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13. ABSTRACT *(Maximum 200 words)*

Breast cancer is the most common cancer occurring in American women. New therapies are needed for treatment of patients who fail standard treatments. We have sought to develop a gene therapy strategy for the treatment of breast cancer utilizing a recombinant adenovirus vector for delivery of the transcription factor E2F-1. We have previously shown that overexpression of E2F-1 can lead to the induction of apoptosis in breast carcinoma cells.

We have demonstrated that E2F-1 can be expressed in the majority of cell lines tested. There is a differential sensitivity to apoptosis which may be related to uptake of the adenovirus vector or due to the genetic background of the cell. Our work demonstrates that the tumor suppresser *p53* is not required for apoptosis. We have studied the interaction between the retinoblastoma tumor suppresser gene and E2F-1 and shown that pRB partially blocks the apoptosis induced by E2F-1. We are beginning to study apoptotic mediators involved in this E2F-induced apoptotic process. Preliminary results demonstrate that bax is not upregulated, however, bcl-2 is markedly upregulated. We are continuing to assess other apoptotic mediators in addition to cell cycle regulatory proteins in order to better understand the mechanism of E2F-induced apoptosis.

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Kelly K. Hunt 8/30/98

PI - Signature Date

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D. Introduction

Apoptosis, or programmed cell death, is an important part of normal cell growth and regulation. One of the mechanisms by which cancer cells develop a growth advantage over normal cells is by circumventing the apoptotic pathway. Breast cancer is the most common cancer occurring in American women and is responsible for the deaths of over 45,000 women each year. These women die because currently available treatments for breast cancer patients are not effective in all cases. Novel treatment strategies that go beyond our current working model of surgery, chemotherapy, and radiation therapy are desperately needed.

Gene therapy allows for the introduction of a gene or genes into the cell that can result in expression of a protein product that has been previously lost or is not normally expressed by the target cells. We chose E2F-1 as our gene of interest because of its important role in controlling cell cycle progression (1). E2F-1 is a member of a family of transcription factors that is inactivated when bound to the protein product of the retinoblastoma tumor suppressor gene. E2F-1 was initially believed to be an oncogene because it can transform fibroblasts (2). However, more recent studies have shown that E2F-1 may actually function as a tumor suppressor gene. E2F-1 promotes apoptosis in fibroblast systems and in tumor cells (3-6). Furthermore, knockout of the E2F-1 gene in mice leads to spontaneous development of tumors in these animals (7, 8).

We chose an adenovirus vector in order to express E2F-1 at high levels in breast carcinoma cells. We have shown that this overexpression leads to apoptosis in most breast carcinoma cell lines tested. This is also true in ovarian carcinoma, leiomyosarcoma, and *p53*-null fibroblasts. Our data confirms that E2F-1 can induce apoptosis in these tumor cell lines without the requirement for *p53*. This E2F-induced apoptosis can only be partially blocked or inhibited by pretreatment with an adenovirus expressing pRb. It is not affected by overexpression of the tumor suppressor gene *p21 waf1,cip1*. We are beginning to study the apoptotic mediators important in E2F-induced apoptosis in addition to other cell cycle regulatory proteins in order to better understand the necessary factors for successful induction of apoptosis through overexpression of E2F-1. Differences in susceptibility to apoptosis may be useful in designing treatment regimens. This is an important step in the process of developing a gene therapy strategy for the treatment of breast cancer patients.

E. Body of Report

Transduction of breast carcinoma cell lines with adenovirus vectors.

In order to determine the uptake of adenovirus among different cell lines, we initially transduced a panel of cell lines with a recombinant adenovirus vector expressing the *E. coli* β-galactosidase gene (AdVβ-gal). Cells were infected with AdVβ-gal at a multiplicity of infection (MOI) of 100 virus particles to 1 cell. Cells were harvested at 48 hours and then stained with X-gal to determine the percentage of blue stained cells. The results of a panel of cells are listed in table 1 below.

Table 1. Transduction of Cell Lines with AdVβ-gal

Cell Line*	% Blue Cells
MDA-MB-361	70
MDA-MB-468	75
2774	65
SKOV3	48
SKBr3	57
MCF-7	50
BT483	50
BT549	61
HBL100	90
MCF10A	50

*Legend
Breast carcinoma cell lines (MDA-MB-361, MDA-MB-468, SKBr3, MCF-7, BT483, BT549)
Ovarian carcinoma cell lines (SKOV3, 2774)
Breast epithelial cell lines (HBL100, MCF10A)

All of the cell lines tested demonstrated at least 50% transduction as assessed by percent blue stained cells with X-gal staining. The transduction efficiency of each individual cell line may affect our ability to determine the effectiveness of E2F-1 following transfer of this gene using a recombinant adenovirus.

Transduction of cell lines with a recombinant adenovirus expressing the transcription factor E2F-1.

Cell lines were transduced with a recombinant adenovirus vector expressing the transcription factor E2F-1 (AdVE2F), an empty adenovirus vector (AdV) or untreated (mock infection). Cells were infected at a MOI of 100, cultured under standard cell culture conditions and then collected for analysis at 48 hours. Cells were suspended in propidium iodide solution and then analyzed for DNA content on a fluorescence activated cell sorting (FACS) instrument (Epics Profile, Coulter Corp., Miami, Fla.). The subdiploid cell population was calculated and recorded as percentage of apoptotic cells. DNA fragmentation assays were performed on each of the cell lines and confirmed apoptotic cell death by demonstration of DNA ladders in the E2F-1 treated cells (data not shown). Cell cycle analysis was also performed on each of the treated and control cell populations. Cells were harvested for protein extraction in order to perform western blot analysis for expression of E2F-1, cell cycle proteins, and apoptotic mediators. Table 2 below shows the percent apoptotic cells in each of the cell lines tested following treatment with AdVE2F. All but one cell line (MCF-7) showed induction of apoptosis over controls.

Table 2. Transduction of Cell Lines with AdVE2F

Cell Line*	% Apoptosis on FACS Analysis			p53 Status
	Control (Mock)	AdV	AdVE2F	
MDA-MB-361	1	7	52	mutated
SKOV3	11	5	22	mutated
MCF-7	4	6	6	wild-type
SKBr3	5	5	44	mutated
BT483	6	6	15	N/A
HBL100	7	6	23	wild-type
MCF10A	11	12	38	wild-type
BT549	15	8	21	mutated
MDA-MB-468	11	9	69	mutated
MDA-MB-453m1	2	2	8	N/A

*Legend - Cell lines are as listed above for Table 1. In addition, MDA-MB-453m1 (breast carcinoma cell line). N/A - not available.

Induction of apoptosis by AdVE2F was most efficient in the Rb-null MDA-MB-468 cell line (69% at 48 hours) but also occurred in cells with wild-type Rb (SKBr3 44%, BT549 21%, SKOV3 22%). Figure 1 shows a representative FACS analysis profilefor the MDA-MB-468 cell line. Within 48 hours following treatment with AdVE2F there was evidence of a large subdiploid cell population (< 2n DNA content) indicative of apoptotic cell death.

All cell lines tested demonstrated overexpression of E2F-1 following transduction with AdVE2F. Mock infected controls and empty adenovirus controls showed minimal expression levels of E2F-1. Figure 2 demonstrates the marked overexpression seen in a representative western blot analysis using 3 breast carcinoma cell lines. Actin levels were also assessed to demonstrated similar protein loading for each of the western blot analyses. Despite the marked overexpression of E2F-1 in the MCF-7 cell line, there is no evidence for induction of apoptosis in this cell line based on FACS analysis and DNA fragmentation assays.

Assessment of changes in cell cycle following transduction with AdVE2F.

We assessed the percentage of cells in S-phase, G1 and G2/M phase following infection with AdVE2F. Cells were treated with AdVE2F at a MOI of 100 and collected as described for FACS analysis. Table 3 below shows the percentage of cells in each phase of the cell cycle in a representative experiment following treatment with AdVE2F. As expected, we were able to demonstrate a significant increase in the population of cells in S-phase following treatment with AdVE2F.

Table 3. Cell Cycle Changes Following Treatment with AdVE2F

Cell Cycle Analysis	MDA-MB-468 Cells - Treatment Group		
	Control (Mock)	AdV	AdVE2F
% G1	39.7	36.3	17.4
% G2	25.9	28.4	30.6
% S-phase	**34.3**	**35.3**	**52.0**

E2F-induced Apoptosis is only Partially Blocked by Pretreatment with pRb.

The protein product of the retinoblastoma tumor suppressor gene (pRb) is known to bind to E2F-1 and can block E2F-initiated cell cycle progression. We therefore sought to determine whether pretreatment with an adenovirus overexpressing pRb could block E2F-induced apoptosis in breast carcinoma cells. Cells were infected with a recombinant adenovirus containing the retinoblastoma gene (AdVRb) followed 24 hours later by infection with AdVE2F, each at a multiplicity of infection (MOI) of 100. Treated cells were assessed for apoptosis using flow cytometry after propidium iodide staining (subdiploid population). Protein expression was confirmed by Western Blot analysis. Results are summarized in table 4 below:

Table 4. Apoptosis Following Adenovirus Infection

		% Apoptosis		
Cell Line	*RB* status	AdVRb	AdVE2F	AdVRb followed by AdVE2F
MCF-7	Wild-type	9	10	10
SKBr3	Wild-type	7	40	31
MDA-MB-468	Null	20	60	63

Assessment of Apoptotic Mediators Following Infection With AdVE2F

We hypothesized that overexpression of E2F-1 in human breast cancer cell lines induces apoptosis through upregulation of *bax* and downregulation of *bcl-2*. We found, however, that E2F-1 apoptosis occurs without the requirement for wild-type *p53* and without upregulation in *bax* (see Figure 3) and despite significant upregulation in *bcl-2* (Figure 4). We are now investigating the role of other members of the *bcl-2* family to determine their role in E2F-induced apoptosis.

F. Conclusions

We have demonstrated that E2F-1 can be expressed in the majority of breast carcinoma cell lines tested using a recombinant adenovirus. The ability of AdVE2F to induce apoptosis amongst the different cell lines tested may vary depending on the efficiency of adenoviral transfer (transduction efficiency) and the genetic background of the cells. Our work demonstrates that the tumor suppressor gene *p53* is not required for E2F-induced apoptosis in breast carcinoma cell lines. This is contrary to previously published literature which suggests that *p53* is required for apoptosis in fibroblast systems. Although the retinoblastoma tumor suppressor gene is responsible for binding to E2F-1 and controlling E2F-initiated cell cycle progression, we have shown that AdVE2F continues to drive the cells into S-phase and results in apoptotic cell death despite pretreatment with AdVRb. The downstream mediators of E2F-induced apoptosis remain unclear. We did not find upregulation in the proapoptotic mediator bax. We did however find marked upregulation in the antiapoptotic mediator bcl-2. Despite this significant upregulation in bcl-2, AdVE2F treatment results in apoptotic cell death within 48 hours following treatment. It is our plan to continue to explore the apoptotic mediators involved in addition to cell cycle regulatory genes. This mechanistic approach will allow us to better design a gene therapy treatment strategy for breast cancer patients utilizing the transcription factor E2F-1.

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I. Listing of Personnel

1. Principal investigator salary support only.

Appendix A: Figures

Figure 1. Flow Cytometry Profile in MDA-MB-468. Cells were evaluated following mock infection (control) or treatment with AdVE2F.

Figure 2. E2F is Overexpressed in Breast Carcinoma Cell Lines Following Infection with Ad5E2F. Western blot analysis was performed to confirm overexpression in each of the cells lines tested.

Figure 3. *Bax* Expression is Unchanged Following Overexpression of E2F-1. Western blot analysis was performed using antibodies against the proapoptotic mediator *bax*. Cell lines were evaluated 12 hours, 24 hours, and 48 hours following treatment with AdVE2F.

Figure 4. *BCL-2* is Upregulated Following Overexpression of E2F-1. Western blot analysis was performed using antibodies to the antiapoptotic mediator *bcl-2*. Cell lines were evaluated 12 hours, 24 hours, and 48 hours following treatment with AdVE2F.

Figure 1

Flow Cytometry Profile in MDA-MB-468

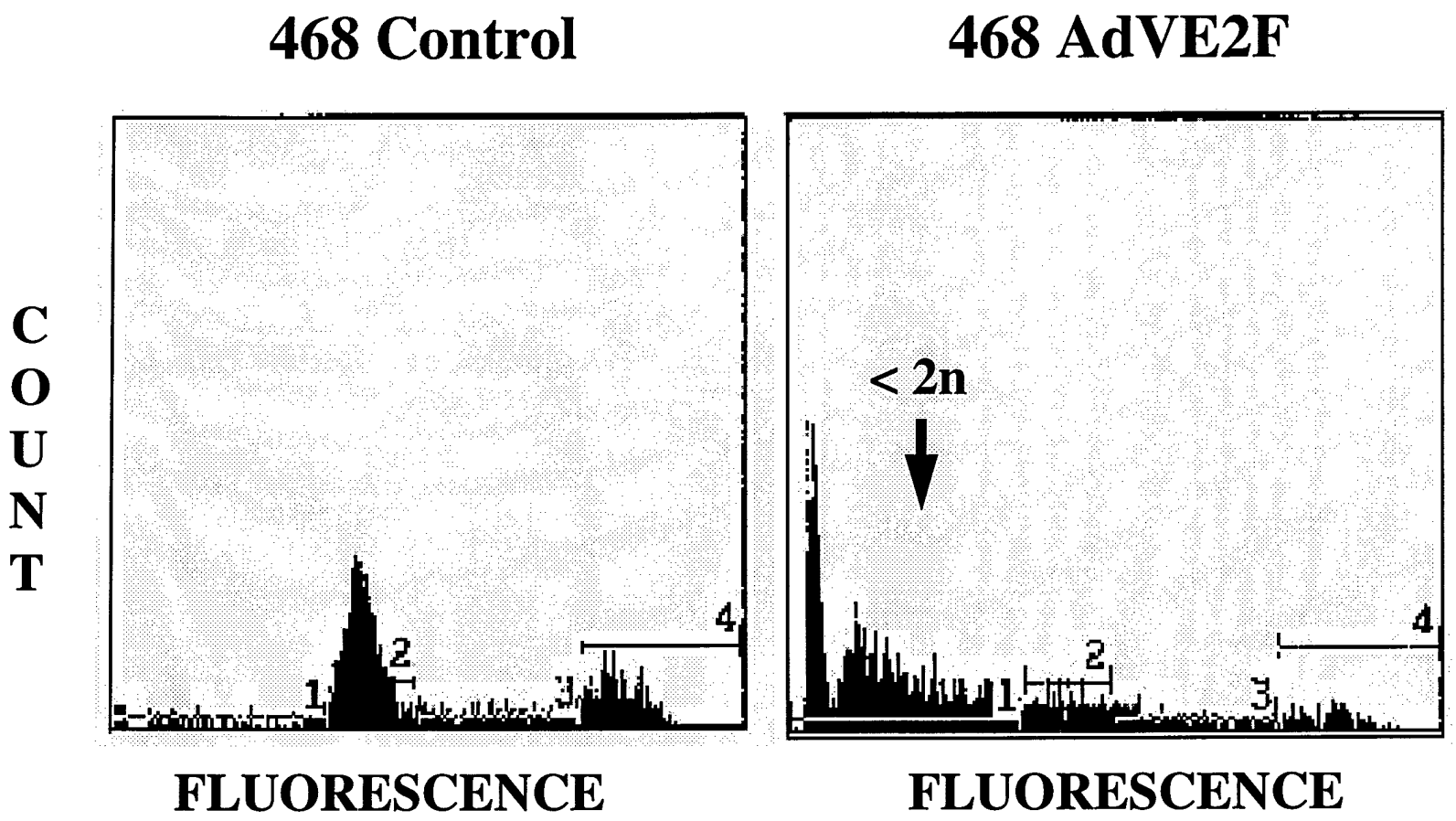

Figure 2

E2F is Overexpressed in Breast Carcinoma Cell Lines Following Infection with Ad5E2F

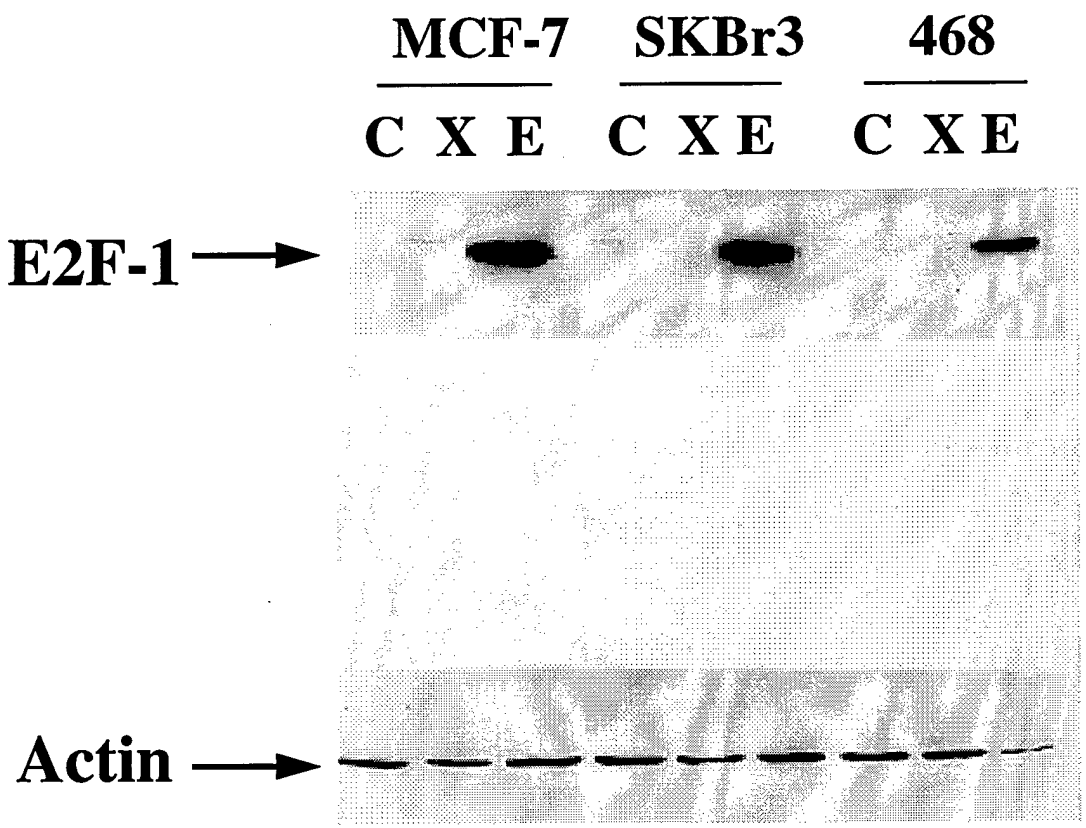

Figure 3

Bax Expression is Unchanged Following Overexpression of E2F-1

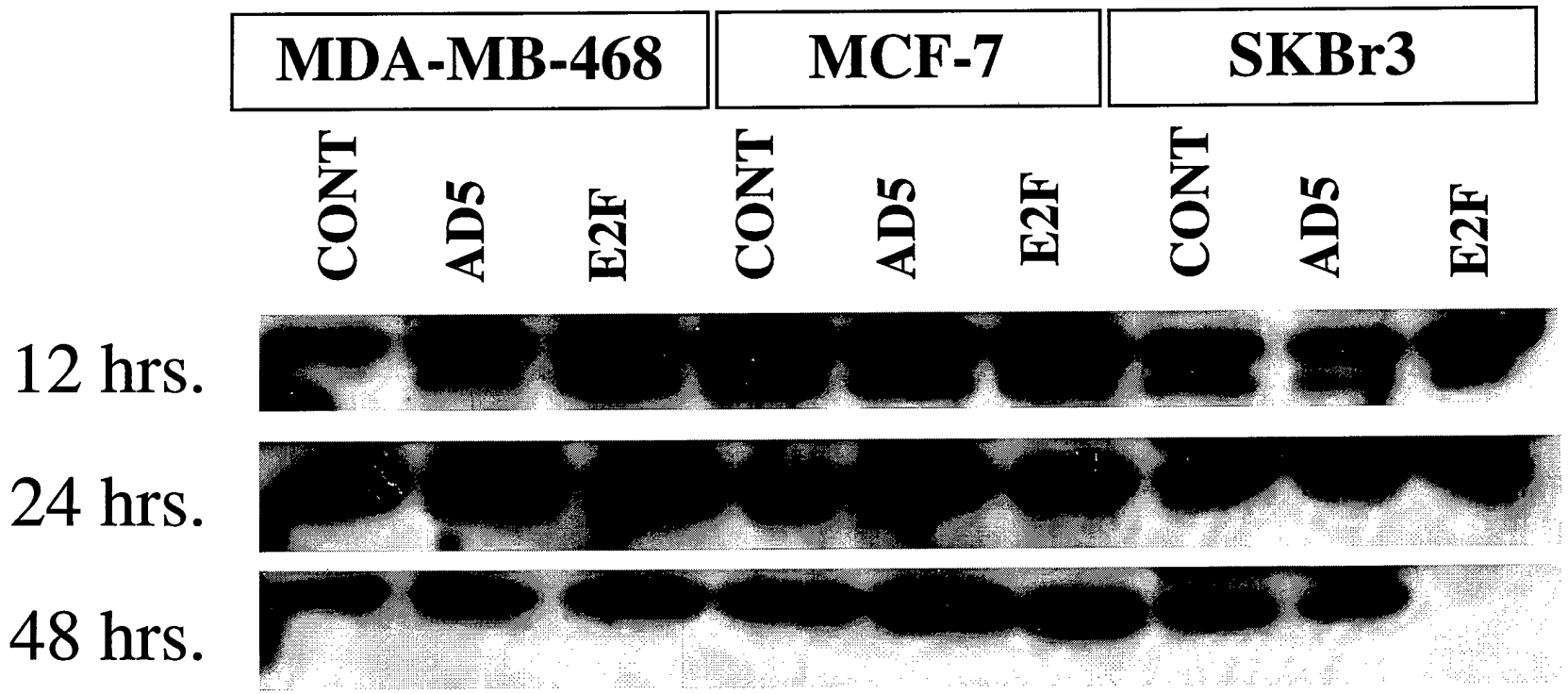

Figure 4

BCL-2 is Upregulated Following Overexpression of E2F-1