AD________________

Award Number: DAMD17-02-1-0623

TITLE: Exploring a Link Between NF-κB and G_2/M Cell Cycle Arrest in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Shelly M. Davis

CONTRACTING ORGANIZATION: University of Wisconsin-Madison
Madison, Wisconsin 53706-5014

REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040901 044

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
April 2004

3. REPORT TYPE AND DATES COVERED
Annual Summary (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Exploring a Link Between NF-κB and G_2/M Cell Cycle Arrest in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-02-1-0623

6. AUTHOR(S)
Shelly M. Davis

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin-Madison
Madison, Wisconsin 53706-5014

E-Mail: swuerzbe@students.wisc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The purpose of this grant is to understand how activation of the NF-κB/Rel family of transcription factors leads to breast cancer cell survival following treatment with radiation. The NF-κB/Rel family of transcription factors are known to greatly affect survival of various cancer cell types, including breast cancer cells. Our hypothesis is that activation of NF-κB in breast cancer cells contributes to a G_2/M cell cycle arrest, affording these cell extra opportunity to repair damaged DNA and thus allowing them to evade death inducing effects of radiation. Cell cycle analysis and levels of apoptosis were determined following exposure to ionizing irradiation. Cells capable of NF-κB activation efficiently arrested in G_2/M cell cycle phase while those that are not capable of activating NF-κB, do not efficiently arrest. Using RPA analysis, we identified a gene, $p21^{Waf1/Cip1}$, and have now shown that it is involved in maintaining the G_2/M phase cell cycle arrest following IR. Through the use of stable RNAi interference, we found that the G_2/M arrest is partially dependent on $p21^{Waf1/Cip1}$. Understanding how NF-κB is activated and how NF-κB provides protection from cell death will be important for designing strategies to circumvent this resistance mechanism to improve efficacy of radiation therapy.

14. SUBJECT TERMS
NF-κB, apoptosis, cell cycle arrest, breast cancer

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..1

SF 298..2

Table of Contents..3

Introduction..4

Body..4

Key Research Accomplishments..6

Reportable Outcomes..6

Conclusions..6

References..

Appendices...

Annual Summary Report

I. Introduction

Background: The nuclear factor κB (NF-κB) family of transcription factors regulates expression of genes critical for multiple biological processes, including immune responses, inflammatory reactions, cell proliferation, cell differentiation, and apoptosis. Traditionally, activation of NF-κB involves an extracellular signal that disassociates the inhibitor protein IκBα from NF-κB, allowing its translocation to the nucleus. Recently, NF-κB has been shown to be activated by DNA damaging agents which cause DNA double-strand breaks (DSB) in several tumor cell lines, including breast cancer lines. These DNA damaging agents are the same chemotherapeutic agents used in the treatment of breast cancer. NF-κB activation has been linked to both cell survival and cell death by inducing the expression of anti-apoptotic genes and apoptotic genes, depending on cell types and inducing agents. Surprisingly, studies in our laboratory further demonstrated that there are many cancer cell lines that fail to activate NF-κB by DSB-inducing agents, indicating that this activation pathway is not a universal phenotype of all cancer cell types. In addition, many normal human cell types fail to activate NF-κB by several DSB inducing agents. Thus, our studies suggest that NF-κB activation by certain DSB-inducing agents may be an "acquired" phenotype of certain malignant cells.

Objective/hypothesis: The purpose of this proposal is to further understand the mechanisms of NF-κB related G_2/M cell cycle arrest. We believe NF-κB can be activated by DSB-inducing agents and that this activation leads to protection from apoptosis by initiating a cell cycle arrest, allowing more opportunity to repair damaged DNA and resistance to anti-cancer agents.

II. Body

Task 1: To determine the role of NF-κB in cell cycle regulation and radiosensitivity.

Sub-tasks (a), (b), and (c) had been completed and outlined in the previous summary. We continued to work on the other sub-tasks. We determined the amount of apoptosis occurring in both populations by Annexin V staining (sub-task (d)). Table 1 outlines the amount of apoptosis as determined by positive Annexin V staining using the FacsVantage flow cytometer. As expected, less apoptosis is occurring in GFP (+) cells, the cells that have activated NF-κB, versus GFP(-) cells, the cells that have not activated NF-κB. We are currently working on determining radiosensitivity of the MDA and MDA S32/36A cells, those developed in sub-task (b) by colony forming assays.

Table 1:

GFP	Control				TNF				VP16				IR			
	-		+		-		+		-		+		-		+	
	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)	% Total Cells	Annexin (+)
6 Hours	90	7	1.5	5	80	14	7	9	70	37	5	13	68	9	5	9
24 Hours	92	4	1	6	72	14	8	12	47	70	5	42	47	73	6	17
48 Hours	94	9	1	4	80	9	8	9	21	80	2	63	18	78	6	23

Table 1: CEMκB cells were treated with 10ng/ml TNFα, 10μM VP16 or 20 Gy of IR and allowed to recover. At each timepoint cells were fixed in 95% ETOH, labeled with FITC-conjugated Annexin V, and analyzed on the flow cytometer for FITC and GFP expression. The percentage of GFP (+) and (-) cells were determined for each treatment along with the percentage of cells that labeled with Annexin V.

Task 2: To identify the NF-κB target gene which may lead to cell cycle and apoptosis differences.

Last progress report, we identified $p21^{WAF1/Cip1}$ as a target of NF-κB by RPA analysis (sub-task (a)). We had compared CEM cells to CEM S32/36A cells and showed that p21 was induced following irradiation in the CEM cells, but not in the NF-κB deficient S32/36 A cells. We were also able to show that p21 protein expression was induced in MDA cells but not in MDA S32/36A cells. The consensus sequence for the predicted NF-κB site in the p21 promoter is: 5'-gcccctatttgggactccccagtctctttc-3'. We now have shown that NF-κB could bind to a consensus sequence in the p21 promoter (Figure 1, sub-task (b)).

Figure 1:

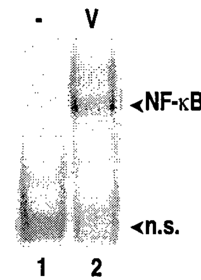

Figure 1: CEM cells were irradiated with 20 Gy of IR. Total cell extracts were incubated with the putative NF-κB binding site in the p21 promoter.

We knocked down $p21^{waf1/cip1}$ expression through the use of stable RNA interference both in CEM T leukemic cells and MDA breast cancer cells. We utilized the pSilencer vector purchased from Ambion Technologies. Due to the low transfection efficiency of MDA cells, traditional RNA interference by transient transfection was not possible. The pSilencer vector containing two DNA oligos designed for knocking down p21 was constructed following the manufacturer's protocal in which the 19-nucleotide sense and antisense (pSil-p21) are linked. A scramble control was also made (pSil-Scr). As seen in Figure 2, we were able to partially inhibit the induction of p21 following treatment with 20 Gy of IR. Cell cycle profiles gathered from the two cell lines displayed a decreased G_2/M arrest when p21 induction was blocked (Figure 3).

Figure 2:

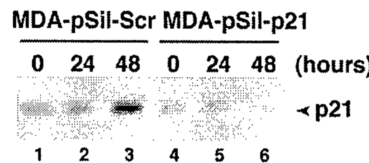

Figure 2: MDA and MDA-pSil-p21 cells were irradiated with 20 Gy of IR for the times indicated. Western Analysis was performed to monitor p21 protein levels.

Figure 3:

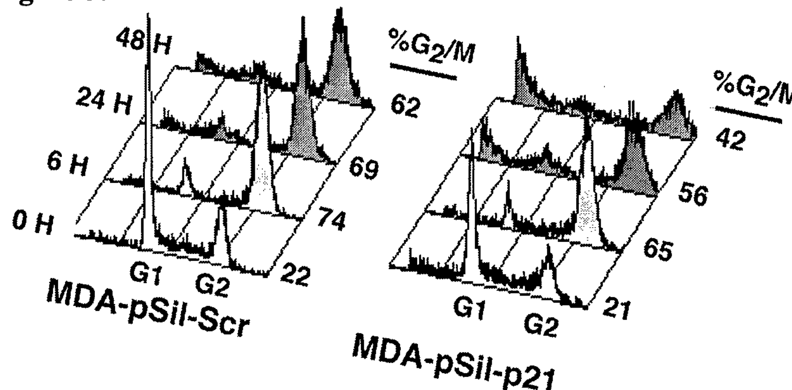

Figure 3: MDA-pSilencer-Scr and MDA-pSilencer-p21 cells were treated with 20 Gy of IR. Cells were fixed at the indicated timepoints and cell cycle profiles were determined by PI staining followed by FACS analysis.

The same phenomenon was observed in CEM human T leukemic cells transfected with pSil-Scr and pSil-p21, indicating that MDA cells were not the only cell line with this phenotype. However, this is not a universal phenotype, because p21 induction is not NF-κB dependent in HEK 293 cells.

III. Key Research Accomplishments and Conclusions

1. NF-κB was able to bind to a putative NF-κB consensus site in the $p21^{WAF1/cip1}$ promoter.

2. A clonal MDA breast cancer cell line and a CEM T leukemic cell line harboring the RNA interference vector specifically knocking down p21 were developed.

3. Cell cycle profiles of MDA versus MDA pSilencer p21 knockdown cell lines following treatment with 20 Gy of irradiation indicated that MDA-pSil-Scr cells display a more abundant and prolonged G_2/M arrest than MDA-pSil-p21 cells, showing a dependence on p21 for the maintenance of the G_2/M arrest.

III. Reportable Outcomes

1. Sequential modification of NEMO/IKKγ by SUMO-1 and ubiquitin mediates NF-κB activation by genotoxic stress. Huang TT, **Wuerzberger-Davis SM**, Wu ZH, Miyamoto S, *Cell.* 2003 Nov 26;115(5):565-76.

2. Enhanced cancer cell survival by the maintenance of G_2/M cell cycle arrest via NF-κB-dependent $p21^{waf1/cip1}$ induction. **Shelly M. Wuerzberger-Davis**, Pei-Yun Chang, and Shigeki Miyamoto (in submission).

V. Conclusions

Task 1: We have shown that there is a NF-κB dependent G_2/M cell cycle arrest in MDA human breast cancer cells. We will continue to work on experiments outlined in the approved statement of work. Annexin V labeling revealed that less apoptosis occurs in GFP(+) versus GFP(-) CEMκB Cells. We are currently working on determining the radioresistance of both cell lines, MDA and MDAS32/36A by colony forming assays along with MDA-pSil-Scr and MDA-pSil-p21 in order to see if there is a growth advantage in cells that maintain a prolonged G_2/M arrest.

Task 2: We have shown that $p21^{WAF1/cip1}$ is a target of NF-κB through promoter analysis and electrophoretic mobility shift assays. Through the use of stable RNA interference, we were able to show that loss of $p21^{WAF1/cip1}$ does correlate with a reduction in the G_2/M cell cycle arrest as does loss of NF-κB activation. However, this reduction is only partial. One reason may be due to the fact that the knockdown produced by the pSilencer vector is not complete. Moreover, we do not believe that $p21^{WAF1/cip1}$ is the only gene involved. We are currently preparing RNA to due affimetrix chip analysis (subtask (c)). We will be looking for differences in gene induction following VP16 treatment between parental cells and those transfected with the super-repressor S32/36A-IκBα construct.

Breast cancer is one of the most common forms of cancer among women. Efficacious treatment of breast cancer patients is one of the most urgent goals. While current treatment regimens are effective, some cancer cells escape the death inducing effects of anticancer treatments. Activation of the transcription factor NF-κB has been implicated as one mechanism contributing to cancer resistance. However, mechanisms by which NF-κB contributes to cancer resistance are not well understood. We have obtained evidence that NF-κB activation by irradiation can cause a prolonged G_2/M cell cycle arrest in the human breast cancer cell line, MDA-MB-231. The elucidation of the mechanisms involved in cell cycle regulation by NF-κB after DNA damage will help us not only better understand how to increase the efficacy of breast tumor treatments, but also lead to the identification of a novel gene, such as $p21^{waf1/cip1}$, whose protein product may prove to be a better target for breast cancer therapy.