Caspase Activation and Changes in Bcl-2 Family Member Protein Expression Associated with E2F-1-mediated Apoptosis in Human Esophageal Cancer Cells

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ABSTRACT

The prognosis for patients with esophageal cancer remains poor, prompting the search for new treatment strategies. Overexpression of E2F-1 has been shown to induce apoptosis in several cancer cell types. In the present study, the effect of adenovirus-mediated E2F-1 overexpression on human esophageal cancer cell lines Yes-4 and Yes-6 was evaluated. Cells were treated by mock infection, infection with an adenoviral vector expressing β-galactosidase (Ad5CMV-LacZ), or E2F-1 (Ad5CMVE2F-1). Western blot analysis confirmed marked overexpression of E2F-1 in Ad5CMVE2F-1-infected cells. Overexpression of E2F-1 resulted in marked growth inhibition and rapid loss of cell viability due to apoptosis, although Yes-6 cells were somewhat more resistant to E2F-1-mediated growth inhibition than Yes-4 cells. Cell cycle analysis revealed that overexpression of E2F-1 led to G2 arrest, followed by apoptotic cell death. p53 expression remained undetectable in both cell lines after E2F-1 overexpression. The apoptosis inhibitor proteins of the Bcl-2 gene family, Bcl-2, Mcl-1, and Bcl-XL, decreased at 48 h after infection in Yes-4 cells, but remained unchanged in Yes-6 cells. Levels of retinoblastoma gene product (pRb) declined at 48 h after E2F-1 infection in Yes-4 cells, at which apoptosis predominated, whereas pRb expression remained constant in Yes-6 cells. Expression of p14ARF did not change after E2F-1 infection in either cell line. Involvement of caspase 3 and caspase 6 in E2F-1-mediated apoptosis was demonstrated by cleavage of caspase 3/PP32 and poly-ADP-ribose polymerase, as well as fragmentation of the caspase 6 substrate, lamin B. These results indicate that the sensitivity of esophageal cancer cells to E2F-1-mediated apoptosis may be related to differential expression of Bcl-2 family member proteins and suggest that the adenovirus-mediated E2F-1 gene therapy may be a promising treatment strategy for the treatment of this disease.

INTRODUCTION

Human esophageal cancer is a major cause of cancer-related mortality worldwide (1–3) and constitutes about 1.5% of newly diagnosed invasive malignancies and 2% of all cancer-related deaths in the United States annually. The incidence of the disease has been increasing in recent years. Approximately 12,000 new cases were diagnosed in the United States in 1995, and >10,000 of these newly diagnosed patients were ultimately expected to die of their disease. Despite intensive multimodality surgery, radiation therapy, and chemotherapy, prognosis remains poor with a 5-year survival rate of only 5–10% (4–6). New treatment strategies are clearly needed.

Esophageal cancers contain a variety of gene mutations that disrupt cell cycle regulation. Of particular relevance in this regard are mutations that directly perturb the pRB3-mediated G1 restriction point (4–9). pRB is thought to exert its cell cycle regulatory effects by binding and sequestering the transcription factor, E2F-1 (10). E2F-1 has unique and somewhat paradoxical activities. E2F-1 promotes cellular proliferation by stimulating expression of a number of genes that promote transition from G1 to the S phase. For example, overexpression of E2F-1 stimulates quiescent cells to enter into the S phase, whereas inhibition of E2F-1 prevents entry into the S phase (10–14). However, overexpression of E2F-1 has also been shown to induce apoptosis in several cell types, indicating that E2F-1 plays a role not only in regulating cell growth, but in coordinating programmed cell death (15, 16). Furthermore, recent studies with E2F-1 knockout mice suggested that E2F-1 functions as a tumor suppressor gene (17–19).

Adenovirus-mediated E2F-1 gene transfer has been evaluated as a cancer gene therapy strategy in melanoma, breast, ovarian, head, and neck cancer as well as glioma (20–23). Although adenovirus-mediated gene expression of p16 has been investigated as a potential strategy for esophageal cancer (24),
the effects of E2F-1 overexpression have not been evaluated. Results of the present studies indicate that E2F-1 gene transfer induces apoptotic cell death in esophageal squamous cell carcinoma cells. Furthermore, resistance to E2F-1-mediated apoptosis may be explained by differential expression of cell death inhibitory proteins of the Bcl-2 family.

MATERIALS AND METHODS

Cell Culture and Culture Conditions. Human esophageal squamous carcinoma cell lines, Yes-4 and Yes-6, were the generous gift of Dr. M. Oka (Yamaguchi University School of Medicine, Yamaguchi, Japan; Refs. 25 and 26). Both cell lines were cultured in DMEM. All cell culture reagents were obtained from Life Technologies, Inc. (Bethesda, MD). All of the media were supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (100 units/ml). Cells were cultured in 5% CO₂ incubator at 37°C, and the medium was changed every 3 days.

Adenoviral Vectors. Two replication-defective recombinant adenoviral vectors were used. The AdCMV-E2F-1 vector has been deleted in the adenoviral E1 subunit and contains the transgene E2F-1 under the control of the cytomegalovirus promoter (21). Ad5-CMV-nls-LacZ (referred to herein as transgene E2F-1 under the control of the cytomegalovirus promoter) (27). Both vectors were propagated in the 293 cell line and titered using standard plaque assays (28). For infection, 1 × 10⁶ cells were plated in 10-cm tissue culture plates. The following day, the media was removed, and cells were infected by adding the adenoviral vectors in 1 ml of α-MEM at an MOI of 100 plaque-forming units/cell. Mock infection was performed by treatment of cells with vehicle (media) only. One h after incubation at 37°C, the medium was removed, and 10 ml of fresh α-MEM with 5% fetal bovine serum were added. Cells were harvested at specific time points for analysis.

β-Galactosidase Assay. The cell lines infected with Ad5CMV-Lacz were assayed for β-galactosidase expression by the X-gal staining method as described previously (29). Brieﬂy, 48 h after infection, the cells were washed with PBS and ﬁxed in 2% (v/v) glutaraldehyde in PBS (pH 7.4) for 5 min at 4°C. The cells were then washed and stained with X-gal solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mm K₃Fe(CN)₆, 5 mm K₃Fe(CN)₆, 2 mm MgCl₂] in PBS (pH 6.5) for 12–18 h at 37°C. Blue staining of cell nuclei identiﬁed transduced cells. Mock-infected cells and cells transduced with other adenoviral vectors served as controls.

PCR Ampliﬁcation and Sequence Analysis of p53. Total ribonucleic acid (RNA) was isolated from exponentially growing cells at 80% conﬂuence using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Reverse-transcription of 1 μg of total RNA was analyzed by RT-PCR using an RNA PCR kit (Promega, Madison, WI) according to the manufacturer’s instructions. For amplification of p53 exon 5 to exon 9 (this region contains a high frequency of mutation in esophageal cancer, as reported; Refs. 5 and 7), the following primers were used to amplify a 703-bp fragment: the upstream primer (5'-TTCTTTGACATTTCTGCAGACCC-3') and the downstream primer (5'-GCCTATTAGGATCTGAGGAC-3'). For analysis of the p53 basal promoter region in exon 1, the following primers were used to amplify a 150-bp fragment from genomic DNA isolated from Yes-4 and Yes-6 cell lines: the upstream primer (5'-AGTCTAGAGCCACCGTCCAG-3') and the downstream primer (5'-CGTGACTCAGAGGACTCAT-3'). As a control, the human sarcoma cell line OsaCL, which expresses wild-type p53, was used. PCR products were puriﬁed with QIAEX II Gel Extraction Kits (Qiagen, Germany) and directly sequenced with an automated DNA sequencing system (Applied Biosystems, model number 377, Foster, CA; sequenced by Macromolecular Structure Analysis Facility, Lexington, KY).

Western Blot Analysis. Cells were treated by mock infection or infection with Ad5CMVE2F-1 or Ad5CMV-LacZ at an MOI of 100. Cells were harvested at selected time points and lysed in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with a protease inhibitor cocktail (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transpeptidase selenicel-phospholipid (4-guanidino) butane (E-64), bestatin, leupeptin, and aprotonin (10:10; Ref. 6; SIGMA, Saint Louis, MI) for 30 min. Cell lysates were centrifuged, and protein concentration was determined by BIO-RAD DC protein assay (BIO-RAD, Hercules, CA). Equal amounts (100 μg/lane) of cellular protein were electrophoresed in 8% (Rb, E2F-1, PARP, and lamin B) or 12% (CPP32, Bax, Bcl-2, Bcl-XL, Bak, p53, and Mcl-1) SDS-polyacrylamide gels and transferred to a Hybond-PVDF membrane (Amersham, Arlington Heights, IL). The membrane was ﬁrst incubated with the following primary antibodies: mouse antihuman E2F-1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), mouse antihuman lamin B and mouse-anti-p53 mAb (CalBiochem, Oncogene Research Products, Cambridge, MA), mouse antihuman CPP32 mAb (Transduction Lab, Lexington, KY), rabbit antihuman Bax pAb, rabbit antihuman Bak pAb, rabbit anti-Bcl-2 pAb, and rabbit antihuman CPP32 pAb (PharMingen, San Diego, CA), and then with antimouse immunoglobulin or antirabbit immunoglobulin, peroxidase-linked, species-specific whole antibody (Amersham). Enhanced chemiluminescence reagents were used to detect the signals according to the manufacturer’s instructions (Amersham).

Cellular Proliferation and Viability Assays. Cell proliferation was assessed 24, 48, 72, and 96 h after infection by measuring the conversion of the tetrazolium salt WST-1 to formazan according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Brieﬂy, cells were plated into 12-well plates and infected with the adenoviral vectors 24 h later. At each time point, 50 μl of WST-1 were added to each well and cultured at 37°C for 1.5 h. The supernatant from each plate was collected for measurement of absorbance at 415 nm and 650 nm (the latter as a reference wavelength). Under the experimental conditions of the present studies, there is a direct correlation between the absorbance at 415 nm and the cell numbers. The results were expressed as the percentage of the absorbance of control (uninfected) cells. Cell viability was assessed by the trypan blue exclusion. Cells were stained with 0.4% trypan blue for 5 min and counted using a hemacytometer.

Cell Cycle Analysis. Both adherent and nonadherent cells were harvested, washed once with PBS, and fixed in 70%
Transduction Efficiency of Adenoviral Vectors. To estimate the transduction efficiency of adenoviral vectors, cell lines Yes-4 and Yes-6 were infected with Ad5CMV-LacZ at the MOI of 100. Transduction efficiency was determined by measuring the percentage of blue cells after cytochemical staining and Western blot analysis. Control mock infection resulted in dose-dependent inhibition of cell growth (Fig. 2A). Ad5CMVE2F-1 infection resulted in marked inhibition of cell proliferation and loss of cell viability, which was more pronounced in the Yes-4 cells (Fig. 2B and C; Fig. 3). Similar cell viability results have been obtained from human esophageal adenocarcinoma cell lines (data not shown).

Effects of Ad5CMVE2F-1 Infection on Cell Cycle. To further investigate the mechanism of E2F-1-induced esophageal cancer cell death, cell cycle distribution after the AdCMV-E2F-1 infection was analyzed. E2F-1 overexpression in both cell lines was associated with G2 arrest at 24 h, followed by a pronounced subdiploid peak suggestive of apoptotic cell death at 48 h. By 72 h, the subdiploid population predominated in Yes-4 cells, but was less pronounced in Yes-6 cells. Neither mock infection nor Ad5CMV-LacZ-infected cells demonstrated substantial changes in cell cycle profiles (data not shown). Quantitation of cell cycle data is shown in Table 1. These data suggest that overexpression of E2F-1 is associated with early G2 arrest from which the cells do not recover, leading to subsequent apoptotic cell death.

E2F-1-mediated Cell Death Is Due to Apoptosis. Several experiments were performed to verify that E2F-1-induced cell death in both Yes-4 and Yes-6 cells was the result of apoptosis. First, cell morphology demonstrated typical changes characteristic of apoptotic cell death, including cell shrinkage, cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies (data not shown). These changes were not seen in mock-infected or Ad5CMV-LacZ-infected cells.

Apoptosis is characterized by internucleosomal degradation of genomic DNA. Using the in situ TUNEL assay, it is possible to confirm that DNA cleavage has occurred and free 3′-OH groups are generated by cellular endonucleases (31). At 72 h after infection, Ad5CMVE2F-1-infected cells demonstrated abundant TUNEL staining (Fig. 4). In contrast, neither mock-infected nor Ad5CMV-LacZ-infected cells showed significant evidence of internucleosomal DNA fragmentation.

p53 in Yes-4 and Yes-6 Cell Lines. In Yes-4 and Yes-6 cells, p53 expression was not detectable after mock infection or infection with AdCMV-LacZ or AdCMV-E2F-1 (Fig. 5A). Therefore, it appears that apoptosis induced by E2F-1 overexpression in these esophageal cancer cells is not associated with a significant increase in p53 levels, and differences in p53 expression do not explain the differential sensitivity of the Yes-4 and Yes-6 cell lines to E2F-1-mediated apoptosis.

Cancer cells that express mutant p53 frequently demonstrate overexpression of p53 (3, 34, 35). Despite multiple experiments, p53 expression could not be conclusively demonstrated in either Yes-4 or Yes-6 cells, suggesting that mutant p53 expression is not likely. To characterize the p53 expression of these cell lines further, the cells were treated with UV radiation. Under normal circumstances, wild-type p53 accumulates after treatment with DNA-damaging agents, including γ- and UV-irradiation (36). However, treatment with UV-irradiation did not increase p53 levels in these cells to detectable levels (Fig. 5B). To determine the reasons for the lack of p53 expression, RT-PCR analysis of p53 exon 5 to exon 9 was analyzed in Yes-4
and Yes-6 cells. In multiple experiments, normal p53 mRNA expression was undetectable by RT-PCR analysis in Yes-4 cells. Yes-6 cells demonstrated p53 mRNA expression, albeit at a lower level compared to OsACL cells, which have normal wild-type p53 expression (Fig. 5C). Sequence analysis revealed a point mutation in the p53 basal promoter region in exon 1 (base 951, from T to G) in Yes-4 cells. Such promoter mutations have been found to affect p53 expression in esophageal cancer cells (37). Yes-6 cells, however, demonstrated wild-type sequence in this promoter region. Taken together, these data indicate that p53 is not expressed normally in either Yes-4 or Yes-6 cells, which is common in esophageal cancer cell lines (37).

Changes in pRb. Some evidence suggests that overexpression of the pRb protein can rescue cells from E2F-1-induced apoptosis (38). pRb protein expression decreased at 48 h after the E2F-1 infection in Yes-4 cells, but remained constant at all of the time points in Yes-6 cells (Fig. 6). These data suggested that down-regulation of pRb expression may contribute to the increased sensitivity of Yes-4 cells to E2F-1-induced apoptosis. Expression of p14ARF did not change after E2F-1 infection in either cell line, indicating that p14ARF may not be involved in this difference of sensitivity.
Apoptosis can result from a relative change in the signals is the concomitant stimulation of E2F-1 and p53 activity. Previous studies have shown that E2F-1 and p53 cooperate to mediate apoptosis (15, 23, 42, 43). For example, E2F-1-induced apoptosis in fibroblasts is potentiated by high levels of endogenous wild-type p53 (15, 42). There is also evidence that overexpression of E2F-1 induces the accumulation of p53, again implicating p53 in E2F-1-mediated apoptosis (44, 45).

It is now clear, however, that p53 is not always required for E2F-1-mediated apoptosis (22, 23, 46, 47). A recent report demonstrated that E2F-1 induced p53-independent apoptosis in Saos-2 cells (p53 and Rb null) and p53 null mice (48). Furthermore, adenovirus-mediated E2F-1 gene transfer recently has been shown to induce apoptosis in human melanoma, glioma, breast cancer, ovarian cancer, and head and neck cancer cells in a p53-independent manner (20–23). In the present study, p53 protein levels remained undetectable by Western blot analysis after E2F-1 overexpression, suggesting that E2F-1-mediated apoptosis in these cells did not involve increased p53 expression.

The incidence of p53 mutations in human esophageal cancer is >60% (5, 7). In the present studies, p53 expression was not normal in either cell line. Yes-4 cells contain a point mutation in p53 transcription. p53 expression in Yes-6 cells was not normal, although it contains a wild-type p53 basal promoter sequence that was demonstrated. The reasons for abnormal p53 expression may be related to the alterations of transcription, mRNA stability, translation, post-translational modifications, or protein stability (34, 35, 37). Of course, additional p53 mutations may exist, which were not demonstrated in this study.

Interestingly, Yes-4 cells were more sensitive than Yes-6 cells to apoptosis after adenovirus-mediated E2F-1 infection, as noted in the growth curves, TUNEL assay, CPP32, PARP, and lamin B assays. Although this difference of sensitivity is not completely known, the present data provide some clues.

First, pRb may be involved in this process. It has been shown that pRb can inhibit apoptosis induced by various agents, including γ-irradiation (49). pRb is known to interact with E2F-1 and negatively regulate its transactivation function (50). Thus it was thought that pRb may inhibit E2F-1-mediated apoptosis through its inhibition on the transcriptional activity of E2F-1.

### Table 1 Cell cycle analysis

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Fig. 4 In situ TUNEL assay for apoptosis. At 72 h after infection, cells were harvested for in situ TUNEL assay. Diaminobenzidine reacts with the labeled cells to generate a brown substrate at the site of DNA fragmentation. Brown staining, therefore, indicates apoptotic cells. Cells were counterstained with methylgreen to aid in the morphological evaluation of normal and apoptotic cells. A, Yes-4, mock infection; B, Yes-4, Ad5CMV-LacZ infection; C, Yes-4, Ad5CMV-E2F-1 infection; D, Yes-6, mock infection; E, Yes-6, Ad5CMV-LacZ infection; F, Yes-6, Ad5CMV-E2F-1 infection.
Fig. 4  D–F Continued.
E2F-1. In the present study, there was a decrease in pRb at 48 h after adenovirus-mediated E2F-1 infection in Yes-4 cells, at a time when apoptosis predominates. This may imply that differential regulation of pRb expression contributes, at least in part, to the differences in sensitivity of Yes-4 and Yes-6 cells to the E2F-1-induced apoptosis.

Second, this differential sensitivity of these cell lines may be attributable to the activity of the Bcl-2 family proteins. Some of the members of this family are blockers of cell death (Bcl-2, Mcl-1, and Bcl-XL), whereas others are promoters of apoptosis (Bax, Bak, and Bcl-XS). Furthermore, in many cases these proteins can physically interact with each other in a network of homo- and heterodimers in which the relative proportions of the antiapoptotic members of this family determine the ultimate sensitivity or resistance of cells to cell death stimuli (51–53).

Overexpression of Bax has been associated with apoptosis in many kinds of cells. However, Fueyo et al. (22) found that E2F-1 overexpression in human glioma cells resulted in a decrease in intracellular levels of Bax, whereas, in our studies, Bax expression remained comparatively constant in both Yes-4 and Yes-6 cells after E2F-1 infection. This response involving Bax is probably cell-type specific. It has been reported that Bak, like Bax, primarily promotes apoptosis (54). However, unlike Bax, Bak has also been shown to inhibit cell death. For example, expression of Bak in a human lymphoblastoid cell line provided protection from apoptosis induced by serum deprivation and the oxidant menadione, implying that the function of Bak may be context dependent (55).

Third, the differences in esophageal cancer cell sensitivity to E2F-1 may also result from differences in activation of caspases, specifically, the caspase 6. It has been shown that Lamin B, a substrate for the caspase 6, is critical to maintain the integrity of the nuclear envelope and cellular morphology and whose degradation is required for packaging of the condensed chromatin into apoptotic bodies (56). In the present studies, cleavage of Lamin B after E2F-1 infection was demonstrated in Yes-4 cells, but not in Yes-6 cells. Recent data suggest that the Bcl-2 family members may regulate the mitochondrial membrane permeability, releasing mitochondrial proteins (e.g., cytochrome C), which are capable of activating the caspase cascade (57).

In conclusion, overexpression of E2F-1 by adenovirus-mediated gene transfer results in apoptosis in esophageal cancer cells. E2F-1-mediated apoptosis was associated with changes in expression of Bcl-2 family proteins and activation of the caspase pathway. Differential regulation of Bcl-2 family proteins may mediate tumor-specific sensitivity to E2F-1-mediated apoptosis.

**Fig. 5** p53 expression. A, Western blot analysis of p53 at indicated time points after Ad5CMV-LacZ or AdCMV-E2F-1 infection. p53 remains undetectable at all time points in both Yes-4 and Yes-6 cells. The positive control (+ control) for p53 in each experiment is a protein extract from V710 cells, which express a temperature sensitive mutant of p53, grown at the permissive temperature. B, p53 expression after UV-irradiation. The positive control is the same as A. α-Actin was used to show the equal loading per lane. C, RT-PCR analysis of p53. Quantitative real-time RT-PCR for p53 exon 5 to exon 9 was performed in Yes-4 and Yes-6 cells as described in “Materials and Methods.” OsACL cells (containing a wild-type p53) were used as a control.

**Fig. 6** Expression of pRb. Western blot analysis of pRb at indicated time points after Ad5CMV-LacZ or Ad5CMV-E2F-1 infection. pRb decreased at 48 h after E2F-1 infection in Yes-4 cells, but remained constant at all time points in Yes-6 cells. α-Actin was used to show the equal loading per lane.
Adenovirus-mediated E2F-1 gene transfer may be a promising strategy for esophageal cancer gene therapy.

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