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13. ABSTRACT (Maximum 200 words)
   There is a compelling need for better ways to select cytotoxic therapy for a given patient with breast cancer. The role of the members of the type 1 growth factor receptor family (erbB1-4) and their ligands in predicting response to chemotherapy is still unknown. Clinical data support the role of the erbB2 receptor in resistance to some chemotherapeutic agents, mainly alkylators, while a dose-response effect to a doxorubicin-containing regimen has been seen. One potential explanation for this finding is our observation of upregulation of topoisomerase II (topo II) and sensitivity to doxorubicin, in cells in which the erbB2 and erbB3 and erbB4 receptors have been activated by the growth factor heregulin.

   Using EGFR-erbB chimera cells activated by EGF, we will determine which receptor is responsible for the phenotype of change in topo II levels and drug sensitivity. In addition, breast cancer cell lines with known expression of erbB receptors will be stimulated with EGF, amphiregulin and heregulin growth factors and topoisomerase changes will be measured. We also propose to examine the expression and activity of the topo II enzyme after erbB2 overexpressing cells are exposed to anti-erbB2 antibodies, many of which cause receptor phosphorylation. This allows us to explore the mechanism of topo II upregulation in cells with activated erbB receptors using a product with potential therapeutic efficacy. We will attempt to elucidate the mechanism of changes in topo II with erbB signalling by examining levels of DNA repair enzymes, changes in cell cycle and topo II promoter regulation. These studies will allow selection of erbB2 positive patients appropriate therapy to improve outcome and minimize toxicity.

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FOREWORD

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Introduction

There is a compelling need for better ways to select cytotoxic therapy for a given patient with breast cancer. The members of the type 1 growth factor receptor family
(ErbB1-4) appear to have a role in predicting benefit from chemotherapy, particularly ErbB2. The mechanisms by which these growth factors alter cellular pathways to change drug response is poorly understood, however. The objective of this proposal is to identify how this family of growth factors, particularly ErbB2, can be used to predict and control drug sensitivity in patients with breast cancer.

While it is well established that growth factors and their receptors play a role in the prognosis of breast cancer, recent data suggest they may have a role in predicting response to therapy as well. In general, the EGFR (Epidermal Growth Factor Receptor) and ErbB2 receptors are associated with resistance to some chemotherapeutic agents, especially alkylator-based regimens such as cyclophosphamide, methotrexate and 5-fluorouracil (CMF). Interestingly, benefit from a doxorubicin (Adriamycin®)-containing regimen was limited to ErbB2 overexpressors in three large randomized cohorts of patients, the CALGB 8869/8541, SWOG and NSABP-B14 companion studies. One potential explanation for this finding is our observation of up regulation of the DNA modifying enzyme and target of doxorubicin, topoisomerase II (topo II), in cells in which the ErbB2, ErbB3 and ErbB4 receptors have been activated using the ligand, heregulin 1. These cells become more sensitive to doxorubicin and etoposide, both topo II inhibitors. We hypothesize that signaling through one or a combination of the ErbB receptors in breast cancer cells increases topoisomerase II which then sensitizes cells to doxorubicin. This predicts that alteration of ErbB signaling by various agents (natural or synthetic) may control response to doxorubicin in a predictable fashion in vitro and in vivo. In this proposal, we are examining the relationship between activation of the ErbB receptors using various ligands (EGF, Heregulin and anti-receptor antibodies) and topoisomerase II regulation.

We propose that the biology of the change in topo II activity may be understood by global changes in DNA repair enzymes, brought about by signaling through the EGFR superfamily. During the time of this award we have shown that ErbB2 signaling has an effect on cell cycle distribution of the topo II enzyme and its phosphorylation state, leading to increased sensitivity to doxorubicin but resistance to the alkylator cyclophosphamide 2. We have also made the novel discovery that topo II is phosphorylated on tyrosine residues as opposed to serine/threonine as previously described 3. This effect is seen in response to ErbB2-mediated signaling, but not ErbB3, and we believe that this phosphorylation event may be important to determining sensitivity to doxorubicin.

This information will allow the use of biological agents with cytotoxic therapy in combination to produce highest tumor response rates. It will also allow selection of appropriate patients (ie those which over express ErbB2 or other members of the ErbB family, or possibly topo II itself) for a given biological therapy directed at a specific molecular target. Patients whose tumors overexpress a given ErbB receptor, particularly if the receptor can be shown to be activated, may benefit from doxorubicin-containing
regimens. By investigating the role of type I growth factors in predicting response to therapy we draw closer to better selecting patients who will benefit from our treatments and avoiding toxicity in those who do not.

Technical objectives (specific aims as listed in 1996 proposal)

Aim 1: To determine if one of the ErbB2/3/4 receptors is responsible for the increased topoisomerase II and change in drug sensitivity seen when herregulin is transfected into cancer cells we propose to examine these endpoints in cell lines in which the individual receptors are activated.

Aim 2: To demonstrate whether specific ligands (antibodies, growth factors or compounds which modulate tyrosine kinase phosphorylation) can predictably alter sensitivity of breast cancer cells to doxorubicin and alkylators by topoisomerase II modulation mediated through ErbB receptor phosphorylation.

Aim 3: To determine the mechanism of topoisomerase II accumulation in ErbB receptor-activated cells we will examine three alternative explanations:

3a) Topoisomerase II upregulation is part of a global effect on DNA repair machinery

3b) Distribution of cell cycle in breast cancer cells is altered by activation of the ErbB2 receptor to produce an accumulation of topoisomerase II.

3c) Accumulation of topoisomerase II in breast cancer cells with activation of the ErbB receptors is due to increase activity of the topo II promotor directly.

Results

During the first two years of the proposal we determined that ErbB2 signaling led to increased topoisomerase II levels. We also made the novel discovery that signaling through this receptor leads to hyperphosphorylation of topo II which appeared to be on tyrosine residues. This discovery is unique as it has heretofore been thought that topo II activity is regulated by serine/threonine phosphorylation of the protein. We further characterized this relationship by evaluating both the cell cycle distribution of topo II levels and phosphorylation in ErbB positive vs negative cells lines (see 1998 annual report). We also evaluated the effect of these changes in topo II on its enzymatic activity in response to signaling by ErbB receptors. We found that topo II was upregulated and its ability to cleave DNA was increased in response to ErbB signaling. In addition, topo II was a more sensitive target for doxorubicin and etoposide in cells with ErbB2 activation but not with ErbB3 alone. Alkylator (cyclophosphamide) resistance seemed to be common to activation of either receptor, however. We postulated that the tyrosine phosphorylation of topo II (seen on only upon ErbB2 activation) was responsible for the doxorubicin sensitivity whereas alkylator resistance might be cause by changes in topo II levels alone.
Tyrosine Phosphorylation on Topo IIα

During this third year we have focused our efforts on attempted characterizing tyrosine phosphorylation on Topo IIα following ErbB2 signaling. We did this by using PTP-1B, (Protein Tyrosine Phosphatase 1B, Upstate Biotechnology) a tyrosine phosphatase, looking for a band shift in topo IIα which would be supportive evidence of tyrosine phosphorylation of topo IIα. These experiments did not yield evidence of a band shift, although tyrosine-specific antibodies did reveal a specific band at the appropriate size for topo IIα (see Fig. 1).

Explanations for this finding are: 1) lack of tyrosine phosphorylation on topo IIα; 2) Lack of mobility shift in topo IIα upon dephosphorylation 3) Endogenous phosphatases present which had dephosphorylated topo IIα during the nuclear extraction procedure, therefore no change in phosphorylation would be seen upon phosphatase treatment.

We attempted to identify a candidate nuclear tyrosine kinase which might be responsible for phosphorylation of topo IIα. Using a kinase assay with c-abl as the kinase (R. Quackenbush) and purified N-terminal topo IIα as the substrate (provided by Dr. Tao Hsieh), we did not see specific phosphorylation of topo IIα.

ErbB2-Inducible System

We attempted to create an inducible system for ErbB2 using the Tet-off® system (see Fig 2). We were able to create stable transfectants in MCF-7 cells with the pTet-off regulator plasmid and demonstrate, using a lac-z that the system could be induced. However, after co-transfection with pBI-SUPER-erbB2 and selection of dozens of clones, we were unable to demonstrate clones which were inducible for ErbB2 protein. We concluded that our difficulty was peculiar to our construct.

METHODS

Nuclear Protein Extraction for Phosphatase Assay

Ref. The Journal of Biological Chemistry vol. 272, No. 2, 91283-91290

1. Wash cells twice in ice-cold PBS, scrape the cells with a rubber policeman, and collect the cells. Centrifuge for 10 min. at 2000 rpm at 4 deg. C. (Note: Cells should be kept on ice.)

2. Quickly wash the cells in buffer A.

<table>
<thead>
<tr>
<th>Buffer A</th>
<th>Stock</th>
<th>For 25ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM Hepes pH 7.4</td>
<td>.5M pH 7.6</td>
<td>.5ml</td>
</tr>
<tr>
<td>1.5M MgCl2</td>
<td>2M</td>
<td>18.75ul</td>
</tr>
<tr>
<td>10mM KCl</td>
<td>1.2M</td>
<td>208.5ul</td>
</tr>
<tr>
<td>0.5mM DTT</td>
<td>1M</td>
<td>12.5ul</td>
</tr>
<tr>
<td>50uM PMSF</td>
<td>100mM</td>
<td>12.5ul</td>
</tr>
</tbody>
</table>

4. Suspend pellet in buffer A + 0.1% Nonidet P-40. Incubate on ice for 10 min. or more until no cytoplasm is present upon examination under microscope.

5. Collect nuclei by spinning at 2000 rpm at 4 deg. C for 10 min. Then suspend the nuclei in 500ul Buffer B.

<table>
<thead>
<tr>
<th>Buffer B</th>
<th>Stock</th>
<th>For 10ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM Hepes pH 7.4</td>
<td>.5M pH 7.6</td>
<td>400ul</td>
</tr>
<tr>
<td>1.5M MgCl2</td>
<td>2M</td>
<td>7.5ul</td>
</tr>
<tr>
<td>420mM NaCl</td>
<td>5M</td>
<td>840ul</td>
</tr>
<tr>
<td>1mM DTT</td>
<td>1M</td>
<td>10ul</td>
</tr>
<tr>
<td>50uM PMSF</td>
<td>100mM</td>
<td>5ul</td>
</tr>
<tr>
<td>.2mM EDTA</td>
<td>.5M</td>
<td>4ul</td>
</tr>
<tr>
<td>25% Glycerol</td>
<td>100%</td>
<td>2.5ml</td>
</tr>
<tr>
<td>dH2O</td>
<td></td>
<td>6.23ml</td>
</tr>
</tbody>
</table>

6. Incubate on ice for 30 min.

7. Ultraspin at max. speed for 15 min. at 4 deg.

8. Collect supernatant and quantitate protein.

**Immunoprecipitation of Nuclear Extract with Topo II ab**

1. Thaw nuclear protein on ice. Use PBS + phosphatase and protease inhibitors to bring volume up to 500ul.

<table>
<thead>
<tr>
<th>PBS + Inhibitors</th>
<th>Stock</th>
<th>Final [L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ml PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100ul PMSF</td>
<td>100mM (in ethanol)</td>
<td>1mM</td>
</tr>
<tr>
<td>10ul Leupeptin</td>
<td>1mg/ml</td>
<td>1ug/ml</td>
</tr>
<tr>
<td>10ul Aprotinin</td>
<td>1mg/ml</td>
<td>1ug/ml</td>
</tr>
<tr>
<td>10ul Pepstatin</td>
<td>1mg/ml (in methanol)</td>
<td>1ug/ml</td>
</tr>
<tr>
<td>50ul Na3VO4</td>
<td>200mM</td>
<td>1mM</td>
</tr>
<tr>
<td>50ul NaF</td>
<td>200mM</td>
<td>1mM</td>
</tr>
</tbody>
</table>
2. Add 40ul of protein A agarose beads and rock for 1 hr. at 4 deg. C.

3. Centrifuge for 15 min. at 4 deg. C at 15,000 rpm.

4. Collect sup., add PBS + inhibitors up to 1ml so protein conc. is approx. 0.5-1.0ug/ul.
   Add Topo II ab (~20ul) [Topogene] or rabbit IgG (~2.5ul of 3mg/ml stock).

5. Rock O/N at 4 deg. C.

6. Add 100 uL of protein agarose beads. Rock for 2 hrs. at 4 deg. C.

7. Spin for 5 min. at 4 deg. C at 15,000 rpm.

8. Discard sup. and wash beads 3X in cold modified RIPA buffer.

9. Resuspend beads in 80ul of 2X sample buffer [ISS] and heat for 5 min. at 100 deg. C,
   vortexing 2X during heating.

10. Microcentrifuge for 5 min. and load sup. on gel.

**Digestion of Phosphoproteins with Protein Tyrosine Phosphatases.**

   Sample containing 10 to 100 μg total protein
   50 mM imidazole, pH 7.5
   Protein tyrosine phosphatase (e.g., PTP-1B or SH-PTP)
   2x SDS-PAGE sample buffer (see Basic Protocol 1) or 100 mM sodium vanadate

1. Incubate the sample (containing 10 to 100 μg total protein) in 50 mM imidazole, pH
   7.5, for 10 min at 37°C.

2. Add 1 to 5 U protein tyrosine phosphatase and incubate 15 to 30 min at 37°C.

   _One unit of phosphatase activity hydrolyzes 1 nmol p-nitrophenyl phosphate (PNPP) in 1 min at 37°C and pH 7.0._

3. Terminate the dephosphorylation reaction by adding an equal volume of 2x SDS-
   PAGE sample buffer or 100mM sodium vanadate to a final concentration of 0.1 mM.

4. Analyze digested material by gel electrophoresis and immunoblotting.
Kinase Assays

Radioactive kinase assays were carried out in 40 μl kinase assay buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂) containing 10 μCi [γ-³²P]ATP for 15 min at 30°C. Reactions were terminated by addition of an equal volume of SDS sample buffer, boiled and analyzed by SDS-PAGE. Sf9 cells that had been co-infected with viruses encoding CDC2K33 and cyclin B were lysed by brief sonication in kinase assay buffer containing okadaic acid, sodium vanadate and protease inhibitors, the lysate being clarified by centrifugation at 70000 g for 20 min. Aliquots of the supernatant (20 μl, 15 mg/ml) were supplemented by addition of an ATP regenerating system and mixed with an equal volume of He La cells lysate (20 mg/ml) or with anti-c-Abl immune precipitates (derived from 100 μl 20 mg/ml HeLa cell lysate). Reactions were incubated at 30°C for 15 min and terminated by addition of SDS. The products were run on 12% Laemmli gels and analyzed by anti-phosphotyrosine blotting using antibody from UBI and the ECL detection system (Amersham). The final concentrations of the ATP regenerating system were 2 mM ATP, 20 mM creatine phosphate, 100 μl/ml creatine phosphate kinase.

Creation of Tet-off® Transfectants (see Figure 2)

Summary

This year has not been as productive for several reasons. First, a number of different experimental approaches used to further characterize tyrosine phosphorylation on topo II either gave negative results or we were not able to get the technique working. We were unable to create stable transfectants of inducible ErbB2 in the Tet-off system. Finally, a move of the laboratory to the Dana-Farber from Duke University led to a temporary interruption of experimental work.

My laboratory is still very devoted to this work and will continue to investigate the ErbB2-topo II connection in the following ways:

Future Directions

1. In our further experiments we plan to do phosphoaminoacid analysis of topo IIα by performing immunoprecipitation of topo followed by digestion with residue-specific proteases. We will then analyze by gel electrophoresis and detect with our tyrosine specific antibodies. We will perform 2-D gel electrophoresis if there is any ambiguity as to the source of the phosphorylated residues. We anticipate the phosphorylated tyrosines will be in the N-terminal fragment, where the candidate residues for tyrosine phosphorylation lie based on 3-D structural analysis (see Fig 3).

2. Given our lack of success with the tet-off® inducible system at creating an ErbB2 inducible cell line we will attempt infection with a recombinant adenovirus
containing erbB2 (in collaboration with Dr. Alex Miron, PhD) and look at short-term transfection of ErbB2 for changes in topo II and drug sensitivity in breast cancer cells which do not express ErbB2 endogenously.

3. We believe that the increase in topo II activity seen after ErbB2 signaling is part of a global effect on DNA repair activity. Using pulse-field electrophoresis (in collaboration with Dr. David Livingston’s lab) and the excision/repair plasmid assay we will evaluate the DNA damage/repair response in cell lines after ErbB2 activation.

4. Using Northern blotting and CAT assays we will evaluate transcriptional activity of topo II α after ErbB2 activation in both chimeric receptor cells and breast cancer cells which overexpress ErbB2.

---


APPENDIX

FIGURES
Figure 1

- HMW Marker
- PTP
- NaVO4
- +
- +
- +
- +

- Ptyr Marker
- Topo II Marker

P170
**a) Creation of MCF-7/erbB2-Tet-off® Transfectants:**

ErbB2 inducible cells were cotransfection with the plasmid pB1-SUPER-erbB2 and pTK-Hyg into MCF-7 cells which have previously been transfected with pTet-off regulator plasmid as detailed in the manufacturer’s instructions for the Tet-off® Gene Expression System, Clontech, CA (see figure 4). The cells will be grown in the presence of hygromycin selection medium and stable clones will be isolated.

Each clonal cell line was tested for induction of erbB2 gene activity by removing tetracycline from the media and evaluating expression of erbB2 message or protein level/activity. Expression of erbB2 message was assessed using Northern blot probing.

Twenty micrograms of mRNA will be run on 1% agarose/formaldehyde gel by electrophoresis. Intact 18S and 28S bands seen by ethidium bromide staining are used to verify integrity of mRNA and the gel will be subject to Northern blot analysis. After extensive washes under standard conditions bands will be visualized by autoradiography. Loading and transfer efficiency will be normalized with a housekeeping gene, GAPDH, under the same conditions.

Protein expression was assessed using antibody 3B5 (Calbiochem, MA) under conditions optimized for Western blotting. Whole cell extracts will prepared from MCF7 transfectants and protein separated using 4-20% SDS-PAGE, transferred onto nitrocellulose and incubated with a mouse monoclonal Antibodies (Ab) against either ErbB2 intracellular domain (Calbiochem, San Diego, CA) or antiphosphotyrosine antibody 4G10 (Upstate Biotechnology, New York) to assess tyrosine kinase activity of the erbB2 receptor. Cells with high number of receptors per cell (equal or greater expression than SKBR3 breast cancer cells which have erbB2 amplification) or high levels of tyrosine phosphorylation of erbB2 on Western blot will be selected for further experiments. Cells will be maintained in tetracycline containing medium to suppress erbB2 gene expression prior to experiments.
Prokaryotic DNA Gyrase
Eukaryotic DNA TOPO IIα