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A recent CALGB 9344 trial (INT 048) examined the addition of paclitaxel therapy to standard chemotherapy regimes. The results of this trial demonstrated addition of paclitaxel to standard adjuvant therapy, doxorubicin and cyclophosphamide, was associated with proportional reductions in both relapse risk and risk of death. Subgroup analyses were conducted to identify tumor characteristics that would predict response to paclitaxel treatment. These analyses showed that the benefit of paclitaxel treatment was restricted to ER- patients (CALGB central statistics office), indicating that there was an association between estrogen receptor status and paclitaxel sensitivity. The results reported here demonstrate that the differences in growth between ER+ and ER- breast cancer cells reflect the differential sensitivity to paclitaxel observed both *in vitro* and in the clinic.

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Michele K Doughty 8/14/00

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The project funded by this grant involved determining the function of the insulin like growth factor 1 (IGF1R) in estrogen receptor negative (ER-) breast cancer. While establishing an *in vitro* model of IGF1R+/ER- breast cancer, a second project was initiated examining the sensitivity of ER+ and ER- breast cancer to paclitaxel treatment. The interest in this particular question arose from the results of the recent CALGB 9344 trial (INT 048) that examined the addition of paclitaxel therapy to standard chemotherapy regimes. The results of this trial demonstrated addition of paclitaxel to standard adjuvant therapy, doxorubicin and cyclophosphamide, was associated with proportional reductions in both relapse risk and risk of death. Subgroup analyses were conducted to identify tumor characteristics that would predict response to paclitaxel treatment. These analyses showed that the benefit of paclitaxel treatment was restricted to ER- patients (CALGB central statistics office), indicating that there was an association between estrogen receptor status and paclitaxel sensitivity. The results reported here demonstrate that the differences in growth between ER+ and ER- breast cancer cells reflect the differential sensitivity to paclitaxel observed both *in vitro* and in the clinic.

BODY

Defining breast tumor characteristics that will aid in predicting response to treatment is an area of considerable interest when designing new therapeutic strategies. Clearly, the most valuable predictive marker currently available to clinicians is the presence or absence of ER. Estrogen receptor expression is predictive for response to hormone therapy in the majority of breast cancer patients (1). Predicting tumor response to cytotoxic chemotherapy has proven to be more difficult due to heterogeneity of breast cancer. Currently, the only cellular component that is predictive of response to certain types of chemotherapy is the ErbB 2 receptor (2,3). However, certain tumor characteristics have been identified that indicate whether a patient is more likely to respond to the effects of cytotoxic chemotherapy.

The kinetic characteristics of breast cancer have proven to be valuable for identifying patients with a better prognosis as well as helping to define a group of patients which may respond more favorably to chemotherapy treatment. Initial comparisons of the growth characteristics of ER+ and ER- breast cancer indicated that ER- cancers were associated with a increased proliferative rate as determined by thymidine labeling index and S-phase fraction (4,5). These observations have been extended in several analyses to demonstrate that increased proliferative rate is associated with a decreased time to relapse (6,7,8,9) and decreased overall survival rates in breast cancer patients (9). Increased proliferative rate is positively correlated with increased survival following chemotherapy (4,10). A large retrospective analysis also indicated that there is increased benefit of chemotherapy treatment for women with ER negative breast cancer, particularly among post-menopausal women (11).

Figure 1a

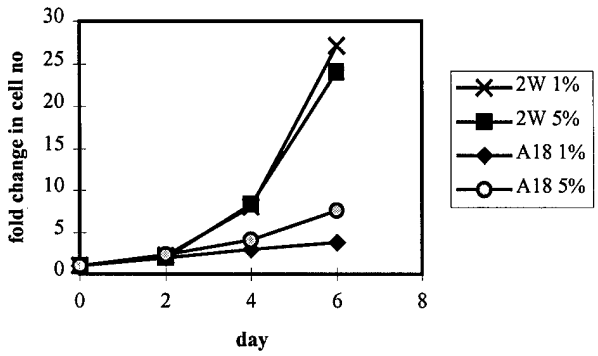

Figure 1b

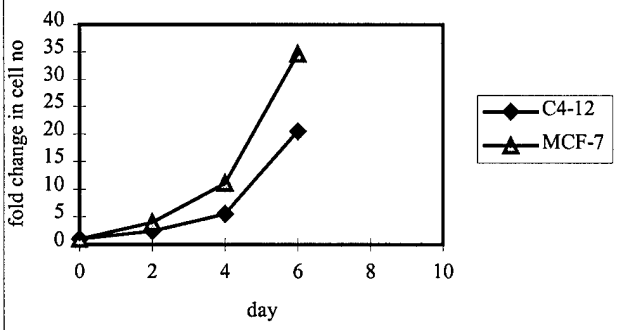

Figure 1. A) cells were plated in 1% or 5% FBS and counted using a Coulter counter at the indicated time points. Results are expresssed as fold change in cell number. B) MCF-7 cells were plated in 10% FBS and C4-12 cells were plated in 10% CCS. Cells were quantified as in A.

Since many chemotherapeutic agents exert cell cycle specific effects, tumors with higher proliferative rates are likely to be more susceptible to the effects of these drugs. Paclitaxel in particular is a cell cycle specific drug as it stabilizes microtubules, causing cells to be blocked in mitosis and subsequently undergo apoptosis (12,13,14) . The results reported for the CALGB 9344 trial indicate that ER- tumors are more susceptible to the effects of paclitaxel treatment. Since the effects of paclitaxel are cell cycle specific, we hypothesized that ER negative breast cancers

are more susceptible to the cytotoxic effects of paclitaxel due to an increase in proliferation of these tumors, resulting in the increased survival rates observed for ER- patients treated with paclitaxel.

Two *in vitro* models of ER- breast cancer were used to evaluate the sensitivity of ER+ and ER- cells to paclitaxel. An ER positive T47D cell line, T47D:A18 (A18), and an ER negative clone, T47D:C4:2 W (2W) generated by limited dilution cloning in the absence of estrogen provided one model of ER- breast cancer cell growth (15). MCF-7 cells and an ER- clone generated in a similar manner provided a second model (Gift of Dr. W. Welshons and Dr. E. Curran, Univ. of Missouri). Proliferation was evaluated by quantifying cell number over time. Cell viability and apoptosis following paclitaxel treatment at low and high serum concentrations was assessed using the Wst-1 assay and Cell Death ELISA, respectively.

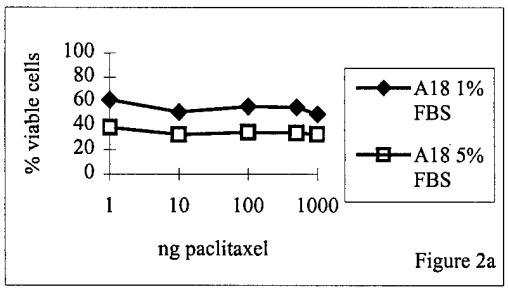

Figure 2a

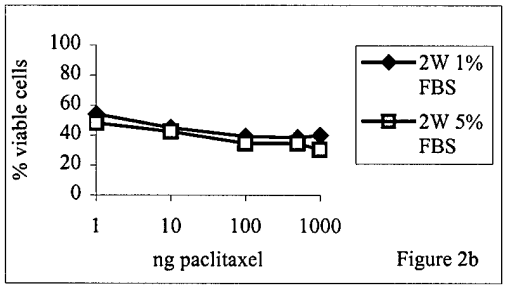

Figure 2b

Figure 2. A) A18 cells were plated in 1% or 5% FBS,paclitaxel treatment was inititated 24 hours later. Cell viability was assessed at 48 hours with the Wst-1 assay. Percentage of viable cells following treatment was calculated from triplicate wells. B) 2W cells were plated in 1% or 5% FBS and treated as in A.

Growth assays were utilized to determine differences in growth between the ER positive T47D:A18 (A18) and ER negative T47D:C4:2W (2W) cells and ER+ MCF7 cells and ER- ECMCF-7 C4-12 (C4-12) cells. The results of these assays demonstrate that there were significant differences in growth between A18 and 2W cells in both low (1% FBS) and high(5% FBS) serum conditions (Fig 1a). Additionally, A18 cells have a decreased doubling time in low serum conditions (96 hours) compared to high serum conditions (48 hours) indicating that a dependence on serum associated growth factors such as estrogen. This model demonstrates that *in vitro* ER- cells have a significantly higher growth rate than ER+ cells. ER negative C4-12 cells have an increased doubling time when compared to parental MCF-7 cells, while this model

does not demonstrate the increased proliferation characteristic of ER- cell lines, it is a useful model to examine the correlation between growth and paclitaxel sensitivity in breast cancer (Fig 1b).

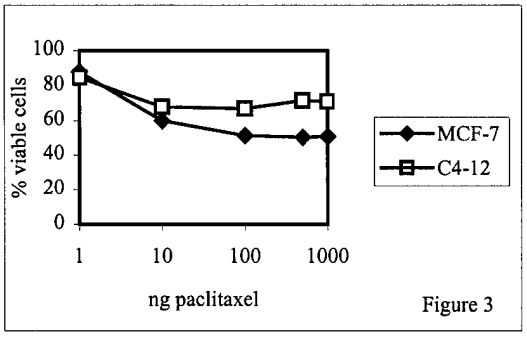

Figure 3. MCF-7 cells were treated in 10% FBS and C4-12 in 10% CCS with paclitaxel for 48 hours. Cell viability was determined with the Wst-1 assay. Results are expressed as percentage of viable cells following taxol treatment.

Cell viability and apoptosis were assessed following paclitaxel treatment. Cell viability was determined with the Wst-1 assay, a reagent that is metabolized by intact mitochondria and quantified photometrically and is directly proportional to cell number. The results of these experiments demonstrate that ER positive A18 cells are sensitive to a range of paclitaxel concentrations in 5% FBS , but are less sensitive when treated in 1% FBS, a condition where the cells have are proliferating more slowly (Fig 2a and 1a). However, ER negative 2W cells grow equally well in low and high serum conditions and display a similar level of sensitivity to paclitaxel in both 1% and 5% FBS (Fig 2b and 1a). Similarly, parental MCF-7 cells are more sensitive to paclitaxel than are C4-12 cells, which have decreased growth when compared to MCF-7 cells (Fig 3 and 1b). These results suggest breast cancer cells with slower proliferative rates are less sensitive to the cytotoxic effects of paclitaxel than are cells with a rapid proliferative rate.

The Wst-1 assay quantifies viable cells, to measure cell death induced by paclitaxel, apoptosis was evaluated with a Cell Death ELISA (Roche). Histone bound DNA cleaved during apoptosis is detected in cellular lysates with a primary antibody against histone and a secondary antibody against DNA conjugated to a reporter enzyme. Processing of added substrate allows photometric quantification of apoptosis. Apoptosis induced by paclitaxel treatment was greater in A18 cells treated in high serum compared to the level of apoptosis induced in low serum conditions. Paclitaxel treatment of 2W cells induces high levels of apoptosis in both low and high serum conditions. Collectively these data indicate that in this model the paclitaxel

sensitivity of these breast cancer cell lines is related to proliferation. MCF-7 and C4-12 cells will also be evaluated for induction of apoptosis using the cell death ELISA. Additionally, an ER- clone (LCC3) of the ER+ ZR-75-1 cell line has recently been obtained (gift of Dr. R. Clarke, Lombardi Cancer Center, Georgetown University). The ZR-75-1 parental ER+ cell line and the ER- clone, LCC3, will be evaluated for growth and paclitaxel sensitivity to confirm the results presented here.

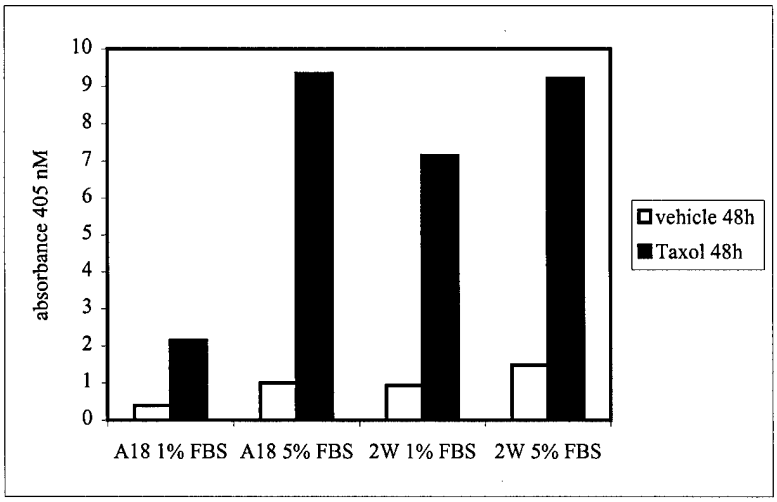

Figure 4. Apoptosis was determined by quantifying the amount of histone-bound DNA in cytoplasmic extracts of A18 and 2W following 48 hour exposure to 100nM paclitaxel or vehicle control in 1% and 5% FBS. .

CONCLUSIONS

The conclusion drawn from this data identifies an important factor impacting paclitaxel sensitivity in breast cancer. In this model, the effect of estrogen receptor expression on paclitaxel sensitivity is related to the growth regulatory properties of ER. ER negative 2W cells have achieved estrogen independence, allowing for increased proliferation. While these cells can survive in poor growth conditions (1% FBS), this adaptation has rendered the cells more susceptible to the effects of paclitaxel, a cell cycle specific drug. The breast cancer cell lines utilized in this project provide important models for studies detailing progression to estrogen independence in breast cancer. The remainder of this project will focus on evaluating the changes in growth factor signaling pathways and cell cycle components that allow these cells to achieve estrogen independence.

Progression of breast cancer from an estrogen-dependent, ER positive status to an estrogen-independent, ER negative status is the focus of much current research. Observations made both from *in vitro* models and clinical research indicates that upregulation of growth factor signaling pathways is correlated with progression to estrogen independence and loss of estrogen receptor expression. Increased levels of MEK and Raf-1 as well as increased expression and activity of MAP kinase has been observed in breast cancer, indicating that hyperactivation of growth signaling pathways is an important factor in breast cancer progression (16,17). Additionally, increased expression of both the erbB2 and EGF receptors is observed in ER- breast cancer (18, 19, 20,21). *In vitro* models where these growth factor receptors have been over-expressed in the ER+ MCF-7 cell line suggest that increased signaling through growth factor receptors is an important step in the progression to estrogen independence (22,23). More recently, increased MAP kinase activity has been shown to contribute to both estrogen-independent growth and the loss of ER- expression *in vitro* (24,25).

The T47D:A18, MCF-7 and ZR-75-1 cell lines along with their ER negative counterparts, all derived by selecting for cell growth in the absence of estrogen, provide models for further examining the status of growth factor signaling pathways in ER- breast cancer. Preliminary data suggests that the ER- 2W, LCC3 and C4-12 cell lines have decreased MAP kinase activity compared to the parental ER + cell lines but maintain MAP kinase expression at levels similar to the parental cell lines (data not shown). This observation indicates these cells may not be dependent on the MAP kinase pathway for growth. Further evaluation of these cell lines using western blot analysis has revealed that both A18 and 2W cells have increased levels of p70/S6 kinase activity (data not shown). p70/S6 kinase is a component of the PI-3-kinase pathway and is activated by ser/thr phosphorylation (26, 27). Both Akt and phosphatidyl inositide dependent kinase I (PDKI) have been shown to mediate activation of p70/S6 kinase (27, 28). Activation of p70/S6 kinase facilitates phosporylation of the ribosomal S6 subunit and subsequently protein

synthesis (29 30). p70/S6 kinase is primarily activated during the G0/G1 and G1/S phase transition, suggesting a role in cell cycle progression (31). Additionally, a recent analysis has demonstrated gene amplification in 59% of breast tumors examined and this amplification was associated with a poorer prognosis (32). While no correlation with ER status was observed, this observation suggests that S6 kinase may play an important role in breast cancer progression (32). Expression and activity of S6 kinase in breast cancer contributes to breast cancer progression and represents an alternative growth pathway by which breast cancer cells can achieve estrogen independence.

SPECIFIC AIMS

1. Evaluate expression and activity of p70/S6 kinase in *in vitro* models of breast cancer
   A)Utilize phosphorylation-site specific western blotting and p70/S6 kinase activity assays to determine the activity of p70/S6 kinase in the T47D, ZR-75-1 and MCF-7 cell lines and ER- counterparts

2. Evaluate the dependence of ER+ and ER- breast cancer on p70/S6 kinase activity by inhibit activity using pharmacologic inhibitors of S6 kinase activity and expression of dominant-negative p70/S6 kinase constructs and determine the impact on growth and estrogen receptor expression.
   A) Evaluate the effect of rapamycin, a pharmacologic inhibitor of p70 S6/kinase activity, on growth of ER+ and ER- breast cancer cells *in vitro* and expression of the estrogen receptor.
   B) Express a dominant-negative p70/S6 kinase construct in ER- breast cancer cells and assess growth and estrogen receptor status.

STATEMENT OF WORK

Task 1
Aim 1 (Months 12-18) Evaluate expression and activity of p70/S6 kinase in T47D, Zr-75 and MCF-7 ER+ and ER- breast cancer cells

Task 2
Aim 2A (Months 18-24) Perform growth assays to determine the effect of rapamycin treatment on growth of ER+ and ER- breast cancer cells in culture. Perform immunohistochemistry to determine the effect of rapamycin treatment on the expression of ER in ER+ and ER- breast cancer cells.
Aim 2B (Months 18-36) Stably express a dominant negative p70/S6 kinase in ER- breast cancer cell lines, confirm activity of dominant negative p70/S6 kinase and evaluate growth and ER status of stable transfected cell lines.

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