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Human Papillomavirus (HPV) 16 E6 Sensitizes Cells to Atractyloside-Induced Apoptosis: Role of p53, ICE-Like Proteases and the Mitochondrial Permeability Transition

Jacqueline Brown,[1] Hiromi Higo,[1] Ann McKalip,[1] and Brian Herman[1,2]*

[1]Laboratories for Cell Biology, Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599
[2]Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract Infection of cervical epithelial cells with certain high risk HPV genotypes is thought to play an etiologic role in the development of cervical cancer. In particular, HPV type 16 and 18 early protein 6 (E6) is thought to contribute to epithelial transformation by binding to the tumor suppressor protein p53, targeting it for rapid proteolysis, resulting in loss of its cell cycle arrest and apoptosis-inducing activities. Recent data indicate that factors responsible for triggering apoptosis reside in the cytoplasm of cells, and not in the nucleus. In particular, the findings that mitochondria are required in certain cell-free models for induction of apoptosis and that bcl-2 is localized to mitochondria have focused attention on the role of the mitochondrial membrane permeability transition (MPT) in apoptosis. Here we present data to indicate that HPV 16 E6 expression sensitizes cells to MPT-induced apoptosis. We also report that HPV 16 E6 sensitization of cells to MPT-induced apoptosis occurs only in the presence of wildtype (wt) p53 expression. The extent of apoptosis induced by atractyloside (an inducer of the MPT) in normal, temperature-sensitive (ts) p53, and HPV-16 E6 transfected J2-3T3 cells, and the HPV expressing cervical carcinoma cell lines SiHa, Hela and CaSki was determined. C33A cells, which express mutant p53 but not HPV, were also exposed to atractyloside in the presence or absence of HPV 16 E6 expression. Dose-dependent apoptosis induced by atractyloside in normal J2-3T3 cells and cervical carcinoma cells was measured by loss of cell viability, nuclear fragmentation and DNA laddering. The sensitivity of cells to atractyloside-induced apoptosis was found to be: HPV 16 E6-J2-3T3 > CaSki > normal-J2-3T3 cells ≈ ts p53-J2-3T3 ≈ vector-J2-3T3 cells > Hela > SiHa > C33A ≈ C33A 16 E6. Cyclosporin A (CsA), an inhibitor of the MPT, and ICE-I, a protease inhibitor, provided protection against atractyloside-induced apoptosis. These findings indicate that: 1) high risk HPV 16 E6 protein is capable of sensitizing cells to apoptosis; 2) HPV 16 E6 sensitization of cells to atractyloside-induced apoptosis occurs in a p53-dependent fashion; 3) the target of HPV 16 E6 sensitization of cells to atractyloside-induced apoptosis is the mitochondria; and 4) HPV 16 E6 sensitization of cells to atroctycoside-induced apoptosis involves an ICE-like protease-sensitive mechanism, regulating the onset of the MPT. These findings constitute the first evidence that mitochondria play a role in HPV 16 E6 modulation of apoptosis. J. Cell. Biochem. 66:245–255.

Key words: p53; HPV; apoptosis; mitochondrial permeability transition; ICE-like proteases

Apoptosis is an actively regulated process of cell death necessary for proper control of tissue growth. The phenomenon was originally described by Kerr et al. [1972] as an event characterized by plasma membrane blebbing, cell shrinkage, chromatin condensation, and degradation of DNA. Apoptosis is believed to play a role in many physiological processes, and when defective can contribute to the pathogenesis of cancer [Wyllie et al., 1980]. A role for altered apoptosis in cancer comes from recent reports which have documented that inactivation of p53 and/or overexpression of bcl-2 occur in nu-

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Ms. Jacqueline Brown is currently at Spelman College, Atlanta, Georgia 30314.

Dr. Hiromi Higo is currently at Laboratory of Gene Structure, Department of Molecular Biology, National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki 305, Japan.

*Correspondence to: Dr. Brian Herman, Department of Cell Biology and Anatomy, CB #7090, 232 Taylor Hall, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. E-mail: bhgf@med.unc.edu

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merous cancers [Tsujimoto et al., 1985a, 1985b; Tsujimoto and Croce, 1986; Bakhshi et al., 1985; Cleary and Sklar, 1985]. The p53 gene is the most commonly mutated gene in human cancer. Although it was originally thought to be a dominant oncogene, p53 is now considered to be a powerful inhibitor of transformation and tumorogenesis [Finlay et al., 1989; Eliyahu et al., 1989; Chen et al., 1990]. p53 functions as a checkpoint control in the cell cycle following DNA damage [Chen et al., 1990]. In addition to causing cell cycle arrest following DNA damage, p53 has been shown to be required for apoptosis induced by E1A [Lowe et al., 1994], ionizing radiation [Clarke et al., 1993] and etoposide [Lowe et al., 1993]. Apoptosis can also be induced by Bax family members and interleukin 1-β-converting enzyme (ICE)-like proteases [Miyashita et al., 1995; Miura et al., 1993]. Bcl-2, a protein localized to mitochondria but of unknown function, can inhibit apoptosis induced by a variety of agents [Krajewski et al., 1993].

Several tumor-associated viruses have evolved mechanisms to modulate apoptosis of infected cells, allowing them to replicate and spread. One of these viruses, HPV, is associated with cervical cancer. Cervical cancer is the second most prevalent cancer in women worldwide, and epidemiological studies have strongly implicated the sexually transmitted human papillomavirus (HPV) as a causative agent [zur Hausen, 1982]. The ability of the high-risk HPVs to contribute to malignant progression appears to depend on expression of the E6 and E7 oncogenes. The high risk HPV 16/18 E6 oncoprotein, adenovirus E1B, and SV40 T antigen all form complexes and inactivate the cellular tumor suppressor protein p53 [Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow et al., 1982]. HPV-16/18 E6 protein interaction with p53 leads to enhanced degradation of p53 through ubiquitin-dependent proteolysis. Certain HPV-negative cervical cancers contain mutated p53, and in cervical carcinoma cell lines containing inactive p53, Bcl-2 is expressed at high levels [Liang et al., 1994]. Thus, high-risk HPV E6 expression is thought to inhibit p53's major biological activities (G_1 cell cycle arrest, transactivation of gene expression and apoptosis) and thereby lead to cervical cancer.

P53 exerts its tumor suppressor activity as a transcriptional regulator of cell cycle related genes (i.e. p53 has been found to transcriptionally activate the WAF1/Cip1 gene, which has been shown to inhibit cell growth by encoding a 21 kDa protein that binds to and inhibits cyclin/cyclin-dependent kinase [cdk] complexes [Harper et al., 1993]), and is thought to require nuclear localization. However, a number of recent reports have suggested that anucleate cells can be induced to undergo apoptosis, indicating the existence of a cytoplasmic apoptosis pathway that functions independently of the nucleus [Jacobson et al., 1994; Schulze-Osthoff et al., 1994; Nakajima et al., 1995]. Subcellular fractionation studies indicate that mitochondrial enriched fractions participate in control of apoptotic nuclear degeneration [Newmeyer et al., 1994]. Both Bcl-2 (which has been localized to mitochondria) and certain ICE-like proteases have also been found to play a role in cytoplasmic control of apoptosis.

These findings have focused attention on the role of the mitochondria in apoptosis. While it is not yet clear how mitochondria regulate apoptosis, it has been suggested that the opening of a mitochondrial permeability transition (MPT) pore or megachannel of very high conductance results in the loss of mitochondrial membrane potential ($\Delta\Psi$) and mitochondrial functions [Zamzami et al., 1996a]. The MPT is characterized by increased permeability to ions, swelling, collapse of mitochondrial membrane potential, and uncoupling of oxidative phosphorylation [de Macedo et al., 1993; Malis et al., 1990; Pastorino et al., 1993; Petronilli et al., 1993; Sokolove et al., 1993; Takeyama et al., 1993]. Oxidant stress, perturbation of the phospholipid deacylation-reacylation cycle, increased matrix free Ca^{2+}, pH, and membrane depolarization all favor pore opening, whereas acidic pH and phospholipase A_2 inhibitors prevent the permeability transition. In addition, atractyloside, a drug that binds to the adenine nucleotide translocator (ANT), which is thought to structurally constitute part of the MPT, has been found to open the MPT and induce apoptosis [Klingenberg, 1980; Toninello et al., 1984; Halestrup and Davidson, 1990; Marty et al., 1992; Majima et al., 1994]. Cyclosporin A (CsA), a cyclic peptide used as an immunosuppressant, inhibits conductance through the megachannel [Zamzami et al., 1996b].

We have previously reported that p53 protein is localized to the cytoplasm of cervical carcinoma cells via binding to HPV 16/18 E6 protein in the cell cytoplasm, preventing p53 from exert-

ing its tumor suppressor function in the nucleus [Liang et al., 1994]. Based on this finding, we would predict that HPV 16/18 E6 expressing cells would be more resistant to apoptosis. Since nuclei are not required for apoptosis induced by certain stimuli and mitochondria may play a role in induction of apoptosis, we examined the sensitivity of normal and HPV 16 E6 expressing cells to atractyloside-induced apoptosis. Surprisingly, we found that HPV 16 E6 expression sensitized cells to atractyloside-induced apoptosis, and that atractyloside-induced apoptosis required wt p53 activity and could be inhibited by CsA, an inhibitor of the MPT, as well as by ICE-1 inhibitor, a specific inhibitor of the ICE protease.

MATERIALS AND METHODS

Cell Culture

The following human carcinoma cell lines were obtained from the American Type Culture Collection: HeLa (derived from HPV-18 integrated cervical carcinoma), CaSki and SiHa (derived from HPV-16 integrated cervical carcinomas) and C33-A cells (derived from HPV-negative cervical carcinomas). HeLa, SiHa and C-33A cell lines were maintained in MEM/10% fetal bovine serum (FBS) and the CaSki cell line was maintained in RPMI/10% FBS. The J23T3 murine fibroblast cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% bovine calf serum.

Human neonatal foreskins were obtained from UNC Hospitals for isolation and propagation of keratinocytes. Normal human keratinocytes were initiated by floating full-thickness 0.5 cm^2 sections from individual foreskins on solutions of dispase for 12 to 16 h at 4°C. The epidermis was separated from the dermis and the keratinocytes detached from each other by trypsin digestion and gentle agitation. The keratinocytes were plated in Keratinocyte Serum Free Medium (KSFM; GIBCO-BRL, Grand Island, NY) supplemented with 5 ng/ml epidermal growth factor (hrEGF) and 25 µg/ml bovine pituitary extract (complete KSFM).

Transfections

J2-3T3 fibroblasts are a normal mouse fibroblast cell line that expresses wild-type p53. In the experiments reported here, the following J2-3T3 cell types were used: normal cells, cells transfected and expressing just vector, cells transfected and expressing ts p53, and cells transfected and expressing HPV 16 E6. ts p53 was transfected into J2-3T3 with an expression vector containing ts p53. The ts p53 vector expresses p53 in the mutant (dominant negative) conformation at 37°C and in wild type conformation at 32.5°C. J2-3T3 and C33A cells were transfected using lipofectamine. In a 100-mm dish 5×10^5 cells were plated 18–24 h before transfection (≈80% confluent). The DNA to be transfected (~4 µg) was incubated with the lipofectamine reagents for 45 min and then added to cells in 5 ml of OPT MEM1 medium for 5–18 h with gentle shaking. Following transfection, cells were washed and changed into complete medium. Transfected cells were selected in kanamycin and then used for experiments.

Primary human keratinocytes harvested from neonatal foreskins were retrovirally infected with the LXSN vector only or the LXSN vector containing HPV-16 E6 (a generous gift from D. Galloway). Transfected cells were selected and maintained in 50–100 µg/ml G418. Keratinocytes were cultured in complete KSFM with 0.5% FBS added.

Western Blot

Approximately 2×10^7 cultured cells were washed twice in phosphate-buffered saline (PBS) and suspended in 0.5 ml lysis buffer (1% Triton X-100, 0.15 M NaCl, 10 mM Tris [pH 7.5], phenylmethylsulfonyl fluoride [PMSF] 50 µg/ml, aprotinin, 50 µg/ml, and 50 µM leupeptin). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The lysate was boiled in $^{1/4}4\times$ protein loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.002% bromphenol blue) for 10 min, centrifuged for 2 min, and 40 µl of each protein preparation electrophoretically separated on 12% SDS-PAGE gel and subsequently transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS; 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.5% bovine serum albumin (BSA) and 1 mM PMSF for 2 h, incubated at room temperature with anti-p53 antibody for 1 h, washed three times with TBS and incubated with alkaline phosphatase-conjugated rabbit anti-hamster IgG (1:1,500). The positive reaction was visualized with NBT and BCIP.

Apoptosis Induction and Assay

Cells were plated in DMEM medium at 2×10^4 cells per well in Falcon 24-well plates and incubated at 37°C for 24 h before drug treatment. The cells were washed with PBS after removal of medium. Atractyloside (Sigma, St. Louis, MO) was used to induce apoptosis. The following concentrations of atractyloside were made in DMEM medium: 0.5 mM, 1.0 mM, 2.5 mM, 5.0 mM. To each well, 1 mL of the atractyloside/DMEM was added. Experiments were performed in quadruplicate along with two controls, DMEM medium only and a blank (no cells). The cells were treated with this drug for 3 days.

Cell Viability Assay

After the treatment period, viability was assessed by removing medium, washing cells 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 diacetyl fluorescein and hydrolyze it to fluorescein, which is retained. The live cells, but not dead cells, fluoresce green. After FDA addition, fluorescence was measured immediately using a fluorescence multi-well plate scanner (Cytofluor 2300). Viability is expressed as percentage of the untreated control.

To assay apoptosis in the presence of cyclosporin A and ICE-1, cells were pretreated with 1 mL of either 0.5 mM cyclosporin A or 25 mM ICE-1 inhibitor for 30 min at 37°C. After pretreatment, cells were treated as previously described.

Apoptosis Assay: DNA Fragmentation

Cells were treated with atractyloside to induce apoptosis. After treatment, culture medium containing dead floating cells was combined with cells detached from the plate with trypsin. DNA was extracted from cell pellets and resuspended in DNA loading buffer containing 5 mg/ml RNase A and loaded onto a 1.6% agarose gel. Genomic DNA was electrophoresed overnight at 22 V. The gel was stained with 2 µg/ml ethidium bromide in water for 1 h and destained with water for 24 h.

RESULTS

Atractyloside Induces Apoptosis and DNA Fragmentation in J23T3 Fibroblasts

Recently, a number of reports have suggested that when a variety of different cell types are exposed to an assortment of inducers of apoptosis, they undergo a reduction in mitochondrial membrane potential ($\Delta\Psi$) before they exhibit typical nuclear alterations associated with apoptosis [Zamzami et al., 1995a, 1996b; Marchetti et al., 1995]. Coupled with the observation that mitochondria undergoing the permeability transition acquire the capacity to induce nuclear apoptosis in an in vitro system [Zamzami et al., 1996a], these findings suggest that the MPT is a critical component of the apoptotic mechanism. Pharmacologically, the most favored way to regulate the open and closed states of the MPT pore has been through the use of ligands thought to specifically bind to the ANT, a potential component of the MPT pore. These include atractyloside, which opens the pore, and bongkrekic acid, which has been found to inhibit the MPT and reduce apoptotic induction by mitochondria in a cell-free system [Zamzami et al., 1996a]. Because p53 is necessary for induction of certain types of apoptosis [Clarke et al., 1993] and mitochondria appear to play a general role in apoptosis [Newmeyer et al., 1994; Marchetti et al., 1995; Zamzami et al., 1996a, 1996b], we examined the p53-dependence of atractyloside-induced apoptosis in a number of cell types where the functional state of p53 has been altered by expression of ts p53 or HPV 16 E6.

Atractyloside induced a dose-dependent loss of cell viability in normal J2-3T3 fibroblasts expressing endogenous (wt) p53 (Fig. 1). That this loss of cell viability was due to apoptosis

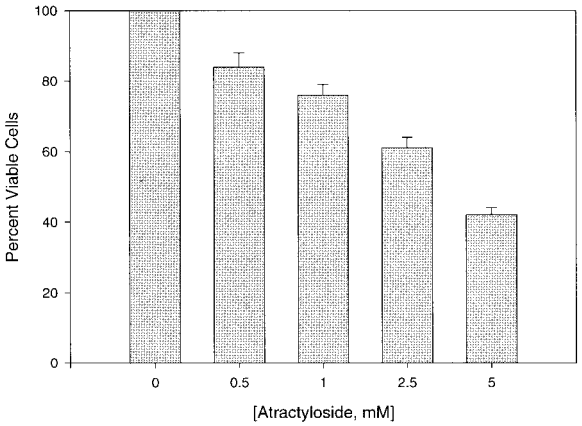

Fig. 1. Effect of atractyloside on J2-3T3 cell viability. J2-3T3 cells were exposed to various concentrations of atractyloside for 3 days at 37°C. Cell viability was determined using fluorescein diacetate and measured using a fluorescence multi-well plate scanner (Cytofluor 2300). Viability is expressed as percentage of the untreated control. Data represent the mean (± SEM) from four trials.

rather than necrosis was shown by the fact that loss of cell viability required prolonged (3 days) exposure to atractyloside, nuclear fragmentation was observed microscopically (data not shown) and atractyloside induced laddering of DNA (Fig. 2) in these same cells under identical conditions of treatment.

HPV 16 E6 Expression Alters Cellular Sensitivity to Atractyloside-Induced Apoptosis

In general, apoptosis can be divided into two categories—p53-dependent vs. p53-independent. For example, agents that cause DNA damage (e.g. mitomycin C, etoposide, cisplatin, etc.) require p53 for induction of apoptosis, whereas other agents (e.g. sodium butyrate, growth factor withdrawal) induce similar levels of apoptosis in the presence or absence or p53 [Clarke et al., 1990]. The role of p53 in atractyloside-induced apoptosis has not been examined to date. Infection of cells with high risk HPV is thought to lead to loss of p53 function, due to high risk HPV E6 binding to p53 and targeting it for rapid degradation via ubiquitin-dependent proteolysis [Werness et al., 1990]. This should result in a defect in p53-dependent apoptosis. Since expression of high risk HPV E6 is known to decrease p53 activity, we transfected J2-3T3 cells with HPV 16 E6 and determined the effect of HPV 16 E6 expression on atractyloside-induced apoptosis. Our prediction would be that if p53 is required for atractyloside-induced apoptosis, then HPV 16 E6 expression should result in lower sensitivity to atractyloside. However, surprisingly, HPV 16 E6 expression was found to increase sensitivity of J2-3T3 cells to atractyloside-induced apoptosis (Fig. 3). At all doses tested, HPV 16 E6 expression resulted in higher levels of apoptosis relative to control or vector expressing J2-3T3 cells. This paradoxical finding suggests that atractyloside-induced apoptosis does not require p53.

To further investigate the role of p53 in atractyloside-induced apoptosis, we examined the level of p53 protein in cells expressing HPV 16 E6 as well as transfecting J2-3T3 cells with a ts p53 expression vector, and determining the sensitivity of these cells to atractyloside treatment at 37°C. HPV 16 E6 expression decreased, but did not result in total loss of p53 protein expression (Fig. 4). Expression of the dominant nega-

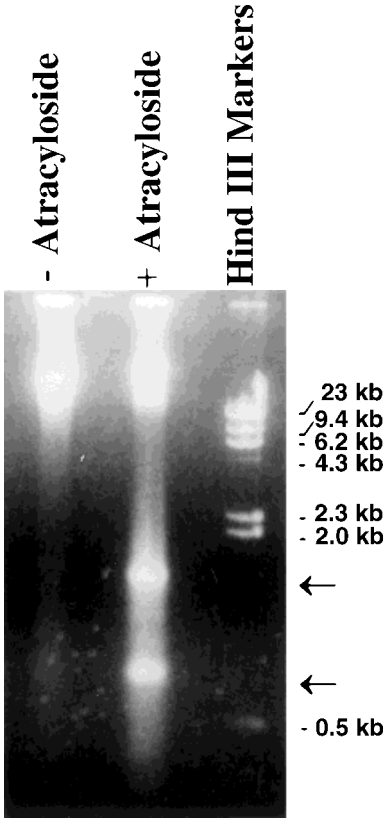

Fig. 2. Treatment of J2-3T3 cells with atractyloside induces DNA fragmentation. Cells were treated with 5 mM atractyloside for 24 h to induce apoptosis. After treatment, culture medium containing dead floating cells was combined with cells detached from the plate with trypsin and DNA was extracted from cell pellets and electrophoresed as described in the Materials and Methods. Atractyloside treatment resulted in DNA fragmentation as indicated by the arrows.

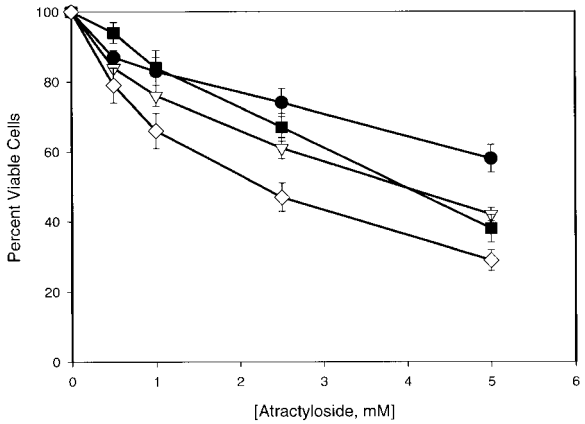

Fig. 3. Stable J2-3T3 cell lines expressing vector alone, dominant negative ts p53 or HPV 16 E6 were generated by transfection with appropriate constructs followed by selection as described in Materials and Methods. Cells were exposed to atractyloside at the concentrations indicated for 3 days and cell viability determined using fluorescein diacetate. Data represent the mean (± SEM) from four trials. Normal, ▽; vector, ●; ts p53, ■; HPV 16 E6, ◇.

tive mutant p53 at the permissive temperature slightly increased cellular sensitivity to atractyloside-induced apoptosis (Fig. 3). At the highest dose employed, ts p53 expression significantly increased cellular sensitivity to atractyloside. Like ts p53, expression of vector alone did not cause a significant increase in cellular sensitivity to atractyloside except at the highest dose employed.

Atractyloside-Induced Apoptosis in Cervical Carcinoma Cells

We also examined atractyloside-induced apoptosis in three cervical carcinoma cell lines (SiHa, HeLa and CaSki), which express various amounts of full length wt HPV 16 (SiHa, one copy/cell; CaSki, 500 copies/cell) or HPV 18 (HeLa, 20–50 copies/cell) protein. These cells have been characterized for functional p53 activity [Butz et al., 1995]. In these cells, atractyloside caused a dose-dependent induction of apoptosis (Fig. 5). CaSki cells were the most sensitive of the three cervical carcinoma cell lines. HeLa and SiHa cells displayed similar levels of sensitivity to atractyloside and were less sensitive than CaSki. This hierarchy in sensitivity correlates with the levels of p53 functional activity in these cells (CaSki ≫ HeLa ≈ SiHa) [Butz et al., 1995].

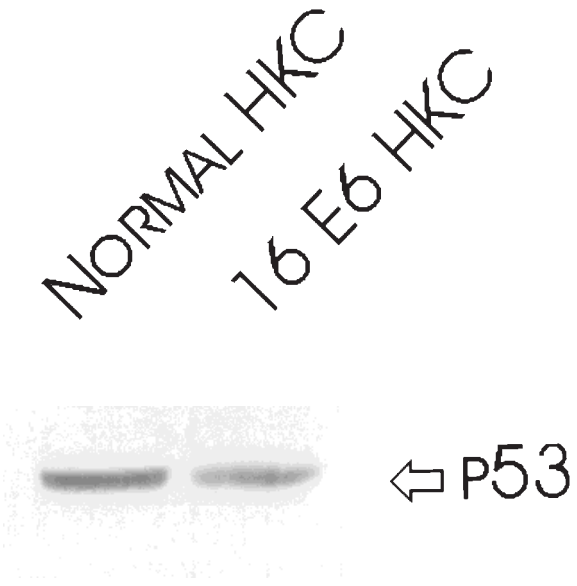

Fig. 4. Effect of HPV 16 E6 expression on cellular levels of p53 protein. Normal human foreskin keratinocytes were retrovirally infected with HPV 16 E6, stably selected and examined for levels of p53 protein expression in the presence and absence of HPV 16 E6 expression.

HPV 16 E6 Sensitization of Cells to Atractyloside-Induced Apoptosis Requires Wild Type p53

The data obtained using the cervical carcinoma cell lines CaSki, HeLa and SiHa in conjunction with those obtained in J2-3T3 cells expressing dominant negative mutant p53 suggest that p53 is required for atractyloside-induced apoptosis. However, expression of HPV 16 E6 increased cellular sensitivity to atractyloside-induced apoptosis, when p53 activity should be decreased. Although HPV 16 E6 expression results in a decreased half-life of wt p53 protein, it is possible that some wt p53 activity could still exist in HPV 16 E6 expressing cells (Fig. 4). To circumvent this concern, we transfected C33A cervical carcinoma cells with HPV 16 E6 and determined the effect of HPV 16 E6 expression on atractyloside-induced apoptosis (Fig. 6). C33A cells are derived from a HPV negative cervical carcinoma and express only mutant non-functional p53. In C33A cells HPV 16 E6 expression did not enhance the sensitivity of cells to atractyloside-induced apoptosis, indicating that HPV 16 E6 requires some wt p53 activity to sensitize cells to atractyloside-induced apoptosis.

Cyclosporin A and ICE 1 Protease Inhibitor Protect Cells Against Atractyloside-Induced Apoptosis

In isolated mitochondria, oxidant chemicals cause a permeability transition of the inner membrane characterized by increased perme-

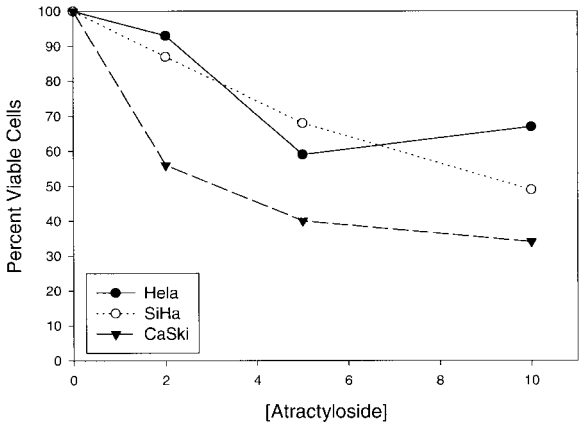

Fig. 5. Cervical carcinoma cells were cultured as described in Materials and Methods, exposed to atractyloside at the concentrations indicated for 3 days and cell viability determined using fluorescein diacetate as described in Materials and Methods. Data represent the mean from four trials.

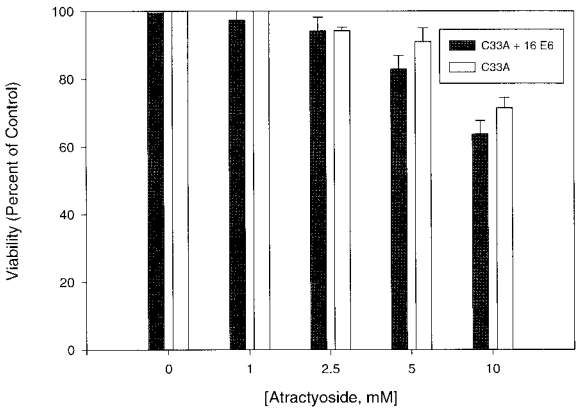

Fig. 6. Effect of HPV 16 E6 expression on atractyloside-induced cell death in C33A cervical carcinoma cells. C33A cells were transfected with HPV 16 E6 as described in Materials and Methods. Cells were then exposed to various doses of atractyloside and viability determined after 3 days in culture. HPV 16 E6 expression did not increase cell sensitivity to atractyloside. Light bars, C33A cells; dark bars, C33A cells expressing HPV 16 E6 protein. Data represent the mean (± SEM) from four trials.

ability to ions, swelling, collapse of mitochondrial membrane potential, and uncoupling of oxidative phosphorylation [de Macedo et al., 1993; Malis et al., 1990; Pastorino et al., 1993; Petronilli et al., 1993; Sokolove et al., 1993; Takeyama et al., 1993]. Onset of the permeability transition is due to activation of a latent permeability transition pore, or megachannel, of very high conductance. Atractyloside favors pore opening by binding to the adenine nucleotide translocator (ANT), thought to be a component of the permeability pore. Cyclosporin A, a cyclic peptide used as an immunosuppressant, inhibits conductance through the megachannel [Bernardi et al., 1994; Broekemeier et al., 1989; Szabo and Zoratti, 1991]. Therefore, we determined the effect of cyclosporin A on atractyloside-induced apoptosis in normal J2-3T3 or J2-3T3 cells expressing vector, ts p53 or HPV 16 E6. In addition, we also examined the effect of an inhibitor of ICE-like proteases (ICE 1 protease inhibitor), as both genetic and biochemical evidence indicates that ICE-like proteases are important in apoptosis [Miura et al., 1993; Martin and Green, 1995].

Both CsA and ICE 1 protease inhibitor protected cells against atractyloside-induced apoptosis (Fig. 7). In normal cells, CsA and ICE-1 protease inhibitor were equally effective in protecting cells (Fig. 7A). In J2-3T3 cells expressing just vector, CsA was slightly less effective than ICE-1 protease inhibitor in protecting cells against atractyloside-induced apoptosis (Fig. 7B). CsA and ICE 1 inhibitor were most effective in protecting cells against atractyloside-induced apoptosis in the presence of HPV 16 E6 expression (Fig. 7C). CsA and ICE 1 protease inhibitor did not provide statistically significant protection against atractyloside-induced apoptosis in ts p53 expressing cells (data not shown).

DISCUSSION

Our interests were to determine whether expression of HPV 16 E6, which is thought to alter p53 levels in cells, resulted in a defect in apoptosis which might play a role in the genesis of cervical cancer. The relationship between mitochondria (and the MPT), p53 status and HPV 16 E6 expression in the regulation of apoptosis has not been examined. We found that high risk HPV 16 E6 protein is capable of sensitizing cells to atractyloside-induced apop-

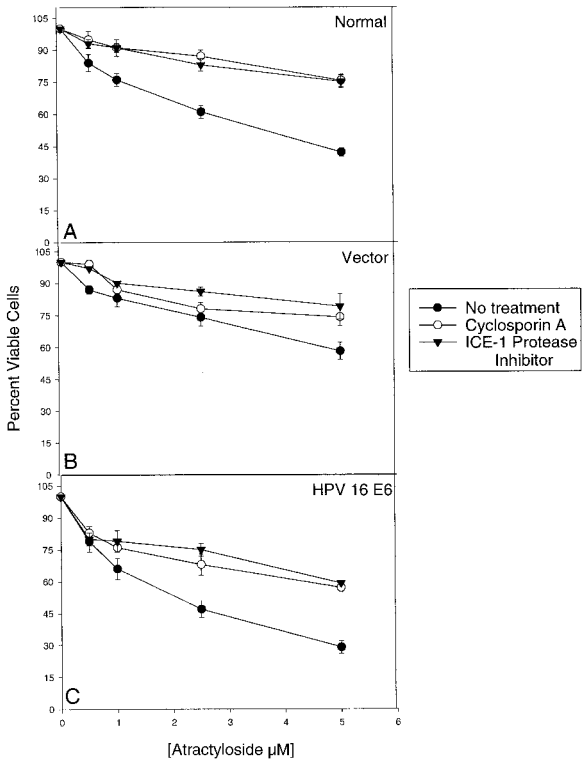

Fig. 7. Effect of cyclosporin A and ICE 1 protease inhibitor on atractyloside-induced apoptosis. Normal J2-3T3 cells (**A**), J2-3T3 cells transfected and expressing vector alone (**B**), or HPV 16 E6 (**C**) were exposed to various concentrations of atractyloside following pretreatment with CsA (0.5 μM) or ICE-1 inhibitor (25 μM) as described in Materials and Methods. After 3 days in culture, cell viability was determined. Data represent the mean (± SEM) from four trials.

tosis in a p53-dependent fashion by targeting the mitochondria through an ICE-like protease-sensitive mechanism. These findings constitute the first evidence for the mitochondria as a target of HPV 16 E6 modulation of apoptosis.

The regulation of apoptosis is complex, with a variety of agents having been found capable of triggering apoptosis, and a number of effector molecules required for induction of apoptosis. Although the initiators, effectors and targets of apoptosis are diverse, the ultrastructural changes that occur in apoptotic cells (e.g. cytoplasmic vacuolization, nuclear fragmentation, plasma membrane blebbing, formation of apoptotic bodies) are remarkably consistent, suggesting a common mechanism. What this common mechanism is is not known, but recent studies have pointed to the mitochondria as potentially providing a crucial common step in the apoptotic pathway. The evidence supporting a role for the mitochondria in apoptosis includes the findings that: 1) a reduction in $\Delta\Psi$ is an early event in many cells undergoing apoptosis [Zamzami et al., 1995a, 1995b, 1996a, 1996b; Marchetti et al., 1995; Wolvetang et al., 1994]; 2) Bcl-2 and Bax, proteins which respectively inhibit or promote apoptosis, are localized to mitochondria [Zha, 1996]; 3) isolated mitochondria induced to undergo the MPT can induce nuclear apoptosis [Zamzami et al., 1996a]; 4) atractyloside, which induces the MPT, causes apoptosis (and conversely, bongkrekic acid, which inhibits the onset of the MPT can prevent apoptosis) [Zamzami et al., 1996a, 1996b]; 5) inhibition of the activity of the mitochondrial protein cytochrome c by a death protease leads to apoptosis in Jurkat cells [Krippner et al., 1996]; 6) glutamate can induce neuronal cell death by either apoptosis or necrosis dependent on mitochondrial function [Ankarcrona et al., 1995]; and 7) Bcl-2 can inhibit the release of an apoptogenic protease from mitochondria induced to undergo the MPT by atractyloside [Susin et al., 1996]. Thus, mitochondria appear to play a crucial role in a variety of cells undergoing apoptosis.

A number of drugs have been found to specifically modulate the MPT. Atractyloside binds to the ANT, favors the opening of the mitochondrial permeability pore [Klingenberg, 1980; Marty et al., 1992; Majima et al., 1994; Zoratti and Szabo, 1995], and has been shown to disrupt $\Delta\Psi$ and induce nuclear apoptosis in a time- and dose-dependent fashion in both intact cells and cell free systems [Zamzami et al., 1996a, 1996b]. Cyclosporin A and the non-immunosuppressive cyclosporin A analogue N-methyl-Val-4-cyclosporin A are transient inhibitors of the mitochondrial permeability pore and act by binding to cyclophilin, inhibiting loss of $\Delta\Psi$ during apoptosis [Pastorino et al., 1993; Petronilli et al., 1993; Zamzami et al., 1996b]. ICE-like protease inhibitor 1 (Ac-YVAD-CHO) is a potent, specific, reversible inhibitor of ICE ($K_i = 0.2 \pm 0.1$ nM for recombinant human ICE). Ac-YVAD-CHO has been shown to inhibit apoptosis induced by a number of agents including respiratory chain inhibitors [Shimizu et al., 1996]. These reagents were used in this study to assess the role of the MPT in HPV 16 E6 sensitization of cells to induction of apoptosis.

Our results indicate that HPV 16 E6 expression sensitizes cells to atractyloside-induced apoptosis and requires wt p53 for this activity. These findings appear to be at odds with current dogma which states that HPV 16 E6 expression should decrease wt p53 activity (i.e. expression of HPV 16 E6 has been used to inhibit wt p53 activity in a variety of studies; based on this activity, results from these studies have been used to identify p53-dependent phenomenon). However, reports from a number of investigators have brought this conclusion into question. For example, in human foreskin fibroblasts HPV 16 E6 expression sensitized cells to apoptosis induced by cisplatin, carboplatin, paclitaxel, melphalan and nitrogen mustard [Hawkins et al., 1996]. HPV 16 E6 expressing cells displayed delayed progression through S phase, and these findings were interpreted to indicate that inactivation of p53 in the absence of other genetic changes increased cellular sensitivity to chemotheraputic agents due to loss of p53-mediated repair of DNA damage (i.e. decreased GADD45 expression). Conversely, while HPV 16 E6 expression has been found to sensitize mammary epithelial cells to apoptosis following DNA damage, mitomycin C, and staurosporine, this apparently occurred in a p53-independent manner [Xu et al., 1995]. 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].

To test the role of p53 in HPV 16 E6 sensitization of cells to atractyloside-induced apoptosis,

we examined apoptosis induced by atractyloside in three different cervical carcinoma cells lines (SiHa, HeLa and CaSki), which express multiple copies of HPV 16 or 18. Even though these cervical carcinoma cells express multiple copies of HPV 16 or 18, they have been shown to contain p53 transactivating activity, and treatment with mitomycin C, cisplatin or ultraviolet radiation led to increased p53 and p21 protein levels as well as G_1 cell cycle arrest [Butz et al., 1995]. Our findings indicate that atractyloside-induced apoptosis in these cervical carcinoma cells is directly correlated to levels of p53 transcriptional activity. These studies suggest that presence of high risk HPV is not functionally equivalent to loss of p53 function, and support the hypothesis that HPV 16 E6 sensitization of cells to atractyloside-induced apoptosis requires some level of wt p53 activity. This is further supported by our findings that HPV 16 E6 expression did not result in total loss of p53 protein expression and that in C33A cells, which lack wt p53 activity, HPV 16 E6 expression was not able to increase cellular sensitivity to atractyloside-induced apoptosis.

CsA was found to protect cells against atractyloside-induced apoptosis. Interestingly, CsA was most effective in cells expressing HPV 16 E6. CsA is a transient inhibitor of the MPT and these findings suggest that HPV 16 E6 protein can modulate the onset of the MPT and appears to require wt p53 activity. The MPT has been found to occur in multiple examples of apoptosis and CsA has been shown by us and others to inhibit onset of the MPT and apoptosis due to agents which specifically affect mitochondrial function. The mechanism(s) by which HPV 16 E6 (and p53) modulate mitochondrial activity is not clear. Possibilities could include a change in the level of a p53-dependent protein which interacts with the mitochondria, or direct interaction between p53 or HPV 16 E6 with the mitochondria. Previous studies from our laboratory have demonstrated that in cervical carcinoma cell lines, HPV 16 or 18 expression results in cytosolic localization of p53 [Liang et al., 1994]. In the findings reported here, these cervical carcinoma cells (which express p53 transcriptional activity) also displayed p53-dependent sensitivity to atractyloside. Thus, p53 nuclear localization does not appear required for atractyloside-induced apoptosis. Whether this reflects changes in protein expression due to loss of p53 nuclear localization or a cytoplasmic target of p53 is not clear. Supporting the possibility of a cytoplasmic (i.e. mitochondrial) target for p53 is the recent report demonstrating that under certain conditions, p53 can associate with HSC70 and GRP75, resulting in localization of p53 to the mitochondria [Merrick et al., 1996].

We also examined the involvement of ICE proteases in atractyloside-induced apoptosis. ICE inhibitor I provided protection against HPV 16 E6 atractyloside-induced apoptosis. This finding is not too surprising, as ICE-like proteases now represent a large family and occupy a central position in the apoptotic pathway [Miura et al., 1993; Martin and Green, 1995; Whyte, 1996]. However, this is the first report which documents that mitochondria may be a target of the ICE proteases. Interestingly, recent evidence has been published that indicates that mitochondria contain an apoptosis-inducing factor (AIF) which is apparently released upon loss of $\Delta\Psi$ and which, in a cell-free in vitro system, can cause isolated nuclei to undergo chromatin condensation and DNA fragmentation [Susin et al., 1996]. AIF is larger than the ICE-like proteases (~50 kDa) but is inhibited by Z-VAD.fmk, an antagonist of ICE-like proteases [Whyte, 1996]. Moreover, preliminary findings appear to indicate that recombinant ICE is a direct inducer of the MPT [Susin et al., 1996]. Collectively, these data suggest that mitochondria contain ICE-like protease activity and that the MPT may be regulated in some fashion by ICE-like proteases. If true then inhibitors of ICE-like proteases would be expected to protect against MPT-mediated apoptosis, as was the case in this study.

In summary, atractyloside, a specific agonist of the MPT, stimulated apoptosis in J2-3T3 fibroblasts. CsA, a inhibitor of the MPT, protected cells against atractyloside-induced apoptosis. Atractyloside-induced apoptosis appears to require wt p53 activity, and expression of HPV 16 E6 sensitized cells to atractyloside-induced apoptosis. In the presence of HPV 16 E6 expression, both CsA and ICE protease I inhibitor protected cells against atractyloside-induced apoptosis. These findings suggest that the mitochondria are a target for HPV 16 E6-mediated apoptosis, and that modulation of the MPT may be the mechanism by which these two proteins affect apoptotic activity in cells.

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