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13. ABSTRACT (Maximum 200) We have previously shown that transfection of heregulin β -2 into MCF-7 breast cancer cells leads to activation of the <i>ErbB2</i> , 3 and 4 receptors and upregulation of topoisomerase II (topo II) with a two log-fold increase in sensitivity to topo II inhibitors (doxorubicin, VP-16). The purpose of this work is to elucidate the relationship between signal transduction through the <i>ErbB</i> family of receptors and chemotherapy response to drugs used in breast cancer. Using our <i>EGFR-ErbB2</i> chimeric receptor model we have confirmed that activation of the <i>ErbB2</i> tyrosine kinase is associated with increase in topo II protein at 48, 72 and 96 hours post receptor activation. We have also observed an increase in topo II phosphorylation on tyrosine, previously unreported. Receptor activation leads to increased topo II cleavage activity and increased sensitivity to doxorubicin. Breast cancer cell lines overexpressing the <i>ErbB2</i> receptor were treated with EGF, heregulin β -2 and 4D5 antibody directed against <i>ErbB2</i> . Although EGF and heregulin can slightly increase tyrosine phosphorylation of these receptors, no significant change in topo II levels or phosphorylation were seen. Interestingly, when 4D5 antibody is applied to these cells there is a direct correlation of decrease in tyrosine kinase activity with topo II protein level and phosphorylation on tyrosine. Drug resistance to doxorubicin is also induced as expected. Taken together, these data suggest that activation of the <i>ErbB2</i> receptor leads to increase in topo II protein level and phosphorylation which corresponds to an increase in sensitivity to doxorubicin. Whether specific residues on topo II are tyrosine phosphorylated upon growth factor activation which may lead to specific response to chemotherapy drugs is intriguing and will be further explored.			
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FOREWORD

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Table of Contents

Introduction	5
Body	6
Conclusions	15
References	16

Annual Report for Grant Number DAMD17-96-1-6133

Introduction

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. The objective of this proposal is to identify how this family of growth factors can be used to predict and control drug sensitivity in patients with breast cancer.

While a great deal of work has focused on the role of growth factors and their receptors in the prognosis of breast cancer, less information is available regarding their role in drug resistance. In general, data support the role of the EGFR (Epidermal Growth Factor Receptor) and *ErbB2* receptors in resistance to some chemotherapeutic agents, especially cyclophosphamide, methotrexate and 5-fluorouracil (CMF)-regimens. Interestingly, a dose-response effect to a doxorubicin (Adriamycin®)-containing regimen was seen in *ErbB2* overexpressors in the CALGB 8869/8541 study(1). One potential explanation for this finding is our observation of up regulation of the DNA modifying enzyme and target of doxorubicin, topoisomerase II α , in cells in which the *ErbB2*, *ErbB3* and *ErbB4* receptors have been activated using the ligand, heregulin(2). These cells also become more sensitive to doxorubicin. We hypothesize that signaling through one or a combination of the *ErbB* receptors in breast cancer cells increases topoisomerase II α 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*. We propose to examine the relationship between activation of the *ErbB* receptors using various ligands (EGF, Heregulin and anti-receptor antibodies) and topoisomerase II α regulation.

We propose that the biology of the change in topoisomerase II α (topo II α) levels seen may be understood by global changes in DNA repair enzymes, brought about by signaling through the EGFR superfamily. One effect of *ErbB* signaling on cell cycle distribution may lead to accumulation of the topo II α enzyme and increased sensitivity to doxorubicin. Alternatively, the topoisomerase II α promotor may be activated specifically through *ErbB* activation. This proposal examines the link between *ErbB*-mediated signaling and topoisomerase II α levels specifically as they relate to drug sensitivity/resistance.

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) for a given biological therapy directed at a specific molecular target. Patients whose tumors over express 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.

Technical objectives (specific aims)

Aim 1: To determine if one of the *ErbB2/3/4* receptors is responsible for the increased topoisomerase II α and change in drug sensitivity seen when heregulin 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 α modulation mediated through *ErbB* receptor phosphorylation.

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

3a) Topoisomerase II α 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 α .

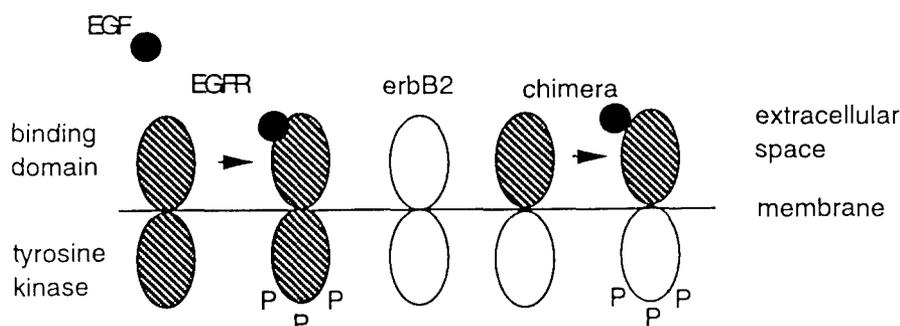
3c) Accumulation of topoisomerase II α in breast cancer cells with activation of the *ErbB* receptors is due to increase activity of the topo II α promotor directly.

RESULTS AND DISCUSSION

Task 1 (0-6 months):

During the first 6 months of the proposal our objective was to create and test the tools to be used for Aim 1. We have shown that activation of the *ErbB2/3/4* receptors by transfection with heregulin β -2 is associated with upregulation of topoisomerase II α levels and increased sensitivity to doxorubicin. In order to determine which, if any, of the specific receptors involved are responsible for this phenotype we have chosen to examine activation of each receptor in isolation using an EGFR-*ErbB* receptor chimera model. This model is unique in that each receptor can be activated artificially by the ligand EGF with subsequent phosphorylation of the *ErbB*-receptor specific intracellular domain tyrosines (3). This is performed in a cell line (NIH-3T3) which does not express any of the *ErbB* receptors endogenously, therefore is a relatively clean system in which to look at *ErbB2/3/4* receptor activation and signaling (fig 1).

FIG. 1



In this proposal we will use NIH-3T3 cells transfected with chimeric receptors for *ErbB2*, 3 and 4 to assess the role of individual receptors in modulation of topoisomerase II α . We have evaluated the first two of these cell lines for expression of receptor, activation of receptor tyrosine kinase evidenced by tyrosine phosphorylation in response to EGF in the following set of experiments:

EGFR/*ErbB2* NIH-3T3 cells

Two single clone populations of this cell line were kindly provided by Dr. C. Richter King and were characterized. NIH-EGFR/*ErbB2*-77 and -82 clones demonstrate tyrosine phosphorylation of the chimeric receptor and upon ligand activation using Western blotting techniques (fig 2) (see methods below).

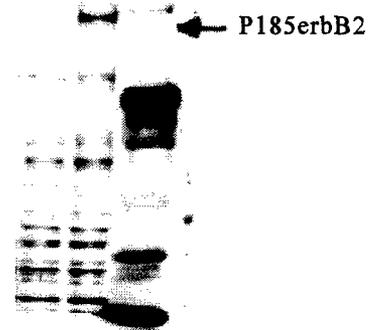


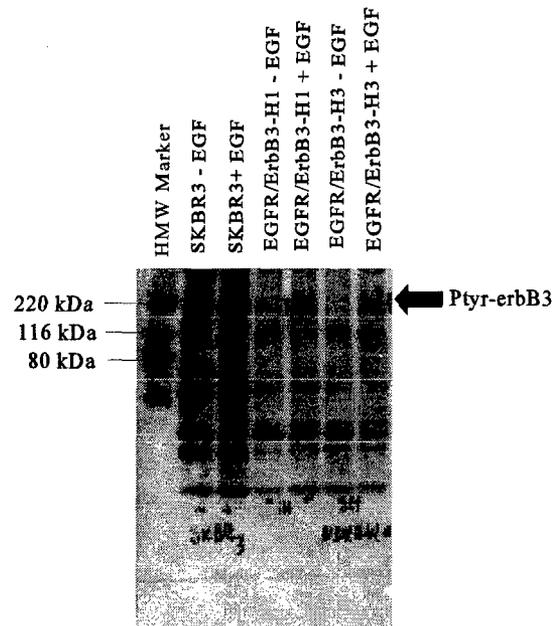
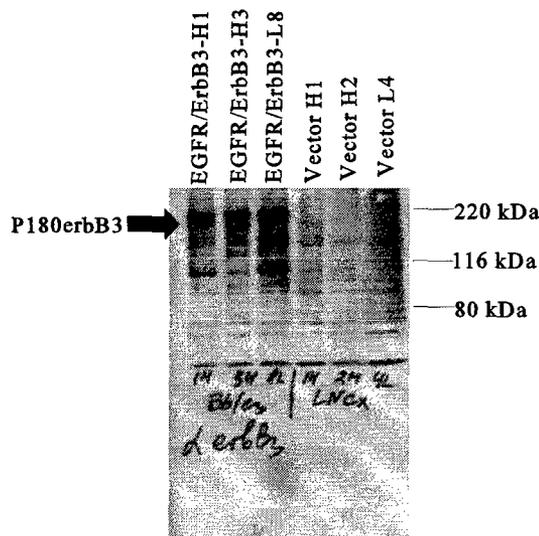
FIG. 2

EGFR/*ErbB3* NIH-3T3 cells

An EGFR/*ErbB3* chimeric receptor construct, kindly provided by Dr. Mattias Kraus, was stably transfected into NIH-3T3 cells using a modified Calcium phosphate precipitation method suggested and selected using G418 (600 ug/ml)(4) Six chimera-expressing clones and five vector-transfected clones were chosen for characterization. All chimera clones express high levels of EGFR/*ErbB3* chimera as compared with vector-transfected cells (fig 3).

FIG. 3

FIG. 4



Growth rates EGFR/*ErbB3* chimeric cells do not seem to reflect amount of receptor expressed in preliminary experiments. Phosphorylation on tyrosine is seen upon receptor activation with EGF 10 ng/ml (fig 4)

EGFR/*ErbB4* NIH-3T3 cells

Unfortunately, the EGFR/*ErbB4* construct did not become available from sources initially proposed therefore this construct is being engineered using the EGFR extracellular domain from the EGFR/*ErbB2* chimeric construct and *ErbB4* transmembrane and intracellular domain using cDNA sequence from the original cloning paper (5) . These experiments are ongoing.

Breast cancer cell lines

In order to demonstrate the relationship of receptor activation to topoisomerase II α modulation in the context of human breast cancer we chose to examine several breast cancer cell lines which are well characterized with respect to *ErbB*-family receptors. The following cell lines were obtained from American Tissue Culture Center (ATCC), Rockville, MD:

Cell Line	EGFR(<i>ErbB1</i>)	<i>ErbB2</i>	<i>ErbB3</i>	<i>ErbB4</i>
SKBR3	++	++++	++	+/-
T47D	+	++	+++	++++
BT-474	+	+++	+++	+++
MDA-361	-	+++	+++	++
MDA-453	-	+++	++	+++
SKOV3	-	++++	-	-
MCF-7	+/-	+	+	++

The ligands chosen are known to phosphorylate either the *ErbB2* receptor alone (anti-*ErbB2* antibodies), *ErbB2* and EGFR concomitantly (EGF) or *ErbB2*, *ErbB3* and *ErbB4* (heregulin β -2).

METHODS

Western Blotting of Whole Cell Extracts for ErbB2,3, anti-Phosphotyrosine (Ptyr)

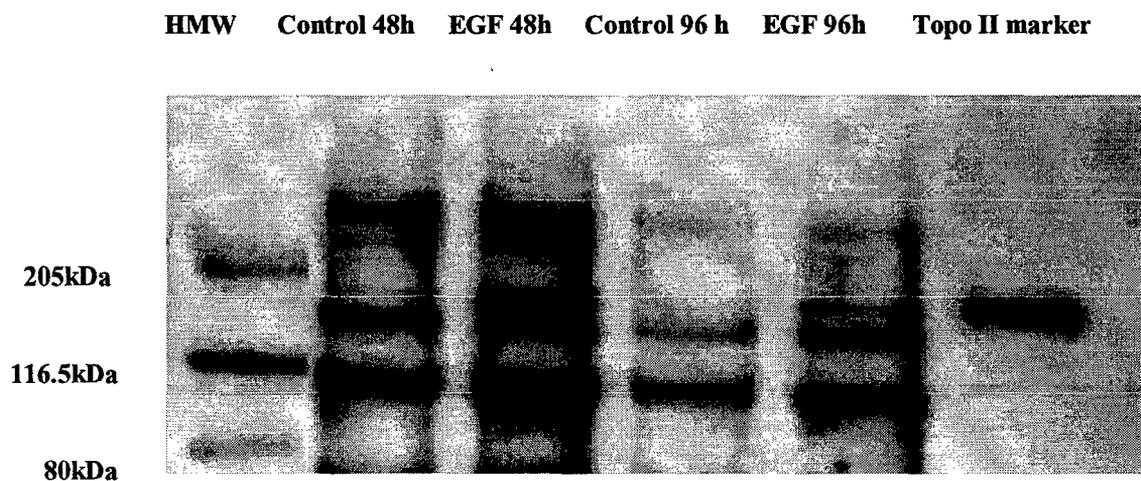
Whole cell extracts were prepared from EGFR-*ErbB2/3* chimera cells after serum starvation for 24 hours and subsequent treatment with and without EGF 10 ng/ml (Becton Dickson, Bedford, MA) for 1 hour. Protein was separated using 4-20% SDS-PAGE, transferred onto nitrocellulose and incubated with a mouse monoclonal Antibodies (Ab) against either *ErbB2* intracellular domain (Calbiochem, San Diego, CA), *ErbB3* (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Ptyr (Upstate Biotechnology, Lake Placid, NY). After incubation with sheep anti-mouse secondary Ab linked to horseradish peroxidase the *ErbB2/3* protein was visualized using Enhanced Chemo-luminescence (ECL-Amersham, Buckinghamshire, England).

TASK 2 (months 6-12)

Topoisomerase II α Activity in NIH3T3 EGFR/*ErbB2* chimera cells

During the second 6 months of the award we planned to evaluate the effect of signaling through the *ErbB* receptors on topoisomerase II α activity. Using our EGFR-*ErbB2* chimeric receptor model we have shown that activation of the *ErbB2* tyrosine kinase is associated with 30% increase topoisomerase II α protein at 48, 72 and 96 hours post receptor activation (fig 5), see methods below. Figure 5 demonstrates this effect for 48 and 96 hours only, however 72 hours shows similar results in both NIH 77 and 82 clones.

Fig 5. Time Course of NIH-3t3 EGFR-*ErbB2* Chimera Cells Showing Induction of Topoisomerase II α upon Receptor Activation



Activation of the chimeric receptor leads to increased sensitivity to doxorubicin in growth assays which corresponds to an 6.5-fold increase in topoisomerase II α cleavage activity (fig 6,7) - methods below.

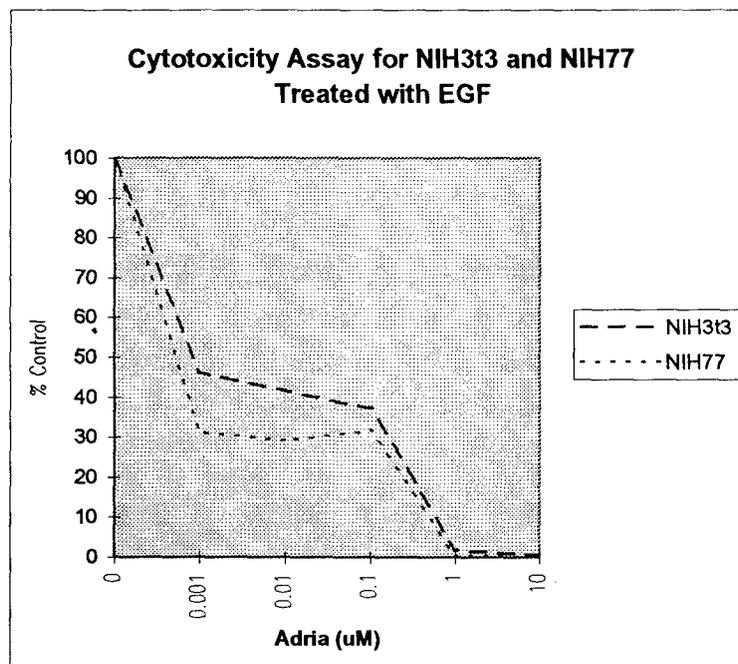


FIG. 6

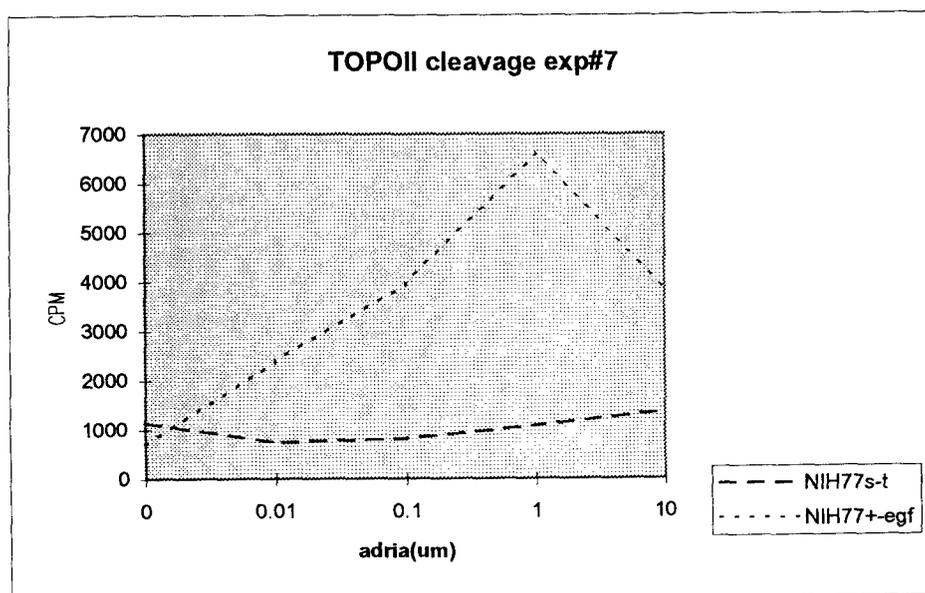
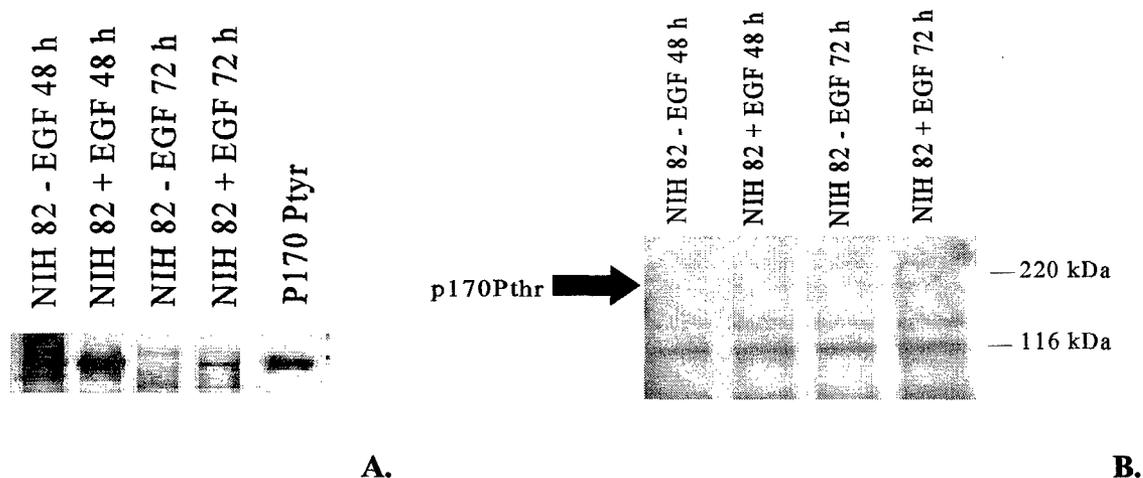


FIG 7

We chose to explore the effect of receptor activation on topo II α phosphorylation due to recent reports that increase in serine and threonine phosphorylation of topo II α increases sensitivity to topo II α inhibitors (6). This appears to be due to increase affinity of the topo II α molecule for DNA. Topo II α serine, threonine and tyrosine phosphorylation were measured by phosphoamino acid-specific antibodies against nuclear protein derived from chimeric cells treated as above. Contrary to what is published in the literature, we observed a 30% increase in topo II α phosphorylation on tyrosine (and not on serine or threonine) upon activation of the EGFR/*ErbB2* chimeric receptor, a phenomenon which has not been previously described (fig 8 a,b).

FIG. 8



Phosphorylation on topo II α tyrosine residues corresponded to the increased levels of topo II α protein seen at 48 and 72 hours. Confirmation of this finding with immunoprecipitation of topo II α protein followed by blotting with specific antiphosphotyrosine Ab is underway. However, our preliminary experiments with the breast cancer cell line SKBR3 which overexpress *ErbB2* at a high level show that topo II α is phosphorylated on tyrosine as assessed by immunoprecipitation and western blot (fig 9). As topo II α phosphorylation has never been shown, to our knowledge, this finding is intriguing and suggests a link between growth factor receptor activation and tyrosine phosphorylation of nuclear proteins.

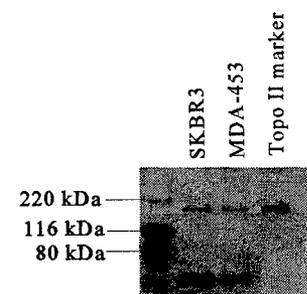
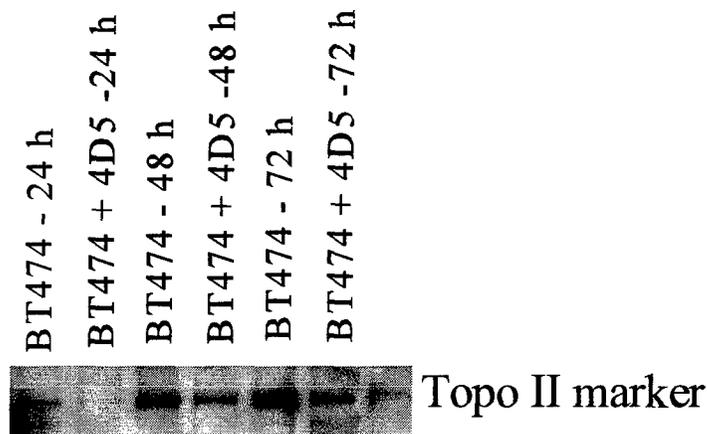


FIG. 9

Topoisomerase II α Activity in Breast Cancer cell lines

Breast cancer cell lines expressing known levels of receptor (see chart above) were tested with the ligands EGF, heregulin β -2 (10ng/ml; kindly supplied by Dajun Yang), and 4D5 antibody (10 ug/ml; kindly provided by Genentech, Alameda, CA). Although EGF and heregulin β -2 can slightly increase tyrosine phosphorylation of these receptors after serum starvation, no significant change in topoisomerase II α levels was seen (data not shown). This may be due to the fact that high levels of topo II α protein are expressed in these cell lines even in the serum

starved condition and further activation of the receptor does not increase these levels. In fact, unlike the NIH 3T3 chimeric receptor model, it is virtually impossible to inactivate *ErbB2* receptor kinase activity by serum starvation. Interestingly, when the 4D5 antibody directed against the extracellular domain of the *ErbB2* receptor is applied to these cells there is a direct correlation of tyrosine kinase activity with topoisomerase II α protein level and



phosphorylation. For example, BT-474 cells show a decrease in *ErbB2* receptor phosphorylation after treatment with 10 $\mu\text{g/ml}$ of 4D5 Ab and also a corresponding 25% decrease topoisomerase II α protein level as well as a 15% decrease in topoisomerase II α tyrosine phosphorylation (fig. 10, 11).

FIG. 10

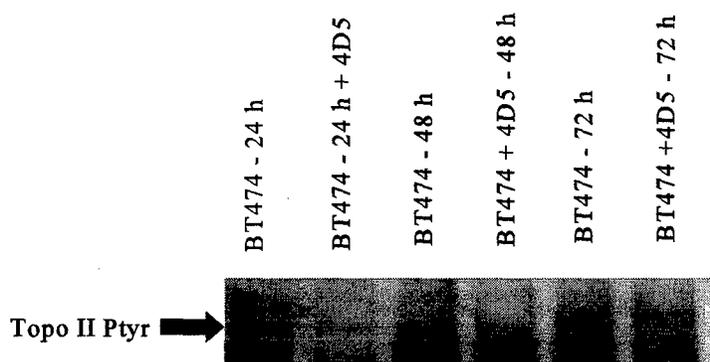


FIG. 11

This is accompanied by a decrease in growth rate of the cells as previously reported (7).

Increased receptor phosphorylation is not the rule, however with Ab treatment in cells which overexpress *ErbB2*. SKBR3 cells, in our hands as well as others, respond to the Ab with an increase in tyrosine phosphorylation as well as activation of the ras-MAP kinase pathway (8)(9). Our experiments demonstrate that this corresponds to an increase in tyrosine phosphorylation on topo II α as one might expect if the effect on topo II α was related to receptor activation (data not shown). Interestingly, SKBR3 cells are still growth inhibited by 4D5 as are all *ErbB2* overexpressing cell lines (6). These observations suggest that the relationship between topo II α and receptor activation is not simply a result of cell growth and division because we still see topo II α upregulation in response to receptor activation in cells which are growth-inhibited by 4D5 Ab. The reasons for these differences between cell lines which express *ErbB2* at a high level are unclear but may lie in the fact that they express different levels of the other *ErbB* receptors. BT-474 cells express high levels of both *ErbB3* and 4 and it has been suggested that it is interference with receptor heterodimerization rather than ligand binding that is responsible for the inhibition of phosphorylation by anti-*ErbB2* antibodies (7). SKBR3, on the other hand, has very low levels of either *ErbB3* and 4 but has significantly more EGFR which may allow for activation of certain signal transduction pathways but not others.

As in the chimeric receptor model, topoisomerase II α phosphorylation appears to be on tyrosine residues but not serine or threonine (data not shown). In addition, topo II α cleavage activity in response to doxorubicin is decreased in BT-474 cells and subsequent cytotoxicity is also reduced after 4D5 treatment (fig 12, 13).

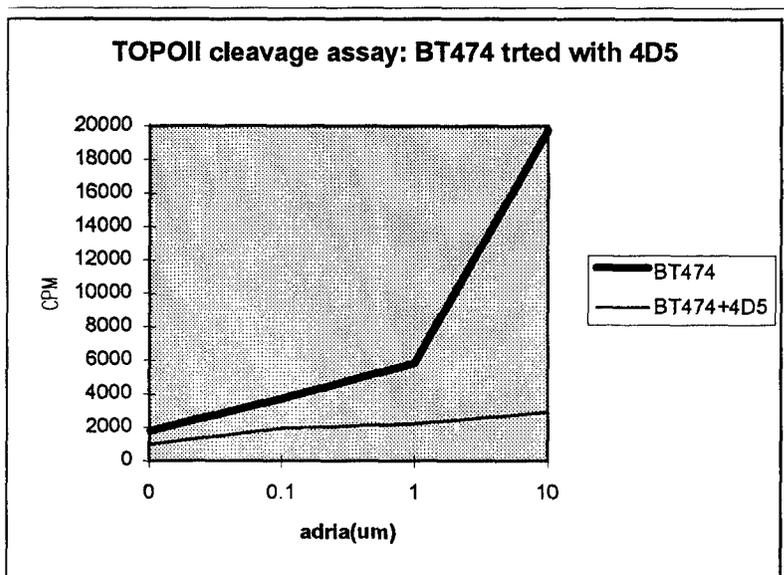


FIG. 12

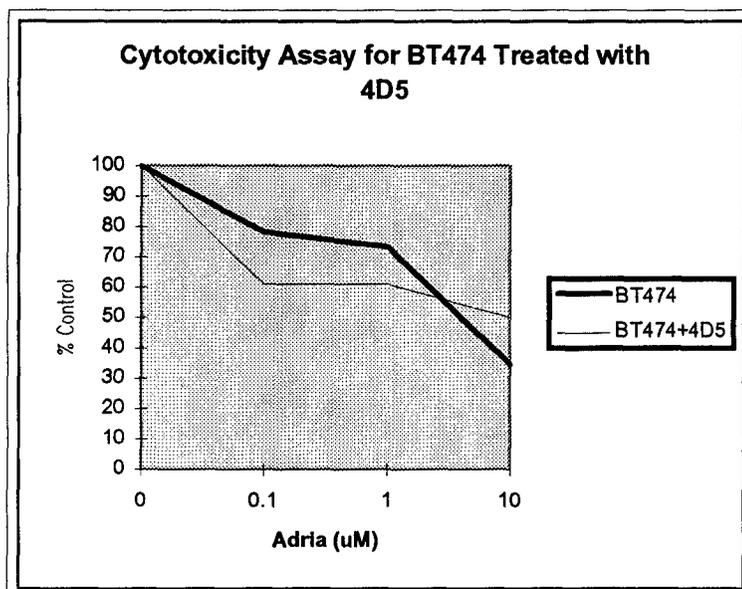


FIG. 13

This further supports the work of Pegram et al where no synergy between 4D5 and doxorubicin was seen in BT-474 cells and is in contrast to experiments in nude mice where synergy between 4D5 and doxorubicin was seen (Pegram et al., AACR Minisymposium, Keystone Colorado, March, 1997) (10).

METHODS

Western Blotting of Nuclear Extracts for Topoisomerase α , anti-Ptyr, anti-Phosphoserine, anti-Phosphothreonine

These experiments were performed by preparation of nuclear extracts from chimeric cells which were serum starved X 24 hours and subsequently incubated + or - EGF 10 ng/ml for 24, 48 and 72 hours respectively. Nuclear extracts were prepared by lysis of cells with high salt buffer with Triton X100 and NP40, pelleting of nuclei, washing and disruption of nuclear envelope using a buffer containing SDS and sonication. Nuclear protein was separated using 4-20% SDS-PAGE, transferred onto nitrocellulose and incubated with a anti-human Topo II α rabbit polyclonal antibody (1U/ml, TopoGEN, Inc, Columbus, Ohio), anti-Ptyr (Upstate Biotechnology, Lake Placid, NY), anti-Pser or Pthr (Sigma, St Louis, MO). After secondary Ab incubation, the p170 kDa protein was visualized using Enhanced Chemo-luminescence (ECL-Amersham, Buckinghamshire, England).

Immunoprecipitation and Western Blot Analysis for Topo II α Protein:

Nuclear protein extracts will be prepared from EGFR-*ErbB*-chimeric NIH-3T3 cells before and after treatment with EGF 10 ng/ml. Extracts were prepared from a non-SDS containing buffer using mechanical disruption of nuclei. After preclearing the extracts with protein A sepharose, samples were immunoprecipitated with an anti-p170 human Topo II α anti-rabbit polyclonal antibody (1U/ml, TopoGEN, Inc, Columbus, Ohio). After incubation on ice, the antigen-antibody complex will be precipitated with 10% protein A-agarose, washed and separated using 4-20% SDS-PAGE. Purified p170 kDa Topo II α will be used as a marker. Protein samples were transferred onto nitrocellulose using Western blot technique. After incubation with a Topo-II α anti-mouse monoclonal antibody (1U/ml), blots are visualized using Enhanced Chemo-luminescence (ECL-Amersham, Buckinghamshire, England).

Cytotoxicity Assays

Growth assays were performed in a standard fashion by exposing cells to increasing doses of doxorubicin (.001-10 μ M) and counting viable cells at 5-7 days post-exposure. Cytotoxicity was expressed as % control of untreated cell number.

Topo II α cleavage assays

Topo II α cleavage assays were performed using the gold standard K-SDS assay for protein/DNA interaction after exposure to topo II α inhibitors. Briefly, cells are serum starved for 24 hours followed by treatment with or without EGF 10 ng/ml X 24 hours. After 3H-thymidine incorporation for 12 hours cells are treated with increasing concentrations of doxorubicin (.1-10 μ M) for a further 12 hours. After the last incubation with the topo II α inhibitor, cells are washed, lysed and DNA-topo II α protein complexes precipitated by 325 mM KCL. The pellet is washed,

resuspended in scintillation fluid and radioactivity of each sample determined. Topo II α cleavage activity is expressed as CPM X [uM doxorubicin] in a standard fashion (11)

CONCLUSIONS AND FUTURE DIRECTIONS

Taken together, these data suggest that activation of the *ErbB2* and possibly other *ErbB* receptors leads to increase in topoisomerase II α protein and increase in sensitivity to doxorubicin. Increased tyrosine phosphorylation but not serine or threonine are seen on a nuclear protein which we believe to be topoisomerase II α 48-72 hours post-receptor activation. This phenomenon has never been described on the topoisomerase II α protein but has recently been observed for other nuclear proteins involved in cell cycle and transcription machinery(12). Whether specific residues on the topoisomerase II α protein are phosphorylated upon growth factor activation is an interesting possibility. In addition, these changes may lead to specific response to doxorubicin and other chemotherapy drugs which may be useful clinically.

In our future experiments we will attempt to continue the Aims outlined in this proposal and will further explore these new findings in the following experiments:

1. We will further characterize changes in topoisomerase II α phosphorylation by immunoprecipitation of the topoisomerase II α protein in NIH-3T3 chimera cells and breast cancer cell lines after treatments discussed above. This finding will be confirmed with 2-d gel phosphaminoacid analysis. We will further evaluate the change in function of topoisomerase II α under these conditions with cleavage activity, topoisomerase II α decatenating assays and global DNA repair activity measured by alkaline elution assays.
2. We will further characterize NIH-3T3 EGFR/*ErbB3* chimera cells as to topoisomerase II α activity (protein and phosphorylation) after receptor stimulation with EGF and we will evaluate drug response by topoisomerase II α cleavage activity and cytotoxicity assays after exposure to doxorubicin and other chemotherapeutic agents.
3. We will transfect the NIH-3T3 EGFR/*ErbB4* construct and characterize these cell lines to determine the role of *ErbB4* in topoisomerase II α regulation.
4. We will transfect the NIH-3T3 EGFR/*ErbB2* chimera into MCF-7 breast cancer cells under the control of a tet-inducible promoter to observe the effect of growth factor signaling in the context of an estrogen-dependent cell line. This will also determine the role of non-specific cell cycle stimulation in topoisomerase II α regulation.
5. We will continue to dissect the role of the individual receptors and their ligands in breast cancer cells by attempting to inhibit each component of the system with anti-sense to *ErbB2* (kindly provided by Jim Vaughn), *ErbB4* ribozymes (kindly provided by Dajun Yang) and inhibitors of specific signal transduction pathways known to be activated by *ErbB* signal transduction.
6. We will pursue changes in transcription and translation of topoisomerase II α where protein levels are seen to be modulated using methods outlined in the original award.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

13 Feb 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statements for Accession Document Numbers listed at enclosure 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. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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