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PRINCIPAL INVESTIGATOR: Lisa R. Williams

CONTRACTING ORGANIZATION: Roger Williams Medical Center
Providence, Rhode Island 02908

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Lisa R. Williams

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Roger Williams Medical Center
Providence, Rhode Island 02908

E-Mail: lisa_nelson_1@brown.edu

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13. ABSTRACT (Maximum 200 Words)
Considerable evidence suggests that poor prognosis in breast cancer may be due to overly-activated growth-factor receptors such as ErbB2, IGF-1, EGFR, and c-Met. A downstream signaling protein common to all these kinases is the adapter protein, Shc. Shc helps activated growth-factor receptors transfer signals to the c-Ras—MAP kinase pathway. Additionally, Shc appears to signal to Jun kinase, PI3kinase and to c-Myc (implicated in cell proliferation, apoptosis and the stress response). Recently we have reported that many human breast cancer cell lines (but not non-cancerous breast epithelial cell lines) not only harbor constitutively activated (phosphorylated on tyronsine 317) Shc proteins, but also breast cancer cells appear to require signaling from Shc in order to proliferate.

A third, 66-kDa splicing isoform, of Shc (p66-Shc) can act as a feedback inhibitor of growth-factor signaling to MAP kinase and to c-fos, and acts as an apoptotic sensitizer for oxidative stress in some cell lines. We have recently reported a strong negative correlation between the cellular levels of activated Shc and cellular expression of the inhibitor p66-Sch isoform. We have also demonstrated that p66-Sch acts as a strong negative growth regulator, as measured by their inability to form colonies in soft agar.

14. SUBJECT TERMS
ErbB2- Her2/Neu, IGF-1-Insulin growth factor receptor, EGFR-epidermal growth factor receptor

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INTRODUCTION

Considerable evidence suggests that poor prognosis in breast cancer is due to the over expression of cell surface receptor tyrosine kinases, such as ErbB2, IGF-1 and EGFR. A molecule downstream in the signaling pathways common to all these receptors is the small adaptor protein Shc. The Shc adapter protein transmits signals from the activated growth factor receptor to Ras.

There are three Shc isoforms of 46, 52, and 66 kDa. The 52- and 46-kDa isoforms, which differ in their 5' initiation site, are ubiquitously expressed[1]. Our laboratory has previously reported that most cell lines derived from breast cancers harbor constitutively tyrosine phosphorylated p46- and p52-Shc. The p66-Shc isoform, expressed through the use of an alternative promoter, contains an additional 110 amino-acid CH2 domain on its amino terminus. Recent studies have suggested that p66-Shc can act as a feedback down-regulator of growth factor signaling to Erk1/2 and *c-fos,* and also can act as an apoptotic sensitizer to oxidative stress[2-4]. In many cell lines, the functions of p66-Shc require phosphorylation in serine-36 of its unique CH2 domain.

Our laboratory has reported a strong negative correlation between the levels of tyrosine phosphorylated p52-Shc and the levels of p66-Shc in cell lines derived from human breast cancers[5]. This suggests the possibility that loss of p66-Shc expression confers a selective advantage for these breast cancer cells.

This research will help me to understand how p66-Shc suppresses tumorgenicity of these breast cancer cell lines by testing the hypothesis that, "*p66-Shc interferes with cell growth and tumorgenicity by downregulating key signaling pathways that regulate cell cycling, cell survival or both*".

BODY

The Body of this progress report is presented in sections according to the approved statement of work. The Specific aims and tasks for each section appear in italics.

Specific Aim 1. To determine the p66-Shc domains and post-translational modifications that are required to inhibit tumorgenicity (as measured by colony formation in soft agar).

I have requested different p66-Shc (S36A mutation) constructs (full length p66-Shc and CH2 domain) from another laboratory but have not yet received those constructs. We have a professor in our department that is well versed in the techniques of making mutants and are planning on setting up a collaboration with his laboratory.

I have worked with Dr. Bryant C. Nelson in analyzing p66-Shc protein samples by Mass Spectrometry. We have been unable to find an optimal procedure for isolation of the p66-Shc protein. I am having difficulty viewing the immunoprecipited p66-Shc protein via the Sypro-red staining procedure. Therefore, I have been unable to successfully isolate and extract the protein band for mass spectrometry analysis.

To trouble-shoot this problem, I increased the amount of cells extracted and was unsuccessful in detecting a band utilizing the Sypro-Red staining procedure. I also attempted to detect immonprecipitated p66-Shc by silver staining but was also unsuccessful.

Specific Aim 2. To elucidate the cell-biological effects of expressing p66-Shc in SKBR3 and MDA-MB-453 cells.

Task 1. Determine if p66-Shc inhibits growth and tumorgenicity by decreasing cell survival, by inhibiting passage through the cell cycle, or both.

I planned on using the TUNNEL assay on p66-Shc expressing cells grown under anchorage independent conditions. I chose to plate cells in methylcellulose over a 1% soft agar underlayer because, 1) the methylcellulose inhibits cell-cell contacts (cell-cell contact confers a growth advantage), and 2) the cells can be easily extracted and used for biochemical analysis.

When I plated the cells I found that they sink through the methlycellulose and adhere very tightly to the 1% soft agar under layer. Our laboratory has developed a procedure to de-attach the cells, but have had problems with soft agar contamination (from the de-attaching procedure) inhibiting downstream analyses. Additionally, since I plate only $1X10^4$ cells per plate, (to minimize the number of intercellular contacts) I find it extremely difficult to isolate enough cells for TUNNEL analysis. To trouble-shoot this problem, I have tried isolating cells from a number of plates, but I am still having problems acquiring enough cells to use for biochemical analysis.

Task 2. Determine subcellular localization of active (vs inactive) forms of p66-Shc and compare this to subcellular localization of p52/p46 Shc. Confocal innunofluorescence microscopy and sub-cellular fractionation will be the major approaches employed.

I have looked at the compartmentalization of p66-Shc in the cells that are forced to express the protein. Preliminary results suggest that p66-Shc is located in both the cytoplasm and the nucleus. This is very surprising; therefore I have obtained well known cytoplasmic, nuclear, and plasma membrane protein markers to ensure that I am not getting the cross-contamination of compartments. I expect to have those answers in the coming weeks.

I plan on doing the confocal microscopy studies after I obtain results from the cell fractionation experiments. The cell fractionation results may shed light on the unique function of p66-Shc in our breast cell line.

Specific Aim 3. To identify biochemical mechanisms whereby p66-Shc inhibits cell proliferation and tumorgenicity.

Task 3. Use of the novel "protein array chip" technology to provide leads to signaling pathways and cellular processes that have been affected by p66-Shc overexpression. Confirm by immoprecipitation.

We have not yet addressed protein interactions using the novel "protein-array" chip technology. Dr. Eugene Chin, a professor in my department, is working with this method. As stated in my grant proposal, I plan on collaborating with him on this aspect of the project. I plan to address this issue in the next year.

Task 4. Determine the effects of p66-Shc on the binding of proteins (e.g. Grb2) to endogenous p52/p46 Shc isoforms. Isolate and identify proteins (by immunoblotting and by Mass Spectrometry) that interact with active and inactive forms of p66-Shc.

Preliminarily, by co-immunoprecipitation, my data suggests that p66-Shc does not compete with p52-Shc for binding to Grb2. My data suggests that Grb2 binds p66-Shc and p52-Shc to the same extent when cells are grown under both adherent and non-adherent conditions. I have been unable to immunoprecipitate Shc and stain for Grb2 because of contaminating low molecular weight immunoglobulin bands which run similarly to Grb2 in SDS-PAGE.

Since it appears that Grb2 sequestration of p66-Shc is not the mechanism for p66-Shc inhibition of cell growth in our cell line, it is possible that some aspect of the inhibitory phenotype is due to other unknown proteins preferentially binding to p66-Shc. I have begun to explore this possibility by metabolically labeling p66-Shc with ^{35}S-Met. Five proteins, p22, p28, p46, p50, and p85 appear to be preferentially binding to p66-Shc. Since I have not yet acquired p66-Shc and CH2 domain mutants, I have been unable to address differences in p66-Shc interacting proteins compared with those proteins that interact with mutants. I hope to address this issue in the next 12-18 months, or as soon as I obtain those mutant constructs from the other laboratory.

Recent data suggests that under conditions of oxidative stress, p66-Shc causes apoptosis in a number of cell lines [4]. In *p66shc*$^{-/-}$ cells the activity of the mammalian forkhead homolog, FKHRL1, is increased and forkhead inactivation is reduced. The activation of FKHRL1 allows for shuttling of the protein to the nucleus to activate the oxygen radical scavenger Catalase. Catalase functions to neutralize oxygen radicals, and therefore, inhibits apoptosis in these cells [6]. It is thought that the presence of p66-Shc induces the phosphorylation of FKHRL1 either directly or indirectly through AKT, which inactivates FKHRL1 causing the protein to remain in the nucleus. I have assayed this pathway in our cell line forced to express p66-Shc by looking at the phosphorylation of AKT (pAKT) and Forkhead (pFKHRL1). My data suggests that p66-Shc expression does not affect the levels of pAKT or pFKHRL1 when cells are plated under anchorage dependent or anchorage independent conditions (after 24 hours of growth under anchorage independent conditions) in our breast cancer cell lines.

Task 5. Prepare manuscript for publication and successfully write and defend my doctoral dissertation.

I have not yet prepared a manuscript for publication. I have attended a number of seminars that are required by the department of Molecular Biology, Cellular Biology and Biochemistry to fulfill requirements for the PhD degree. I have attended weekly departmental seminars. Our department hosts a number of speakers throughout the academic year to speak about their research to the Brown University students, faculty and staff. I have also attended and presented in a weekly student-led journal club. Students find and prepare a 30 minute presentation of an article of great significance.

I have attended in a number of hospital based seminars which have added to my scientific development. I have attended and presented in monthly signal transduction meetings which are held at Rhode Island Hospital. In this meeting, professors, researchers and students present original research. I have also participated and presented in a weekly hospital-run journal club where members of the hospital, from varying disciplines, come together and present original work or journal articles that within varying fields. I have also attended hospital wide seminars and learned about apoptosis and stem cell development.

I have attended and participated in the annual Molecular Biology, cellular biology and Biochemistry retreat, which includes presenting original research in a poster session. I have also met with my graduate committee in May of 2002 where I successfully wrote and defended a grant proposal for my graduate research.

Specific Aim 4. To study the effects of expressing p66-Shc in SKBR3 and MDA-MB-453 cells on their tumor biology. Questions that will be addressed include: Does p66 expression inhibit tumorgenicity in mouse xenografts, as well as colony formation in soft agar? How is it that p66-Shc does not inhibit tumorgenicity of MCF-7 or MDA-MB-231 breast cancer cells? What is the

mechanism whereby cell aggregation on polyHEMA plates seems to partially circumvent p66-Shc's ability to block anchorage independent growth?

I have not yet addressed this specific aim. I plan on addressing these issues in the coming months.

KEY ACCOMPLISHMENTS

- Data detailed in last years grant application suggested that forced expressional of p66-Shc inhibits growth of certain breast cancer cell lines when grown under anchorage independent conditions. Preliminary evidence suggests that p66-Shc also confers a growth advantage when cells are grown anchorage independently under semi-hypoxic (2% O_2) conditions. When p66-Shc expressing MDA-MB-453 cells were grown under anchorage independent conditions in a semi-hypoxic environment, those cells grew while the parental MDA-MB-453 did not grow. This is opposite to what happens under normal conditions.
- Using commercially available phospho-serine p66-Shc monoclonal antibody, primary evidence suggests that p66-Shc (in MDA-MB-453) is not serine phosphorylated in cells in our cells grown under anchorage-dependent and anchorage-independent conditions.

REPORTABLE OUTCOMES

- Poster presentation at the annual Molecular Biology, Cellular Biology and Biochemistry retreat which takes place at Heffenreffer Estate in Bristol, Rhode Island.
- Published abstract for the Era of Hope Department of Defense Breast Cancer Research Meeting. The abstract number is P37-8.
- Post presentation (P37-8) at the Era of Hope Department of Defense meeting in Orlando Florida.

CONCLUSIONS

We have shown that forced expression of p66-shc inhibits breast cancer cell growth when they are grown under anchorage independent conditions in normoxic (20% oxygen) conditions. Under semi-hypoxic conditions (2% oxygen), p66-Shc appears to confer a growth advantage to MDA-MB-453 cells. Additionally, we have demonstrated that p66-Shc does not compete with p52-Shc for binding with Grb2. Also, pAKT levels are similar in parental and p66-shc expressing cells. Likewise, our data suggests that there are no changes in Forkhead phosphorylation levels in cells that are forced to express p66-Shc.

In addition to the proposed work, I have begun investigating other mechanisms for the growth inhibitory phenotype. First, I am studying growth factor receptor downregulation and tyrosine phosphorylation. I am interested in whether breast cancer cells forced to express p66-Shc contain fewer growth factor receptors, or more likely, are less able to respond to growth factor signaling using immunoprecipation techniques. Second, I am investigating $p21^{cip1/waf1}$ expression levels. p21 has been shown to have a role in cell growth inhibition and apoptosis in HER2/NEU overexpressing cell lines [7-9]. I am now asking questions to determine whether p21 plays a pivotal role in the inhibition of growth phenotype in breast cancer cells forced to express p66-Shc. Third, I am investigating whether p66-Shc cells have different growth factor expression levels. Utilizing conditioned media experiments, I am looking at the growth response of cells forced to express p66-Shc.

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