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UV-Vulnerability of Human Papilloma Virus Type-16 E7-expressing Astrocytes Is Associated with Mitochondrial Membrane Depolarization and Caspase-3 Activation

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The human papilloma virus-type 16 (HPV-16) E6 and E7 proteins interact with the p53 and pRb tumor suppressor proteins, respectively. The effect of E6 or E7 expression on UV irradiation (5 and 20 J/m^2)-induced genotoxic injury of confluent primary murine astrocytes was determined. Retroviral vectors were used to overexpress E6 and E7. Astrocytes expressing E7 showed increased vulnerability to UV-induced apoptosis while E6 over expressing astrocytes were protected from the same insults. Cell death in the E7 overexpressing cells was apoptotic because it showed DNA ladders, activation of caspase-3, formation of apoptotic bodies and decreased DNA content to less than the G_0 level. After UV-irradiation the level of E2F1 in E7-expressing astrocytes was higher than E6-, LXSN- or mock-infected cells, and caspase-3 was activated to a greater extent. E7-expressing astrocytes showed the highest levels of Bax under normal growth conditions. The mitochondrial membrane potential of E7-expressing astrocytes was depolarized by 90% after UV-irradiation while the depolarization in control cells was about 50%. E6 overexpression decreased while E7 overexpression increased UV-induced astrocyte apoptosis.

Keywords: Apoptosis; Astrocyte; Bax; Caspase-3; Human Papilloma Virus E6 and E7 Genes; UV Irradiation.

Introduction

The human papilloma virus (HPV) E6 and E7 genes are both essential and sufficient to immortalize and transform cells (Davies *et al.*, 1993; Harry and Wettstein, 1996; Hwang *et al.*, 2002). HPV E6 and E7 proteins interact with p53 and pRb tumor suppressor proteins, respectively (Xu, 1996). HPV16-E6 and/or E7 were previously found to enhance the survival of cortical astrocytes stressed by exposure to hydrogen peroxide or glucose deprivation, both injury paradigms involving oxidative stress and having characteristics of necrotic cell death (Lee *et al.*, 1998). E6 stimulates the degradation of p53 and reduces the apoptotic sensitivity of cells to genotoxic damage (Clarke *et al.*, 1994; Halbert *et al.*, 1991; Scheffner *et al.*, 1990), but also acts independently from p53 (Thomas and Banks, 1998). E7 binds pRb and facilitates its dissociation from E2F1. Free E2F1 can activate regulatory genes such as DNA polymerase, cdc-2 and myc, which are critical for cell cycle progression and DNA synthesis (Dyson *et al.*, 1989; Farnham *et al.*, 1993; Nevins, 1992) but can also induce apoptosis (Sears and Nevins, 2002). Evidence for a role for the Rb/E2F pathway in apoptosis was found as an extensive programmed cell death was observed in Rb-deficient embryos (Morgenbesser *et al.*, 1994). Selective degradation of cellular regulatory proteins may influence the likelihood of a cell undergoing apoptosis.

Another set of proteins important in the regulation of apoptosis are the members of the Bcl-2 family (Farrow *et al.*, 1995; Han *et al.*, 1996). This family consists of proapoptotic family members such as Bax, and antiapoptotic members such as bcl-2. The balance between proapoptotic and antiapoptotic members can decide the balance be-

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Abbreviation: HPV-16, human papilloma virus-type 16.

tween survival and apoptosis. These proteins are in part localized to mitochondrial membranes and influence mitochondrial membrane potential (Datta *et al.*, 1997). Work from several laboratories suggests that the mitochondrial membrane potential can influence the initiation of apoptosis (Kroemer *et al.*, 1997; Zamzami *et al.*, 1996). Because altering the threshold for triggering apoptosis may have implications in the treatment of cancer, and radiation is a common treatment strategy, we investigated the effect of E6 or E7 expression on genotoxic injury by UV irradiation in primary cultures of mouse astrocytes. We determined the effect of E6 or E7 on several genes involved in regulation of the cell cycle and apoptosis: pRb, E2F1, cyclin D1, Bax, and caspase-3, as well as measuring the relative change in mitochondrial membrane potential.

Materials and Methods

Materials Endotoxin-free water, glutamine and MEM were obtained from Gibco-BRL (USA). Fetal bovine serum and horse serum came from Hyclone Laboratories (USA). Falcon plasticware came from Becton Dickinson (USA). ICR Mice were from Samtako (South-Korea).

Cell culture Primary astrocyte cultures were prepared from postnatal 1−3 d mice as previously described (Lee *et al.*, 1998). The cortical hemispheres from brains of 1−2 d postnatal mice were dissected free of the meninges and treated with 0.09% trypsin for 30 min at 37°C. Dissociated neocortical cells were plated in Falcon Primaria 24-well plates at a density of 2 hemispheres per well in plating medium containing 10% fetal bovine serum (Hyclone, USA). The cultures were incubated in a 37°C, 5% CO_2 incubator for 2 d and infected with retroviral vectors when approximately 10−15% confluent.

Expression of HPV genes in astrocytes Retroviral vectors LXSN16E6 and LXSN16E7 were used to express the E6 or E7 genes of HPV-16, respectively, in addition to the neomycin-resistance gene. The construction and generation of the retroviruses has been described by Halbert *et al.* (1991). Controls included mock-infected cells and cells infected with the same retroviral vector LXSN without the gene of interest. Astrocytes expressing the viral genes were selected in 400 mg/ml G418 for 5 d. Cells were used for experiments after 25−30 d *in vitro.*

UV irradiation For UV-irradiation, cells were grown to 60−70% confluence, and the medium was changed to phosphate buffered saline (pH 7.4, PBS) and the cells irradiated with the UV-cross linker adjusted to deliver 5 or 20 J/m^2.

Evaluation of astrocyte injury Astrocyte injury was evaluated morphologically by phase-contrast light microscopy and staining of non-viable cells with trypan blue or propidium iodide. Live cells were stained with Hoechst 33258 dye (Sigma). Cell lysis was quantitated by assay of lactate dehydrogenase (LDH) activity released into the culture medium (Koh and Choi, 1988). Total LDH release corresponding to complete astrocyte death was determined at the end of each experiment following freezing at −70°C and rapid thawing. Control cells washed with $BSS_{5.5}$ but not irradiated showed ~10% maximal LDH release at the end of the experimental period.

DNA laddering Cells were washed with cold-PBS (pH 7.4), scraped and pelleted. The pellets were digested with DNA lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA, and 0.2% SDS), and the DNA extracted with phenol-chloroform. DNA fragments were separated on 0.8% agarose gels and visualized with ethidium bromide staining.

Flow cytometric analysis Cells were collected from the dish and a drop of 0.25% trypsin and 0.02% EDTA (ethylenediamine tetraacetic acid) in DMEM was added to the cell suspension and mixed gently. Approximately $1-2 \times 10^6$ cells were washed with ice-cold 1× PBS and centrifuged at $50-70 \times g$ for 5 min and the supernatant was aspirated with a pasteur pipette. The cell pellets were resuspended in 1 ml ice-cold 70% ethanol and fixed overnight at −20°C. For staining the cells were pelleted and resuspended in propidium iodide (PI) staining solution [PI (50 mg/ml), RNase (10 mg/ml), 1× PBS] and incubated in the dark, at room temperature for 30 min. Measurements of fluorescence were made with a FACSalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, USA) using a 488 nm argon ion laser. Data were acquired using selective gating to exclude doublet cells and the extent of DNA degradation was analyzed with CELLQuest software. DNA fragmentation was recognized as the sub-G1 population. In each experiment, the data from 5,000 to 10,000 cells were collected, stored and analyzed.

Immunoblotting Cultured astrocytes were prepared in 60 mm dishes, washed with cold-PBS (pH 7.4), resuspended with a rubber policeman and pelleted. The pellet was suspended in cold RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). The immunoblotting was performed with anti-Rb rabbit polyclonal antibody (Santa Cruz, USA) or E2F1 (Santa Cruz, USA) primary antibodies according to the manufacturer's protocol (Santa Cruz Biotechnology, USA).

Cell extracts were electrophoresed on 10 or 15% SDS-PAGE and electrotransferred onto nitrocellulose membrane (Millipore Co., USA). The membranes were blocked with 10 mM Tris buffered saline (TBS, pH 8.0) containing 5% nonfat dry milk overnight at 4°C, then probed for 2 h with 1:1,000 diluted E2F1 or Rb primary antibodies respectively. The membranes were washed three times with TBS containing 0.05% tween 20 and incubated with a 1:1,500 dilution of anti-rabbit Ig-horseradish peroxidase (Amersham, USA) for 1 h. Following three washes with TBS, bound antibody was visualized with the ECL system (Amersham, USA). After staining, the membrane was washed with TBS and blocked again with TBS containing 5% nonfat dry milk overnight at 4°C and incubated with 1:1,000 diluted-E2F1

or Rb primary antibodies according to the multiple detection method (Krajewski *et al.*, 1996), respectively. After washing with TBS, the membranes were incubated with a 1:1,500 diluted-anti-rabbit Ig-horseradish peroxidase and exposed to the ECL system. pRb (Santa Cruz, USA) and HeLa-phorbol nuclear extract (Santa Cruz, USA) were used as positive controls for the Rb and E2F1 antibodies, respectively. Western blot assays for cyclin D1, caspase-3, Bax and actin were performed by the method of Bergeron *et al.* (1996).

Caspase-3 assay Cells were irradiated by 20 J/m^2 and washed and collected in PBS. Cells were pelleted and resuspended in 50 µl of cold cell lysis buffer. The CaspACETM Colorimetric Assay System (Promega, USA) was employed to detect caspase-3 protease activity. The specific activity of caspase-3 is indicated as pmole pNA (chromophore, p-nitroaniline) liberated/ h · µg protein at 37°C.

Measurement of mitochondrial membrane potential Rhodamine 123 (500 nM; Molecular Probes, USA) was added to the astrocytes and incubated at 37°C in the dark for 30 min. At the end of the incubation period, astrocytes were observed on an inverted Nikon Diaphot microscope, and mitochondrial membrane potential was measured by fluorescence intensity using Axon Imaging Workbench 2.2 software, and analyzed on a Windows98 PC with a Pentium 3 processor. After a baseline level of rhodamine 123 fluorescence was established, astrocytes were exposed to 500 µM of the uncoupling agent carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma, USA) in order to determine maximum mitochondrial depolarization.

Results

To confirm expression of the HPV-16 E6 and E7 genes after infection with the retroviral vectors, RT-PCR was carried out using primers specific for E6 or E7. The expected size of PCR-products were found using RNA isolated from retrovirus infected astrocytes expressing E6 or E7 and not from RNA isolated from uninfected or LXSN infected astrocytes. A 322 bp fragment of the E6 gene was obtained while a 142 bp fragment of the E7 gene was amplified as previously described (Lee *et al.*, 1998). HPV-16 E7 expressing astrocytes were more vulnerable to UV irradiation (20 J/m^2), than mock infected or E6 expressing astrocytes (Fig. 1A). Exposure of E7 expressing cells to UV irradiation at doses of 5 and 20 J/m^2 resulted in approximately 27 and 43% cell death when injury was assessed by LDH release (Fig. 1A). The empty retroviral vector LXSN-infected astrocytes showed increased injury at the higher dose (20 J/m^2).

To determine whether UV-induced cell death was due to apoptosis we looked for the presence of nuclear fragmentation by staining with Hoechst and propidium iodide (PI) dyes. After UV-irradiation, E6 expressing cells and control mock infected cells showed few PI positive cells (Fig. 1B). E7 expressing astrocytes showed nuclear fragmentation and highly fluorescent and condensed nuclei, a hallmark of apoptosis (Fig. 1C). Many of the cells had already lyzed as determined by PI staining (Figs. 1C and 1D). Cells infected with the empty retroviral vector LXSN showed increased cell death relative to the other groups, but the morphology showed PI positive nuclei without evidence of fragmentation, consistent with necrotic cell death (Fig. 1D).

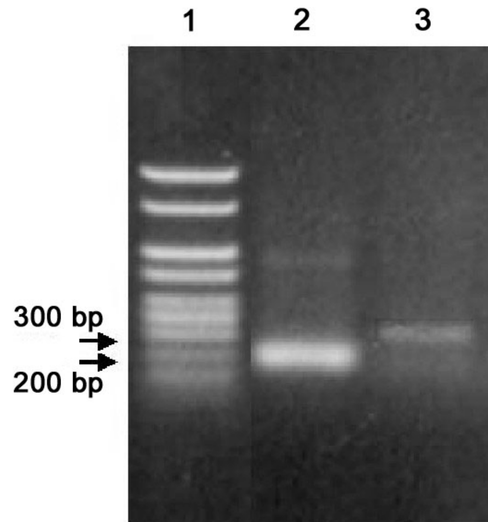

Fig. 1. Amplification of the E6 and E7 transcripts by RT-PCR. The gels were stained with ethidium bromide and photographed with a UV light. Lane 1, low DNA ladder; lane 2, RT-PCR amplification with RNA extracted from astrocytes expressing E7. E7 genes were amplified using E7 primers (180 bp). Lane 3, DNA amplified from E6-expressing cells using E6 primers (320 bp).

To further characterize the UV-induced cell death, fragmentation of the DNA into oligonucleosomal fragments was sought. DNA was extracted 24 h after UV irradiation and analyzed by agarose gel electrophoresis (Fig. 2). DNA laddering with production of fragments of approximately 200 bp multiples was prominent in E7 expressing astrocytes irradiated at a dose of 20 J/m^2. We also evaluated the DNA content of cells after UV-irradiation by flow cytometry after PI staining. As shown in Figs. 3 and 4, the percentage of cells containing sub-G$_0$ amounts of DNA, consistent with apoptosis, was markedly increased in the astrocytes overexpressing E7 after uv-irradiation.

Caspase activation is another hallmark of apoptotic death so caspase-3 expression and activation were determined after UV-irradiation. Protein levels of active caspase-3 by immunoblot were highest in E7 expressing cells after 20 J/m^2, although pro-caspase-3 was detected in control, E6 and E7 cells (Fig. 5A). However, the level of caspase-3 is very low in cells under normal growth conditions

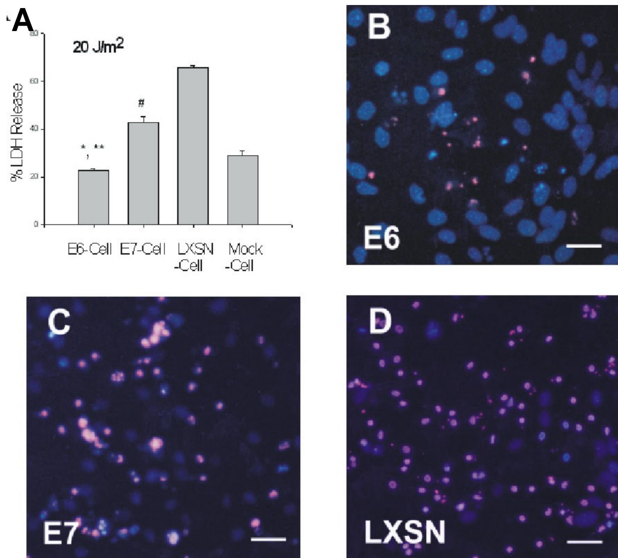

Fig. 2. Effect of HPV-16 E6 and E7 genes on survival of primary astrocyte cultures after UV-irradiation. **A.** Cell death was quantitated by release of LDH 24 h after UV-irradiation by 20 J/m^2. HPV-16 E6 expressing astrocytes were protected, while E7 and LXSN astrocytes were more injured than control. *, $p < 0.05$ comparing to Mock-cell; **, $p < 0.01$ comparing to E7- and LXSN-cell; #, $p < 0.01$ comparing to Mock-cell. (B)–(D), Micrograph of astrocytes 24 h after UV exposure (20 J/m^2). **B.** HPV-16 E6-overexpressing astrocytes stained with Hoechst-propidium iodide, bar = 60 µm. **C.** E7 gene expressing astrocytes, bar scale = 67 µm. **D.** LXSN-astrocytes, bar scale = 67 µm.

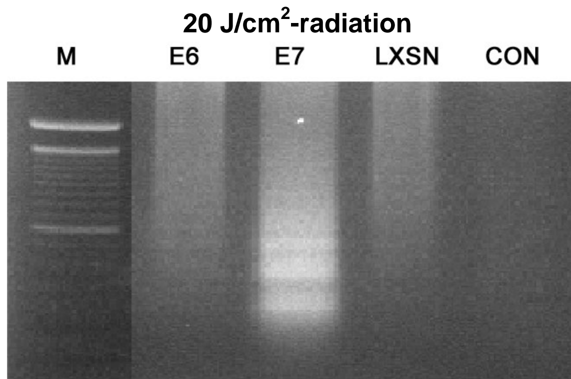

Fig. 3. DNA fragmentation of UV (20 J/m^2) irradiated astrocytes. A DNA ladder was seen following uv-irradiation in E7 expressing astrocytes.

(data not shown). Caspase-3 activity was measured using a fluorescent substrate, and only E7 expressing cells after 20 J/m^2 showed significant activity (Fig. 5B). We previously showed that E7 expressing cells had higher levels of E2F1, Rb and p21 proteins under normal growth conditions (Lee *et al.*, 2001b). The level of E2F1, although reduced after irradiation in all groups, was still higher in E7

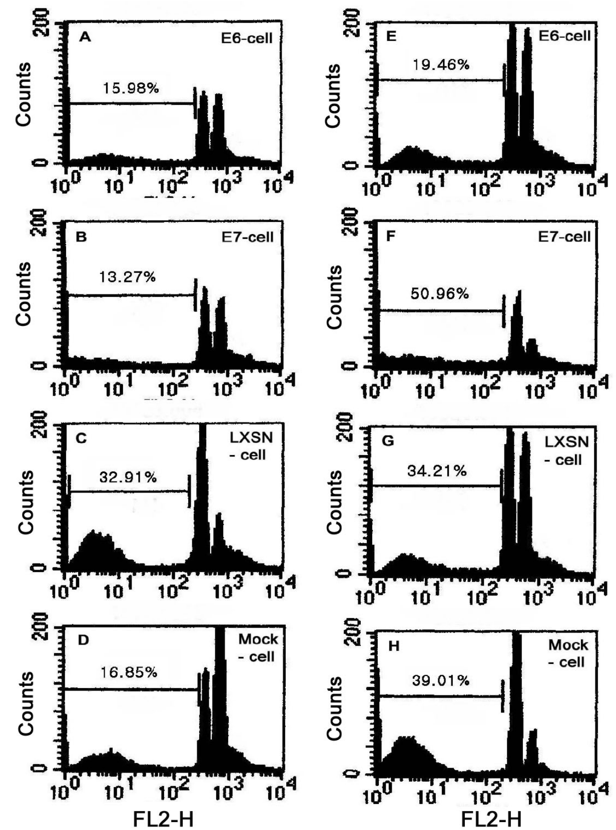

Fig. 4. Flow cytometric histograms for DNA content. (A)–(D), Under normal growth conditions. (E)–(H), After UV-irradiation injury (20 J/m^2). (A) and (E), Astrocytes expressing E6. (B) and (F), Astrocytes expressing E7. (C) and (G), LXSN-infected astrocytes. (D) and (H), Mock-infected control cells. The Y axis indicates the number of cells, X axis indicated level of fluorescence with propidium iodide.

expressing cells (Fig. 6A). Rb and cyclin D were present at comparably low levels after UV-irradiation in all groups (data not shown). By Western blot the level of the pro-apoptotic protein Bax was elevated under growth conditions in the astrocytes expressing E7, relative to the other groups (Fig. 6B) and after UV irradiation also (data not shown). However, p53 was not expressed in all kinds of astrocytes, even after UV irradiation, suggesting that p53 is not involved in UV-induced astrocyte apoptosis (Fig. 6C).

Rhodamine 123 was used to assess changes in mitochondrial membrane potential $\Delta\Psi_m$ after UV-irradiation. The mitochondrial membrane potential of E7-expressing and LXSN-infected astrocytes was 80–90% depolarized 24 h after UV-irradiation, while the extent of depolarization seen in the E6 and control cells was not significant (Fig. 7). The E6-expressing astrocytes and control cells maintained $\Delta\Psi_m$ at partially depolarized levels even after 48 h while at that point the E7 cells were fully depolarized (data not shown).

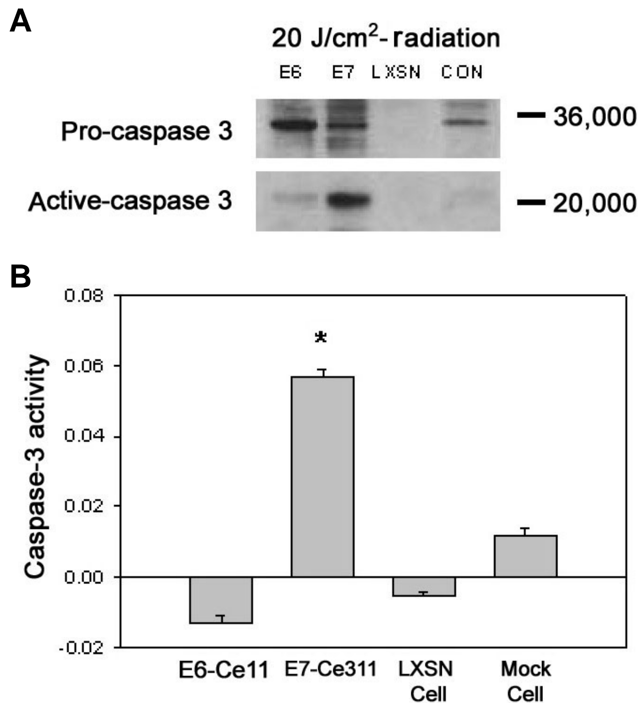

Fig. 5. A. Western blot after UV (20 J/m^2)-irradiation of astrocytes for pro-caspase-3 and activated caspase-3. **B.** Assay of caspase-3 activity after UV-irradiation (20 J/m^2) using a flourescent substrate. *, p < 0.01 comparing to E6-, LXSN- and mock-infected cells.

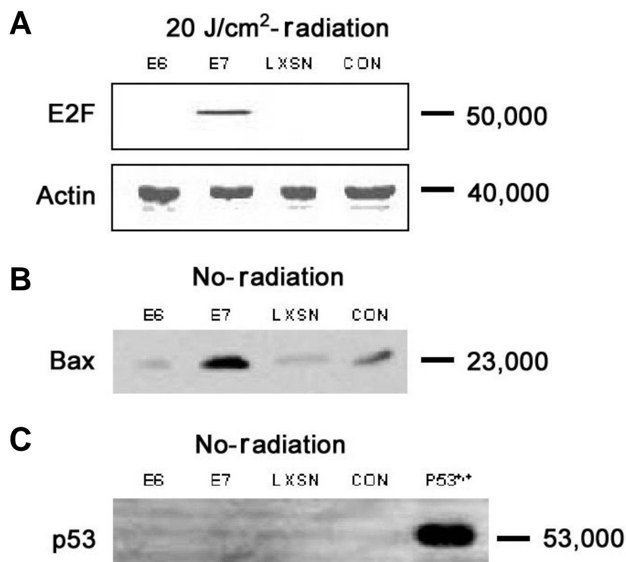

Fig. 6. A. Western blots after UV (20 J/m^2)-irradiation of astrocytes for E2F1 and actin. **B.** Western blot of astrocytes under normal growth conditions for Bax. **C.** p53.

Discussion

The human papilloma virus (HPV16) E6 and E7 genes had

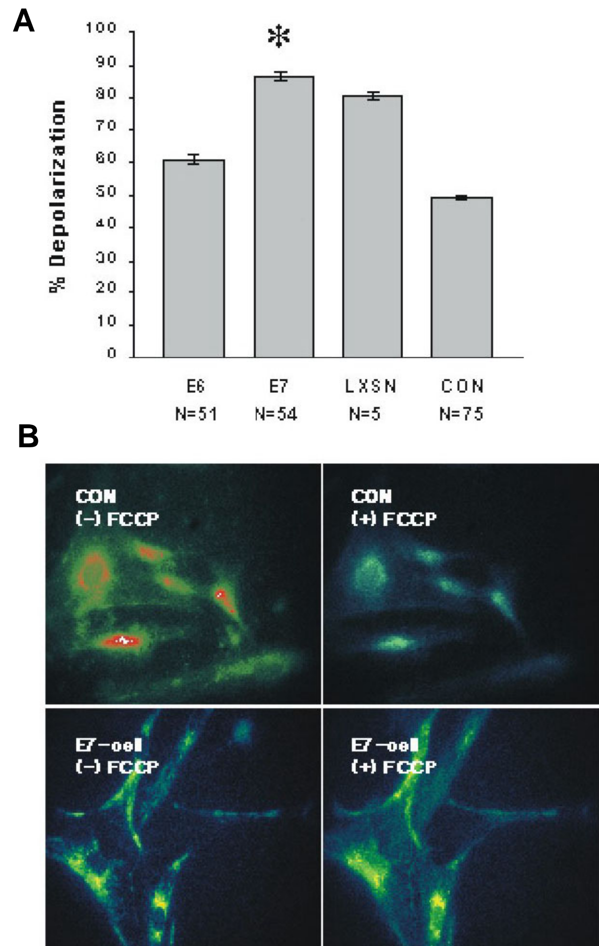

Fig. 7. Mitochondrial depolarization measured with Rodamine123. **A.** Relative membrane potential was determined with Rodamine123 and compared to fluorescence levels after addition of FCCP (= 100% depolarization). **B.** The mitochondrial membrane potential of E7 gene-expressing astrocytes was almost fully depolarized by 24 h after UV-irradiation (20 J/m^2). *, p < 0.01 compared to E6 and CON.

opposite effects on astrocyte survival after UV-irradiation. E6 expressing cells were protected while E7 overexpression was associated with much higher levels of apoptosis. This is in contrast to earlier observations in which both genes enhanced survival after exposure to hydrogen peroxide or glucose deprivation, both injury paradigms involving oxidative stress and having characteristics of necrotic cell death (Lee *et al.*, 1998; 2001b). E6 can stimulate the degradation of p53 and reduces the apoptotic sensitivity of cells to genotoxic damage, but it also decreases apoptosis by mechanisms independent of p53 (Lee *et al.*, 2001a; Yeo *et al.*, 2000). E7 binds pRb and faciliates its dissociation from E2F1. After UV-irradiation, E7 expressing astrocytes showed DNA ladders and nuclear morphology consistent with apoptosis including condensed nuclei and the formation of apoptotic bodies.

Additional characteristics of apoptosis found in UV irradiated E7 expressing cells included increased numbers of cells with sub-G_0 DNA content by flow cytometry and higher levels of Bax.

It is unlikely that the mechanism of E6 protection from UV injury is due to induced degradation of p53, since p53 was undetectable in these primary cultures by western. Another likely explanation is the ability of E6 to directly inhibit apoptosis by inhibiting the proapoptotic protein Bak. Interaction between E6 and Bak results in degradation of Bak (Thomas and Banks, 1998). Furthermore, in mice which were null for p53, HPV-16 E6 was still able to prevent the induction of apoptosis (Pan and Griep, 1995). It has also been shown that E6 will inhibit drug induced apoptosis in cells lacking p53 (Steller *et al.*, 1996).

The E7 protein preferentially binds to underphosphorylated pRb and facilitates its dissociation from E2F1, promoting the G1 to S phase transition in the cell cycle (Nevins, 1992; Pagano *et al.*, 1992). It has been demonstrated that E7 contributes to the deregulation of pRB-dependent E2F1 repression and to the further activation of E2F1 independent of pRb (Hwang *et al.*, 2002). However, UV-irradiation blocks the activation of E2F1 expressed independently of pRb. This may be partly responsible for the increased vulnerability to UV-induced apoptosis only found in astrocytes expressing the E7 gene. E2F1 knockout mice crossed with Rb deficient mice show partial rescue of the apoptotic phenotype, and E2F1 expression has been demonstrated to induce apoptosis under conditions where serum growth factors are limiting (Tsai *et al.*, 1998).

E7 expressing cells have higher levels of E2F1 and p21 proteins under normal growth conditions (Lee *et al.*, 2001a), and after UV-irradiation (20 J/m^2). E2F1 can induce apoptosis in a p53 independent manner. E2F1 has been shown to specifically induce expression of Apaf1, which in combination with cytosolic cytochrome C and caspase 9 forms the so-called apoptosome. This ternary complex then activates the downstream caspases including caspase-3 (Morgenbesser *et al.*, 1994). Consistent with this pathway the activity of caspase-3 was increased in astrocytes expressing the E7 gene. Caspase activation is important for the appearance of the morphological signs of apoptosis such as nuclear condensation, as well as DNA degradation and cell membrane changes (Salvioli *et al.*, 1997). Caspase-3 was activated only in E7 expressing astrocytes. A recent report suggests that the p53- and Bax-mediated apoptosis of UV-irradiated U937 cells results from caspase-3 activation. In that case an increased Bax/Bcl-2 ratio is due to Bax upregulation and Bcl-2 downregulation (Kimura *et al.*, 1998). In this paper the level of the pro-apoptotic protein Bax was increased by E7 expression even without added stress. Thus the Bax-caspase-3 pathway is implicated in uv-induced apoptosis of E7 expressing astrocytes.

Mitochondrial membrane potential, $\Delta\Psi_m$ changes have recently been implicated in apoptosis, and thought to be involved with opening of the mitochondrial permeability transition pore [for a review, see Kroemer *et al.* (1997)]. This process has been suggested to release apoptogenic molecules into the cytosol (Susin *et al.*, 1997; Zamzami *et al.*, 1996). The mitochondrial membrane potential of E7 expressing astrocytes was largely depolarized by 24 h after UV-irradiation. The reduction in $\Delta\Psi_m$ has been found by some authors to be an event early in apoptosis committing the cell to death, but others have found it to be a later step, namely, to occur after the activation of caspases (Bossy-Wetzel *et al.*, 1998). Moreover, a recent report suggests that an increase in $\Delta\Psi_m$ occurs early in apoptosis, preceding the later final reduction (Vander Heiden *et al.*, 1997).

Radiation induced apoptosis is well known from the widespread use of radiotherapy in cancer treatment. Apoptosis can be utilized as a therapeutic method to target and destroy tumor cells. Treatments designed to alter the apoptotic threshold have the potential to change the balance between apoptosis and viability, and the study of proteins that alter susceptibility to apoptosis may therefore have clinical relevance.

In conclusion E6 expressing astrocytes are protected from uv radiation induced injury while E7 expressing astrocytes are more injured. The E7 expressing cells die by apoptosis, associated with upregulation of Bax and increased caspase-3 activation.

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