Adenovirus-Mediated Overexpression of the Transcription Factor E2F-1 Induces Apoptosis in Human Breast and Ovarian Carcinoma Cell Lines and Does Not Require p53

Kelly K. Hunt, Jiong Deng, Ta-Jen Liu, Marcia Wilson-Heiner, Stephen G. Swisher, Gary Clayman, and Mien-Chie Hung

Departments of Surgical Oncology (K. K. H., M. W.-H., M.-C. H.), Tumor Biology (J. D., T.-J. L., M.-C. H.), Thoracic and Cardiovascular Surgery (S. G. S.), and Head and Neck Surgery (T.-J. L., G. C.). The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Apoptosis is a mode of cell death that is carefully regulated based on cellular and environmental signals. The ability to modulate the individual cellular machinery and thereby to promote apoptosis is an important strategy in cancer therapy. It has previously been shown that overexpression of the transcription factor E2F-1 can induce apoptosis in quiescent rat embryo fibroblasts. This effect has been reported to occur in a p53-dependent manner. To investigate whether overexpression of E2F-1 could also induce apoptosis in human cancer cells, a recombinant adenovirus vector containing the transgene E2F-1 under control of the cytomegalovirus promoter (Ad5CMVE2F) was used to induce high levels of the E2F-1 protein in human breast and ovarian carcinoma cell lines. Significant morphological changes occurred in four of the five cell lines within 48 h of transduction with the Ad5CMVE2F. These changes were consistent with apoptosis, which was confirmed further by DNA fragmentation assay and fluorescence-activated cell sorting analysis. On the basis of these assays, which show that apoptosis occurred in those cell lines with mutations in the p53 gene, we suggest that the induction of E2F-1-mediated apoptosis does not require wild-type p53 when E2F-1 is overexpressed using an adenovirus-based strategy.

Introduction

Cancer cells can develop survival advantages over normal cells by increased proliferation and/or evasion of normal cellular controls, such as programmed cell death (apoptosis). Cancer cells may evade apoptosis by genetic changes that lead to increased expression of survival signals or decreased expression of death signals. The induction of apoptosis in cancer cells may therefore be possible by the introduction and overexpression of genes that are normally transcribed, such as transcription factors, which have the potential to "switch on and off" the expression of many genes.

E2F-1 is a member of a family of transcription factors that activate genes required to enter S phase. Although early studies with E2F-1 suggested that it functioned as an oncogene, recent studies with E2F-1 knockout mice suggest that E2F-1 may actually function as a tumor suppressor gene (1-4). E2F-1 knockout mice demonstrate lymphocyte defects with failure of thymocyte apoptosis and increased lymphocyte proliferation (3). Overexpression of E2F-1 in quiescent fibroblasts has been shown to induce S-phase entry and apoptosis (5). Furthermore, this E2F-induced apoptosis appeared to occur through a p53-dependent pathway (5). Because p53 is commonly mutated in many types of cancers, we evaluated the effect of overexpression of E2F-1 in malignant cells. We found that adenoviral transduction of cell lines from breast and ovarian cancer with Ad5CMVE2F resulted in high but transient levels of E2F-1 protein production. This overexpression of E2F-1 was associated with the induction of apoptosis in the majority of cells tested (four of five cell lines). Four of the five cell lines were p53 mutated, suggesting that the mechanism of E2F-1-induced apoptosis in breast and ovarian cancer cells does not require wild-type p53 when E2F-1 is overexpressed using an adenovirus-based gene transfer strategy.

Materials and Methods

Cell Culture and Adenovirus Vectors. Human breast and ovarian carcinoma cell lines were maintained as described previously (6). Cells were grown in DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS.

Cells were treated with replication defective recombinant adenovirus vectors at a MOI of 100 virus particles to 1 cell (100:1). Adenovirus 5 (Ad5) is a vector control with deletions in E1A and E1B without a transgene. The Ad5CMVE2F vector has been deleted in the E1 subunit and contains the transgene E2F-1 under control of the CMV promoter. Cells were untreated (mock infected), treated with vector control (Ad5), or treated with Ad5CMVE2F and allowed to incubate under standard cell culture conditions for 48 h prior to testing.

Western Blot Analysis. Cells were harvested for Western blot analysis 48 h after treatment with the recombinant adenovirus vectors. After washing the adherent cells twice with PBS, the cells were collected in lysis buffer by scraping the adherent cells from the tissue culture plates. The cell extracts were then centrifuged at 14,000 rpm for 15 min, and the supernatant was collected. Extraction was conducted using the Bio-Rad protein determination method (Bio-Rad, Hercules, CA). Fifteen μg of protein from each sample was loaded onto a 10% acrylamide gel. Gel electrophoresis was performed, and then the protein was transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with a mouse monoclonal IgG antibody to E2F-1 (Santa Cruz Biotechnology, Santa Cruz, CA). An antismouse peroxidase-conjugated antibody was then added for 1 h, followed by incubation with the enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science, Buckinghamshire, England). The nitrocellulose membranes were then exposed to X-ray film, and autoradiographs were obtained. Autoradiographs were used to determine protein expression of E2F-1.

Cell Morphology. Cell morphology was evaluated by standard light microscopic examination of cell cultures. Cells were evaluated for morphological changes consistent with apoptosis at both 24 and 48 h. Morphological changes were scored on a scale of 0 to 3+, with 0 representing no change and 3+ representing significant apoptotic changes (e.g., shrunk cells, apoptotic bodies, floating cells).

The abbreviations used are: MOI, multiplicity of infection; FACS, fluorescence-activated cell sorting; Ad5, adenovirus 5; CMV, cytomegalovirus.
Flow Cytometry. Cell lines were trypsinized and then washed twice with PBS, followed by fixation of the cell pellet in 80% ethanol. At the time of FACS analysis, the cells were pelleted and washed with PBS and then suspended in propidium iodide solution. Cells were then analyzed for DNA content on a fluorescence activated cell sorting instrument (Epics Profile, Coulter Corp., Miami, Fla.). The subdiploid population was calculated and recorded as percentage of apoptotic cells.

DNA Fragmentation Assay. Cells were trypsinized after 48 h and washed with PBS. DNA was isolated using a standard phenol-chloroform extraction procedure. The genomic DNA was analyzed for degradation by gel electrophoresis. The agarose gels were photographed and examined for evidence of a DNA ladder (DNA oligonucleosomal fragmentation).

Results

E2F-1 Transgene Expression. To confirm overexpression of the transgene E2F-1 following treatment of breast and ovarian carcinoma cell lines with Ad5CMVE2F, we performed Western blot analysis to confirm protein expression. In Fig. 1, Western blot analysis of three of the breast carcinoma cell lines demonstrates significant overexpression of E2F-1 in the Ad5CMVE2F-treated cells over mock-infected or vector control-treated cells. The same samples were incubated with an antibody to β-actin to demonstrate similar levels of protein loading in each Western blot analysis. We found high levels of E2F-1 protein expression in all cell lines tested within 48 h after treatment with Ad5CMVE2F.

Changes in Cell Morphology. Transduction of breast and ovarian carcinoma cells with Ad5CMVE2F induced morphological changes within 48 hours in four of the five cell lines tested (Table 1). These morphological changes were not seen in mock-infected control cells or in cells transduced with the Ad5 vector control (Fig. 2). The breast carcinoma cell line MDA-MB-468 showed the most marked changes with shrunken dense-appearing cells and numerous small cells that suggested apoptotic bodies. The Ad5CMVE2F-treated cells showed marked morphological changes in all the cell lines except the MCF-7 breast carcinoma cell line.

Apoptosis Assays. To determine whether these morphological changes were due to apoptosis, DNA fragmentation assays were performed. As illustrated in Fig. 3, the Ad5CMVE2F-treated cells showed significant DNA laddering consistent with oligonucleosomal fragmentation. Neither the mock-infected or the vector control-treated cells showed evidence of DNA laddering. Again, the MCF-7 cell lines showed no evidence of apoptosis as measured by DNA fragmentation assay.

Additional evidence to confirm apoptosis in the Ad5CMVE2F-treated cells was obtained after propidium iodide staining and FACS analysis. The cell lines that showed morphological changes suggestive of apoptosis had a significant subdiploid population of cells, consistent with programmed cell death. Fig. 4C illustrates the flow cytometry profile seen in the Ad5CMVE2F-treated MDA-MB-468 cells versus mock-infected (Fig. 4A) or adenoviral vector control-treated cells (Fig. 4B).

The flow cytometry profile of the MCF-7 cell line (data not shown) did not change following infection with Ad5CMVE2F, consistent with the absence of morphological changes and DNA laddering.

Relationship of p53 and Rb to E2F-induced Apoptosis. Despite evidence of significant levels of transgene expression in all the breast and ovarian carcinoma cells treated with Ad5CMVE2F, we noted a difference across cell lines in this sensitivity to induction of apoptosis. We evaluated whether this might be due to the p53 or Rb status of the cells. As demonstrated in Table 1, all but one of the cell lines tested had mutations in the p53 gene. The only cell line that was resistant to induction of apoptosis, the MCF-7 breast carcinoma cell line, which has wild-type p53. These data suggest that E2F-1-induced apoptosis can occur through a p53-independent pathway when overexpressed using adenovirus-mediated gene transfer. Furthermore, deletion of Rb was also not required for successful induction of apoptosis through overexpression of E2F-1.

Discussion

It has previously been shown that overexpression of E2F-1 in fibroblasts can lead to their entry into S-phase and to induction of apoptosis (7, 8). Our current study demonstrates for the first time that adenovirus-mediated overexpression of E2F-1 can also induce significant apoptosis in malignant cells. Four of the five breast and ovarian carcinoma cell lines tested demonstrated morphological changes consistent with apoptosis within 48 h of treatment. The morphological changes present in the Ad5CMVE2F-treated cells were not seen in mock-infected cells or adenovirus vector control-treated cells. Using FACS analysis and DNA fragmentation assays, we confirmed that these morphological changes were indeed due to apoptosis. The Ad5CMVE2F-treated cells showed a significant subdiploid population on FACS analysis and produced characteristic DNA ladders using a DNA fragmentation assay in all but one of the cell lines. Furthermore, because apoptosis occurred in cell lines with mutated p53, E2F-1-induced apoptosis does not appear to require p53 when delivered in an adenovirus-based strategy.

To confirm that the E2F-1 protein was overexpressed in cells following treatment with Ad5CMVE2F, we performed Western blot analysis on all the cell lines tested. Using a MOI of 100, we were able to induce significant overexpression of E2F-1 in all the Ad5CMVE2F-treated cells. Even the MCF-7 breast carcinoma cell line, which was resistant to induction of apoptosis, showed overex-

Table 1 Relationship of p53 and Rb status in individual cell lines to adenovirus-mediated E2F-induced apoptosis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 status</th>
<th>Rb status</th>
<th>% apoptosis (FACS)</th>
<th>Morphological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB668*</td>
<td>Mutated</td>
<td>Deleted</td>
<td>70</td>
<td>3+</td>
</tr>
<tr>
<td>SKBr3*</td>
<td>Mutated</td>
<td>Wild type</td>
<td>42</td>
<td>2+</td>
</tr>
<tr>
<td>SKOV3*</td>
<td>Mutated</td>
<td>Wild type</td>
<td>22</td>
<td>2+</td>
</tr>
<tr>
<td>BT549*</td>
<td>Mutated</td>
<td>Wild type</td>
<td>21</td>
<td>2+</td>
</tr>
<tr>
<td>MCF-7*</td>
<td>Wild type</td>
<td>Wild type</td>
<td>5</td>
<td>0</td>
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</tbody>
</table>

* Breast carcinoma cell line.  
* Ovarian carcinoma cell line.

Fig. 1. Overexpression of E2F-1 through adenoviral gene transfer in human breast carcinoma cell lines. The MCF-7, SKBr3, and MDA-MB-468 cell lines were mock infected (control), treated with the vector control without a transgene (Ad5) at a MOI of 100, or treated with the Ad5CMVE2F vector (E2F) at a MOI of 100. In each of the cell lines, there was significant overexpression of E2F-1. β-Actin is shown to demonstrate similar levels of protein loading for each of the samples.
expression of the E2F-1 transgene. Therefore, we could not attribute differential sensitivity to E2F-induced apoptosis to an inability of the cells to express the E2F-1 transgene.

The mechanism of E2F-1-induced apoptosis is not very clear. It is believed that overexpression of E2F-1 can force cells to enter the cell cycle and that this event then progresses to activate apoptotic pathways (9, 10). Previous reports have suggested that wild-type p53 is required for E2F-1-induced apoptosis (11). These previous studies were performed in murine embryo fibroblast systems with coexpression of p53 and E2F-1. In the human breast and ovarian carcinoma cells used in our study, the only cell line that was resistant to Ad5CMVE2F-induced apoptosis was a wild-type p53 breast carcinoma cell line (MCF-7). O'Connor et al. (12) have shown that p53 can inhibit E2F transcriptional activity through direct physical interactions and by up-regulation of p21. This induction of p21 prevents phosphorylation of pRb, which then results in inactivation of E2F.
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transcriptional activity. The ability of p53 to inactivate E2F through Rb-dependent and -independent pathways may be important in regulating transcriptional activity and in the induction of apoptosis.

Recently, Bargou et al. (13) found that blocking of the E2F function in nonmalignant human breast epithelial cells by introducing dominant negative mutants of E2F-2 or DP-1 (a related protein that forms heterodimers with E2F and promotes E2F transcriptional activity) can lead to enhanced resistance to apoptosis and to induction of tumors in nude mice. This suggests that E2F-mediated apoptotic activity is linked directly to its tumor suppressor function. Furthermore, dysregulation of E2F-mediated apoptotic pathways may be an important step in mammary tumor development and progression.

Many breast cancers fail to respond to conventional chemotherapy, because the cancer cells develop drug resistance and the apoptotic pathways are often disrupted. Thus, strategies designed to efficiently induce apoptosis in cancer cells are critical to cancer therapy. Using E2F-1 as a therapeutic gene may provide several advantages. First, E2F-1 is a tumor suppressor that can induce apoptosis in cancer cells, as demonstrated by our present study. Secondly, E2F-1 may preferentially induce apoptosis in cancer cells that have mutations in other tumor suppressor genes such as p53 or Rb. Indeed, in our present study, the cell lines tested that had p53 and Rb mutations had more extensive apoptosis than did cells that had wild-type p53 or wild-type Rb. The mechanism of E2F-induced apoptosis in malignant cells appears to differ from that of fibroblasts. It is possible that E2F-1-induced apoptosis in breast and ovarian carcinoma cell lines occurs through a p53-independent pathway. A second possibility is that adenovirus-mediated overexpression of E2F-1 provides additional genes from the adenovirus genome that allow for E2F-induced apoptosis without p53.

We have demonstrated that recombinant adenovirus-mediated gene therapy with E2F-1 is a powerful inducer of apoptosis in human breast and ovarian carcinoma cell lines. Because this adenovirus-based strategy does not require p53, it may prove to be an important novel therapy for patients with breast and ovarian cancers that frequently harbor mutations in p53.

<table>
<thead>
<tr>
<th>MDA-MB-468</th>
<th>SKBr3</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ad5</td>
<td>E2F</td>
</tr>
<tr>
<td>Control</td>
<td>Ad5</td>
<td>E2F</td>
</tr>
<tr>
<td>Control</td>
<td>Ad5</td>
<td>E2F</td>
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Fig. 3. DNA fragmentation confirms apoptosis following treatment with Ad5CMVE2F. DNA laddering was seen in each of the cell lines tested except the MCF-7 breast carcinoma cell line. (Data not shown for SKOV3, BT549). Shown here are samples from the MDA-MB-468, SKBr3, and MCF-7 cell lines. Cells were mock infected (Control), treated with the Ad5 vector control at a MOI of 100 (Ad5), or treated with the Ad5CMVE2F vector at a MOI of 100 (E2F).

Fig. 4. FACS analysis demonstrates a subdiploid cell population consistent with apoptotic cell death. The flow cytometry profile is shown here for the MDA-MB-468 cell line. There was no significant difference in the control cells (Fig. 4A) or the Ad5 vector control (Fig. 4B)-treated cells. The MDA-MB-468 cell line showed a significant subdiploid cell population 48 h following treatment with Ad5CMVE2F (Fig. 4C; arrow, subdiploid cells). The MCF-7 cell line showed no significant change in the flow cytometry profile (data not shown) and no evidence of a subdiploid cell population despite high levels of E2F-1 protein expression 48 h following treatment with Ad5CMVE2F.

Acknowledgments

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References

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