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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 β1 integrin, in that inhibition of the β1 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. Inhibitiors 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 β_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 β1 integrins

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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 anallysis 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 5μg/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 TGFβ. 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 TGFβ. 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 TGFβ. 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 β1 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 affect 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 florescence 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 phosphoyrlation 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 obtained antibodies that have been designed to specifically recognize 6 phosphotyrosine residues on FAK. Analysis with these antibodies suggests that that the residues at the C-terminus responsible for association of FAK with p130cas may be differentially regulated in Myc83 cells in the presence or absence of EGF and dependent on Collagen IV adhesion. This work will need to be confirmed.

B. **Design and use PCR primer to mutate Tyr^{925} 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 TGFα. (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 designed antisense oligonucleotides to pp125^{FAK}, however we are still optimizing the transfection conditions for c-myc / TGF-α cell line. We have stopped using the cell-death ELISA because it is semi-quantitative. We are now evaluating apoptosis by *in situ* TUNEL analysis and Hoescht DNA stain for evaluation of condensed nuclei.

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 used the pIND and ecdysone system. We felt it would be best to try an initial evaluation with a constitutive promoter expression construct. If we didn't see activity, it would save time and effort. If we did see activity and we needed to evaluate this effect further we could examine an inducible promoter system. Since there were no antinbodies that could distinguish transfected FRNK from endogenous FRNK, we created epitope tagged FRNK constructs. The first was FLAG-FRNK. This makes use of a unique 7 amino acid epitope placed at the N-terminus of the protein. We created the construct pCDNA3-FLAG-FRNK. We have transfected pCDNA3FLAG-FRNK into COS-7 cells, myc-83 cells, MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-361, T47D, A1N4, ZR75 cells, and the immortalized breast epithelial cell lines MTSV 4.1, MRSV. We have demonstrated expression of FRNK by immunofluorescence microscopy, and western blot analysis using both antibodies to the FLAG epitope and the C-terminal portion of FAK. The immunoblot analysis has demonstrated that we are expressing the appropriate sized chimeric

protein. Immunofluorescence has localized FLAG-FRNK to the cell membrane particularly in regions of membrane ruffling. Through these procedures, we have also determined that overexpression of FRNK does promote apoptosis in Myc83, MCF-7, A1N4, and immortalized breast epithelial cell lines.

Interestingly, FLAG-FRNK did not induce apoptosis in MDA-MB-231 or MDA-MB-435 cells. Transiently transfected FLAG-FRNK could be expressed in proliferating MDA-MB-435 cells for up to 4 passages. Since it did not appear the FRNK interfered with proliferation of MDA-MB-435 cells, we attempted to stably express FRNK in MDA-MB-435 cells. To accomplish this we cloned FLAG-FRNK into a vector that stably expressed the Green Fluorescent Protein. The GFP-FRNK chimera was expressed in MDA-MB-435 cells. Expression was confirmed by flow cytometry for the GFP protein, western blot analysis with antibodies for GFP and the C-terminal end of FAK, and immunofluorescence for GFP. Stably transfected cell populations expressing either GFP or GRP-FRNK were isolated by fluorescent cell sorting and selection in G418. The resultant cell populations expressed strong levels of GFP or the GFP-FRNK chimera. These cells proliferate faster than the parental MDA-MB-435 cells. Further characterization of MDA-MB-435 cells expressing GFP and GFP-FRNK will be performed.

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. We did perform cell cycle analysis of MDA-MB-435 cells overexpressing GFP and GFP-FRNK and found that they had slightly decreased G1 population and slightly increased S and G2 M phase fraction which is consistent with an increased proliferative rate.

Bulleted list of Key Research Accomplishments:

- Collagen IV inhibits the apoptosis of c-myc overexpressing Myc83 breast cancer cells.
- Survival is mediated through β1 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 TGFβ.
- 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.
- Inhibitiors 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.

- Expression of the C-terminal end of FAK induces apoptosis in Myc83 cells as well as imortalized breast epithelial cell lines MTSV 4.1 and A1N4.

- Expression of the C-terminal end of FAK (FRNK) does not induce apoptosis in two models of tumor progression.

- Stable expression of FRNK in MDA-MB-435 cells actually promoted proliferation.

Reportable Outcomes:

No patents have been applied for or granted based on the work performed under this contract.

Abstracts (Full Abstracts follow in the Appendix).

1. **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
2. Wang, J.-K., Johnson, M.D., **Rosfjord, E.C.**, Jamerson, M.H., and Dickson, R.B. EGF-dependent survival signaling pathways in c-myc overexpressing mouse mammary tumor cell lines; role of Erk1/Erk2 and PI3K pathways. Presented at the 90th Annual Meeting of the American Association for Cancer Research, Philadelphia, PA. April 10-14, 1999. Abstract #1093.
3. **Rosfjord, E.C.**, and Dickson, R.B. Role of focal adhesion kinase (FAK) in metastatic breast carcinoma cells assessed by expression of FRNK in a mouse xenograft model. Presented at the 91st Annual Meeting of the American Association for Cancer Research, SanFrancisco, CA. April 1-5, 2000.
4. **Rosfjord, E.C.**, and Dickson, R.B. Cellular Attachment as a Target for Promoting the Programmed Cell Death of Breast Cancer Cells. Presented at the Department of Defense Breast Cancer Research Program Era of Hope Meeting, Atlanta, GA. June 8-12, 2000.

Cell Lines

Developed MDA-MB-435 GFP and MDA-MB-435 GFP-FRNK cell lines that may be useful for evaluating the role of FAK in metastatic progression. In addition, the MDA-MB-435 GFP cell line may be useful for the evaluation of hematogenous dissemination of tumor cells away from the primary tumor and for evaluating the role of other oncogenes on metastatic progression of breast cancer.

Employment

Partially as a result of the training received under this award, I obtained employment evaluating tyrosine kinase inhibitors as anti-tumor agents for breast cancer cell lines. I now work in the oncology department of Wyeth-Ayerst pharmaceuticals. I ended my postdoctoral training at Georgetown University in November, 2000.

Appendicies – Meeting Abstracts

[PROC. AMER. ASSOC. CANCER RES. 40, March 1999]

#1093 EGF-dependent survival signaling pathways in c-Myc-overexpressing mouse mammary tumor cell lines; Role of Erk1/Erk2 and PI3K pathways. Wang, J.-K., Johnson, M.D., Rosfjord, E.C., Jamerson, M.H., and Dickson, R.B.Department of Cell Biology/Lombardi Cancer Center, Georgetown University Medical Center, Washington DC, 20007.

Expression of both c-Myc and EGF family members has been implicated in mammary tumorigenesis. To further investigate cooperative mechanisms of c-Myc and EGF, a c-Myc overexpressing mouse mammary epithelial carcinoma cell line, Myc83, has been established from a mammary tumor of a c-Myc-transgenic mouse. Myc83 cells grow well in the presence of EGF, but many of these cells undergo apoptosis after withdrawal of EGF (Nass et al. BBRC 227:248-256, 1996). This feature of Myc83 cells provides a useful model system to study the EGF-dependent survival pathway of c-Myc-induced apoptosis. In order to examine the possible survival and apoptosis signaling pathways in Myc83, we have treated Myc83 cells with different kinase inhibitors: PD98059 (inhibitor of MEK), LY294002 (inhibitor of PI3 kinase), and PD153035 (inhibitor of EGF receptor kinase). In the presence of EGF, only 1-2% Myc83 cells undergo apoptosis within 48 hr; however, removal of EGF resulted in apoptosis of approximately 15% of Myc83 cells within 48 hr. Treatment with either LY294002 or PD98059 in the presence of EGF results in apoptosis of approximately 10% of Myc83 cells by 48 hr, suggesting that both PI3 kinase and Erk1/Erk2 pathways are necessary for EGF-mediated survival in c-Myc overexpressing mammary epithelial cells. (Supported by NCI R01AG1496)

[PROC. AMER. ASSOC. CANCER RES. 40, March 1999]

#2144 **Expression and tyrosine phosphorylation of pp125^{FAK} in human breast cancer cell lines and the effect of overexpression of epitope-tagged pp41/43FRNK on malignant cell growth. Rosfjord**, E.C., Herrell, R.W., and Dickson, R.B. Georgetown University Medical Center, Lombardi Cancer Center, Washington, D.C. 20007.

Prior analysis of breast cancer pathology specimens has determined that focal adhesion kinase (pp125^{FAK}) mRNA and protein is expressed at high levels in over 80% of invasive and 100% of metastatic breast cancers, while it is not expressed or expressed at low levels in normal breast tissue and benign breast disease. These studies have suggested that the overexpression of pp125^{FAK} is associated with a transition to malignant disease. We have examined the expression and activation (tyrosine phosphorylation) of pp125^{FAK} in both immortalized breast epithelial cell lines and breast cancer cell lines. These studies have identified pp125^{FAK} expression in both immortalized breast epithelial cells and malignant cell lines, with high expression of pp125^{FAK} observed in the metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435. Both immortalized breast epithelial cell lines and malignant breast cancer cell lines such as MDA-MB-435, MDA-MB-468, and BT549 activate pp125^{FAK}. Prior studies have demonstrated that pp125^{FAK} activity is negatively regulated by the competitive inhibitor, pp41/43FRNK (FAK-related non kinase), that competes for integrin binding with pp125^{FAK} but lacks kinase activity. To examine how activation of pp125^{FAK} affects the growth and survival of immortalized breast epithelial cells and the malignant breast cancer cell lines; MDA-MB-435, MDA-MB-468, and MDA-MB-231, we have transfected these cells with an epitope (FLAG)-tagged pp41/43FRNK chimera. We demonstrate that overexpression of FLAG-FRNK affects the growth of these cells, suggesting that pp125^{FAK} activation and kinase activity may be a useful target for regulating the growth of malignant breast cancer cells. E.C.R. was supported by DAMD 17-98-1-8096 a postdoctoral fellowship sponsored by the U.S. Army.

[PROC. AMER. ASSOC. CANCER RES. 41, March 2000]

 # 5523 ROLE OF FOCAL ADHESION KINASE (FAK) IN METASTATIC BREAST CARCINOMA CELLS ASSESSED BY EXPRESSION OF FRNK IN A MOUSE XENOGRAFT MODEL. Rosfjord, E.C., and Dickson, R.B. *Georgetown University Medical Center, Lombardi Cancer Center, Washington, D.C. 20007*

Prior studies of breast cancer pathology specimens have determined that focal adhesion kinase (FAK) is expressed at high levels in over 80% of locally invasive and 100% of metastatic breast cancers. In contrast, FAK is not expressed or expressed at low levels in normal breast tissue and in benign breast disease. These studies have suggested that the overexpression of FAK is associated with dissemination of the disease away from its primary site. I have examined the expression and activation (tyrosine phosphorylation) of FAK in highly and poorly invasive breast cancer cell lines. These studies identified strong expression of FAK in the highly invasive breast cancer cell lines MDA-MB-231, MDA-MB-435, and BT549 with lower expression of FAK in the poorly invasive breast cancer cell lines T47D, MDA-MB-453, BT474, and ZR75. Immunocoprecipitation of FAK and analysis of tyrosine phosphorylation has determined that FAK is phosphorylated in highly invasive breast cancer cell lines but not in poorly invasive breast cancer lines. To further evaluate the role of FAK in metastasis, we have stably transfected MDA-MB-435 cells with either green fluorescent protein (GFP) or GFP tagged to FRNK, a competitive inhibitor of FAK (GFP-FRNK). Expression of GFP or GFP-FRNK was confirmed by flow cytometry, immunocytochemistry, and immunoblot analyses. GFP-FRNK-transfected MDA-MB-435 cells proliferate *in vitro* as well as MDA-MB-435 cells and 3-fold faster than GFP-transfected MDA-MB-435 cells. All three cell types formed fast-growing moderately vascularized tumors when injected subcutaneously in nude mice. MDA-MB-435 cells and GFP transfected MDA-MB-435 cells formed necrotic tumors that resulted in open wounds. GFP-FRNK tumors were less necrotic and the skin remained intact even when tumor volume reached 2 cm^3. MDA-MB-435 tumors and GFP-FRNK tumors reached limiting tumor burden by 42 days. GFP-FRNK tumors produced macroscopic metastases in the lung and liver. These studies suggest that for the metastatic breast cancer cell line MDA-MB-435, overexpression of FRNK does not inhibit cell growth, tumor growth or ability to form distant metastases. E.C.R. was supported by postdoctoral fellowship DAMD 17-98-1-8096.

Abstract for the Era of Hope meeting in Atlanta, June 2000

CELLULAR ATTACHMENT AS A TARGET FOR PROMOTING THE PROGRAMMED CELL DEATH OF BREAST CANCER CELLS

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The purpose of our research is to identify and characterize the role of adhesion and **f**ocal **a**dhesion **k**inase (FAK) in the survival of breast cancer cells expressing c-Myc. The c-myc gene encodes a transcription factor important for controlling cell growth. Constitutive overexpression of c-myc deregulates the cell cycle resulting in either uncontrolled proliferation or programmed cell death (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. Similarly, expression of FAK is elevated in breast cancer and breast cancer metastases. Our work utilizes a breast cancer cell line, Myc83, obtained from transgenic mice overexpressing c-myc in their mammary glands. These cells grow well in the presence of epidermal growth factor (EGF), however these cells undergo a rapid apoptotic cell death upon removal of EGF. 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.

Our analysis determined that Myc83 cells adhere well to collagen IV. Flow cytometric analysis of integrins determined that Myc83 cells express β1 integrins on their cell surface. Incubation of Myc83 cells with neutralizing antibodies to β1 integrins inhibited 70% of the adhesion to collagen IV, suggesting that adhesion of Myc83 cells to collagen IV was mediated primarily by β1 integrins. We further determined that growth of Myc83 cells on collagen IV inhibited apoptosis in the absence of EGF. This inhibition of apoptosis could be abrogated by neutralizing antibodies to β1 integrin, suggesting strongly that β1 integrins stimulate a survival pathway in these cells. We have also determined that collagen IV does not stimulate the growth of Myc83 cells and does not change their cell cycle. Lastly, we have used inhibitors to phosphoinositol-3 kinase (PI-3 kinase) to demonstrate that PI-3 kinase participates in adhesion mediated survival. In summary, we have determined that adhesion to collagen IV promotes survival for the c-myc overexpressing cell line Myc83. This survival signal is mediated through the β1 integrin and utilizes the PI-3 kinase pathway.

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