor levels, but this cell density parameter for nuclear receptor in breast cancer cells remains to be tested). Therefore, plating cells at higher densities outside of the optimal range for mER measurement could result in false negative values for mER and thus the prediction that antiestrogen therapy would not be useful for the patient being assessed. Certainly to clarify this point we will need further studies with HCC38 cells of different cell densities as well as with other responsive and unresponsive cell lines. These results will then have to be carefully evaluated with respect to the assays that were used previously to declare these cell lines positive or negative for membrane and/or nuclear receptor.

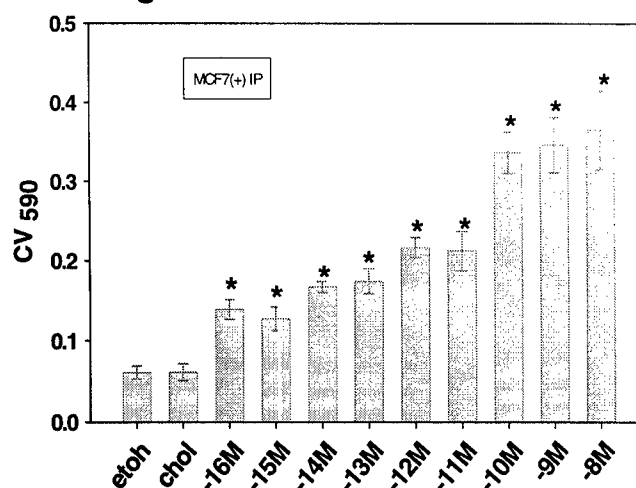
Since we have shown an effect of density on the expression of our receptor, this brings up the issue of how many cells/well should be plated for the functional assay (cell proliferation, see below). Since the lowest number on this density graph stems from wells in which 1000 cells were plated and measured 5 days later, we would expect to get the best mER α -mediated response at this cell density and time point of the growth assay. As you can see from the data reported in the next section, these conditions were appropriate for observing estrogen-induced actions in mER⁺ or mER⁺⁺ cells.

In summary, we have satisfactorily adapted our plate immunoassay to the measurement of mER levels in our MCF-7 cells, including the development of permeabilization conditions for quantitative measurement of both mER and nuclear ER. Based on these studies (and our past studies in pituitary tumor cells), we believe that the plate assay is more sensitive, as it always measures a population of receptors that is not observable via immunocytochemistry. Although cells selected for bearing the mER are enriched for this form of the receptor, mER⁻ cells are clearly not negative for these receptor forms, but they have demonstrably lower levels when measured at low cell densities.

Objective 3. Determine cell number in response to a wide range of concentrations (picomolar to 100 nanomolar) of estrogens in mER⁺, mER⁻, and wild-type in 96-well plates.

Cell proliferation was measured via the crystal violet dye absorption assay described above. For these experiments cells were plated in DMEM with defined supplemented calf serum that had been charcoal-stripped 4 successive times to remove endogenous steroids. In optimizing these experiments we also tried totally defined media and found that the cells did not grow robustly. Therefore other factors in serum besides steroids are required to support basal growth and maintenance of these cells.

Fig 8

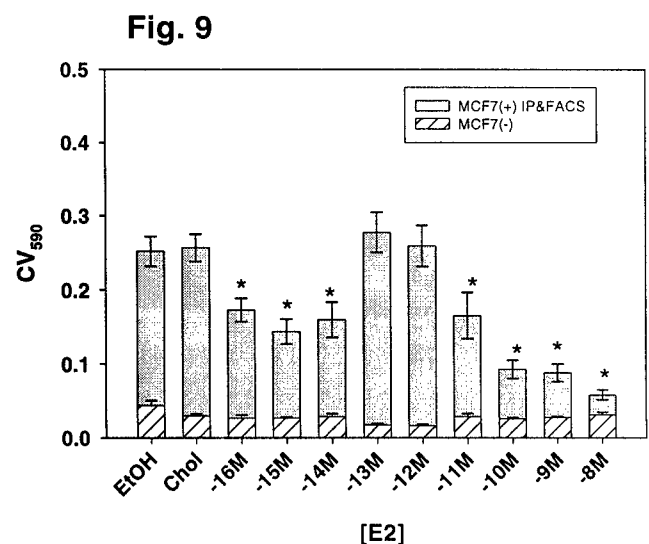


Preliminary experiments (not shown) optimized the assay for the number of days growth in estradiol (days 2-7) and we selected day 5 growth responses for subsequent experiments. Initial experiments also demonstrated that neither cell line responded to low (10^{-8} M) concentrations of cholesterol, showing hormone specificity of these responses. Cells for these experiments were plated at 1000 cells/well of a 96-well plate, as previous optimizations of the assay indicated that this density gave readily observed growth responses over the selected time period and matched conditions in which mER α was expressed.

Figure 8: Cells selected by immunopanning for mER enrichment have a dose dependent and sensitive growth response to estradiol. Cells from passage numbers 5-8 post immunopanning were used for these experiments. Estradiol concentrations from 10^{-16} M to 10^{-8} M were assayed on day 5 of growth. Cholesterol at 10^{-8} M concentration was used as a control to demonstrate hormone specificity. N=16; error bars are SEM from data obtained with different samples using the same reagents. Repeats of these results with different batches of reagents gave similar curves.

MCF-7 cells selected for mER α by immunopanning display a dose dependent increase in cell proliferation with increasing estradiol (E_2) (Figure 8). But when immunopanned cells were further selected for mER α via fluorescence-activated cell sorting, they responded to E_2 treatment by decreasing their numbers, at both high (nM) and low (fM) E_2 concentrations. (A similar bimodal estrogen dose effect was observed previously in our laboratory when we investigated the effect of E_2 and E_2 -BSA on prolactin secretion in GH3 cells.) Since cells decreased their numbers at some concentrations, we tentatively assign this response to cell killing which may or may not be via the apoptotic mechanism. Others have shown that over-expression of transfected ER α (Kushner PJ, Hort E, Shine J, Baxter JD, Greene GL, Mol Endocrinol 4:1465-1473, 1990) results in cell death at physiological E_2 concentrations. Our cells selected for increased mER α respond as do engineered over-expressers of ER α . As we expected, mER α cells were not influenced by E_2 .

Figure 9: Cells highly selected for mER by immunopanning plus fluorescence-activated cell sorting (FACS) have a dose dependent and sensitive growth inhibitory response to estradiol. These cells were used at passages 6-9 after isolation. Cells isolated via immunopanning plus FACS as mER α did not respond to E_2 at any concentration (used at passage 8-10 after selection). E_2 concentrations from 10^{-16} M to 10^{-8} M were assayed on day 5 of growth. Cholesterol at 10^{-8} M concentration was compared to demonstrate hormone specificity. N=16; error bars are SEM using values for samples assayed with the same reagents. Repeat



experiments with different batches of reagents gave similar growth curves.

So we think that our highly selected cells may be showing characteristics of cells over-expressing ER α . Interestingly, other cells overexpressing ER α have been shown to also express a small amount of mER which can function in modifying the activity of MAP and Jun kinases (M. Razandi, A. Pedram, G. L. Greene, and E. R. Levin. *Mol Endocrinol* 13:307-319, 1999). Clearly, establishing the quantitative level differences in these different sublines will be important for understanding the growth vs. apoptosis response.

Optional Objective 4 – Does estradiol activate MAP kinase in mER⁺ cells? We began our studies under this funding and proposed that we would only be able to do initial determinations of the presence of MAP kinase and some aspects of its activation by a physiological concentration of estradiol.

In summary, our preliminary results for this exploratory study strongly indicate the existence of a rapid estrogen effect on activation of MAPKs in our MCF-7 mER⁺ breast cancer cell line. An increase in phosphorylated MAPK was evident after 6 minutes of 17 β -estradiol treatment. This early increase was observed only with the 44,000 molecular weight size of the ERK 1 protein (p44), while p42 (ERK 2) showed a pronounced increase in phosphorylation after 20 minutes of treatment (still relatively rapid). Only the picomolar concentration of estradiol was effective at the shortest time. The nanomolar concentration of estradiol produced the effect after fifteen minutes of treatment. So this effect is very sensitive to estradiol. This demonstrates that MAP kinases are likely to be a signaling mechanism through which mER can stimulate cell proliferation. We are reporting further details of these results under other funding which supported more extensive studies.

KEY RESEARCH ACCOMPLISHMENTS

- Adaptation of immunocytochemistry visualization of mER α for MCF-7 cells
- Adaptation of quantitative plate assay for mER α vs. nuclear ER α to MCF-7 cells. Saturable antibody recognition of membrane receptor and appropriate permeabilization parameters
- Repetition of estrogen-induced growth responses in several types of cells selected for presence (different levels) or absence of mER α expression. Cells expressing the highest levels of mER (assessed via immunocytochemistry) are growth inhibited by estradiol. Cells with a moderate level of mER are very sensitive to the growth promoting effects of estradiol. Cells with no mER do not respond to estradiol.
- Demonstration of the activation of the p44 form (ERK 1) of MAP kinase in MCF-7 cells which have mER α

REPORTABLE OUTCOMES:

- Abstract for the 8th annual Texas Forum on Female Reproduction: Zivadinovic D, Hawkins B, Gametchu B, and CS Watson The membrane form of the estrogen receptor- can be quantitatively measured and correlated to very sensitive growth control of MCF-7 cells. Texas Forum on Female Reproduction. Houston May 2002.

- Abstract for the 2nd FASEB Summer Conference on Steroid Receptors in the Plasma Membrane: Zivadinovic D, Hawkins B, Gametchu B, and CS Watson Quantitative measurement of the membrane form of the estrogen receptor-- predictions for estrogen-controlled growth Snow Mass Colorado, June 2002.

- Abstract for the DOD Era of Hope Meeting: Watson CS, Zivadinovic D, Hawkins B, Gametchu B. The membrane form of the estrogen receptor-alpha in MCF-7 cells can be quantitatively measured, correlated to estradiol-induced sensitive growth, and linked to activation of MAP kinases. ERA of Hope Breast Cancer Meeting, Orlando FL, September, 2002

A manuscript reporting these results is currently being drafted. Before submission for publication, we would like to finish our studies on several cell lines reported to be receptor negative or positive to validate our system in conditions of appropriately low cell densities.

CONCLUSIONS

We have made significant progress in characterizing and measuring the mER α of MCF-7 cells both by immunocytochemical and antigen quantitating methods. Our initial observations encourage us that this unique form of the receptor can be specifically compared to the nuclear receptor and that measurements could be done conveniently in a clinical lab setting.

Because estrogen also induced activation (and some deactivation) of mitogen-activated protein (MAP) kinases on a very fast time scale, these nongenomic responses can be linked mechanistically to cell proliferation which occurs on a slower time course. Activation of these kinases occur in cells that proliferate in response to estradiol and thus the presence of these receptor subtypes may be connected to this mechanism of estrogen-induced cell growth in breast cancer cells. Our future studies will further refine these conclusions and their applicability to predicting tumor cell behavior. Such information should assist in designing more relevant estrogen receptor measurement protocols for predicting successful breast cancer patient treatment strategies.

FUTURE PLANS – BENEFIT OF THIS MECHANISM TO PROMOTING NEW STRATEGIES FOR BREAST CANCER

We would like to add that the existence of this form of the estrogen receptor has been, and continues to be, very controversial, making it very difficult to obtain funding for these studies. Through the use of these seed funds we have been able to more carefully characterize this membrane steroid receptor in combination with functional consequences for breast cancer cell proliferation. The ability to more fully support our novel hypothesis and produce enough data for a publication will be crucial for increasing our chances for further funding in this area. We think that this mechanism of giving good ideas a chance to prove themselves is an invaluable service to public health. It fills a critical need in developing new knowledge which may lead to better diagnoses and treatments for serious and prevalent diseases such as breast cancer.



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REPLY TO
ATTENTION OF

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15 May 03

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
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