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The purpose of the research described in this proposal is to identify and characterize the role of adhesion and pp125^{FAK} in the survival of breast cancer cells expressing c-Myc. This work utilizes a breast cancer cell line Myc83 obtained from transgenic mice overexpressing c-myc. These cells undergo a rapid apoptotic cell death upon removal of EGF. The major findings of this work to date are that type IV collagen promotes the survival of Myc83 cells in the absence of EGF. Adhesion based survival is mediated by the $\beta 1$ integrin, in that inhibition of the $\beta 1$ integrin using neutralizing antibodies promotes the rapid apoptotic cell death of Myc83 cells. We have also determined that collagen IV does not stimulate the growth of Myc83 cells and does not change their cell cycle. Inhibitors of actin polymerization promote the apoptosis of Myc83 cells. Lastly, growth of Myc83 cells on collagen IV slightly increases the expression of the cell death inhibitor Bcl-2 and that inhibition of collagen IV adhesion by neutralizing antibodies to $\beta 1$ integrins increased expression of the cell death inducer Bax. These results strongly suggest that collagen IV functions as a survival factor for Myc-83 cells and that this activity is mediated by $\beta 1$ integrins.

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FOREWORD

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TABLE OF CONTENTS

| | |
|---|-----|
| Front Cover | 1 |
| Standard Form (SF) 298 Report Documentation Page | 2 |
| Foreword | 3 |
| Table of Contents | 4 |
| Introduction | 5 |
| Body – Statement of Work | 5-8 |
| Appendicies | 9 |

INTRODUCTION

The c-myc gene encodes a transcription factor important for regulating cell proliferation. Constitutive over expression of c-myc deregulates the cell cycle resulting in either uncontrolled proliferation or apoptosis. Activation of c-myc is thought to function in the development of breast cancer since it is amplified and/or over expressed in human breast tumors. C-myc amplification is associated with a high proliferation index in mammary tumors and may correlate with poor prognosis. We are examining a cell line (Myc83) established from a transgenic mouse that constitutively over expresses c-myc. In the presence of epidermal growth factor (EGF), Myc83 cells proliferate rapidly; however, removal of EGF results in rapid apoptotic cell death of these cells. We have used this model system to examine how growth factors promote the survival of c-myc overexpressing breast epithelial cells. **The purpose of the present study is to determine whether adhesion to extracellular matrix proteins and signaling pathways stimulated by adhesion can serve as a survival signal and inhibit the apoptosis of c-myc overexpressing breast cancer cells.** The work performed in this proposal will identify extracellular matrix molecules that Myc83 cells adhere to. The work will then go on to identify the integrins responsible for the adhesion and characterize the role of focal adhesion kinase FAK in the survival of these cells. This research will examine the role of endogenous FAK in the survival of these cells as well as examine the constitutively active FAK and a competitive inhibitor of FAK to fully evaluate the role of FAK in adhesion based survival.

ANNUAL SUMMARY

STATEMENT OF WORK

Task 1. Examine the inhibition of apoptosis by extracellular activation of pp125^{FAK} in c-myc overexpressing breast cancer cells (Months 1-16)

- A. **Evaluate the expression of β_1 integrins on the three c-myc overexpressing cell lines by western blot analysis and FACS analysis.** To determine what integrins may be responsible for the adhesion of Myc83 cells to collagen IV we performed flow cytometric (FACS) analysis with antibodies to some of the common epithelial integrins. FACS analysis of Myc83 cells determined that they express β_1 integrin, α_2 integrin and α_6 integrin on their cell surface. $\alpha_2\beta_1$ integrin is a integrin heterodimer, common in breast epithelial cells, which can function as a receptor for either collagen I or collagen IV. $\alpha_6\beta_1$ is also a common integrin in breast epithelial cells and functions as a receptor for laminin.
- B. **Evaluate the ability of c-myc overexpressing cells to adhere to collagen IV. Determine which neutralizing antibodies to β_1 integrins to inhibit adhesion to collagen IV.** Since $\alpha_2\beta_1$ integrin was identified on the surface of the Myc83 cells, we utilized a neutralizing antibody to β_1 integrin to determine whether β_1 integrin functioned in the adhesion to collagen IV. Treatment of Myc83 cells with a neutralizing monoclonal antibody to β_1 integrin inhibited adhesion to collagen IV in a dose dependant manner with a maximal inhibition of 70% of adhesion to collagen IV at 5 μ g/ml antibody. These data suggest that the β_1 integrin is the major collagen IV receptor on these cells. We are attempting to identify which α integrin subunit is responsible for the adhesion, however, there are few neutralizing antibodies to mouse integrin subunits and we have not been able to identify a particular a subunit responsible for adhesion to collagen IV. We are contacting commercial antibody suppliers and the literature to obtain additional antibodies.
- C. **Determine to what extent growth of c-myc overexpressing cell lines on collagen IV inhibits apoptotic cell death and effects cell proliferation.** We have examined whether adhesion of Myc83 cells to collagen IV could inhibit apoptosis. Cells were grown on collagen IV, laminin, or untreated tissue culture plastic, in the presence or absence of EGF. Subsequent analysis of apoptosis by an ELISA method that identifies the presence of nucleosomal DNA fragments, determined that growth of Myc83 cells on collagen IV inhibited their apoptotic cell death approximately 50%. Growth on laminin in the presence or absence of EGF actually increased the amount of apoptosis over growth of Myc83 cells on plastic in the absence of EGF. This is most likely due to the inability of these cells to

adhere to laminin. Collagen IV did not inhibit apoptosis promoted by TGF β . Further analysis suggested that the enhanced survival of cells on collagen IV is being mediated through adhesion by the β_1 integrin, because incubation of Myc83 cells with neutralizing antibodies to β_1 integrin resulted in apoptotic cell death, even in the presence of EGF. These results suggest that adhesion can function as a survival signal for Myc83 cells and that this signal functions irrespective of other growth factor survival signals. These analyses have been repeated with flow cytometry, and immunocytochemical methods for examining apoptosis. These analyses support a survival role for collagen IV and support the role of β_1 integrin in survival of the Myc83 cells.

To further characterize the influence of collagen IV on Myc83 cells, we evaluated whether collagen IV was acting as a mitogen to promote cell growth, or merely as a survival factor preventing cell death. To evaluate this characteristic, cells were plated on collagen IV or tissue culture plastic and grown in the presence or absence of EGF or in the presence of TGF β . Analysis of growth at multiple time points, up to 96 hours after seeding, determined that collagen IV does not function as a mitogen for Myc83 cells, and that the major determinant of cell growth was the presence or absence of EGF or TGF β . Most importantly, collagen IV did not promote growth of Myc83 cells in the absence of EGF.

- D. **Perform cell cycle analysis on cells growing on collagen IV to determine whether adhesion mediated survival requires arresting cell growth at a specific cell-cycle checkpoint.** Further analysis was performed to determine whether growth of cells on collagen IV altered cell cycle distribution. Myc83 cells grown on collagen IV or tissue culture plastic in the presence or absence of EGF were analyzed by flow cytometric DNA ploidy / cell cycle analysis. As with the proliferation assay (C above) the major determinant for progression through the cell cycle was the presence or absence of EGF. Interestingly, EGF appears to promote DNA synthesis even in the presence of neutralizing antibodies to β_1 integrin, further supporting the mitogen activity of EGF and a survival function for collagen IV. Together, these analyses suggest that while collagen IV may inhibit apoptosis, it does not function as a growth stimulus. These experiments have determined that extracellular activation of β_1 integrin inhibits the apoptosis of c-myc-overexpressing breast cancer cells. Specifically, collagen IV can act, in part, as a survival factor to inhibit c-myc-induced apoptosis through a survival signal initiated by β_1 integrins.
- E. **Examine whether specific inhibitors of calpain (calpeptin or calpain inhibitor I) inhibit c-myc-induced apoptosis and proteolytic degradation of pp125^{FAK} in the absence of EGF. Evaluate pp125^{FAK} cleavage by western blot analysis, and apoptotic cell death by ELISA.** Prior studies have suggested that FAK is cleaved during apoptosis of Myc83 cells. We examined the cleavage of FAK during the death of Myc83 cells and the effect of calpain inhibitor I, inhibitor of μ -calpain (N-Ac-Leu-Leu-norleucinal) and calpain inhibitor II, inhibitor of m-calpain (N-Ac-Leu-Leu-methioninal). We were able to confirm that FAK cleavage did occur when Myc83 cells underwent apoptosis upon removal of EGF, however, we were not able to inhibit the cleavage using calpain inhibitor I. The calpain inhibitors affected Myc83 cell survival. Calpain inhibitor I decreased Myc83 cell survival and allowed FAK cleavage, while calpain inhibitor II increased cell survival and inhibited FAK cleavage. Effects observed were statistically significant with ANOVA analysis. These results suggest that m-calpain could be involved in FAK cleavage, however they do not point to a direct effect. We have attempted to demonstrate a direct effect by immunoprecipitating FAK from the Myc83 cells and attempting to cleave it using purified calpain (Calbiochem, San Diego, CA). All attempts to cleave FAK with calpain have failed and we hypothesize that the effects observed with calpain are an indirect effect due to the inhibition of the apoptotic process. Recent evidence has identified FAK as a substrate for the apoptosis related proteinase caspase-3 (Gervais et al., 1998, J. Biol. Chem. 273:17102-17108; van de Water et al., J Biol Chem 1999 274:13328-13337). Together these analyses suggest that cleavage of FAK is an effect of apoptosis and not a cause of apoptosis.
- F. **Identify the formation of focal adhesions in cells grown on collagen IV using rhodamine phalloidin to stain f-actin and immunocytochemistry to identify focal adhesion associated proteins. Evaluate using fluorescence microscopy and/or confocal microscopy.** (Months 9-13) Recently, Aplin and Juliano, (J Cell Sci 1999,112:695-706) presented data that argues that actin polymerization and organization is necessary for EGF receptor phosphorylation in fibroblasts. In

their system, inhibition of actin polymerization with cytochalasin D or latrunculin A results in a loss of EGF receptor phosphorylation even in the presence of EGF. These studies suggest that actin filament formation originating from focal adhesions may directly affect EGF receptor activity. Therefore, we hypothesized that since EGF receptor activity is necessary for the survival of Myc83 cells, inhibition of actin polymerization could promote apoptosis even in the presence of EGF. Myc83 cells were grown on collagen IV and treated with increasing amounts of cytochalasin D for 18 hours in media containing EGF and then assayed for apoptotic cell death. Cytoskeletal changes were assessed by staining with Rhodamine phalloidin, a fluorescent dye that binds to filamentous actin. Myc83 cells showed markedly increased apoptosis at 0.2 μM cytochalasin D, and apoptosis increased in a dose dependent manner. Treatment with cytochalasin D resulted in marked morphological changes, with a loss of actin stress fibers and organized cell structure. Treatment with 1.0 μM cytochalasin D resulted in cells that were completely rounded, with no discernable cell structure. Short-term treatment with cytochalasin D is reversible; cells treated with 10 μM for 6 hours were washed and grown in fresh media for an additional 12 hours. These cells appeared normal on phase contrast microscopy and had low background levels of apoptosis. These studies suggest that cytoskeletal organization may play an important role in promoting the adhesion- based survival of c-myc-overexpressing breast cancer cells. Further examination of the activity of the EGF receptor in these cells will be made to determine if cytoskeletal organization is critical to EGF receptor function.

- G. **Quantitate expression of cell death associated proteins Bcl-2, Bcl-x_L, Bcl-x_S, and Bax by western blot analysis to determine whether adhesion based survival mechanisms utilize the same bcl-family members as EGF based cell survival growth stimulatory mechanisms.** We examined whether extracellular activation of FAK influences the Bcl family of cell death inducer genes in the same manner as EGF. Prior studies by in our laboratory have examined the regulation of the Bcl family of cell death regulatory proteins in c-myc-overexpressing breast carcinoma cells. These studies determined that c-myc overexpressing cells express high levels of the cell death inducer Bax. In the presence of EGF, there is also high expression of the cell death suppressor Bcl-x_L, but upon removal of EGF or addition of TGF- β there is a decrease in the protein levels of Bcl-x_L concurrent with the onset of apoptosis. We performed immunoblot analysis to evaluate the relative expression of cell death inhibitor Bcl-2 and the cell death inducer Bax. These analyses determined that growth on collagen IV slightly increases the expression of the cell death inhibitor Bcl-2 and that inhibition of collagen IV adhesion by neutralizing antibodies to β_1 integrins increased expression of the cell death inducer Bax. Further analysis will be performed to examine the other members of the Bcl family.

Task 2. Identify pp125^{FAK} initiated signal transduction pathways that participate in adhesion-mediated survival of c-myc overexpressing breast cancer cells. (Months 16-28)

- A. **Assess the activation of pp125^{FAK} from cells growing in the presence or absence of ECM. Evaluate kinase activity of endogenous pp125^{FAK} by immunoprecipitation of pp125^{FAK}, pp60^{src}, paxillin, tensin, or p130^{Cas} and immunoblot using anti-phosphotyrosine antibody. (Months 16-19)** We have begun this analysis. New antibody reagents have become available specific for 5 different sites of phosphorylation on FAK. We have begun to analyze which sites on FAK become activated upon adhesion of Myc83 cells to collagen IV.
- B. **Design and use PCR primer to mutate Tyr⁹²⁵ the site of binding by Grb2 within pp125^{FAK} cDNA of pCD2-FAK to create CD2-FAK-925*. Confirm by sequencing (Months 19-20).** We have not begun this analysis.
- C. **Stably cotransfect c-myc overexpressing cells with CD2-FAK or CD2-FAK mutants and pCNCX (a construct encoding G418 resistance). Select CD2FAK transfectants by growth in G418 and evaluation by FACS analysis and sterile sorting using a monoclonal antibody to human CD2 (Months 19-22).** I have obtained from Sandro Aruffo (Bristol Meyers Squibb, Seattle WA) an expression plasmid for CD2FAK. CD2 FAK is a fusion protein containing the extracellular and cytoplasmic domains of the human CD2 protein (a lymphocyte marker) and the full-length pp125^{FAK} cDNA. This protein chimera is expressed at the cell membrane and, as a result, functions as a constitutively active pp125^{FAK}. Myc83 cells do not express CD2. Myc83 cells were transfected

with 8, 12 or 16 μg of CD2FAK by lipofectamine and analyzed by flow cytometry 72hrs later with a monoclonal antibody to CD2. The results indicate that with 12 or 16 μg CD2FAK there was an approximate 2% transfection efficiency. Expressing cells (15,000 - 16,000) were sterile sorted and grown to confluence. Flow cytometric analysis of these cells determined that expression of the CD2 chimera protein was unstable. Further experiments are underway to optimize transfection conditions and increase the likelihood for stable expression of the CD2-FAK chimera.

- D. Evaluate apoptosis and formation of focal adhesions by CD2-FAK and CD2-FAK mutant transfected cell lines in the presence or absence of EGF. (Months 21-26)** We have not begun this analysis.
- E. Perform cell cycle analysis on transfectants to determine whether CD2-FAK mediated survival occurs at a similar cell-cycle checkpoint as adhesion mediated survival. (Months 26-27)** We have not begun this analysis
- F. Quantitate expression of cell death associated proteins in CD2-FAK transfectants by western blot analysis. (Months 26-27).** We have not begun this analysis.
- G. Examine kinase activity of CD2-FAK by immunoprecipitation of pp60^{src}, paxillin, tensin or CD2-FAK (using anti-CD2) and immunoblot using anti-phosphotyrosine antibody (Months 27-28).** We have not begun this analysis.

Task 3. Examine the effect of pp125^{FAK} inhibition on apoptosis in bitransgenic mammary tumor cells overexpressing c-myc and TGF α . (Months 27-37)

- A. Synthesize antisense oligonucleotides to pp125^{FAK}, treat bitransgenic c-myc/TGF- α cells with oligonucleotides and evaluate inhibition of pp125^{FAK} protein expression by western blot analysis. Evaluate apoptosis in treated cells by cell-death ELISA.(Months 27-29)** We have not begun this analysis.
- B. PCR amplify pp41/43^{FRNK} from the murine pp125^{FAK} cDNA and clone into pIND to create pIND-FRNK. (Months 29-30).** We have amplified pp41/43^{FRNK} from the murine FAK cDNA. We have cloned it into pCR2000 (Invitrogen) and into pCDNA3. We have confirmed expression and size by expressing it in COS-7 cells. We have also verified that it is expressed in focal adhesions by expressing it in MCF-7, MDA-MB-435, MDA-MB-231 breast cancer cells.
- C. Cotransfect bitransgenic c-myc/TGF- α cells with the plasmids pVgRXR and pIND-FRNK and select stable transfectants with the antibiotics Zeocin (for pVgRXR) and G418 (for pIND-FRNK). (Months 31-33)** We have not begun this analysis.
- D. Select clones based on their expression of pp41/43^{FRNK} in the presence of the inducer, muristerone A, by identification of a 41-43 kD protein by western blot analysis. (Months 34-35)** We have not begun this analysis.
- E. Evaluate kinase activity of endogenous pp125^{FAK} by immunoprecipitation and western blot analysis using anti-phosphotyrosine. Evaluate apoptosis induced by expression of pp41/43^{FRNK}. (Months 35-36)** We have not begun this analysis.
- F. Quantitate expression of cell death associated proteins by western blot analysis during muristerone A induced expression of pp41/43^{FRNK}. (Months 36-37)** We have not begun this analysis.
- G. Perform cell cycle analysis on cells transfected with pp41/43^{FRNK} to determine whether apoptosis occurs at a specific cell-cycle checkpoint. (Months 36-37)** We have not begun this analysis.

Appendix

Bulleted list of key research accomplishments:

- Collagen IV inhibits the apoptosis of c-myc overexpressing Myc83 breast cancer cells.
- Survival is mediated through β_1 integrins.
- Collagen IV does not function as a mitogen for Myc83 cells. The major determinant of cell growth was the presence or absence of EGF or TGF β .
- Collagen IV does not impact the cell cycle of Myc83 cells. The major determinant for progression through the cell cycle was the presence or absence of EGF.
- Calpain inhibitors affect Myc83 cell survival. Calpain inhibitor I decreased Myc83 cell survival and allowed cleavage of FAK
- Calpain inhibitor II increased cell survival and inhibited FAK cleavage.
- Inhibitors of actin polymerization promote the apoptosis of Myc83 cells.
- Growth of Myc83 cells on collagen IV slightly increases the expression of the cell death inhibitor Bcl-2 and that inhibition of collagen IV adhesion by neutralizing antibodies to β_1 integrins increased expression of the cell death inducer Bax.

Reportable Outcomes:

Manuscripts

Rosfjord, E.C. and Dickson, R.B. β_1 integrin and pp125^{FAK} mediated inhibition of apoptosis in c-Myc overexpressing mammary epithelial cells. Manuscript in Preparation.

Abstracts

1. **Rosfjord, E.C.** and Dickson, R.B. Adhesion to collagen IV *via* β_1 integrins inhibits apoptosis of c-myc overexpressing breast cancer cells through a PI-3 kinase dependent mechanism. Presented at the 21st annual San Antonio Breast Cancer Symposium, December 12-15 1998. Abstract #264 *Breast Cancer Research and Treatment* **50**:271.
2. **Rosfjord, E.C.**, Herrell, R.W., and Dickson, R.B. Expression and tyrosine phosphorylation of pp125^{FAK} in human breast cancer cell lines and the effect of overexpression of epitope-tagged pp41/43^{FRNK} on malignant cell growth. Presented at the 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, PA. April 10-14, 1999. Abstract # 2144