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Award Number: DAMD17-97-1-7162

TITLE: Induction of Apoptosis in Human Breast Cancer by
Adenoviral-Mediated Gene Transfer of the Transcription
Factor E2F-1

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REPORT DATE: August 2000

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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20010504 111

REPORT DOCUMENTATION PAGEForm 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**
August 2000**3. REPORT TYPE AND DATES COVERED**
Annual (1 Aug 99 - 31 Jul 00)**4. TITLE AND SUBTITLE**

Induction of Apoptosis in Human Breast Cancer by Adenoviral-Mediated Gene Transfer of the Transcription Factor E2F-1

5. FUNDING NUMBERS

DAMD17-97-1-7162

6. AUTHOR(S)

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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**

This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

We have previously shown that the transcription factor E2F-1 results in apoptotic cell death in breast cancer cells when overexpressed in using an adenoviral-mediated system. In order to determine whether the E2F-1 transgene is effective in sensitizing cells to chemotherapy-induced apoptosis, we utilized a recombinant adenovirus in combination with Taxol or doxorubicin and evaluated our results using a two-dimensional isobologram statistical analysis. We observed marked synergistic growth inhibitory effects in breast cancer cell lines treated with a low-dose of adenovirus E2F-1 and low doses of Taxol or doxorubicin. In conclusion, adenovirus-mediated expression of E2F-1 can inhibit breast cancer cell growth synergistically in combination with low-dose Taxol. This combination of gene therapy and chemotherapy may lower the dose of chemotherapeutic agents necessary in the treatment of breast cancer patients and thus may reduce the adverse effects seen with chemotherapy treatments. We plan to explore this further in an animal model using breast cancer xenografts in nude mice.

14. SUBJECT TERMS

Breast Cancer, Apoptosis, E2F-1, Chemotherapy

15. NUMBER OF PAGES

20

16. PRICE CODE**17. SECURITY CLASSIFICATION OF REPORT**

Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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Introduction

Breast cancer continues to be one of the leading causes of death in American women today. Recent changes in the treatment strategies of breast cancer patients have improved the survival rates in American women who develop breast cancer. We continue to see however, over 45,000 deaths each year from metastatic breast cancer. Systemic therapy has been more frequently employed in the treatment of breast cancer patients and recently the overview analysis published by the Early Breast Cancer Trialists Group has shown that systemic chemotherapy can significantly reduce the risk of recurrence and death from breast cancer in both premenopausal and postmenopausal women¹. Current systemic agents utilized are combination regimens with doxorubicin-based chemotherapy and paclitaxel (Taxol) alone or in combination with Adriamycin. These chemotherapeutic agents result in improvements in overall survival and disease-free survival in breast cancer patients, however they are not without significant morbidity. We have previously shown that the E2F-1 transcription factor can result in apoptotic cell death in breast cancer cells *in vitro* when overexpressed using a recombinant adenovirus vector². We have also shown that E2F-1 results in marked apoptotic cell death in breast cancer cells independent of the *p53* status and independent of the *Rb* status. In this report we discuss results obtained in year 3 of this project where we sought to evaluate the results of combination therapies of E2F-1 (AdVE2F) and chemotherapeutic agents on the growth of breast cancer cells *in vitro*. We show here that when E2F-1 is delivered in low-doses it acts synergistically with Taxol and doxorubicin to result in growth inhibition in breast cancer cells.

Body

I. Methods

Three different breast carcinoma cell lines were utilized for the studies described in this report. The three cell lines are MDA-MB-361, MDA-MB-468, and SKBr3. Experiments performed included XTT assay for cell viability, FACS analysis for percent subdiploid cells and analysis of changes in cell cycle profile, and 2-D isobologram analysis to determine the additive or synergistic nature of combination therapies. For the XTT assay cells were added to a 96 well plate at a concentration of 5×10^4 cells/well. After the cells attached to the wells, they were then infected with a recombinant adenovirus expressing the luciferase reporter gene (AdVLuc) or a recombinant adenovirus expressing the E2F-1 gene (AdVE2F) at a multiplicity of infection (MOI) ranging from 10:1 to 100:1. Eight hours following infection with the recombinant adenoviruses, the chemotherapeutic agents were added at different concentrations. Paclitaxel was added at concentrations of 0, 0.1, 0.5, 1, 2.5, 5, 10, and 20 nanomolar. Doxorubicin was utilized at concentrations of 0, 1, 5, 10, 50 and 100 nanomolar. Each of the chemotherapeutics were added to wells with untreated cells (control), wells containing cells with AdVLuc (Luc) and wells containing cells treated with AdVE2F (E2F). XTT assay was performed 24 hours after the addition of the chemotherapeutic agents. Cells were harvested for FACS analysis at 24 hours following treatment with the chemotherapeutics.

II. Results

- a. **CELL CYCLE ANALYSIS.** Listed below are the results of cell cycle analysis for each of the three breast carcinoma cell lines after treatment with the recombinant adenoviruses and doxorubicin. Cell cycle changes were most marked in the SKBr3 cell line where we noted a marked increase in the G2/M phase. This was most notable at a doxorubicin concentration of 100 nanomolar. There was a concomitant decrease in the percentage of cells in G1 phase and S phase.

MDA-MB-468 Doxorubicin Concentration	% G1 phase					% G2/M phase					% S phase				
	0	1	5	10	50	0	1	5	10	50	0	1	5	10	50
Control	39.1	34.1	22.9	4.9	0.6	22.8	20.6	27.4	33.2	67.7	38.1	45.3	49.7	33.2	31.7
Luc	36.2	37.8	21.9	9.1	1.7	22.6	23.3	35.8	27.4	70.3	41.2	39	42.3	27.4	28
E2F-1(MOI 1:1)	35.8	35.1	25.3	7.3	1.5	23.6	26.6	43.5	55	86	40.6	38.3	43.5	37.7	12.9

MDA-MB-361 Doxorubicin Concentration	% G1 phase							% G2/M phase							% S phase						
	0	1	5	10	50	100	0	1	5	10	50	100	0	1	5	10	50	100			
Control	48.4	58.7	56.6	56	28.8	23.4	20.2	10.9	11	12.2	28.3	14	31.4	30.3	32.5	31.9	43	62.6			
Luc	61.2	58.2	59.4	54.6	31.1	22.3	10.9	10.3	10.3	12	28.8	11.9	27.9	31.5	30.3	33.4	40.1	65.8			
E2F-1	62.1	61.7	58.3	57.1	32.8	27.9	10.7	11.3	10.6	13.7	23.8	24.1	27.2	27	31	29.2	43.4	48			

SKBr3	G1 phase					% G2/M phase					% S phase				
	0	1	5	10	100	0	1	5	10	100	0	1	5	10	100
Doxorubicin Concentration															
Control	55.6	52	49.9	35.3	29.6	13.8	15.8	17.5	37.4	4.6	30.7	32.1	32.6	27.3	65.8
Luc	54.9	53.3	48.8	36.8	22.4	12.3	13.4	17.5	34.7	10.2	32.8	33.3	33.7	28.5	67.4
E2F-1	54.6	56.3	51.5	35	1.8	33.2	33.5	30.7	36.3	68.4	33.2	33.5	30.7	28.7	29.9

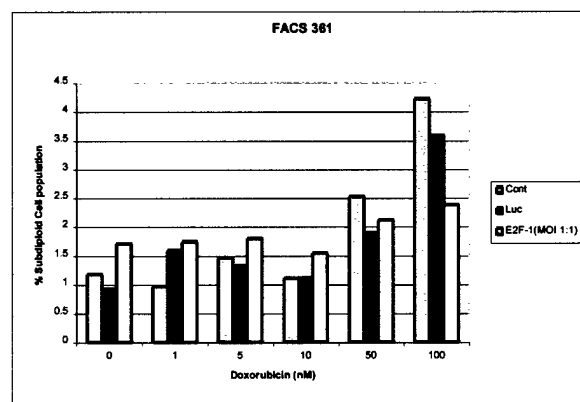
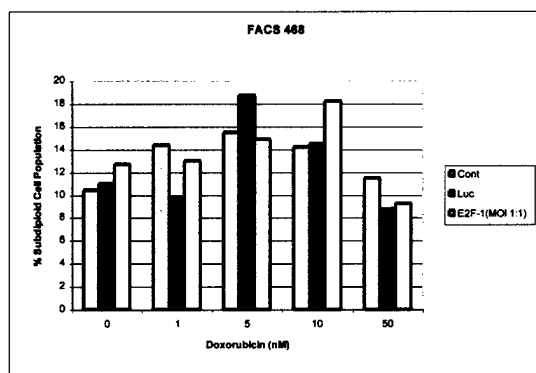
Shown below are the percent changes in cell cycle phases seen in 2 of the breast cancer cell lines following treatment with the recombinant adenoviruses and taxol. Data is shown for the MDA-MB-468 and MDA-MB-361 cell lines. We noted a slight increase in the G2/M phase in the E2F-treated cells.

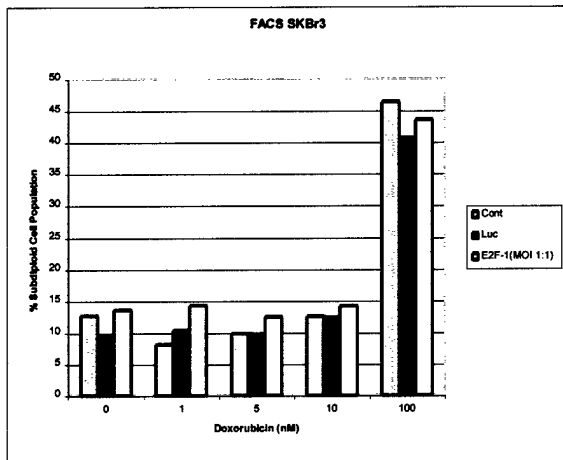
468	% G1 phase				% G2/M phase				% S phase			
	0	0.1	0.5	1	0	0.1	0.5	1	0	0.1	0.5	1
Taxol Concentration												
Control	45.3	48	45.3	44.2	19.3	18.1	19.7	18.9	35.4	33.9	35	37
Luc	43.7	49.4	47.1	49.9	20.7	18.1	20.1	16	20.7	18.1	20.1	16
E2F-1(MOI 1:1)	50.5	46.9	52.7	49.7	16.2	17.3	14.4	18	16.2	17.3	14.4	18

361	% G1 phase						% G2/M phase						% S phase					
	0	0.1	0.5	1	5	10	0	0.1	0.5	1	5	10	0	0.1	0.5	1	5	
Taxol Concentration																		
Control	49.2	53.5	48.2	50.7	32.1	36.2	18.4	12.2	12.1	12.1	12.2	7.6	32.5	34.3	39.7	37.2	55.7	
Luc	44.7	45.4	41.3	42.2	26	20.9	17.9	16.4	17.7	15	20.3	14.8	37.4	38.2	41	42.8	53.6	
E2F-1	53.9	52.4	51	49.7	27.6	20.7	15.7	15.9	14.3	16.5	21.5	23.4	30.4	31.6	34.7	33.8	51	

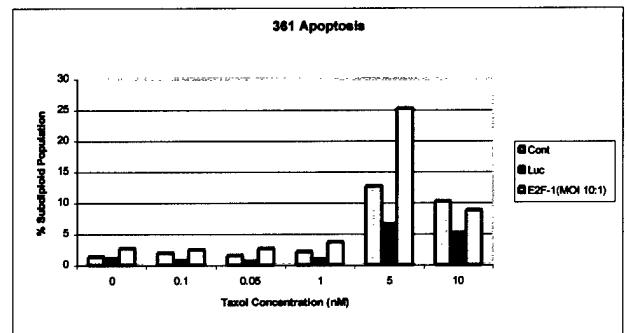
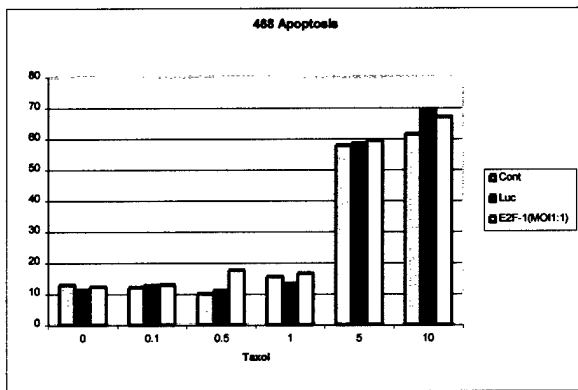
b. ANALYSIS OF SUBDIPLOID CELL POPULATION

Shown below are graphs of the % subdiploid cells following treatment with the recombinant adenoviruses at an MOI of 1:1 followed by treatment with doxorubicin at different concentrations. The cell lines shown are MDA-MB-468, MDA-MB-361, and SKBr3. We did not note a significant increase in subdiploid cells at any of the cell lines except the MDA-MB-468 with doxorubicin concentration at 10 nM. It is possible that we would see an increase in subdiploid cells if we performed the analysis at 48 hours as opposed to 24 hours. We do plan to do these experiments in the near future.





Shown below are graphs of the MDA-MB-468 and MDA-MB-361 cell lines following treatment with the recombinant adenoviruses at an MOI of 1:1 followed by treatment with taxol at varying doses. We did note a significant increase in subdiploid cells in the MDA-MB-361 cell line at a taxol concentration of 5 nM.

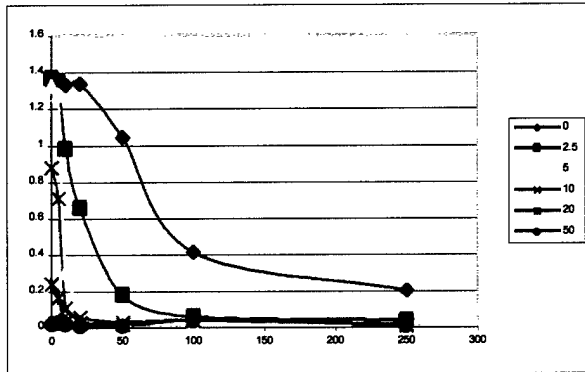


We plan to perform additional assays to evaluate for evidence for apoptotic cell death including TUNEL staining of cytospin preparations and western blot analysis for PARP cleavage. Preliminary cytospin analyses do show an increase in TUNEL positive cells in the breast carcinoma cells pretreated with E2F-1 followed by doxorubicin or taxol.

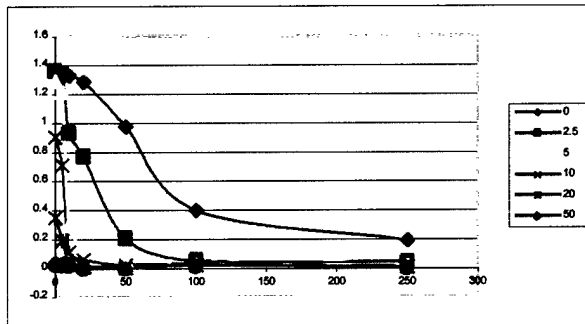
c. Isobolograms

We evaluated all of our combination chemotherapy and recombinant adenoviruses results with 2-D isobolograms and then evaluated for statistical significance using a Wilcoxon Signed-Ranks test. We found that there was synergistic killing when E2F-1 was used in combination with doxorubicin or taxol ($p < 0.05$). Shown below are the 2-D isobolograms.

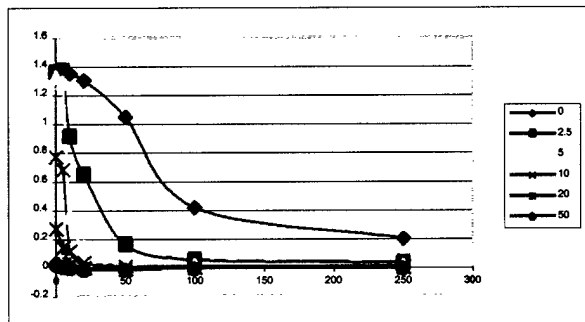
MDA-MB-468 cell line treated with E2F-1 and doxorubicin



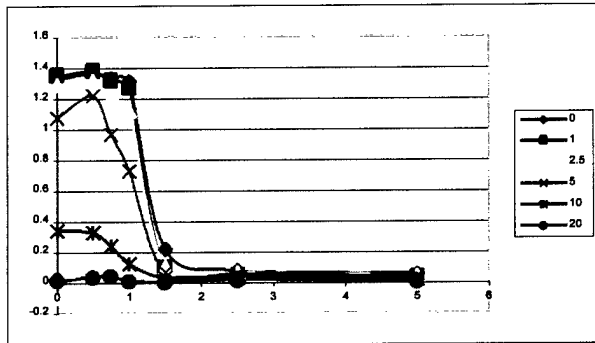
MDA-MB-361 cell line treated with E2F-1 and doxorubicin



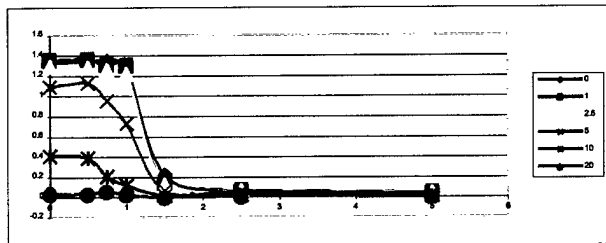
SKBr3 cell line treated with E2F-1 and doxorubicin



MDA-MB-468 cell line treated with E2F-1 and taxol



MDA-MB-361 cell line treated with E2F-1 and taxol



d. Conclusions

Adenovirus mediated overexpression of E2F-1 combined with paclitaxel and doxorubicin results in synergistic cell killing. The synergistic cell killing allows for a decrease in the doses of both the adenovirus E2F-1 and the chemotherapeutic agents. Combining gene therapy strategies with standard chemotherapeutics may improve rates of tumor cell kill and provide alternative treatments for patients who fail standard therapy.

Key Research Accomplishments

- Combination therapy of AdVE2F-1 with taxol and doxorubicin results increased cell death in breast cancer cell in vitro.
- Using a 2-D isobologram method of analysis, we demonstrate here that E2F-1 acts synergistically with taxol and doxorubicin to result in breast cancer cell death.

Reportable Outcomes

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Conclusions

Adenovirus mediated overexpression of E2F-1 combined with paclitaxel and doxorubicin results in synergistic cell killing. The synergistic cell killing allows for a decrease in the doses of both the adenovirus E2F-1 and the chemotherapeutic agents. Combining gene therapy strategies with standard chemotherapeutics may improve rates of tumor cell kill and provide alternative treatments for patients who fail standard therapy.

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