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Induction of apoptosis by hydrogen peroxide in HPV 16 positive human cervical cancer cells: involvement of mitochondrial pathway

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Abstract Cervical cancer is the second most common malignant neoplasm in women, in terms of incidence and mortality rates worldwide, and is associated with excessive inflammation. This involves the expression of both pro- and anti-apoptotic proteins that have varied effect on tumor growth and metastasis. The objective of the present study was to elucidate the effect of hydrogen peroxide (H_2O_2) on apoptotic signal molecules in vitro in SiHa and CaSki cell lines expressing the human papilloma virus 16 E6 protein, which causes the ubiquitin-mediated degradation of p53 protein and is thus p53 deficient. The p53 is known to act as a cellular stress sensor and triggers apoptosis. We demonstrate, here, that in HPV 16 positive cell lines apoptosis is triggered by upregulation of p73, which causes activation of pro-apoptotic Bax accompanied by down regulation of anti-apoptotic Bcl xl, release of cytochrome c from mitochondria and activation of caspases-9 and -3.

Keywords SiHa · CaSki · H_2O_2 · p73 · Bax · Apoptosis

Introduction

Human papilloma viruses (HPVs) are associated with cervical cancer in most instances and the early genes E6 and E7 from high risk HPVs play a crucial role in tumor formation, by altering pathway in cell cycle control, downregulating the regulatory function of tumor suppressors p53 and Rb [1]. There is loss of p53 function in cervical cancer as it undergoes degradation by ubiquitination by high-risk HPV's viral E6 protein. The p53 has been established as an important player in inducing growth arrest and DNA repair, irreversible growth arrest, terminal differentiation, or apoptosis in response to oncogenic stress such as DNA damage [2].

The p73 belongs to a family of p53 related nuclear transcription factors that include p53, p73, and p63. Unlike p53, mutations in p73 in human tumors are rare. p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in cell cycle checkpoint control through transactivating an overlapping set of p53/p73 target genes. p73 is induced and stabilized in response to a subset of DNA damaging agents in a way that is distinct from that of p53 [3]. It becomes relatively important in tumors having loss of p53 function such as in cervical carcinoma.

Apoptosis can be induced by diverse stimuli, common signaling mediators, including reactive oxygen species (ROS), which induce DNA damage [4]. It has been shown that high concentration of hydrogen peroxide induces necrosis, whereas low concentration induces apoptosis [5]. The extrinsic receptor pathway of apoptosis is triggered by Fas and TNF (tumor necrosis factor) family leading to activation of initiator caspase-8, followed by cleavage of downstream effector caspases. The intrinsic pathway is triggered by release of cytochrome c from mitochondria and results in the activation of the initiator caspase-9, which then cleaves and activates caspase-3 [6].

Reactive oxygen species exist in biological cells and their concentration is determined by a balance between the rates of clearance by various antioxidants and enzymes. Redox signaling involving ROS requires that this balance be disturbed either by increase in ROS concentrations or down regulation of antioxidant enzymes. Cancers

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commonly show a pro-oxidative shift in the systematic thiol/disulfide redox state. This condition is referred to as "Mitochondrial oxidative stress" or a second condition referred to as "Inflammatory oxidative condition", which is associated with excessive stimulation of NAD(P)H oxidase activity by cytokines or other agents induced by infection [7].

Cervical neoplasia is associated with excessive inflammation as a result of oxidative stress. Inflammation may have varied effect on tumor growth with various pro-apoptotic and anti-apoptotic proteins acting in an antagonistic manner. Cell stress and cell death have multiple points of regulatory cross talk and the balance between these two pathways depends on the specific nature and intensity of stress. In the present study we investigated the effect of H_2O_2 mediated oxidative stress on apoptotic signaling pathways in HPV 16 positive cervical cancer SiHa and CaSki cells. We demonstrate that H_2O_2 induced apoptosis in these cells by upregulation of Bax, p73 and downregulation of Bcl-XL. This was accompanied by release of cytochrome c from the mitochondria and the activation of caspases-9 and -3.

Material and methods

Cell culture

The human cervical cancer cell lines SiHa and CaSki were obtained from the National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma, USA) or RPMI (Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone) and antibiotics, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The cells were treated with 125 μM H_2O_2 for 1 and 3 h.

Antibodies

Antibodies against p53, p73, ERK, JNK, Bcl xl, Bax, Ras, c-Myc and Hsp 70 as well as secondary AP conjugated antibodies were obtained from Santa Cruz, USA.

Flow cytometery

SiHa and CaSki cells (1×10^4 cells) were incubated with 125 μM Hydrogen peroxide for 1 and 3 h and then harvested. Cells were fixed in 70% ethanol and left overnight at -20°C. Cells were then washed with PBS and incubated in staining solution (20 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100 and 0.1 mM EDTA) for 2 h at 4°C in dark. The DNA content was measured by flow cytometer (Becton Dickenson, USA) using Cell Quest program, and the percentage apoptosis was analyzed using Win mdi programme [8].

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Apoptotic cells were visualized by the Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique using the Dead End Colorimetric Cell Death Detection kit (Promega Inc, USA), as described earlier [9]. The apoptotic index (AI) was determined by microscopic examination of randomly selected fields containing at least 500 cells.

Western blot analysis

The level of expression of various proteins was determined in control and H_2O_2 treated cells by Western blotting as described previously [8]. Briefly, cells were washed twice with PBS and lysed in RIPA lysis buffer. Total protein was determined by the Bradford assay. Equal amount of protein was loaded and run on 10–15% SDS-polyacrylamide gels. The proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA, followed by hybridization with respective primary and secondary antibody. Final detection was performed with BCIP/NBT substrate (Promega, USA). The bands were analyzed and quantified using α image scanner densitometer (Alpha innotech, USA). The density of control was taken as 1 and results of treatment are expressed in relation to the control in terms of relative unit (RU).

Cytochrome c release from mitochondria

The cells were harvested after the respective treatments, washed once with ice-cold PBS. For isolation of mitochondria and cytosol, the cells were sonicated in buffer containing 10 mM Tris–HCl pH 7.5, 10 mM NaCl,175 mM sucrose and 12.5 mM EDTA and the cell extract centrifuged at 1,000 *g* for 10 min to pellet nuclei. The supernatant thus obtained was centrifuged at 18,000 *g* for 30 min to pellet the mitochondria, which was purified as described [10]. The resulting supernatant was termed as the cytosolic fraction. The pellet was lysed and protein estimated in both fractions by Bradford's method. The purity of the fractions was confirmed by assaying the marker enzymes succinate dehydrogenase for mitochondria, lactate

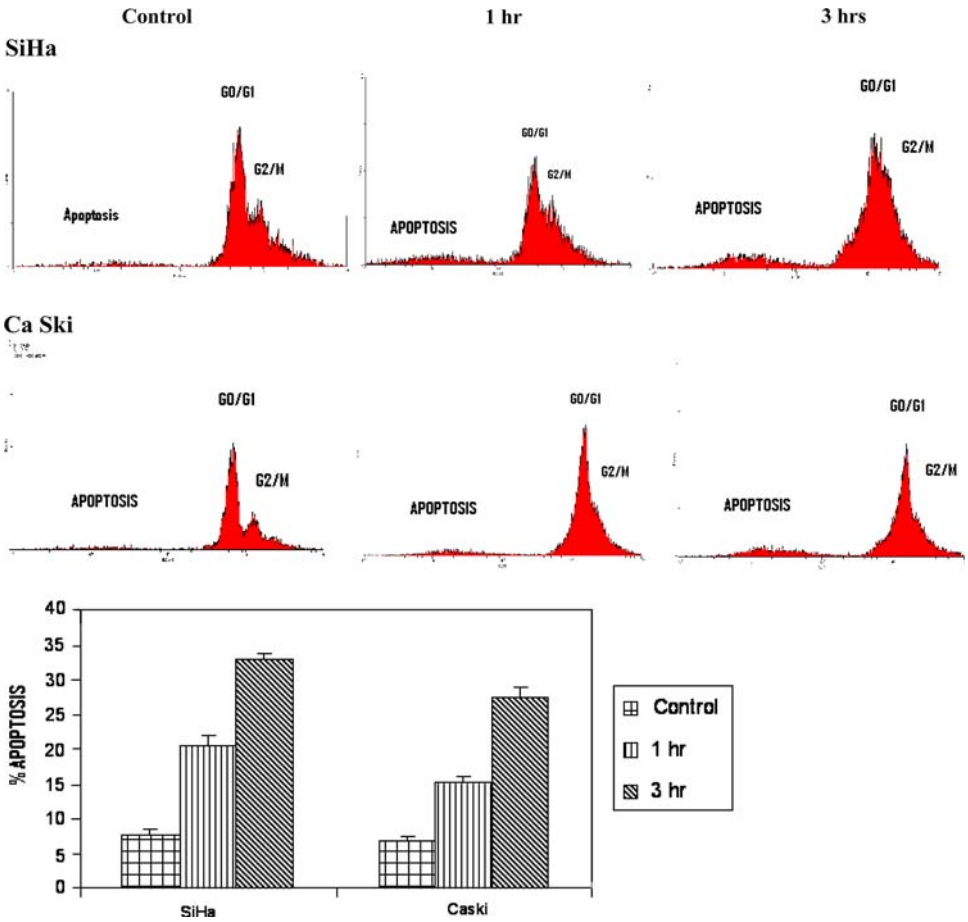

Fig. 1 Flow cytometric analysis of apoptosis in SiHa and CaSki cells on treatment with 125 μM Hydrogen peroxide. (**a**) The % Apoptosis shown in the bar diagram as mean ± SD of three individual experiments

dehydrogenase for the cytosol as described earlier [10]. Cytochrome c determination in cytosolic and mitochondrial fractions was done by Western blotting [8].

Assay of caspase-9 and -3 activity

Caspase-9 and -3 activity was measured by the direct assay for caspase enzyme activity in the cell lysate using synthetic fluorogenic substrate (Ac-DEVD-AFC; substrate for caspase-3; MBL Bioscience, USA; Ac-LEHD-AFC, substrate for caspase-9; Genotech, USA) as described by the manufacturer. The amount of fluorogenic AMC/AFC moiety released was measured using a spectrofluorimeter (ex. 380 nm, em. 420–460 nm for caspase -3; ex. 400 nm, em. 490–520 nm for caspase-9). The results are expressed in arbitrary fluorescence units/mg protein [9].

Statistical analysis

Results are expressed as mean of three individual experiments ± standard deviation (SD) was calculated using Microsoft excel.

Results

Hydrogen peroxide induces apoptosis in cervical carcinoma cell lines

The apoptosis induced by H_2O_2 was measured using flow cytometery and TdT-mediated dUTP nick end labeling (TUNEL) assay (Figs. 1 and 2). In DNA histograms sub G0/G1 fraction indicates apoptosis. As shown in Fig. 1, treatments with 125 μM hydrogen peroxide resulted in 20.44% apoptosis after 1 h and 30.44% apoptosis after 3 h in SiHa cells while CaSki showed 15.08% and 27.47% apoptosis at 1 and 3 h respectively. TUNEL data was in agreement with the flow data (Fig. 2).

Hydrogen peroxide induced activation of MAPK

Since JNK and ERK protein play an important role in apoptosis, hence we studied their expression by Western blotting using phospho-specific pERK and JNK antibodies. Treatment of SiHa and CaSki cells with 125 μM hydrogen peroxide resulted in a marked time-dependent increase in phosphorylation pattern of both ERK and

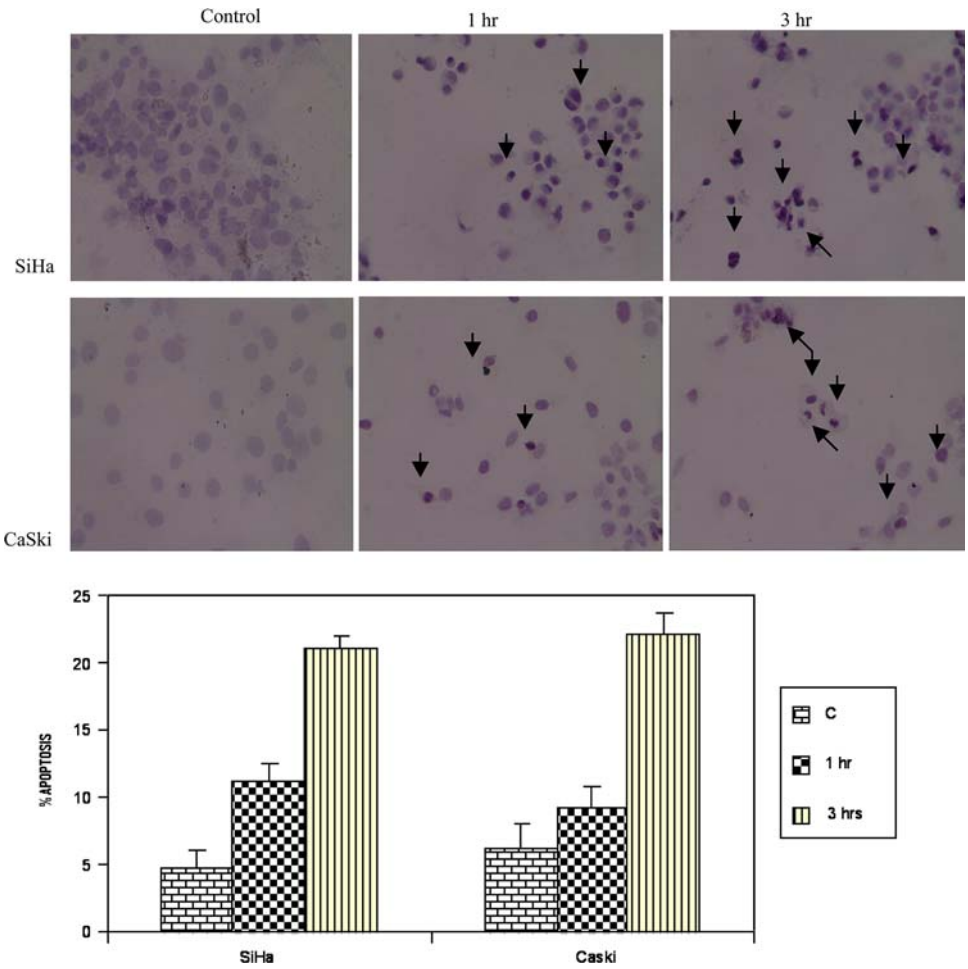

Fig. 2 TUNEL assay for apoptosis in SiHa and CaSki cells on treatment with 125 μM hydrogen peroxide. The % apoptosis shown in the bar digram are mean ± SD of three individual experiments

JNK. SiHa cells showed an 18% and 30% increase in expression of JNK, and 55% and 97% increase in ERK at 1 and 3 h. CaSki cells showed an increase of 11% and 35% in expression of JNK and 125% and 167% increase in ERK after treatment for 1 and 3 h (Fig. 3).

Activation of p53 and p73 in cervical carcinoma cells by H_2O_2

The p53 family of genes plays a central role in apoptosis with p53, p63, and p73 acting as stress sensors and triggering the activation of various pro-apoptotic genes. As shown in Fig. 4, a slight increase in p53 expression was seen. Significant activation of p73 was obtained on carrying out the same treatment. SiHa showed 10% and 25% increase in expression of p53 in comparison to 69% and 75% increase in expression of p73 post-H_2O_2 treatment for 1 and 3 h. CaSki showed a significant increase of 37% and 78% in p73 and a 27% increase in p53 at both 1 and 3 h.

Upregulation of Bax and down regulation of Bcl XL

Having obtained an activation of upstream apoptotic gene p73 we next looked at the effect of hydrogen peroxide on pro-apoptotic and anti-apoptotic genes whose interplay decides the fate of cell following stress conditions. As shown in Fig. 4 hydrogen peroxide caused increase in Bax expression in a time-dependent manner with maximum activation occurring at 3 h. This was accompanied by a simultaneous decrease in anti-apoptotic Bcl XL in both the cell lines. SiHa showed an increase of 50% and 70% in Bax, accompanied by 21% and 34% decrease in Bcl XL at 1 and 3 h. CaSki cells showed an increase of 35% and 65% in Bax accompanied by 28% and 42% decrease in Bcl XL at 1 and 3 h.

Induction of protein expression of c Myc and H Ras

Expression of c Myc transcription factor sensitizes cells to diverse apoptotic stimuli. The c Myc induces apoptosis via mitochondrial release of cytochrome c by

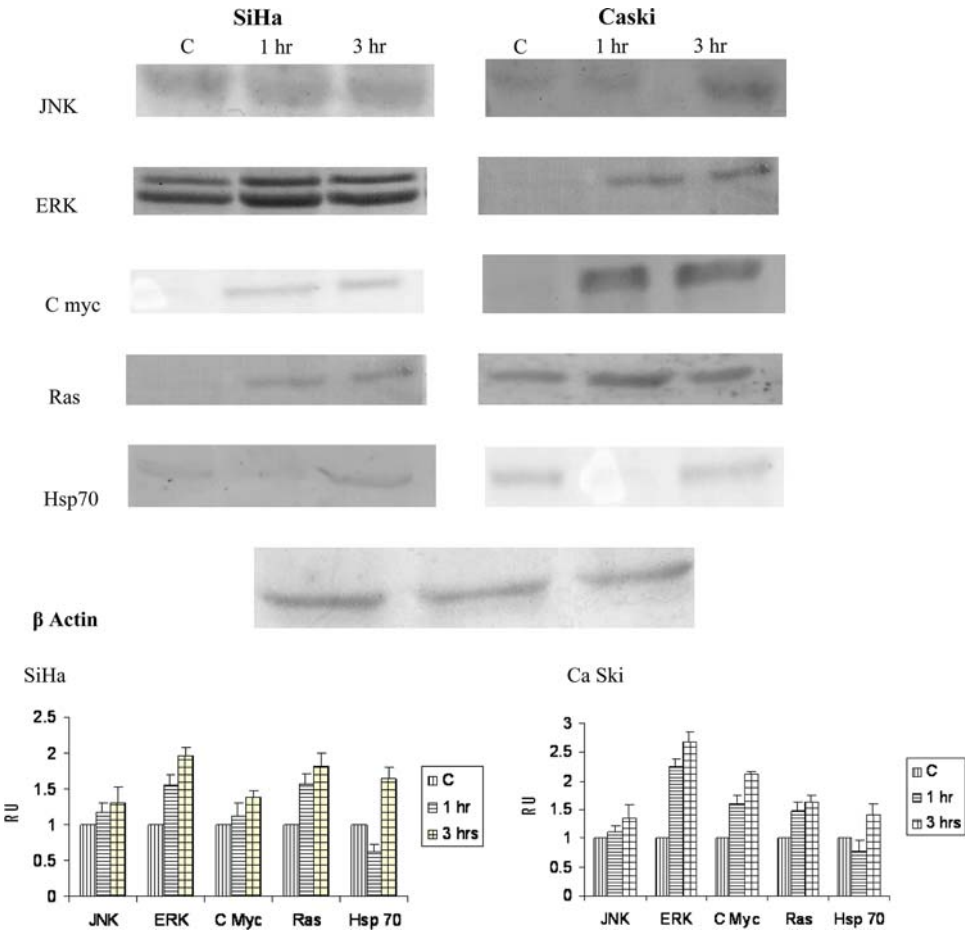

Fig. 3 Effect of 125 μM Hydrogen peroxide on the protein expression of various proteins in SiHa and CaSki cells. **Lane 1** control, **Lane 2** H_2O_2 treatment for 1 h, **Lane 3** H_2O_2 treatment for 3 h. The results shown are mean ± SD of three individual experiments

cooperating with another protein like Max, Mad. As shown in Fig. 3 sustained increase in expression of c Myc was seen in both the cell lines. The role of c Myc protein has also been implicated in malignant transformation of cells along with another oncoprotein Ras. Hence, we also probed the effect of hydrogen peroxide on Ras. Distinct time-dependent activation of Ras was obtained on treatment with hydrogen peroxide. SiHa cells showed a 12% and 40% increase in c Myc and 57% and 83% increase in Ras at 1 and 3 h. CaSki cells showed 60% and 112% increase in c Myc and 48% and 63% increase in Ras.

Activation of Hsp 70 by H_2O_2

Hsp 70, a molecular chaperone plays a critical role during cell stress. An initial decrease at 1 h in expression of Hsp 70 was observed in both the cell lines, which was followed by an increase in Hsp 70 expression at 3 h. SiHa showed an initial decrease of 40% followed by an increase of 65% at 3 h. CaSki showed a 22% decrease at 1 h followed by a 42% increase at 3 h (Fig. 4).

Hydrogen peroxide induces the mitochondrial release of cytochrome c

Cytochrome c release from mitochondria is a critical step in the apoptotic cascade as this activates downstream caspases. As shown in Fig. 5, we obtained a consistent increase in cytochrome c content in cytosol after treatment with hydrogen peroxide. This was accompanied by a simultaneous decrease in cytochrome c in mitochondrial fraction, indicating that there is a time dependent release of cytochrome c in SiHa cells exposed to oxidative stress such as hydrogen peroxide. There was a 17% and 27% decrease in the mitochondrial cytochrome c level at 1 and 3 h in SiHa cells. This was accompanied by a simultaneous increase in cytochrome c level in the cytosol of 48% and 59%. However, there was no significant release of cytochrome c in CaSki cells.

Activation of caspase-9 and -3 activities

As shown in Fig. 6, a time-dependent increase in activity of caspase-9 and caspase-3 was observed in both the cell

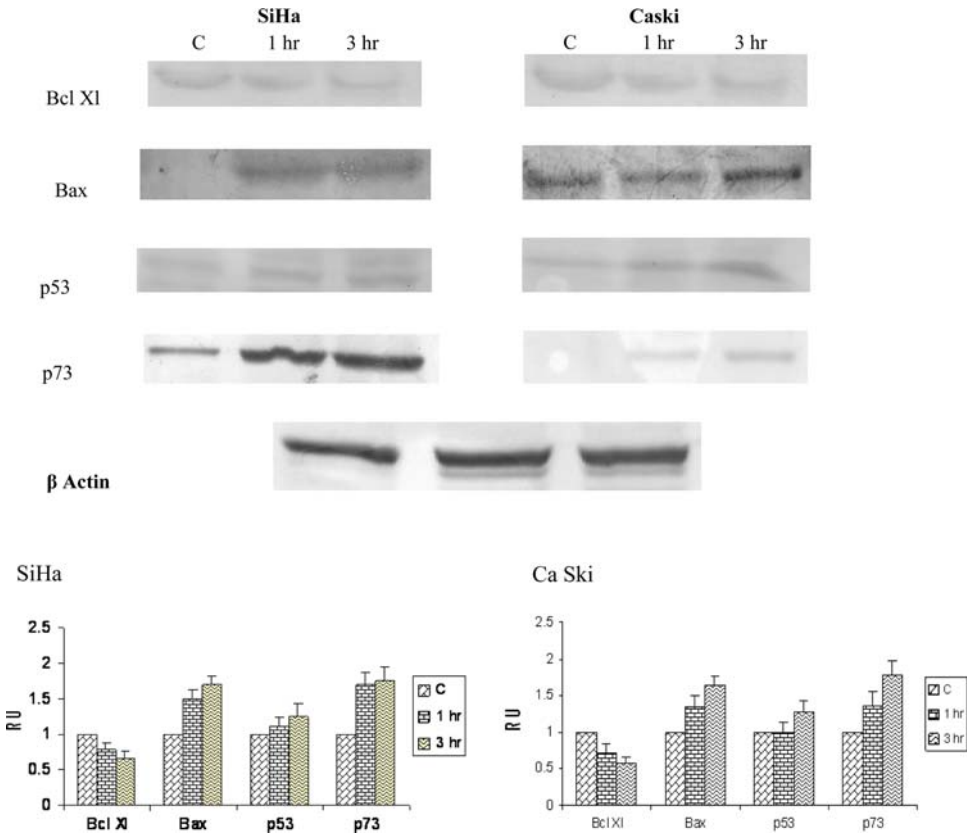

Fig. 4 Effect of 125 μM hydrogen peroxide on the expression of various proteins in SiHa and CaSki cells. **Lane 1** control, **Lane 2** H_2O_2 treatment for 1 h, **Lane 3** H_2O_2 treatment for 3 h. The results shown in bar digrams are mean ± SD of three individual experiments

lines, suggesting that hydrogen peroxide induced apoptosis in these cells by the mitochondrial pathway. SiHa cells showed an initial 1.8-fold increase in caspase-9 activity at 1 h followed by a 6.8-fold increase at 3 h. Caspase-3 showed an initial 1-fold increase followed by a 3.5-fold increase in activity after 3 h. CaSki cells showed an initial 2-fold increase in caspase-9 activity at 1 h, followed by a 3.4-fold increase after 3 h. Caspase-3 showed an initial 0.5-fold increase followed by a 1-fold increase at 3 h.

Discussion

Apoptosis by oxidative stress has been implicated in several biological and pathological processes like aging, inflammation and carcinogenesis [11]. However, the mechanism of cell death by oxidative stress are not yet clarified some of the effects of oxidative damage are well known such as mitochondrial failure, alternation in calcium homeostasis and impairment of energy metabolism. H_2O_2 acts on mitochondria causing disruption of mitochondrial membrane potential and release of cytochrome c. Though apoptosis and cell survival are exclusively different phenomenon, apoptotic signal and anti-apoptotic signal occur simultaneously. Cellular protein damage activates ERK and JNK kinase pathway, which are involved in controlling the cellular decision to proliferate, arrest or die depending on severity of the stress. The decision to live or die in turn depends on the relative strength of cellular survival and apoptotic signals.

The cellular response to diverse external stimuli is controlled via a complex array of phosphorylation cascades. The extracellular signal regulated protein kinase (ERK) cascade is a prominent component of mitogen activated protein kinase (MAPK) family that in particular plays an integral role in both growth factor and stress signaling. Interestingly at least some stress signals eg UV radiation utilize the same signaling pathway for ERK activation as do mitogens. The sequential activation of the GTP binding protein Ras then ensues [12]. ERK activation culminates in the phosphorylation of downstream cytosolic and nuclear factors that control a variety of cellular processes. However ERK along with Ras has been implicated in tumor progression Paradoxically, it along with JNK has also been shown to induce apoptosis [13, 14]. Simultaneous activation of both JNK and ERK was observed in this study. JNK induces apoptosis by phosphorylation of BCL 2 family of proteins including anti-apoptotic Bcl 2, Bcl XL and pro-apoptotic Bax, Bak and Bid [15]. The phosphorylation cascade activate pro-apoptotic proteins and inactivates anti-apoptotic proteins thereby activating apoptotic pathway as has been observed in both the cell lines in this study.

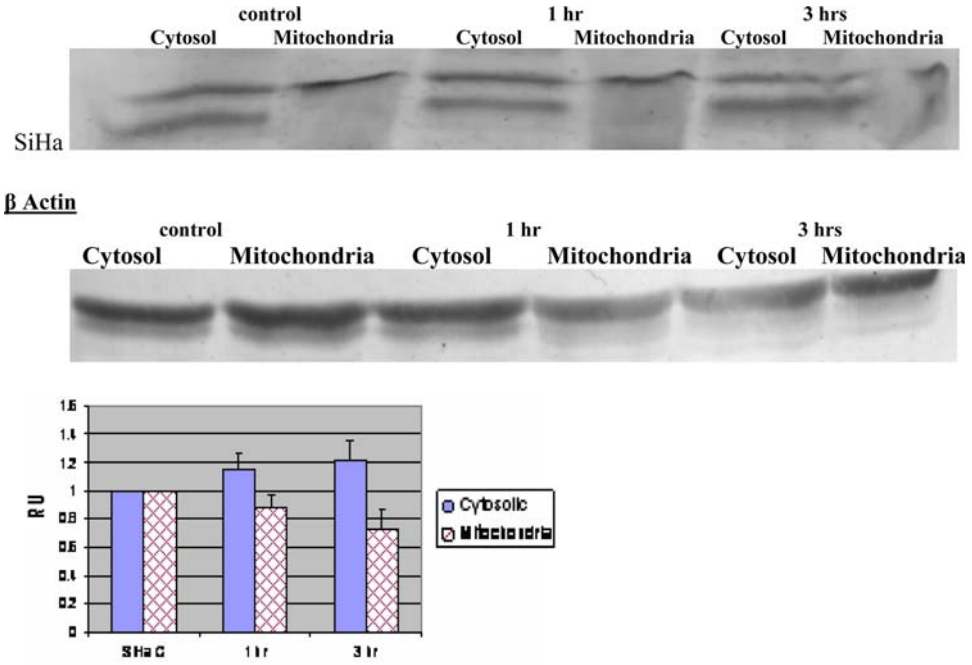

Fig. 5 Effect of 125 μM hydrogen peroxide on release of cytochrome c from mitochondria. The results shown in the bar diagram are mean ± SD of three individual experiments

The transcription factor and tumor suppressor p53 and its two homologues p63 and p73 form a family of proteins. For over 20 years p53 has been recognized as a guardian against cellular stressors particularly those that inflict DNA damage. It is a transcription factor with a modular structure comprising trans activation (TA) DNA binding and oligemerization domain and exerts it's protective effect by inducing cell cycle arrest (with possibility of repair) or apoptosis. Both the cell lines used in current study are HPV 16 positive with SiHa possessing a single copy of HPV while CaSki having the copy number in range of 60–600 with traces of HPV 18 as well. The high risk HPV's encode for E6 protein, which causes ubiquitin mediated degradation of p53 protein [16, 17]. Thus HPV infected cell lines are relatively p53 deficient. The slight but nonsignificant increase observed in p53 may be due to activation of p53 gene due to oxidative stress resulting in increased translation of p53 protein. Another member of p53 family, p73 has been shown to have functions similar to p53 and induces both G1 cell cycle arrest and apoptosis [18–20]. Though It has been reported that p73 induces apoptosis via mediation of PUMA protein, which in turn activates Bax, which induces mitochondrial translocation and cytochrome c release [21]. p53 is mutated in nearly 50% of cancers and functionally inactivated in futher 20%, whereas p73 and p63 mutation are extremely rare. The observed increase in p73 in a time dependent manner suggests that perhaps p73 is taking over the function of p53 in SiHa and CaSki cells. It appears that it is p73, which is taking over the function of p53 and causing downstream activation of Bax and inactivation of Bcl-XL.

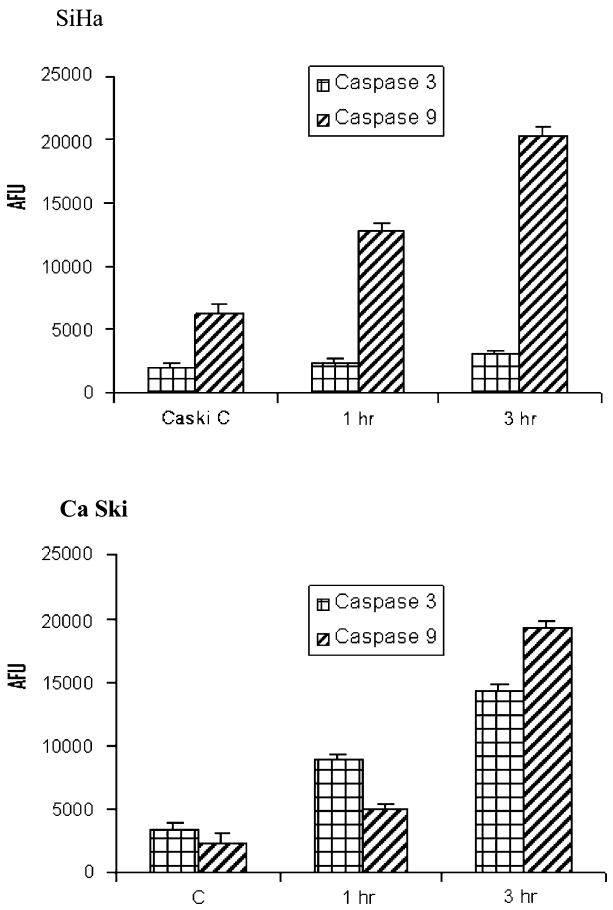

Fig. 6 Caspase-3 and -9 activity in SiHa, and CaSki treated for the indicated times with 125 μM H_2O_2. Data shown are mean ± SD of three independent experiments

Many of the signals that elicit apoptosis converge on the mitochondria, which respond to pro-apoptotic signals by releasing cytochrome c a potent catalyst of apoptosis. Bcl-2 family of genes code for membrane channel forming proteins that have critical role in apoptosis [22]. Members of Bcl-2 family of proteins whose members have either pro-apoptotic (Bax, Bak, Bid, Bim) or anti-apoptotic (Bcl-2, Bcl XL, Bcl W) function act in part by governing mitochondrial death signaling through cytochrome c release [23]. The p53 tumor suppressor can elicit apoptosis by upregulating expression of pro-apoptotic Bax in response to sensing DNA damage, which in turn stimulate mitochondria to release cytochrome c and the activation of caspases [24]. It appears that in SiHa cell line H_2O_2 may promote the translocation of Bax from cytosol to the mitochondria leading to release of cytochrome c. However, in CaSki though downregulation of activity of Bcl XL was seen no distinct release of cytochrome c was seen suggesting that activation of caspases is ensuing without release of cytochrome c thereby showing difference in apoptotic pathways in these cell lines. An increase in expression of pro-apoptotic Bax and a simultaneous decrease in expression of anti-apoptotic Bcl-xl was seen in both the cell lines suggesting that apoptosis involves activation of pro-apoptotic proteins and inhibition of anti-apoptotic proteins.

The Myc family of protooncogene is believed to be involved in many type of human malignancy. The members of this family have been shown to function as transcription factors and through a designated target sequence bring about continued cell cycle progression, cellular immortalization and blockage to differentiation into a particular cell lineage [25]. However, it has been shown that c myc is effective in transcription repression as well as it has a role in programmed cell death [26]. A time dependent increase in expression of c Myc was seen in both the cell lines suggesting its role in the observed apoptosis.

Many key components of survival and apoptotic pathways are regulated by interactions with heat shock proteins (Hsp). They are a class of proteins that interact with diverse protein substrates to assist in their folding and play a critical role during cell stress to prevent the appearance of folding intermediates that lead to misfolded or otherwise damaged molecules [27, 28]. We obtained an increase in expression of Hsp-70 after exposure to hydrogen peroxide for 3 h, suggesting that it acts as sensor and regulator of stress induced apoptosis.

Mitochondria function as sentinels that receive death signals and commit cells to apoptosis by releasing cytochrome c [29], Smac/Diablo [30, 31], AIF [32]. Once released into cytosol during apoptosis cytochrome c binds to Apaf, thus forming a complex called apoptosome, which recruits and activates procaspase-9. We conclude that in HPV 16 positive cervical carcinoma cell lines, apoptosis is triggered by H_2O_2 by up regulation of JNK but, more markedly of ERK, and also of c-Myc and Hsp-70. In addition, it stimulates apoptosis through activation of p73 and inhibition of downstream Bcl-XL, accompanied by activation of Bax, release of cytochrome c from mitochondria in SiHa but not in CaSki and recruitment and activation of caspase-9, which cleaves inactive procaspase-3 to active caspase-3, thereby providing a link between the mitochondria and H_2O_2 induced apoptosis in HPV 16 positive cells.

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