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Human Papillomavirus Type 16 E6 and HPV-16 E6/E7 Sensitize Human Keratinocytes to Apoptosis Induced by Chemotherapeutic Agents: Roles of p53 and Caspase Activation

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Abstract We and others have previously reported that human papillomavirus (HPV)-16 E6 protein expression sensitizes certain cell types to apoptosis. To confirm that this sensitization occurred in HPV's natural host cells, and to explore the mechanism(s) of sensitization, we infected human keratinocytes (HKCs) with retroviruses containing HPV-6 E6, HPV-16 E6, HPV-16 E7, or HPV-16 E6/E7. Apoptosis was monitored by DNA fragmentation gel analysis and direct observation of nuclei in cells stained with DAPI. Exposure of HKCs to etoposide, cisplatin, mitomycin C (MMC), atractyloside, and sodium butyrate, resulted in a time and dose-dependent induction of apoptosis. Expression of HPV-16 E6 and HPV-16 E6/E7, but not HPV-6 E6 or HPV-16 E7, enhanced the sensitivity of HKCs to cisplatin-, etoposide- and MMC-, but not atractyloside- or sodium butyrate-induced apoptosis. Expression of both HPV-16 E6 and HPV-16 E6/E7 decreased, but did not abolish, p53 protein levels relative to normal HKCs, and resulted in cytoplasmic localization of wt p53. P53 induction occurred in HPV-16 E6 and HPV-16 E6/E7 expressing cells after exposure to cisplatin or MMC, though never to levels found in normal untreated HKCs. P21 levels were decreased in HPV-16 E6 and HPV-16 E6/E7 expressing HKCs, and no induction of p21 was seen in these cells following exposure to cisplatin or MMC. Caspase-3 activity was found to be elevated in HPV-16 E6-expressing HKCs following exposure to cisplatin and MMC as documented by fluorometric and Western Blot analysis. Expression of wt CrmA or treatment of HPV-16 E6 expressing HKCs with the caspase-3 inhibitor DEVD.fmk prevented HPV-16 E6-induced sensitization in HKCs. These results suggest that HPV-16 E6 and HPV-16 E6/E7 expression sensitizes HKCs to apoptosis caused by cisplatin, etoposide and MMC, but not atractyloside or sodium butyrate. The data also suggest that wt p53 and caspase-3 activity are required for HPV-16 E6 and HPV-16 E6/E7-induced sensitization of HKCs to DNA damaging agents. J. Cell. Biochem. 78:334–349, 2000.

Key words: apoptosis; human papillomavirus; p53; caspases; DNA damage

In the treatment of cancer, resistance of tumor cells to radiation and chemotherapy remains a significant obstacle. This resistance is thought to arise due to defects in apoptosis, (also termed "programmed cell death"), which is a physiological process of cell deletion that functions as an essential mechanism of normal tissue homeostasis, but also plays a critical role in cancer and other disease states.

Cervical cancer is the second leading cause of death from cancer in women worldwide. The "high risk" HPVs (mainly types 16 and 18) have been found to be strongly associated with the carcinoma of the uterine cervix [for review, see Zur Hausen, 1996], and protein products of these viruses are thought to underlie the development of cervical cancer. The E6 and E7 genes of these HPVs are responsible for the transforming and immortalizing activity of the

Abbreviations used: HPV, human papillomavirus; HKC, human keratinocyte; MMC, mitomycin C; MPT, mitochondrial permeability transition.

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viral genome. Co-expression of high risk E6 or E7 with an activated oncogene (e.g., ras) leads to immortalization of rodent cells. Combined expression of high risk E6 and E7 efficiently immortalizes primary HKCs, although co-expression does not induce the tumorigenic phenotype directly.

The oncogenic properties of E6 and E7 viral proteins correlate with their ability to interact respectively with two cellular tumor suppressor proteins, p53 and Rb. HPV-16/18 E6 protein, like adenovirus E1B and SV40 T antigen, forms a complex with p53 and stimulates its degradation through ubiquitin-dependent proteolysis. Similarly, the transforming activity of E7 protein is at least partly due to its interference with Rb/E2F interaction and the consequent loss of cell cycle control functions of the Rb protein.

The p53 tumor suppressing protein is involved in several different aspects of cell cycle control including cell cycle arrest, control of genome integrity, DNA repair, as well as triggering apoptosis [Ko and Prives, 1996; Levine, 1997]. Stimuli such as DNA damage, withdrawal of growth factors and expression of myc or E1A can cause p53-dependent apoptosis. The mechanisms by which p53 mediates apoptosis in response to different types of stress remain unknown. Recently, several papers have indicated that activation of caspase-3 and other caspases may play a role in p53-dependent apoptosis [Chandler et al., 1997; Fuchs et al., 1997; Ding et al., 1998]. In addition, the pro-apoptotic proteins Bax and IGF-BP3 are transcriptional targets of p53, suggesting that transactivation of these proteins by p53 is important in modulating apoptosis in some circumstances. However, other data, employing cell-free systems, suggest that p53 can induce apoptosis independent of its transcription function [Chen et al., 1996; Haupt et al., 1995; Cadlles et al., 1994; Wagner et al., 1994].

We and others have previously reported that HPV-16 E6 or HPV-16 E7 sensitizes fibroblasts to apoptosis induced by several kinds of agents [Brown et al., 1997; Xu et al., 1995; Hawkins et al., 1995; Morgenbesser et al., 1994; Pan and Griep, 1995]. This is consistent with the findings that cellular apoptosis has been observed to varying degrees in most cases of cervical cancer examined. To determine whether this sensitization occurs in HPVs natural host cells, human genital epithelial cells and to determine the mechanism of this sensitization, we infected HKCs with HPV-16 E6 or HPV-16 E6/E7-containing retroviruses, then treated these HKCs with different apoptosis-inducing drugs. Our results show that both HPV-16 E6 and HPV-16 E6/E7, but not HPV-6 E6 or HPV-16 E7 alone, sensitize HKC to apoptosis induced by cisplatin, etoposide, and mitomycin C, but not by atractyloside or sodium butyrate. This HPV-16 E6 and HPV-16 E6/E7 sensitization was found to be: a) p53-dependent; b) associated with cytoplasmically localized p53; c) defective in nuclear translocation of p53 following exposure of cells to apoptosis-inducing agents; and d) accompanied by loss of p53-dependent p21 expression observed in normal HKCs after exposure to apoptosis inducing agents. The HPV-16 E6- or HPV-16 E6/E7-induced sensitization could be blocked by the expression of wt-CrmA or treatment of cells with the caspase-3 inhibitor DEVD.fmk. Lastly, HPV-16 E6 and HPV-16 E6/E7 expression were found to increase caspase 3 activity especially after exposure of HKCs to apoptosis inducing agents. These findings indicate that HPV-16 E6 or HPV-16 E6/E7 expression sensitizes HKCs to certain apoptotic inducing agents (i.e., DNA damaging agents) and that this sensitization may require a transcriptionally independent function of p53 and caspase-3 activity.

MATERIALS AND METHODS

Cell Culture

Human neonatal foreskins were obtained from UNC Hospitals for isolation and propagation of keratinocytes. The isolation methods were as previously described [Demers et al., 1994]. HKCs and HKCs infected with HPV-6 E6 or HPV-16 E6 or HPV-16 E6/E7 (HPV-16 E6-HKCs and HPV-16 E6/E7-HKCs) were maintained in Keratinocyte-Serum Free Medium (K-SFM, GIBCO-BRL, Grand Island, NY) supplemented with 5 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract. C33-A cells (derived from an HPV negative cervical carcinoma and which expresses mutated p53) were maintained in MEM/10% fetal bovine serum (FBS) with supplemented with 0.1 mM non-essential amino acids and 1 mM sodium pyruvate.

Vector Constructions and Transfection

The HPV-16 E6 gene, cloned into pLXSN retroviral vector, was kindly provided by Dr.

Denise Galloway (University of Washington). A 1.3 Kb fragment (EcoRI/PstI) containing the HPV-16 E6/E7 region of HPV-16 was inserted into pLXSN retroviral vector. A 1.2 Kb wt crmA and mt crmA (with a mutation of T to R in the amino acid 280) from pFLAG-crmA and pFLAG-mt crmA (kindly provided by Dr. Guy Salvesen, Burnham Cancer Research Institute) were subcloned into the pLXSH retroviral vector. Bcl-2 was subcloned into the pBabepure retroviral vector. According to the Retroviral Gene Transfer and Expression Protocols (CLONTECH), the constructs were transfected into PA317 packaging cells with the use of lipofectamine (GIBCO). Virus-containing supernatants were collected and used to infect HKCs or C33A cells. Transfected HKCs and C-33A cells were stably selected with media containing 75–100 μg/ml G418 (for pLXSN constructs) or 50–70 μg/ml hygromycin (for pLXSH constructs) for 10–14 days. Multiple dishes of cells expressing the same construct that survived selection were pooled and used for further studies. The HPV 16 E6 expressing HKCs were able to undergo up to 50 passages in culture. All constructs were confirmed by sequencing.

Apoptosis

The percent apoptotic cells as a function of time after induction of apoptosis was determined by examination of nuclei in the fluorescence microscope following staining with the fluorescent stain 4′, 6-diamidino-2-phenylindole, hydrochloride (DAPI). Cells were fixed in methanol and stained for 10 min with DAPI (0.5 μg/ml in H_20). The percentage of cells containing apoptotic nuclei (as defined by their smaller size and the appearance of irregular patches of irregularly dispersed brightly staining condensed chromatin) was determined. For documentation of DNA fragmentation, DNA was isolated from keratinocytes using a modified Hirt procedure which allows selective isolation of low molecular weight degraded DNA from high molecular weight intact chromosomal DNA [White et al., 1984]. Hirt supernatant fractions from the cells were equalized with respect to original cell number, analyzed by electrophoresis in a 1% agarose gel, and visualized by ethidium bromide staining. The appearance of low-molecular weight DNA in the Hirt DNA supernatant fractions was used as an indication of the induction of DNA degradation.

RT-PCR

Total RNA was isolated from 1 million cultured cells using Boehringer Manngeim's High Pure RNA Isolation Kit. RT-PCR was carried out using Promega's Access RT-PCR System. The primers used were: for E6-5′-GGATCCATGCACCAAAAGAGAACTGCA-3′ (encompassing nucleotides 63–83 of the published HPV-16 sequence) and 5′-GGATCCTTACAGCTGGGTTTCTCTACG-3′ (encompassing nucleotides 559–539); for crmA-5′ CAT TTC TCC ACC GTC AAT CTC GT; 3′ AGT TGT TGG AGA GCA ATG TCT ACC; for E7 (or HPV-16 E6/E7)- 5′ TCA TGC ATG GAG ATA CAC CTA CAT TGC AT; 3′ GTT TCT GAG AAC AGA TGG GGC ACA C. The protocol for RT-PCR was: 48°C, 45 min; 94°C, 2 min for synthesizing first strand cDNA and RNA/cDNA/primer denaturation; for second strand cDNA synthesis and PCR amplification, 94°C, 30 s; 60°C, 1 min; 68°C, 2 min; 30–40 cycles; final extension for 7 min in 68°C.

Southern Blot

RT-PCR products were detected using Southern Blot. Agarose gels containing RT-PCR products were transferred to nitrocellulose membrane using capillary blotting. The DNA was fixed for 2 h at 80°C in a vacuum oven. A 253 bp E7 fragment (pBlueHPV-16/DpnI) and 1.2 Kb crmA fragment (pFLAGcrmA/NcoI-XhoI) were labeled using Amersham's Probe Labeling and Hybridization System. Signals were detected employing the ECL Western Blotting Detection System (Amersham, Arlimgton Heights, IL).

Western Blotting

Cultured cells were washed twice in PBS and suspended in lysis buffer (50 mM Tris-HCL, pH 6.8; 2% SDS; 10% glycerol and 1 pellet of protease inhibitor cocktail [Boehringer Mannheim, Indianapolis, IN] per 25 μl buffer). The lysates were boiled for 5 min and then passed through a 25 Gauge Syringe needle four to five times. Protein concentrations were determined using the BCA Protein Assay (Pierce Co., Rockford, IL). Equal amounts of protein lysates were loaded onto a 10–20% SDS-PAGE Precast Gel (Bio-Rad, Richmond, CA) and subsequently transferred to a PVDF Membrane (Bio-Rad). Membranes were blocked in 5% of fat-free milk in PBST overnight at 4°C on a shaker, incubated at room temperature with primary anti-

bodies for 1 h, washed four to five times with PBST for 1 h; incubated with secondary antibodies (Anti-mouse IgG or Anti-rabbit IgG, horseradish peroxidase conjugated, Amersham Co.), and washed four to five times with PBST for 1–1.5 h. A positive reaction was visualized with ECL Western Blotting Detection System. Antibodies used were anti-p53 monoclonal antibody (Calbiochem Co., San Diego, CA), anti-p21 monobody antibody (Pharmingen Co., San Diego, CA), anti-Caspase-3 monoclonal antibody (Transduction Laboratories, Lexington, KY) and anti-Bcl-2 monoclonal antibody (Pharmingen Co.).

Apoptosis Induction and Cell Viability Assay

Cells were plated in Costar 24-well plates at 2×10^4 cells per well, incubated at 37°C for 18–24 h, and then treated with cisplatin, mitomycin C or atractyloside (Sigma Co., St. Louis, MO) at various concentrations. To study DEVD.fmk's effect on the cell viability, 7.5 μM DEVD.fmk (ClonTech, Palo Alto, CA) was added before the addition of cisplatin or mitocycin C. Experiments were performed in quadruplicate along with two controls, medium only and no cells. After treatment, viability was assessed by removing the medium, washing cells once with PBS, and applying 5 μg/ml fluorescein diacetate (FDA) in PBS from a stock solution of FDA (10 mg/ml) in DMSO. Viable cells take up diacetylfluorescein and hydrolyze it to fluorescein, which is retained. Live cells, but not dead cells, fluoresce green. After FDA addition, fluorescence was measured using a fluorescence multi-well plate scanner (Cytofluor 2300). Viability is expressed as percent of untreated control.

Caspase-3 Assay

Cells were treated with drugs to induce apoptosis, trypsinzed and washed in PBS. One $\times 10^6$ cells were pelleted and resuspended in 50 μl of chilled cell Lysis Buffer. The ApoAlert™ Caspase-3 Fluorescent Assay Kit (Clontech) was employed to detect Caspase-3 protease activity. After reaction with the Clontech fluorescence-conjugated substrate, cell lysates were transferred to a 96-well plate. Fluorescence was measured using a fluorescence multi-well plate scanner (FluoStar).

Indirect Immunofluorescence Assay

Cells grown on glass coverslips were washed with PBS and fixed with cold acetone. The cells were either stored fixed at −20°C or used immediately. After blocking with 3% bovine serum albumin (BSA), cells were incubated with anti-p53 monoclonal antibody (1:100 with 3% of BSA, Ab-2, Oncogene, Manhasset, NY) for 30 min, rinsed in PBS and incubated with fluorescein-conjugated anti-mouse secondary antibody (1:200, Molecular Probes, Eugene, OR) for 30 min. Excess antibody was washed off and the cells were additionally stained with 0.2 μg/ml 4′ 6-diamidino-2-phenylindole, hydrochloride (DAPI; Molecular Probes) and mounted with SlowFade Light Antifade Medium (Molecular Probes). Cells were imaged using an Olympus IX-70 epifluorescence microscope coupled to a Hamamatsu Orca charged coupled device (CCD) camera and a Universal Imaging Metamorph digital image analysis system. The excitation and emission wavelengths used were 470–490 nm/510–530 nm for fluorescein and 360–370 nm/420 nm for DAPI.

RESULTS

Expression of HPV-16 E6 and HPV-16 E6/E7, But Not HPV 6 E6 or HPV 16 E7, Sensitizes HKCs to Apoptosis Induced by Cisplatin, Etoposide, and MMC, But Not Atractyloside or Sodium Butyrate

HPV-16 E6 has been shown to sensitize many cell types to apoptosis [Brown et al., 1997; Xu et al., 1995; Hawkins et al., 1995]. To confirm these findings in HPV's natural host cells, we introduced HPV-16 E6 and HPV-16 E6/E7, as well as the HPV-16 E7 or HPV-6 E6 gene into human keratinocytes isolated from neonatal foreskins, and determined the effect of the expression of these various proteins on cellular sensitivity to a variety of apoptosis inducing agents (Fig. 1). The expression of the HPV proteins was confirmed by both RT-PCR and Southern Blot (data not shown). Figure 1 demonstrates the effect of the expression of the various HPV proteins on HKC sensitivity to etoposide- (Fig. 1A), MMC- (Fig. 1B), and sodium butyrate- (Fig. 1C) induced apoptosis. All three of these agents induced dose-dependent increases in apoptosis. Interestingly, expression of HPV-16 E6 increased the sensitivity of HKCs to etoposide and MMC, but not sodium

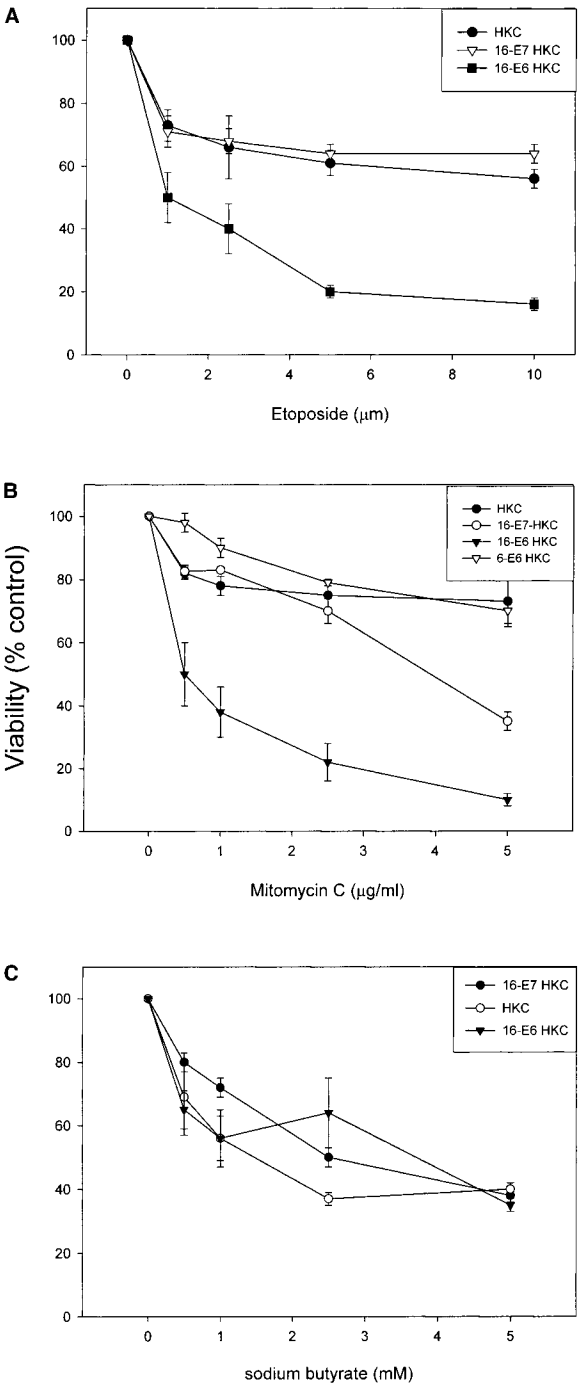


Fig. 1. Dose-dependent induction of apoptosis in normal HKC, HPV-16 E6-HKC, and HPV-16 E6/E7-HKC by etoposide (**A**), MMC (**B**), or sodium butyrate (**C**). HKCs were exposed to the various DNA damaging agents for 72 h. Viability is expressed as percentage of the untreated control. Data represent the mean (± SEM) from four trials.

butyrate-induced apoptosis. Neither HPV-16 E7 or low risk HPV-6 E6 expression alone altered the sensitivity of HKCs to etoposide or MMC-induced apoptosis, suggesting that the increased sensitivity observed is due to the expression of HPV-16 E6 protein and not a more general effect of genetic instability.

Apoptosis is characterized by a series of morphological and biochemical events, which include plasma membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, and degradation of DNA. The morphology of HPV-16 E6 HKC nuclei following exposure to cisplatin is shown in Figure 2A. Nuclear fragmentation is clearly seen (arrows). Figure 2B illustrates DNA degradation following exposure of HPV-16 E6 expressing HKCs to various doses of cisplatin. Both of these figures illustrate classic apoptotic processes in HPV-16 E6 expressing HKCs following exposure to cisplatin. Similar changes were seen in HKCs treated with sodium butyrate, etoposide, MMC, and atractyloside [Brown et al., 1997, and data not shown].

Combined expression of high risk E6 and E7 efficiently immortalizes primary HKCs, although co-expression does not induce the tumorigenic phenotype directly. We therefore examined the effect of co-expression of HPV-16 E6 and E7 in HKCs on sensitivity to inducers of apoptosis (Fig. 3). The DNA damaging agents cisplatin, MMC and atractyloside (an agent thought to open the mitochondrial permeability pore), all caused dose dependent increases in apoptosis. However, HPV-16 E6 and HPV-16 E6/E7 only sensitized cells to apoptosis induced by cisplatin and MMC, but not to atractyloside, with HPV-16 E6/E7 providing greater sensitization than that of HPV-16 E6 alone.

Role of p53

Many DNA damaging agents are thought to induce apoptosis through a p53-dependent pathway. HPV-16 E6 is thought to bind to and accelerate p53 degradation, and many investigators functionally equate HPV-16 E6 expression with loss of p53 function. However, we and others have been able to detect not only p53 protein expression but also p53 transcriptional activity in HPV expressing cells and cervical carcinomas [Brown et al., 1997; Butz et al., 1995; Liang et al., 1993]. To determine whether p53 was required for HPV-16 E6 sensitization of HKCs to apoptosis, we examined the level of

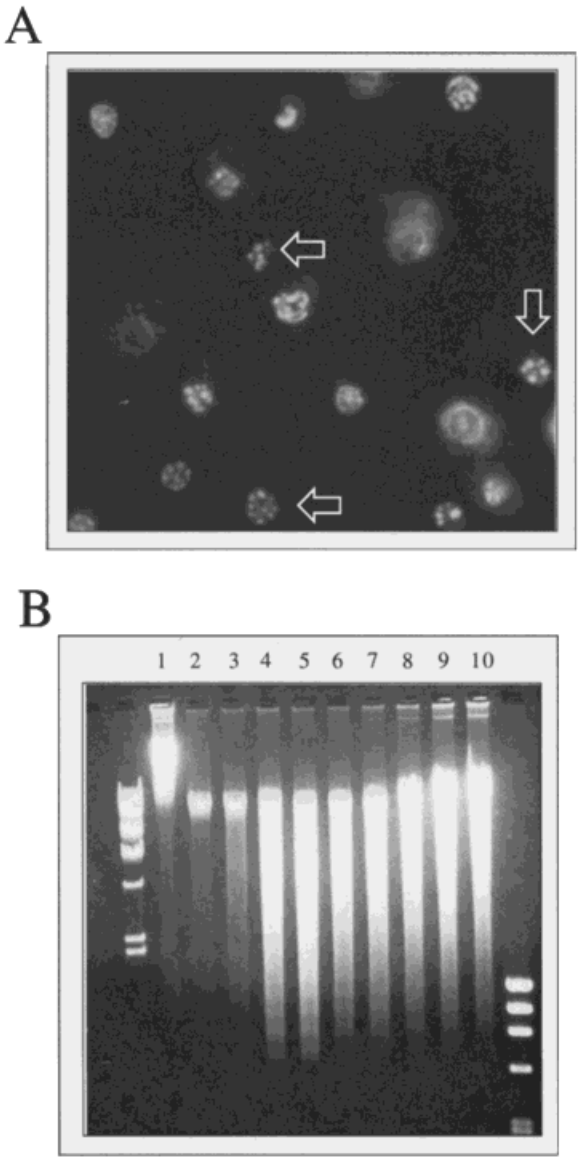


Fig. 2. Morphological and gel electrophoretic demonstration of cisplatin-induced apoptosis in HPV-16 E6-HKCs. **A**: Nuclear fragmentation visualized by DAPI staining (see arrows). HPV-16 E6 expressing HKCs were exposed to 10 μg/ml cisplatin for 48 h. **B**: DNA gel electrophoresis assay. HPV-16 E6 expressing HKCs were treated with different concentrations of cisplatin for 24 h. The concentrations used were 0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0 μg/ml (**lane 1–10**).

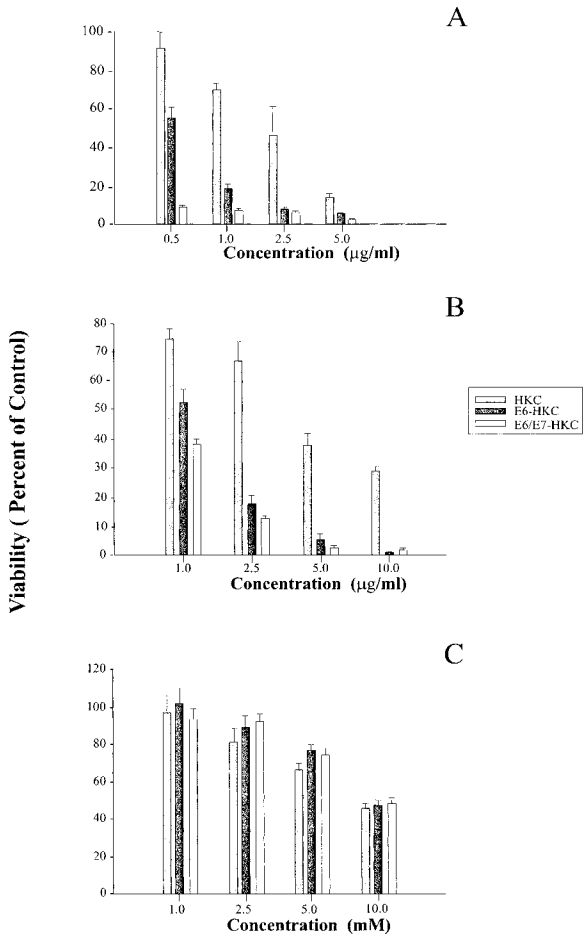


Fig. 3. Dose-dependent induction of apoptosis in normal, HKC-16 E6 and HPV-16 E6/E7 expressing HKCs. HKCs were exposed to cisplatin (**A**), MMC (**B**), or atractyloside (**C**) for 72 h. Viability is expressed as percentage of untreated control. Data represent mean (± SEM) from four trials.

p53 protein in normal, HPV-16 E6 and HPV-16 E6/E7 expressing keratinocytes before and after exposure to cisplatin or MMC (Fig. 4A,B). Expression of HPV-16 E6 or HPV-16 E6/E7 resulted in a decrease (but not total loss), of p53 protein in HKCs. Incubation of both normal (4 P HKC) as well as HPV-16 E6 and HPV-16 E6/E7 expressing HKCs with cisplatin or MMC elevated p53 protein expression. Most importantly, p53 levels increased in both HPV-16 E6 HKCs and HPV-16 E6/E7-HKCs, although total cellular p53 protein levels were lower compared with those of normal non-treated HKCs. p53 levels did not change after treatment with atractyloside or sodium butyrate (data not shown). In contrast to HPV 16 E6 expression, HPV 16 E7 expression led to an increase in the level of p53 expression in the absence of DNA damage (data not shown).

These data indicate that HPV-16 E6 or HPV-16 E6/E7 expression in HKCs may sensitize these cells to apoptosis induced by cisplatin and MMC via upregulation of p53 protein levels. As a transcriptional regulatory factor, p53 activates or suppresses the expression of downstream 'effector' genes (i.e., p21). Thus,

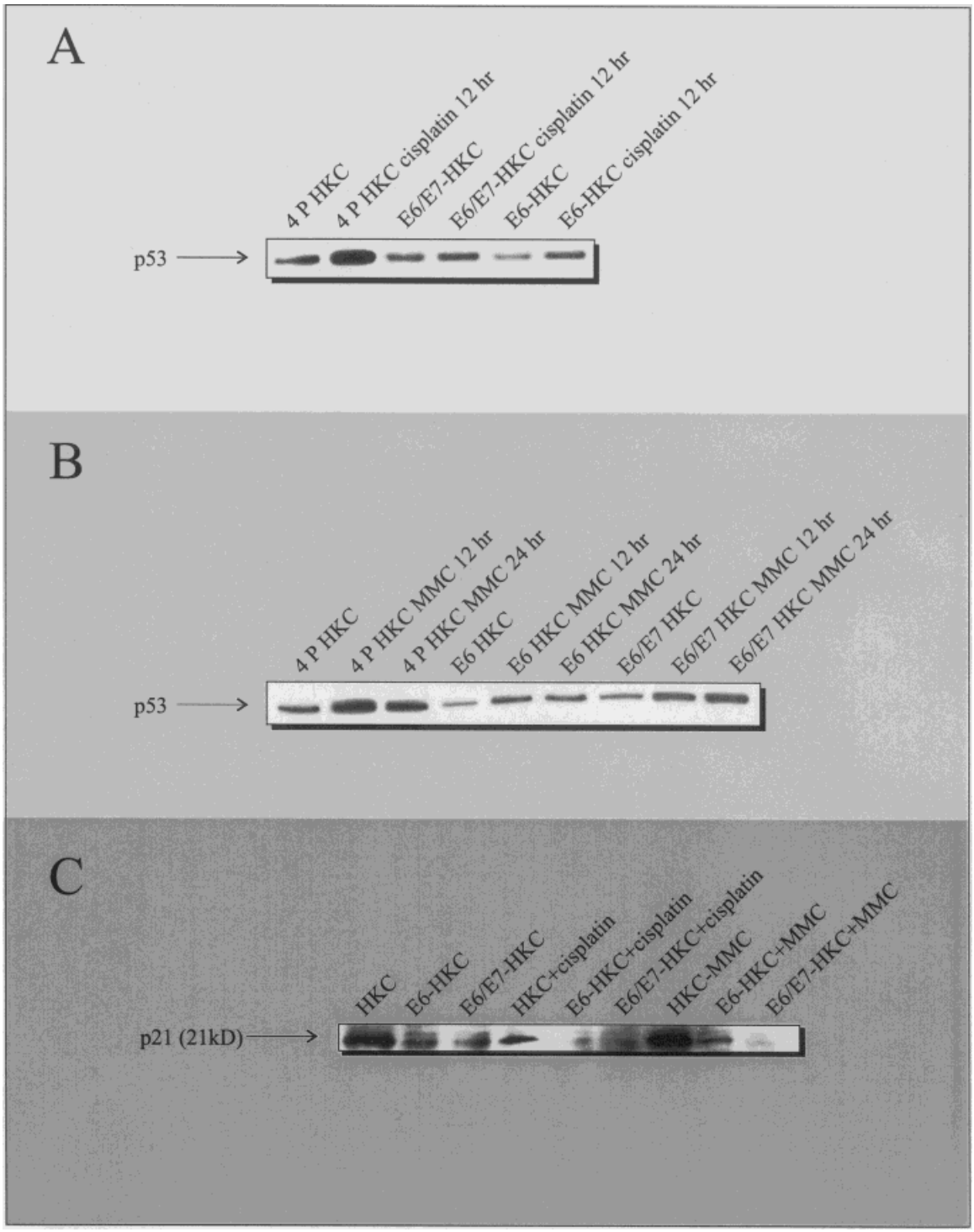


Fig. 4. Effect of HPV-16 E6 or HPV-16 E6/E7 expression on cellular levels of p53 and p21 protein. HKCs were treated with cisplatin (5 μg/ml) or MMC (10 μg/ml) for 24 h. Four P HKC- normal HKCs passaged four times.

alterations in the level or activity of p53 could alter the regulation of these downstream effector genes, leading to a change in the cellular sensitivity to inducers of apoptosis. To determine whether the alterations in p53 protein levels in HPV expressing following induction of apoptosis had any effect on downstream p53-mediated gene expression, we examined the

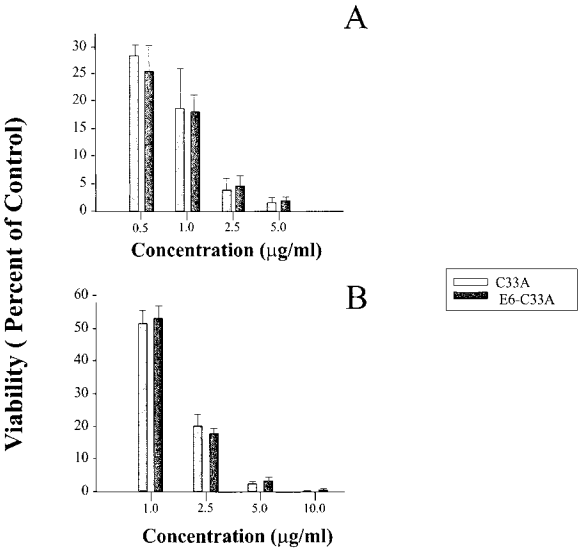


Fig. 5. Viability of normal, HPV-16 wt E6 and HPV-16 mt E6 expressing C33A cells following induction of apoptosis. Cells were exposed to a various concentrations of cisplatin (**A**) or MMC (**B**) for 72 h. Data represent mean (± SEM) from four trials.

expression of $p21^{WAF1/Cip-1}$ (Fig. 4C). HPV-16 E6 and HPV-16 E6/E7 expression decreased p21 expression in HKCs. MMC but not cisplatin, induced p21 expression in normal HKCs, but p21 expression, already very low, was not induced in HPV-16 E6 and HPV-16 E6/E7 expressing HKCs after exposure to either cisplatin or MMC. These data indicate that the normal p53/p21 pathway is disrupted in HPV-16 E6 and HPV-16 E6/E7 expressing HKCs.

In order to investigate the effect of E6 on apoptosis in the absence of all p53 activity, we used untransfected and E6-transfected C-33A cervical carcinoma cells. C-33A cells are derived from an HPV-negative cervical carcinoma and express a mutant, nonfunctional form of p53. As a control, we used C-33A cells transfected with a mutant form of E6 that retains the ability to degrade p53 but lacks transformation activity [Nakagawa et al., 1995]. We found that there was no significant effect of E6 on apoptosis induced by MMC, etoposide, or cisplatin in C33A cells (Fig. 5). This would suggest that a certain level of wt p53 expression/activity is necessary for HPV 16 E6-induced sensitization of HKCs to apoptosis induced by cisplatin, etoposide, and MMC. These data also indicate that in the absence of p53 activity, the p53-independent transformation activity of HPV-16 E6 is not sufficient for HPV-16 E6-induced sensitization to DNA damage-induced apoptosis.

p53 Localization in HPV-16 E6 and HPV-16 E6/E7 Transformed HKCs

p53 is a transcription factor that normally resides in the nucleus of cells. However, we have previously demonstrated that p53 is located in the cytoplasm of HPV expressing cells [Liang et al., 1993]. Employing immunofluorescence microscopy, we examined the distribution of p53 in normal, HPV 16 E6 and HPV-16 E6/E7 expressing HKCs (Fig. 6). In normal HKCs, p53 is localized mainly in the nucleus with a limited amount in the cytoplasm. In HPV-16 E6 and HPV-16 E6/E7 expressing HKCs, p53 was found exclusively in the cytoplasm in a punctuate distribution. Treatment of cells with cisplatin or MMC also did not change the localization of p53 in normal HKCs, HPV 16 E6 or HPV 16 E6/E7 expressing HKCs (data not shown).

Role of Caspase in HPV-16 E6 and HPV-16 E7-Induced Sensitization of HKCs to Apoptosis

A key regulator of apoptosis is the growing family of caspases, cysteine proteases related to interleukin 1β-converting enzyme (ICE/caspase-1), which share sequence homology with *ced-3*, a gene essential for apoptosis in the nematode *Caenorhabditis elegans*. Caspases are involved in apoptosis induced by many cellular stresses and play a role in both the initiation as well as the execution phase of apoptotic pathways.

Studies on caspase activity have identified a number of reagents that can inhibit their activity. Amongst those is CrmA, a gene product from the chicken cowpox virus that has been shown to inhibit activity of certain caspases. We transfected HPV-16 E6 HKCs with wt crmA or mt crmA and documented the expression of this caspase inhibitor using RT-PCR and Southern Blot (data not shown). HPV-16 E6-HKCs stably expressing wt or mt CrmA were then treated with cisplatin, MMC, and atractyloside, respectively. As shown in Figure 7A,B, expression of wt CrmA, but not mt CrmA, provided protection to HKCs against HPV-16 E6's sensitization to apoptosis induced by cisplatin and MMC. CrmA expression also protected HKCs from apoptosis induced by atractyloside, though via a mechanism that did

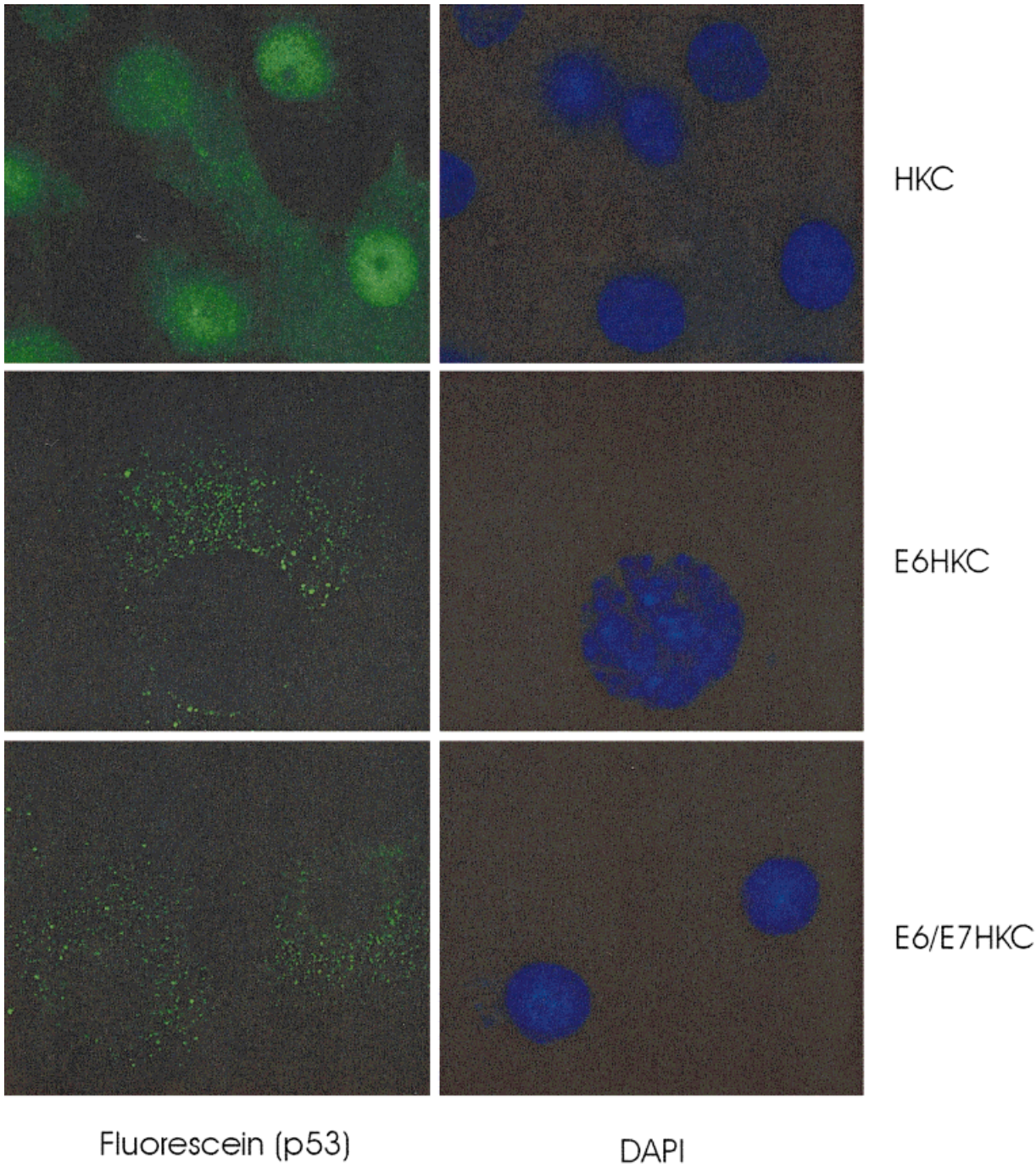


Fig. 6. Distribution of p53 assessed by indirect immunofluorescence microscopy. Anti-p53 monoclonal antibody (1:100, Ab-2, Oncogene) and Fluorescein-conjugated anti-mouse IgG (1:200) were employed. The right row shows the DAPI (0.2 μg/ml)-stained nuclei of the same cell to its immediate left. Magnification 100×.

not involve sensitization by HPV-16 E6 (Figs. 3C, 7C).

To further characterize the role of caspase activity in HPV-induced sensitization of HKC apoptosis, we employed the caspase-3 inhibitor DEVD.fmk (Fig. 8). Caspase-3 activity has been implicated in p53-dependent apoptosis [Chandler et al., 1997]. DEVD.fmk deceased the level of apoptosis induced in normal, HPV-16 E6 and HPV-16 E6/E7 expressing

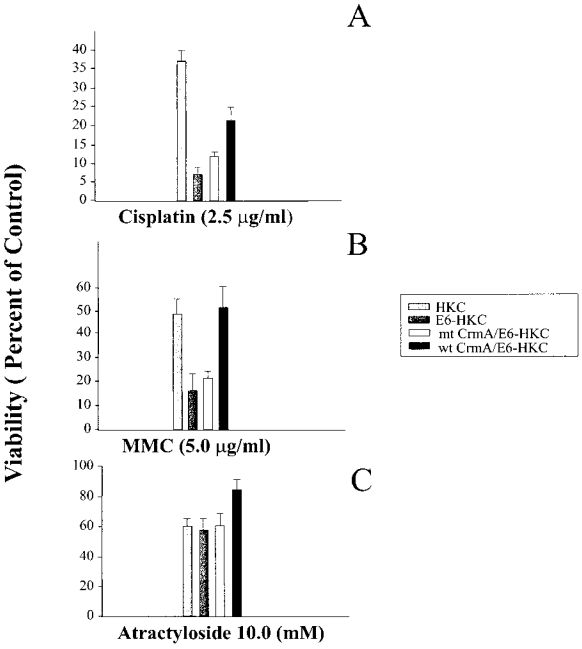


Fig. 7. Effect of wt CrmA and mt CrmA expression on apoptosis induced by cisplatin (2.5 μg/ml; **A**), MMC (5.0 μg/ml; **B**), or atractyloside (10.0 mM; **C**), respectively in normal vs. HPV-16 E6 expressing cells. HKCs were exposed to the various agents for 72 h. Viability is expressed as percentage of the untreated control. Data represent the mean (± SEM) from four trials.

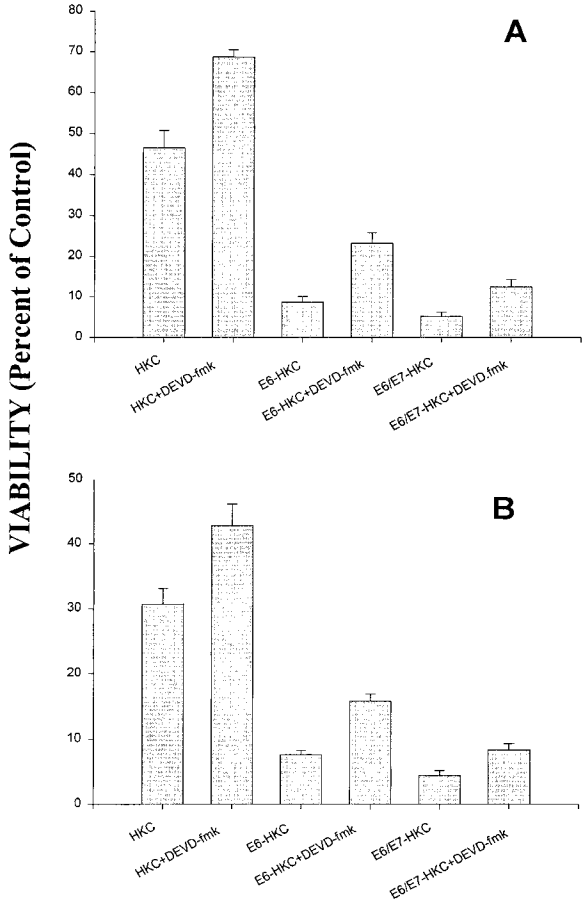
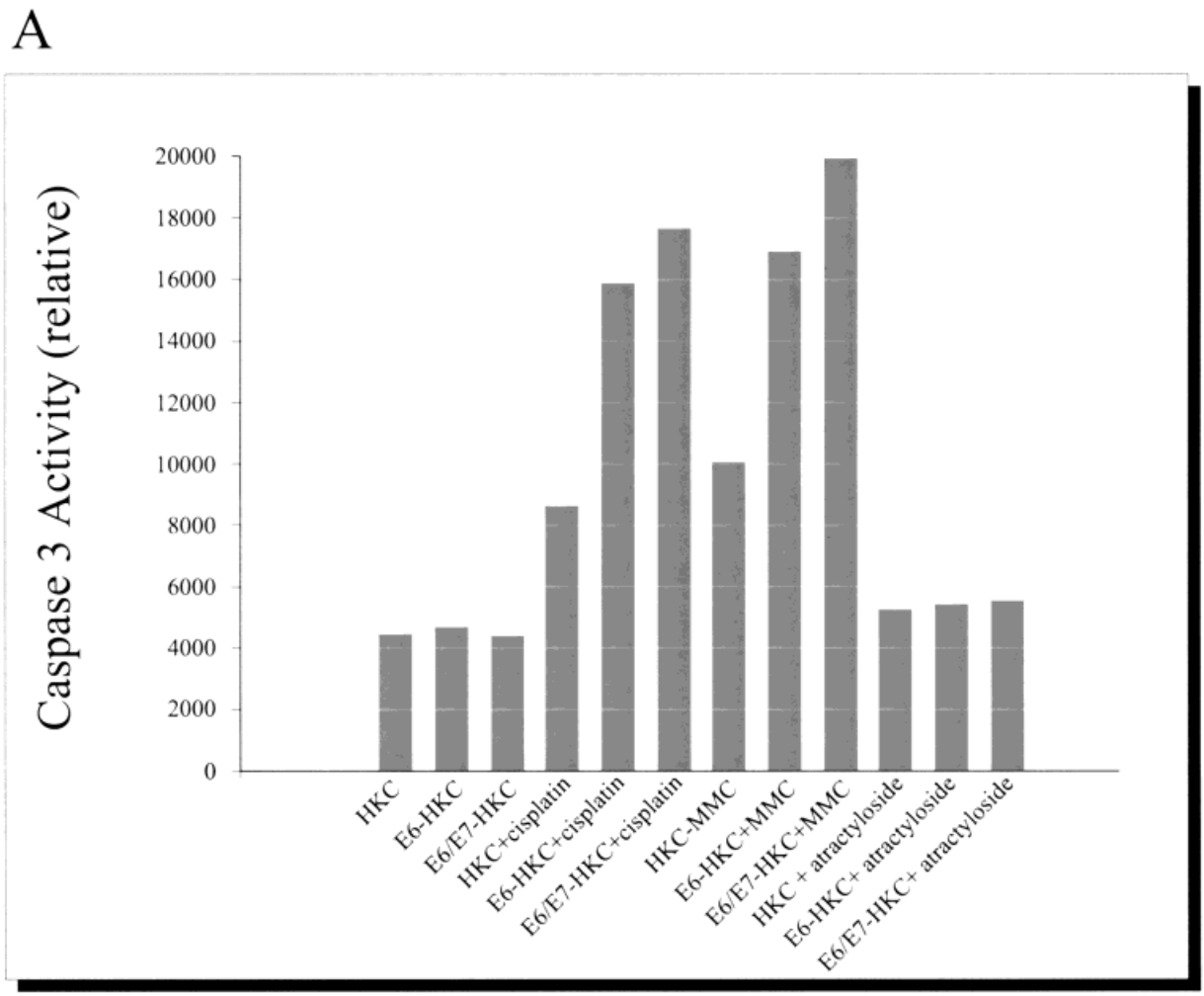


Fig. 8. Effect of caspase-3 inhibitor, DEVD-fmk, on apoptosis induced by cisplatin (**A**) or MMC (**B**). HKCs were treated with 7.5 μM DEVD-fmk and either cisplatin (5 μg/ml) or MMC (10 μg/ml) for 24 h. Viability is expressed as percentage of the untreated control. Data represent the mean (± SEM) from four trials.

HKCs by both cisplatin and MMC. DEVD.fmk inhibited cisplatin-induced apoptosis by 33%, 61%, and 58% in normal, HPV-16 E6, and HPV-16 E6/E7 expressing HKCs, respectively. DEVD.fmk inhibited MMC-induced apoptosis by 30%, 50%, and 38% in normal, HPV-16 E6, and HPV-16 E6/E7 expressing HKCs, respectively. We also monitored caspase-3 activity using a fluorometric assay based upon the red shift in emitted fluorescence of 7-amino-4-trifluoromethyl coumarin (AFC) following cleavage of DEVD-AFC by caspase-3, as well as by observation of loss of intact caspase-3 protein on Western blots (Fig. 9A,B). In normal HKCs, both cisplatin and MMC increased caspase-3 activity. This increase in capase-3 activity was substantially enhanced by the expression of HPV-16-E6 and HPV-16-E6/E7. Interestingly, atractyloside, although it induces apoptosis, either alone or in the presence of HPV-16 E6 or HPV-16 E6/E7 expression, did not increase caspase-3 activity.

DISCUSSION

HPV-16 E6 expression has been shown to prevent or enhance apoptosis depending on the stimulus and cell type. HPV-16 E6 expression increases the apoptotic sensitivity of J23T3 mouse fibroblasts to atractyloside; human mammary fibroblasts to cisplatin, carboplatin, paclitaxel, melphalan, and nitrogen mustard; human foreskin fibroblasts to MMC and staurosporine; and murine fibrosarcoma L929 cells to tumor necrosis factor alpha (TNF-α) [Brown et al., 1997; Hawkins et al., 1995; Liu et al., 1999; Xu et al., 1995]. Here, we extend these findings by demonstrating that HPV-16 E6 and HPV-16 E6/E7 sensitize HPV's natural host cells, human genital keratinocytes, to apoptosis induced by the DNA damaging agents etoposide, cisplatin, and MMC, but not to atractyloside or sodium butyrate. Our data also indicate that HPV-16 E6 and E7 co-expression result in greater sensitization of HKCs to apo-



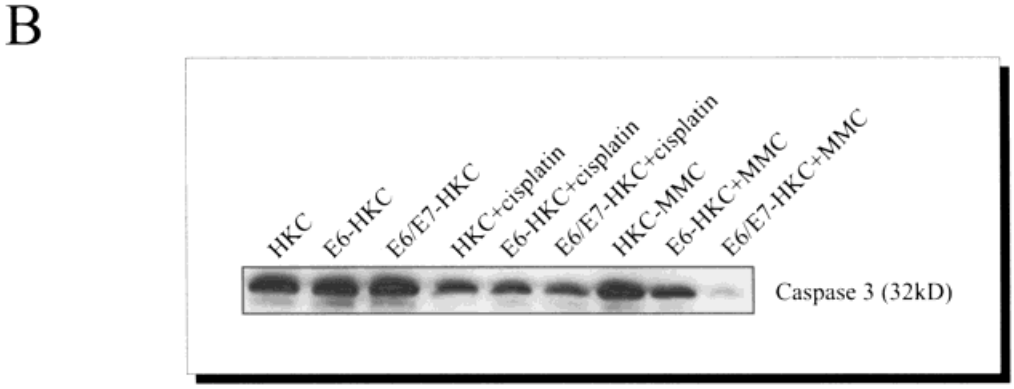


Fig. 9. Effect of HPV-16 E6 or HPV-16 E6/E7 expression on caspase-3 activity in HKCs. Caspase-3 activity was determined using Clontech's ApoAlert Caspase-3 Assay (**A**) and by Western blotting (**B**), using an antibody that recognizes only the prodomain of caspase 3. Cells were treated with cisplatin (5.0 μg/ml), MMC (10.0 μg/ml), or atractyloside (10.0 mM) for 24 h, respectively.

ptosis than expression of E6 alone, and most importantly, that HPV-16 E7 and low risk HPV-6 E6 expression did not sensitize HKCs to apoptosis induced by these agents. While the data presented here and elsewhere clearly demonstrates that HPV-16 E6 can sensitize HKCs to apoptosis, some cells show decreased sensitivity to apoptosis when E6 is expressed [Thomas et al., 1996; Yu et al., 1997]. These apparent inconsistencies in the effect of high risk HPV E6 on apoptosis could be due to differences in model systems used, as each employed different cells types, different reagents to induce apoptosis, and even different assays to measure apoptosis.

While we find that both HPV-16 E6 and HPV-16 E6/E7 expression sensitize HKCs to DNA damaging agent-induced apoptosis, it should be recognized that co-expression of HPV-16 E6/E7 is not equivalent to HPV-16 E6 expression alone. HPV-16 E6 binds and stimulates the degradation of p53 while E7 can increase the expression of p53 [Hickman et al., 1997; Demers et al., 1994]. These two opposing effects counteract each other and thus the overall p53 level in HPV-16 E6/E7 HKCs is not that low, and is intermediate in concentration between that of HPV-16 E6 HKCs and normal HKCs, as shown in Figure 3. In addition, HPV-16 E7, like E1A, can bind Rb protein. Previous studies have demonstrated that expression of HPV-16 E7 was able to overcome p53 induced G1 phase cell cycle arrest, which usually favors apoptosis [Wang et al., 1996]. The increased sensitivity of HPV-16 E6/E7 expressing HKCs relative to just HPV-16 E6 expressing HKCs to apoptosis, may therefore relate to both higher levels of p53 protein expression as well as the binding of E7 to the hypophosphorylated active form of Rb, resulting in release of transcriptionally active E2F-1 from the Rb-E2F-1 complex, leading to loss of cell cycle arrest, entry of cells into S-phase and p53-dependent apoptosis [Hiebert et al., 1995; Wu and Levine, 1994; Almasan et al., 1995].

The mechanisms by which high risk E6 sensitizes cells to apoptotic induction are not understood. High risk HPV E6 is known to target p53 protein for ubiquination-mediated degradation. As a transcriptional regulatory factor, p53 activates or suppresses the expression of downstream 'effector' genes such as p21, mdm2, Gadd45, Bcl-2, and Bax. The products of these effector genes are critical in the regulation of the cell cycle, programmed cell death, DNA repair, and replication [reviewed by Chandler et al., 1997]. Thus, alterations in the level or activity of p53 could alter, via changes in transcriptional regulation of these downstream genes, cellular sensitivity to inducers of apoptosis. Preliminary findings from our laboratory indicate that HPV-16 E6 expression reduces endogenous Bax expression in both HKCs and C33A cells (data not shown), and that HPV-16 E6 expression in HKCs prevented cisplatin and MMC-induced increases in Bax expression (data not shown). These data indicate that HPV-16 E6 can decrease Bax expression levels in the presence (HKCs) and absence (C33A cells) of wt p53 activity and that increased Bax expression does not appear to underlie the HPV-16 E6 sensitization of HKCs to DNA damaging agents.

P53 is also a critical regulator of G_1 cell cycle arrest, which in normal cells is activated in response to DNA damage. Failure of cell cycle checkpoints is known to result in genetic instability, and loss of cell cycle arrest potentially, via HPV-16 E6-mediated p53 degradation, could lead to genetic instability and explain the results we obtained in this study (i.e. changes in apoptotic sensitivity are due to genetic instability rather than any specific effect of HPV-16 E6 expression). While it is possible that genetic instability could play a role in the results of our study, we believe the increase in sensitivity of HKCs to the DNA damaging inducers of apoptosis used in this study is specific to HPV-16 E6 expression for the following reasons.

In the current study, as well as in previously published data, we and others have been able to detect both p53 expression and transcriptional activity in cervical carcinoma cells expressing various copy levels of full length HPV-16/18 [Butz et al., 1995; Liang et al., 1993]. In the data reported here, cisplatin and MMC led to an increase in p53 expression in HKCs in a time-dependent manner in normal HKCs, as well as in HKCs transfected with HPV-16 E6 or HPV-16 E6/E7. Thus, even though HPV-16 E6 can binds to and accelerate p53 degradation, this is not functionally equivalent to loss of p53 function.

Further evidence that our findings are specific to HPV-16 E6 expression and not a more general effect of genetic instability come from our findings that HPV-16 E7 (and HPV 6 E6),

expression did not increase HKC sensitivity to the various inducers of apoptosis. Numerous studies have indicated that both HPV-16 E6 and HPV-16 E7 expression disrupt p53-dependent G_1 arrest [Kessis et al., 1996], induce high frequency telomere associations of chromosomes [Wan et al., 1999], and increase mutation frequency in response to mutagenic agents (even in the presence of increased p53, p21 and gadd45 levels) [Liu et al., 1997]. In addition, expression of HPV-16 E7 alone has been shown to prevent suprabasal cells from exiting the cell cycle [Galloway and McDougall, 1996], disrupt quiescence imposed upon suprabasal cells, prevent G_1 arrest induced by DNA damage and inhibition of DNA synthesis caused by treatment with transforming growth factor beta [Demers et al., 1996], overcome p53-mediated G_1 arrest [Jones and Munger, 1997], neutralize the inhibitory activity of CDK inhibitors [Zehbe et al., 1999] and delay senescence in human myoblasts [Lochmuller et al., 1999]. Thus, HPV-16 E7 expression like HPV-16 E6 expression, initiates a number of processes that lead to genetic instability, yet in our studies, HPV-16 E7 expression did not sensitize HKCs to apoptosis as HPV-16 E6 did. It has also been shown that persistent infection with high risk HPV and high passage number are required for genetic instability to manifest itself following HPV infection [Pfister, 1996; Kaufmann et al., 1997]. We infected HKCs with HPV expressing retroviruses following two passages in culture after primary isolation and most of the studies described here utilized cells obtained immediately following antibiotic selection. Thus, the HKCs used in our studies are at relatively low passage numbers (6-10), and have a lower likelihood of being genetically unstable.

We also find that wt p53 activity is required for HPV-16 E6 sensitization based upon our studies employing C33A cells. C33A cells are a cervical carcinoma cell line-expressing mutant, nuclear localized p53. In these cells, expression of either wt HPV-16 E6 or an HVP-16 E6 mutant form that retains the ability to degrade p53 but lacks transformation activity [Nakagawa et al., 1995], did not result in sensitization of these cells to DNA damaging agents. Our data indicate that C-33A cells are more sensitive to apoptosis than HKCs. It might be argued that C-33A cells thus are maximally sensitive to induction of apoptosis and that expression of HPV 16 E6 protein cannot further sensitize these cells to apoptosis. Data previously published from our laboratories demonstrated that C-33A cells were found to be less sensitive than 3T3 fibroblasts to certain inducers of apoptosis, indicating that C-33A cells are not universally more sensitive (i.e., maximally primed) to all inducers of apoptosis. These results support the hypothesis that HPV-16 E6 sensitizes cells to apoptosis in a p53-dependent manner, although HPV-16 E6 has been reported to have p53-independent activity including modulation of transcription [Foster et al., 1994], immortalization of mouse cells in conjunction with ras [Storey et al., 1995] and growth stimulation [Ishiwatari et al., 1994]. Based upon our collective findings that: 1) p53 protein expression is detectable in HKC-16 E6 expressing HKCs; 2) DNA damaging agents induce an increase in p53 expression in HPV-16 E6 and HPV-16 E6/E7 expressing HKCs; and 3) HPV-16-E6 expression does not sensitize cells expressing mutant p53 to DNA damaging agents, we propose that wt p53 activity is necessary for HPV-16 E6s sensitization of cells to apoptotic induction by DNA damaging agents. Sodium butyrate and atractyloside stimulate apoptosis via p53-independent pathways, and this is likely the explanation for the lack of sensitization by HPV 16 E6 of HKCs to these apoptosis-inducing agents.

It is widely believed that p53 exerts its transcriptional activity in the nucleus. Recently, several studies have documented cytoplasmic sequestration of p53 in a subset of human tumor cells and virus infected cells, and suggested that this is related to mutant or inactivated wt p53 [Knippschild et al., 1996; Hwang and Lin, 1997; Moll et al., 1996; Isaacs et al., 1998; Kovacs et al., 1996]. We report here that wt p53 is distributed in the cytoplasm of HPV-16 E6 and HPV-16 E6/E7 expressing HKCs. Overexpression of Bcl-2, wt CrmA or treatment with cisplatin, etoposide, or MMC did not change this distribution. This altered cellular localization of p53 in high risk HPV E6 and HPV-16 E6/E7 expressing cells leads us to hypothesize that p53-dependent HPV-16 E6 sensitization of cells to apoptosis is independent of its transcriptional function. To examine this, we monitored p21 expression in normal, HPV-16 E6 and HPV-16 E6/E7 expressing HKCs following exposure to DNA damaging

agents. Our data demonstrate a loss of p53's ability to induce p21 in HPV expressing cells, suggesting that p53 transcriptional activation of p21 is defective in these cells. In combination with our p53 localization studies, we suggest that wt p53 protein expression, but not p53 transcriptional activity, is required for sensitization of HKCs to DNA damaging agents. Indeed, many forms of p53-dependent apoptosis have been shown to occur in the presence of inhibitors of RNA and protein synthesis as well as in the presence of various p53 mutants lacking transcriptional function [Chen et al., 1996; Haupt et al., 1995; Cadlles et al., 1994; Wagner et al., 1994].

How might p53 play a role in the regulation of apoptosis in the absence of transcriptional activity? Studies examining the regulation of cell death in *C. Elegans* have shown that the product of the *ced-3* gene is essential for normal developmentally regulated apoptosis. The discovery that the *ced-3* gene shares homology to a class of mammalian cysteine proteases has led to the identification of a family of 14 (to date) related proteases termed caspases (cysteine proteinases that cleave after and Asp residue) [Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998]. These caspases exists normally as inactive precursor molecules and can be subdivided into two groups based on the size of their prodomains—those with long prodomains (1,2,4,5,9,11,13), which act as initiator caspases, and those with short prodomains (3,6,7,14), which act as downstream effector caspases.

Recent data indicate that p53-dependent apoptosis involves caspase activity. P53-mediated neuronal cell death due to DNA damaging agents has been found to require caspase activation [Johnson et al., 1999]. Caspase-9 has been found to be an essential downstream component of p53 myc-induced apoptosis [Soengas et al., 1999] and p53-induced apoptosis in Jurkat cells and in human lymphoblasts has been found to involve activation of caspase-3 and other DEVD-sensitive caspases [Gao and Tsuchida, 1999; Yu and Little, 1998]. To further clarify the mechanism of HPV-16 E6 p53-dependent sensitization of HKCs to apoptosis, we investigated the role of caspases and caspase-3 in particular. In support of a role for caspases in HPV-16 E6's sensitization of HKCs to apoptosis, we found that expression of wt, but not mt CrmA, inhibited HPV-16 E6 sensitization of HKCs to apoptosis induced by DNA damaging agents. CrmA is a serpin from cowpox virus, that exhibits cross-class inhibition of both serine and cysteine proteases. Its expression has been found to inhibit apoptosis in a wide range of systems. CrmA can directly or indirectly interact with many kinds of caspases or related molecules and block their apoptotic activity to varying degrees [Turner and Moyer, 1998]. Our data also suggests that expression of HPV-16 E6 and HPV-16 E6/E7 increase caspase activity, in particular caspase-3 activity. Interestingly, caspase-3 activity was only increased in normal or HPV-16 E6 and HPV-16 E6/E7 expressing HKCs when exposed to DNA damaging agents, and not when undergoing apoptosis stimulated by atractyloside or sodium butyrate.

Our findings that HPV-16 E6 and HPV-16 E6/E7 sensitize human keratinocytes to apoptosis induced by DNA damaging agents may have implications for the therapy of cervical carcinoma. As p53 mutation is rarely found in cervical cancers [Crook et al., 1991a,b; Scheffner et al., 1991], p53-dependent sensitization of epithelial cells by HPV-16 E6 and HPV-16 E6/E7 may be an appropriate target in the treatment of cervical cancer.

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