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Original Article

Human Papilloma Virus Type 16 E7 Genes Protect Astrocytes against Apoptotic and Necrotic Death Induced by Hydrogen Peroxide

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Hydrogen peroxide is considered to be a dose- and time-dependent mediator in apoptotic and necrotic death. In this study, we examined the signaling of the E6 and E7 proteins with respect to apoptosis or necrosis after H_2O_2 injury using an *in vitro* model with overexpressed E6 or E7 genes. For this purpose, the E6 and E7 gene expressing astrocytes were exposed to 10 μM and 200 μM H_2O_2 solutions. Twenty-four hours after treatment with the lower dosage(10 μM H_2O_2), control, E6-expressing cells suffered about 45% injury and LXSN-expressing cells decreased by 67% as assessed by LDH release. However, E7-expressing cells showed less injury, resulting in 20 - 30% of LDH release. Astrocytes expressing E6, E7, LXSN and mock-infected cells showed a typical apoptotic death pattern on the DNA gel after treatment with a low-dose of H_2O_2 (10 μM), however they died from necrotic death after a high-dose (200 μM) H_2O_2. Overexpression of HPV-E7 genes protected the cells from apoptotic death after a low-dose of H_2O_2 and from necrotic death after a high-dose of H_2O_2, while the overexpression of E6 genes from the necrotic death. E7 expressing astrocytes showed higher catalase activity and the levels of E2F protein surged more than 100-folds compared with the control astrocytes. We believe that the activity of E7 protein to protect astrocytes from H_2O_2 injury was at least partly due to increased catalase, a scavenger protein.

Key Words: E6 gene, E7 gene, hydrogen peroxide, apoptosis, necrosis, E2F

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INTRODUCTION

Cell death is regulated by environmental factors and affected by injury.[1] Many oncogenes are implicated in the regulation of cell death, such as apoptosis, and in the control of the cell cycle, and several genes such as bcl-2 and bcl-x have been shown to protect cells from cell death directly. The accumulation of wild-type p53 protein results in two pathways, cell cycle G1 arrest by p21QAF1 or apoptosis inhibited by bcl-2. Moreover, an altered cell cycle, apoptosis controlled by p53 or of its mediators may facilitate tumor progression. Hydrogen peroxide (H_2O_2) is considered to be a dose- and time-dependent mediator of apoptotic and necrotic death. However, there is a growing body of evidence that some factors, particularly free radicals, that can induce necrotic cell death in neurons and glia, when applied at the sub-necrotic levels, induce apoptotic mechanisms in these cells.[2-4] Damaging stimuli associated with apoptosis include, viral infection, inappropriate regulation of cell growth control, cellular damage, and the loss of cell-cell or cell-substrate contact.[1] Apoptotic conditions have been hypothesized as influencers of the cell-cycle status of resting cells, via the activation of various kinase pathways, the production of various transcription factors, notably constituents of AP-1, and the transcription of cell cycle regulatory proteins.[5,6] However, these mechanisms are hypothetical and remain to be

demonstrated.

The HPV-16 E6 and E7 genes are essential and sufficient to immortalize and transform cells.[7-10] HPV-16 E6 and E7 proteins interact with p53 and pRb tumor suppressor proteins, respectively. E6 stimulates the degradation of p53 and reduces the apoptotic sensitivity of cells to genotoxic damage.[11-13] In contrast, E7 binds pRb and facilitates its dissociation from E2F.[14-16] Both p53 and Rb negatively regulate the cell cycle and appear to inhibit the G0-G1 and G1-S phase transitions.[17-20] When E6 and E7 proteins were overexpressed in primary astrocytes using retroviral vector, including HPV-16 E6 and E7 genes, E6 and E7 genes protected cells from H_2O_2 injury, in our previous study.[21] Astrocytes expressing the E7 gene showed significantly decreased vulnerability to hydrogen peroxide and glucose deprivation injury, share aspects of oxidative stress. E6 was much less effective at protecting cells from hydrogen peroxide than E7. Oncoprotein HPV-16 E6 proteins interact with p53 and E7 proteins interact with pRb and are related to the regulation of cell death.[22,23] However, function of E6 and E7, and the relationship between E6 and E7 and cell cycle regulation, and cell protection are not known.

In this study, we examined the apoptotic and necrotic signaling effects of E6 and E7 proteins after H_2O_2 injury using an *in vitro* model with overexpressed E6 or E7 genes. Astrocytes expressing the E6 and E7 genes were exposed to 10μM and 200μM H_2O_2 solutions.

MATERIALS AND METHODS

Materials

Endotoxin-free water, glutamine and MEM were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum and horse serum were purchased from Hyclone Laboratories (Logan, UT, USA), and Falcon plastic ware was from Becton Dickinson (Lincoln, NJ, USA).

Cell culture

Primary astrocyte cultures were prepared from postnatal (day 1 - 3) mice as previously described.[21] Cultures were incubated in a 37℃, 5% CO_2 incubator for 2 days and infected with retroviral vectors when the astrocytes were approximately 10 - 15% confluent.

Expression of HPV genes in astrocytes

Retroviruses LXSN16E6E7, LXSN16E6 and LXSN16E7 directing the expressions of the E6E7, E6 or E7 genes of HPV-16 respectively, as well as the neomycin-resistance gene, were used to infect astrocytes.[21,24] Astrocytes expressing the viral genes were selected in 1 mg/ml G418 (Geneticin, GIBCO BRL) for 5 days. Cells were used for experiments after 25 - 30 days *in vitro*.

Hydrogen peroxide exposure

Astrocyte cultures were washed three times with balanced salt solution (BSS5.5) containing (in mM) glucose 5.5, NaCl; 116, $CaCl_2$; 1.8, $MgSO_4$; 0.8, KCl; 5.4, NaH_2PO_4; 1, $NaHCO_3$; 14.7, N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES) 10; and phenol red 10 mg/L at a pH 7.4. A 30% solution of H_2O_2 was diluted in $BSS_{5.5}$ and added to the wells to a final concentration of 10 or 200μM Injury was assessed 2, 5, 10, or 24 hr after the addition of the H_2O_2.

Evaluation of morphology

Astrocyte injury was evaluated morphologically by phase-contrast light microscopy and by staining non-viable cells with trypan blue or propidium iodide, and live cells with Hoechst 33258 dye (Sigma, St. Louis, MO, USA)[25] Staining with the fluorescent dyes propidium iodide and Hoechst 33258 allow apoptotic and non-apoptotic cells to be differentiated on the basis of their nuclear morphologies and by evaluating their membrane integrities.[21] Cells were observed under the OLYMPUS diaphot microscope.

Measurement of LDH activity

Cell lysis was quantified by assaying lactate dehydrogenase (LDH) activity released into the culture medium.[26] Total LDH release corresponding to complete astrocyte death was determined at the end of each experiment following freezing at -80℃ and rapid thawing. Data are expressed as

the means ± S.E.Ms; statistical significance was determined by one-way ANOVA and Student-Newman-Keul's test using SigmaStat (Jandel).

DNA Fragmentation

DNA fragments were separated using 0.8% agarose gels and visualized by ethidium bromide staining. For this, cells were washed with cold-PBS (pH 7.4), scraped and pelleted. The pellet so obtained was 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 was extracted using phenol-chloroform.[27]

Caspase-3 assay

Cells were treated with 20 μM H_2O_2 for 24 hr, collected and washed in PBS. Cells were then pelleted and resuspended in 50 μl of cold cell lysis buffer. Cell extracts were used as an enzyme source. The CaspACE^{TM} Colorimetric Assay System (Promega) was employed to detect caspase-3 protease activity. The specific activity of caspase-3 was calculated as follows: pmole of pNA (p-nitroaniline, chromophore) liberated per hour at 37 per μg protein.

Catalase assay

Cells were lysed with dH_2O 24 hr after the addition of H_2O_2 (10 or 200 μM) and 60 mM of the H_2O_2 was then added to each lysate. After incubation for 1 min, 2 M of H_2SO_4 was added to stop the catalase reaction. For color development, 2.2 mM of $KMnO_4$ was added and catalase activity was measured at 490 nm using an ELISA Reader (SpectaMAX 340, Molecular Devices).

Immunoprecipitation and immunoblotting

Cultured astrocytes were prepared in 60 mm dishes and washed with cold-PBS (pH 7.4), scraped and pelleted. The pellet was then digested with cold RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Immunoprecipitation was performed with anti-rabbit pRb polyclonal antibody (Santa Cruz Biotechnology) or E2F1 (Santa Cruz Biotechnology) primary antibodies, according to the manufacturer's instructions (Santa Cruz Biotechnology).

Cell extracts and immunoprecipitated samples were electrophoresed on 10% or 12.5% SDS-PAGE and electrotransferred onto nitrocellulose membranes (Millipore Co., Bedford, MA, USA). The membranes were probed with E2F, pRb, cyclin D1, p21, Bax and actin primary antibodies respectively. Bound antibody was visualized with the ECL system (Amersham, Arlington Heights, IL, USA).

RESULTS

Cell death by apoptosis frequently occurred in E6-, and E7-expressing cells, LXSN-infected and mock-infected cells under a low dose hydrogen peroxide (10 μM) conditions, while necrotic death was induced by a high dose of hydrogen peroxide (200 μM) (Fig. 1 and 2). Approximately 50% of themock-infected cells were injured after low-doses of H_2O_2, as assessed by LDH release (Fig. 1), and turned out to have undergone apoptosis based on nuclear morphology (Fig. 2). Astrocytes expressing E7 gene were protected from hydrogen peroxide induced apoptotic death but E6 cells were not so protected. Twenty four hours after treatment, mock-infected and E6-expressing cells reduced LDH release by 45% and LXSN-expressing cells reduced LDH release by 67% (Fig. 1A). However, E7-expressing cells reduced LDH release by 20 - 30% (Fig. 1A). Results were assessed by lactate dehydrogenase (LDH) assays. E7 expressing astrocytes were efficiently protected from injury induced by low-dose H_2O_2 compared with the mock-infected astrocytes (Fig. 2). More apoptosis was induced in mock-infected cells, as evidenced by the appearance of DNA fragmentation by Hoechst-PI staining. Apoptosis induced by H_2O_2 injury may involve the p53- dependent pathway. To demonstrate whether low-dose hydrogen peroxide induced apoptotic death in the E7 gene expressing astrocytes, DNA fragmentation assay was performed (Fig. 3). At a dose of 10 μM of H_2O_2, apoptotic cell death was shown in E6, E7, and LXSN expressing astrocytes and mock-infected cells as indicated by typical DNA ladders, which are a hallmark of apoptosis.

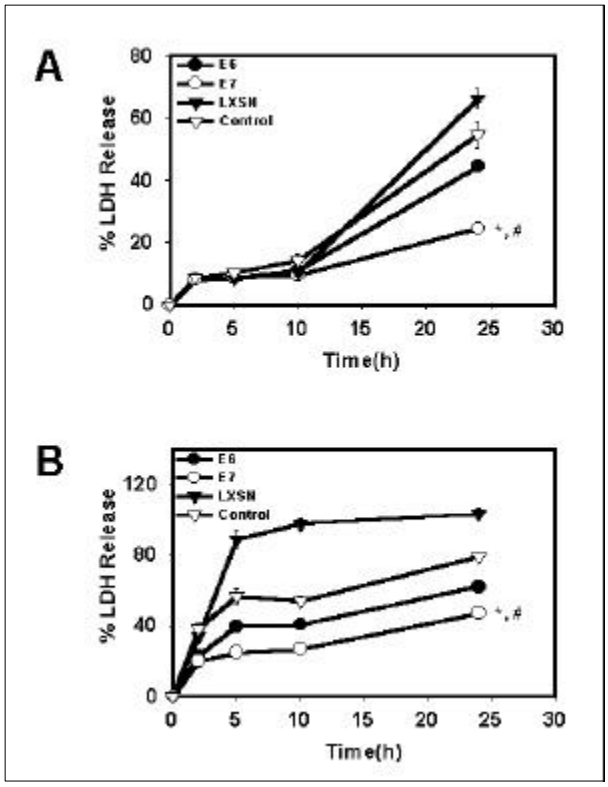

Fig. 1. Time course of hydrogen peroxide-induced cell death in the E6 or E7 expressing astrocytes. (A), 10μM H_2O_2 injury; (B), 200μM H_2O_2 injury. LDH release was assayed after the indicated times. * Represents significant differences ($p < 0.05$) from mock-infected control cell; # indicates difference from LXSN ($p < 0.05$) by one-way ANOVA and Student-Neuman-Keul's test.

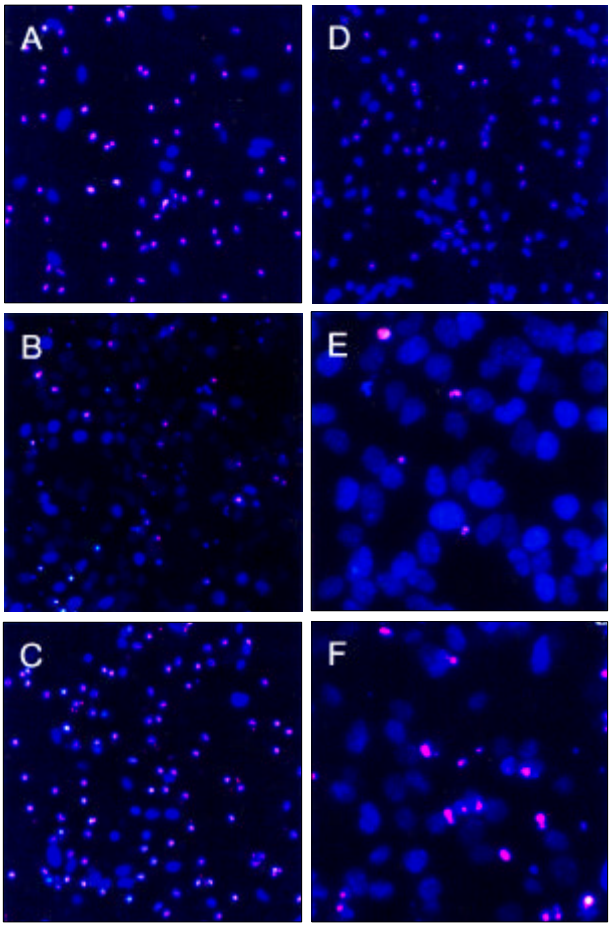

Fig. 2. Effect of E6 or E7 expression on apoptosis induced by low dose hydrogen peroxide (10μM H_2O_2) in the (A) E6, (B) E7, (C) LXSN (vector only) expressing astrocytes and (D) mock-infected cells. Cells were stained with propidium iodide (PI) and Hoechst dye. The large diffusely fluorescent nuclei stained by Hoechst dye were in intact cells by PI exclusion criteria. (E) E7 expressing astrocytes were efficiently protected from injury induced by H_2O_2 compared with mock-infected astrocytes. More apoptosis was induced in (F) mock-infected cells, as determined by DNA fragmentation after Hoechst-PI staining. (A), (B), (C), (D): × 200. (E), (F): × 400.

However, no DNA ladders were found in the gel of the mock-infected control cells, E6 gene expressing cells and LXSN-infected astrocytes after high-doses of hydrogen peroxide. After treatment with higher doses (200μM H_2O_2), both E6 and E7 expressing cells were protected from same insult and all the cells were died from necrosis (Fig. 1B, Fig. 3, data not shown this time, refer to 21). HPV-16 E7 genes protect from both apoptotic and necrotic cell death, whereas E6 genes cannot protect from apoptotic cell death induced by low dosages (10μM H_2O_2) (Fig. 1). Therefore, E6 and E7 proteins protect the cells from hydrogen peroxide injury through different death signaling mechanisms.

To further investigate the involvement of caspase in the protection of HPV-16 E7 from apoptosis induced by low doses of hydrogen peroxide, the relative caspase activity was measured in astrocytes expressing the HPV-16 E6 and the E7 genes. Cell lysates were collected 24 hours after exposure to low doses of H_2O_2 (10μM H_2O_2) and caspase activities were measured by spectrophotometric assay. The caspase activity of the LXSN-infected cells and the astrocytes expressing HPV-16 E7 genes were significantly lowered after exposure to low doses of H_2O_2. In those astrocytes expressing HPV-16 E6 genes, the caspase activities were elevated and no significant change of relative caspase activities were found in the mock-

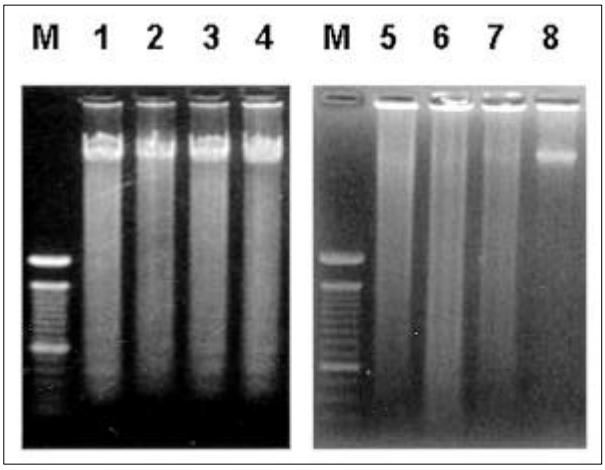

Fig. 3. DNA fragmentation assay of E6 or E7 expressing astrocytes after exposure to hydrogen peroxide. At a low-dose H_2O_2 (10 μM), the apoptotic cell death was showed in E6, E7-expressing, LXSN- and normal astrocytes, as determined by the typical DNA ladders that are a hallmark of apoptosis. Lane 1,5: E6, Lane 2,6: E7, Lane 3, 7: LXSN, Lane 4, 8: Mock-infected control cells. Lane 1-4: cells exposed to a low-dose H_2O_2 (10 μM). Lane 5-8: cells exposed to a high-dose H_2O_2 (200 μM).

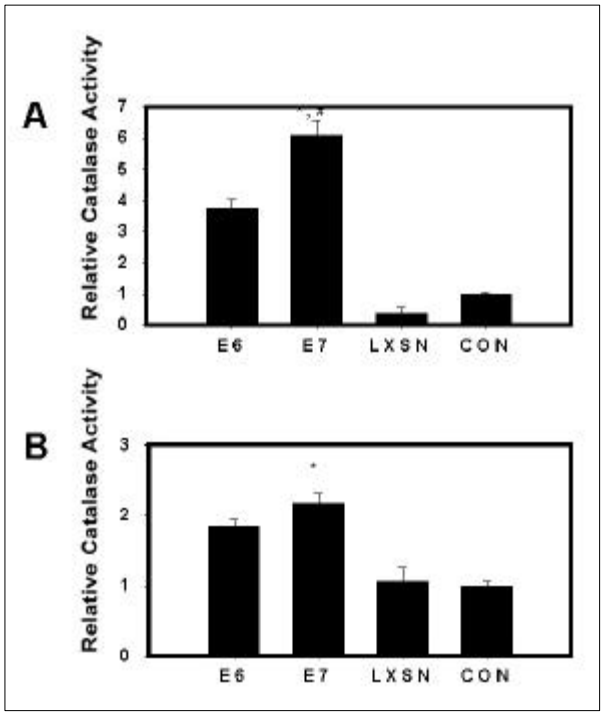

Fig. 4. Relative catalase activity (unit/μg protein) in astrocytes expressing the HPV-16 E6 and E7 genes. Cell lysates were collected 24hr after H_2O_2 exposure. (A), 10 μM H_2O_2 injury; (B), 200 μM H_2O_2 injury. E7 expressing astrocytes show higher catalase activity than E6 or mock-infected cell. *Represents significant differences ($p < 0.05$) from mock-infected control cells and LXSN cells; # from E6 cell ($p < 0.05$) by one-way ANOVA and Student-Neuman-Keul's test.

infected normal astrocytes after exposure to low doses of H_2O_2 (Table 1).

E7 expressing astrocytes showed higher catalase activity, which is one of the scavenger enzyme activity than E6 expressing cells or mock-infected cells (Fig. 4). In order to elucidate the factors responsible for the cellular protective mechanism involved in E7 proteins against H_2O_2 injury and investigate the interaction and regulation between cell protection and cell cycle regulatory protein, Western blot analysis were performed with E2F-1, pRb and p21, which participate in cell cycle regulation and apoptosis, and actin as a control protein (Fig. 5, and 6). When the genes coding for the E7 protein were incorporated in the astrocytes, the levels of the E2F protein surged more than 10 folds compared that of the mock-infected cells (Fig. 5). E2F and p21 induction occurred in normal HPV-16 E7 expressing astrocytes and both of the protein levels were lower in HPV-16 E7 expressing astrocytes after exposure to low (10 μM H_2O_2) and high (200 μM H_2O_2) doses of hydrogen peroxide by Western analysis (Fig. 7).

DISCUSSION

Hydrogen peroxide (H_2O_2), which is generated as a byproduct during both normal and aberrant

Table 1. Caspase Activity in Astrocytes Expressing HPV-16 E6 and/or E7 Genes by Spectrophotometric Assay

Mock-infected Astrocyte		LXSN-Astrocyte	E6-Astrocyte	E7-Astrocyte
No treatment	1.59 ± 0.55	5.65 ± 0.29	3.55 ± 0.23	7.63 ± 0.64
10 M H_2O_2	3.83 ± 0.83	1.82 ± 0.37*	9.61 ± 0.75*#	4.83 ± 0.52*

Specific activity: pmol p-nitroaniline liberated per hr at 37 per μg protein.
*p <0.05 compared with each group untreated, #p <0.05 compared with mock-infected astrocytes which were treated with 10 μM H_2O_2.

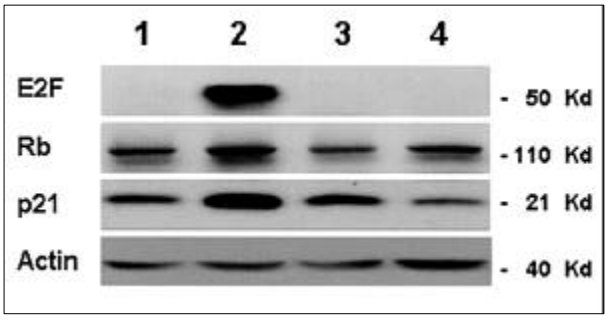

Fig. 5. Cellular levels of E2F, Rb and p21 proteins in astrocytes expressing HPV-16 E6 and/or E7. Lane 1, E6-expressing astrocyte; Lane 2, E7-expressing astrocyte; Lane 3, LXSN-astrocyte; Lane 4, Mock-infected astrocyte. 50μg proteins were loaded in each lane.

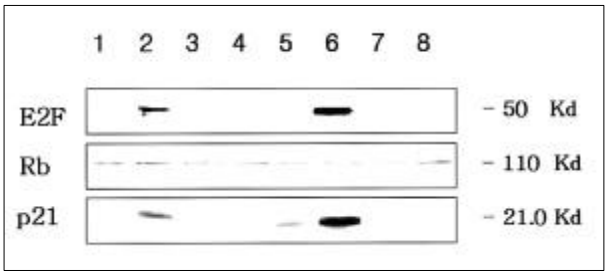

Fig. 6. Immunoblotting of E2F, Rb and p21 in astrocytes expressing E6 and/or E7 gene after hydrogen peroxide injury. Lanes 1 and 5, E6-expressing astrocyte; Lanes 2 and 6, E7-expressing astrocyte; Lanes 3 and 7, LXSN-astrocyte; Lanes 4 and 8, Mock-infected astrocyte. Lanes 1-4, 10μM H_2O_2 added; Lanes 5-8, 200μM H_2O_2 added for 24 hr. 30μg proteins were loaded in each lane.

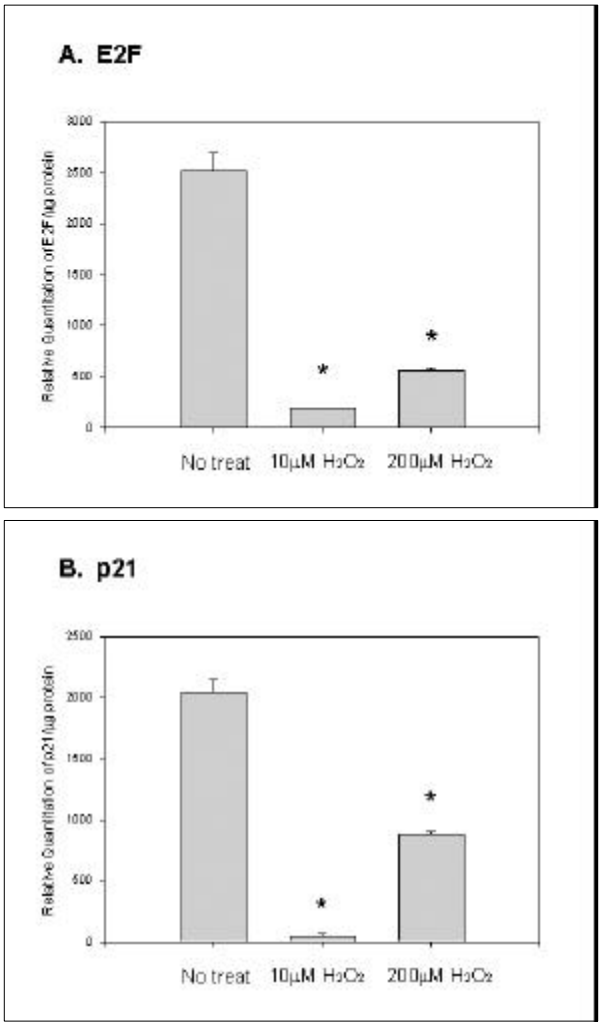

Fig. 7. Relative quantification from immunoblotted gel with E2F and p21 antibodies in astrocytes expressing E7 gene after hydrogen peroxide injury. *Represents significant differences ($p < 0.01$) from control cells (No treatment) by one-way ANOVA and the Student-Neuman-Keul's test.

metabolism, has been shown to elicit cellular injury by initiating lipid peroxidation, protein oxidation, and DNA damage.[28] H_2O_2 is considered to be a dose- and time-dependent mediator of apoptotic and necrotic death and involves the generation of the highly reactive hydroxyl radical (·OH) and other oxidants.[29,30] However, the neurotoxic efficiency of H_2O_2 and the detail mechanism by which it induces apoptosis or necrosis are debatable. When E6 and E7 proteins were overexpressed in primary astrocytes using retroviral vectors, including the HPV-16 E6 and E7 genes, E6 and E7 gene products were able to protect cells from H_2O_2 injury, in our previous study.[21] Astrocytes expressing the E7 gene showed a significant resistance to hydrogen peroxide and glucose deprivation injury, which share aspects of oxidative stress. E6 was much less effective at protecting from hydrogen peroxide than E7. In this study, we examined the signaling of the E6 and E7 proteins with respect to apoptosis and necrosis after H_2O_2 injury using an in vitro model with overexpressed E6 or E7 genes and compared the antioxidative activity of the E6 and E7 proteins. For this purpose, E6 or E7 gene expressing astrocytes were exposed to 10μM and 200μM H_2O_2 solutions. Cell apoptosis occurred under a low dose hydrogen peroxide condition. Approximately 50% of the mock-infected cells underwent apoptosis. Astrocytes expressing the E7 gene were protected from hydrogen peroxide induced apoptotic death but E6 cells were not protected.

E6 and E7 proteins induced different type of death signaling depending on the injury paradigm. After treatment with higher dosage (200 μM H_2O_2), both E6 and E7 expressing cells were protected from apoptosis by the insult but all cells died from necrosis.[21] HPV-16 E7 genes expressing astrocytes were protected from both apoptotic and necrotic cell death, whereas E6 genes expressing astrocytes, which were deprived of p53 proteins were not protected from the apoptotic cell death induced by low doses of (10 μM H_2O_2). The apoptosis induced by H_2O_2 injury may involve a p53-dependent pathway.[31-33] Apoptosis was monitored by DNA fragmentation gel analysis and direct observation of nuclei in cells stained with Hoechst-PI dye. At a dose 10 μM H_2O_2, apoptotic cell death was observed in E7 expressing astrocytes, as determined by the typical DNA ladders that are a hallmark of apoptosis. However, no DNA ladders were found in the gel of the mock-infected control cells, E6 gene expressing cells and LXSN-infected astrocytes after the same insults. The expression of HPV-16 E7, but not of HPV-16 E6 decreased the sensitivity of astrocytes to low-dose hydrogen peroxide-induced apoptosis.

Caspases are involved in the apoptosis induced by many cellular stresses and play a role in both the initiation and the execution phase of the apoptotic pathway.[34,35] To further investigate the involvement of caspase in the protection of HPV-16 E7 to apoptosis induced by low doses of hydrogen peroxide, relative caspase-3 activity was monitored by spectrophotometric assay in astrocytes expressing HPV-16 E6 and E7 genes 24 hours after exposure to low doses of H_2O_2. The caspase activity of the LXSN-infected cells and the astrocytes expressing HPV-16 E7 genes were significantly decreased after exposure to low doses of H_2O_2. Caspase-3 activity was found to be elevated in astrocytes expressing HPV-16 E6 genes, no significant change of relative caspase activities were shown in the mock-infected normal astrocytes after exposure to low doses of H_2O_2. HPV-16 E6 protein expression sensitizes certain cell types, for example, human keratinocytes are sensitive to apoptosis upon decreasing p53 and p21 levels.[36] However, no p53 proteins were expressed in the primary cultured astrocytes (data not shown).

The E7 protein is a major oncogenic protein produced by cervical cancer-associated HPV16. The transforming potential of E7 protein has been ascribed to its ability to bind to, and downregulate, the Rb gene product.[6,14,37] Loss of Rb function causes the release and activation of multiple E2F transcription factor family members, leading to cell cycle progression through the G1/S phase transition.[9,18,38] Expression of E7 proteins can also increase p21 protein levels.[39] Depending upon the degree of E7 expression and the differentiation state of the cell type, increased p21 levels can enhance DNA synthesis and apoptosis or cause growth arrest.[39,40] Therefore, E7 expression increases the apoptotic sensitivity of transformed cells to genotoxic stress and hypoxia.[22,23] In order to elucidate the factors responsible for the cellular protective mechanism involving E7 proteins against H_2O_2 injury and investigate the interaction and regulation between cell protection and the cell cycle regulatory protein, Western blot analysis were performed with E2F, Rb and p21, which all participate in apoptosis; actin was used as a control protein. E2F and p21 induction occurred in normal astrocytes expressing the HPV-16 E7 gene, not the E6 gene or in mock-infected astrocytes. Both of these protein levels were lower in HPV-16 E7 expressing astrocytes after exposure to low (10 μM H_2O_2) and the high (200 μM H_2O_2) doses of hydrogen peroxide. When we incorporated the genes coding for the E7 protein in astrocytes, the levels of the E2F protein surged to more than 10 folds that of the mock-infected cells. It is postulated that the E7 protein dissociates the E2F by binding to the phosphorylated form of Rb. When released, the E2F's do not have that much of an impact upon the overall protective mechanism. However, once the released E2F's reach a certain threshold level of effectiveness, the factor E2F becomes potent in initiating autoregulation.[41] To summarize, E7 protein seems to influence the protection of cells against low and high dose-hydrogen peroxide induced apoptotic and necrotic death.

Reactive oxygen species (ROS) are continuously generated after exposure to low doses of H_2O_2. They cause lipid peroxidation and result in membrane disturbances. ROS, i.e. O_2^-, H_2O_2 and ·

OH, are produced during normal and aberrant metabolism using molecular oxygen and causes tissue damage.[29,36] H_2O_2 readily crosses the cell membrane and breaks down in the presence of ferrous ions, to produce · OH which is one of the most harmful free radicals.[29] Enzymes which act as an antioxidants in brain include, superoxide dismutase (SOD), catalase and glutathione peroxidase. To elucidate which factor is responsible for the cellular protective mechanism involved in E7 proteins against H_2O_2 injury, the antioxidative activity of E6 and E7 proteins were compared with the relative activity of catalase. E7 expressing astrocytes showed higher catalase levels, which is one of the scavenger enzyme activity than E6 expressing cells or normal cell. In this study, the overexpression of E2F and p21 in E7 expressing astrocytes decreased after exposure to low and high doses of H_2O_2. However, the activity of catalase, which is one of the scavenger enzymes was increased after exposure to high doses of H_2O_2. It is considered that ability of E7 protein to protect astrocytes from H_2O_2 injury is due to this increased catalase activity.

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