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Hydrogen peroxide induces apoptosis in HeLa cells through mitochondrial pathway

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Abstract

Cervical cancer is the most common cancer amongst females in India and is associated with high risk HPVs, reactive oxygen species (ROS), and excessive inflammation in most cases. ROS in turn affects the expression of pro- and anti-apoptotic proteins. The objective of the present study was to elucidate the effect of hydrogen peroxide (H_2O_2) on apoptotic signaling molecules *in vitro*. HeLa cell line expresses the Human papilloma virus – 18, E6 oncoprotein which causes the ubiquitin mediated degradation of p53 protein and is thus p53 deficient. p53 is known to act as a cellular stress sensor and triggers apoptosis. p73, a member of the p53 family also induces apoptosis in response to DNA damaging agents but unlike p53, it is infrequently mutated in human tumors. We demonstrate here, that in HeLa cells, apoptosis is triggered by H_2O_2 via the mitochondrial pathway involving upregulation of p73, and its downstream target Bax. This was accompanied by upregulation of ERK, JNK, c-Myc, Hsp-70 and down regulation of anti-apoptotic Bcl-XL, release of cytochrome *c* from mitochondria and activation of caspases-9 and -3.

Keywords: HeLa; H_2O_2; p73; Bax; Apoptosis

1. Introduction

Apoptosis is an inherent cellular response for an effective cellular disposal against development and environmental insults. It can be induced by diverse stimuli, common signaling mediators, including reactive oxygen species (ROS), which induce DNA damage (Rodriguez et al., 1997). It has been shown that high concentration of hydrogen peroxide induces necrosis, whereas low concentration induces apoptosis (Troyano et al., 2003). There are two major apoptotic pathways for activation of caspases (Earnshaw et al., 1999; Strasser et al., 2000). The extrinsic pathway is triggered by Fas and TNF (Tumor necrosis factor) family through receptor mediated pathway, which leads to the 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, which in turn activates a DNase termed CAD (Caspase activated DNAse) (Nagata, 2000; Enari et al., 1998).

Reactive oxygen species exist in biological cells and tissues at low but measurable concentrations (Halliwell, 1992). Their concentration is determined by a balance between the rates of clearance by various antioxidants and enzymes, resulting in a stable state in normal cells. Redox signaling involving ROS requires that this balance be disturbed either by increase in ROS concentrations or down regulation of antioxidant enzymes. Cancers 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.

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Human papilloma viruses (HPVs) are associated with cervical cancer in most instances and the early genes E6 and E7 from high risk HPVs play an important role in tumor formation by downregulating the regulatory function of tumor suppressors p53 and Rb (Sigal and Rotter, 2000). p73, a member of the p53 family of nuclear transcription factors, functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in cell checkpoint control through transactivating an overlapping set of p53/p73 target genes. p73 is induced by DNA damaging agents in a way that is distinct from p53, and becomes important in tumors having loss of p53 function, such as cervical cancer, in which p53 is degraded by HPV viral E6 protein via ubiquitination. p73 mutation is detected in less than 0.5% of human tumors, whereas, more than 50% of cancers carry p53 mutations (Kaghad et al., 1997; Ikawa et al., 1999).

Cervical neoplasia is associated with excessive inflammation as a result of oxidative stress by ROS. Inflammation may have varied effect on tumor growth with various pro-apoptotic and anti-apoptotic proteins acting in an antagonistic manner. Stress signaling initiates two interconnected yet opposing pathways for survival and for apoptosis. 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 cervical cancer HeLa cells. We found that H_2O_2 induced apoptosis in this cell type. Further, we examined the signaling pathways of H_2O_2 induced apoptosis and investigated the role of these pathways in apoptosis. We demonstrate that H_2O_2 induced apoptosis, which is regulated by simultaneous activation of pro-apoptotic p73, Bax and inhibition of anti-apoptotic Bcl-XL. This was accompanied by release of Cytochrome *c* and the activation of caspases-9 and -3.

2. Material and methods

2.1. Cell culture and treatment

The human cervical cancer cell line HeLa was obtained from the National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (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. For all experiments, cells in the log phase were used. The cells were treated with a standardized dose of 125 μM H_2O_2 for 15 min, 1 h and 3 h.

2.2. 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.

2.3. Flow cytometry

HeLa cells were incubated with 125 μM hydrogen peroxide for 15 min, 1 h 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 within the cell was measured by flow cytometer (Becton–Dickenson, USA) using Cell Quest program and the percentage apoptosis was analyzed using Win MDI programme (Sharma et al., 2005).

2.4. 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 (Sen et al., 2005). The apoptotic index (AI) (number of apoptotic cells in 500 cells) was determined by microscopic examination of randomly selected fields containing at least 500 cells.

2.5. Western blot analysis

The level of expression of various proteins was determined in control and treated cells by Western blotting as described previously (Sharma et al., 2005). Briefly, cells were washed twice in PBS and lysed in RIPA lysis buffer containing protease inhibitors. Total protein was determined by the Bradford assay (Bradford, 1976). Equal amount of protein (80 μg) was loaded and run on 10–15% SDS–polyacrylamide gels and 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). Densitometric analysis of the bands obtained was performed using Alpha Imager 2200 (Alpha Innotech, USA). Positive and negative controls were run with each antibody. The density of control was taken as 1 and results of treatment were expressed in relation to the control in terms of relative unit (RU). Beta-actin was used for monitoring equal loading.

2.6. 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 1000*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 (Kuhar et al., 2006). 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 dehydrogenase for the cytosol as described earlier (Kuhar et al., 2006). Cytochrome *c* determination in cytosolic and mitochondrial fractions was done by Western blotting (Sharma et al., 2005).

2.7. Assay of caspase-3, -9 activity

The activity of these caspases 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 manufacturer. 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 (Sen et al., 2005).

2.8. Statistical analysis

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

3. Results

3.1. Hydrogen peroxide induces apoptosis in human cervical carcinoma HeLa cells

Apoptosis was measured using Flow cytometery and TdT mediated dUTP nick end labeling (TUNEL) assay (Fig. 1). DNA content of the cells was estimated using flow cytometery. In DNA histograms sub G0/G1 fraction indicates apoptotic cells. As shown in Fig. 1 we observed that treatment of HeLa cells with 125 μM hydrogen peroxide resulted in 6.89% apoptosis after 15 min, 11.05% apoptosis after 1 h and 28.96% apoptosis after 3 h. The TUNEL data was in agreement with the flow cytometry data.

3.2. Activation of MAPK family in HeLa cells by H_2O_2

MAPK pathway is another pathway that mediates signals stimulated by ROS. It is known that increase in JNK and ERK protein expression plays an important role in apoptosis. Hence, we studied their expression by Western blotting using a phospho specific ERK and JNK antibody. Stimulation of HeLa cells with 125 μM hydrogen peroxide resulted in a marked time dependent increase in phosphorylation pattern of both ERK and JNK which was sustained even till 3 h (Fig. 2), suggesting their involvement in H_2O_2 mediated apoptosis. HeLa showed a 59% and 65% increase in expression of JNK while a 142% and 252% increase in expression of ERK was obtained after treatment for 1 h and 3 h.

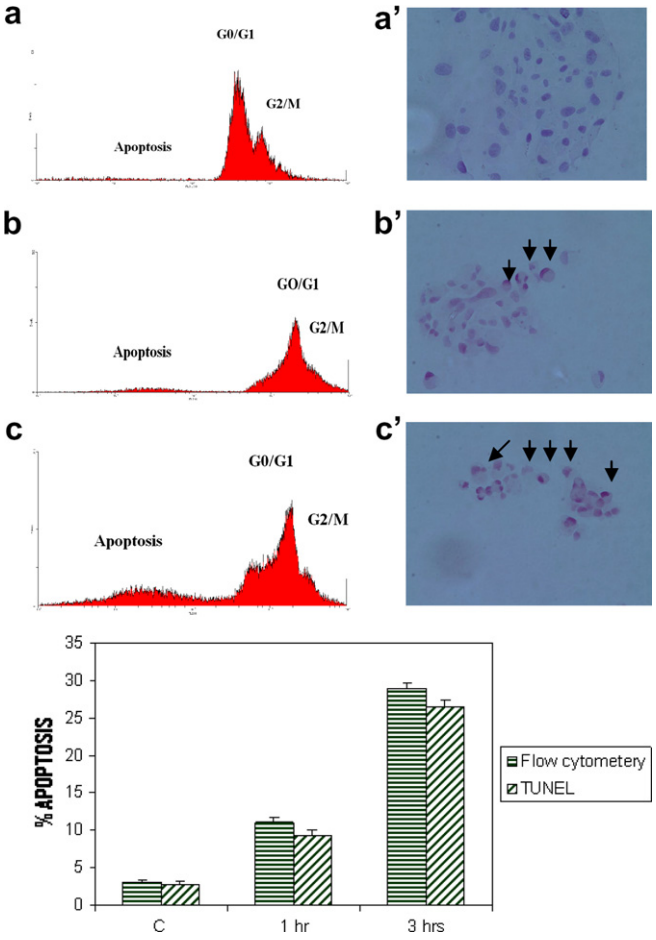

Fig. 1. Flow cytometric and TUNEL analysis of percentage apoptosis in HeLa cells on treatment with 125 μM Hydrogen peroxide. Flow cytometery (a) Control cells. (b) H_2O_2 treatment for 1 h. (c) H2O2 treatment for 3 h. TUNEL (a′) Control cells. (b′) H_2O_2 treatment for 1 h. (c′) H_2O_2 treatment for 3 h. The results shown in the bar diagram are means ± SD of three individual experiments.

3.3. Hydrogen peroxide induces p73

The p53 family of genes play a central role in apoptosis with p53, p63 and p73 acting as stress sensors of the cell and triggering the activation of various pro-apoptotic genes. Western blotting was done to check the expression of p53 and p73 in response to hydrogen peroxide. As shown in Fig. 2 we obtained a slight increase in p53 expression in HeLa cells treated with 125 μM hydrogen peroxide in a time dependent manner, but this was not statistically significant. However, a significant increase in p73 was obtained (Fig. 2) HeLa showed a 25% and 32% increase in expression of p53 while a 51% and 72% increase in expression of p73 was obtained after treatment for 1 h and 3 h.

3.4. Modulation of Bax and Bcl-XL in H_2O_2 induced apoptosis in HeLa cells

Having established the activation of upstream apoptotic genes we set out to find the effect of hydrogen peroxide on downstream pro-apoptotic and anti-apoptotic genes whose

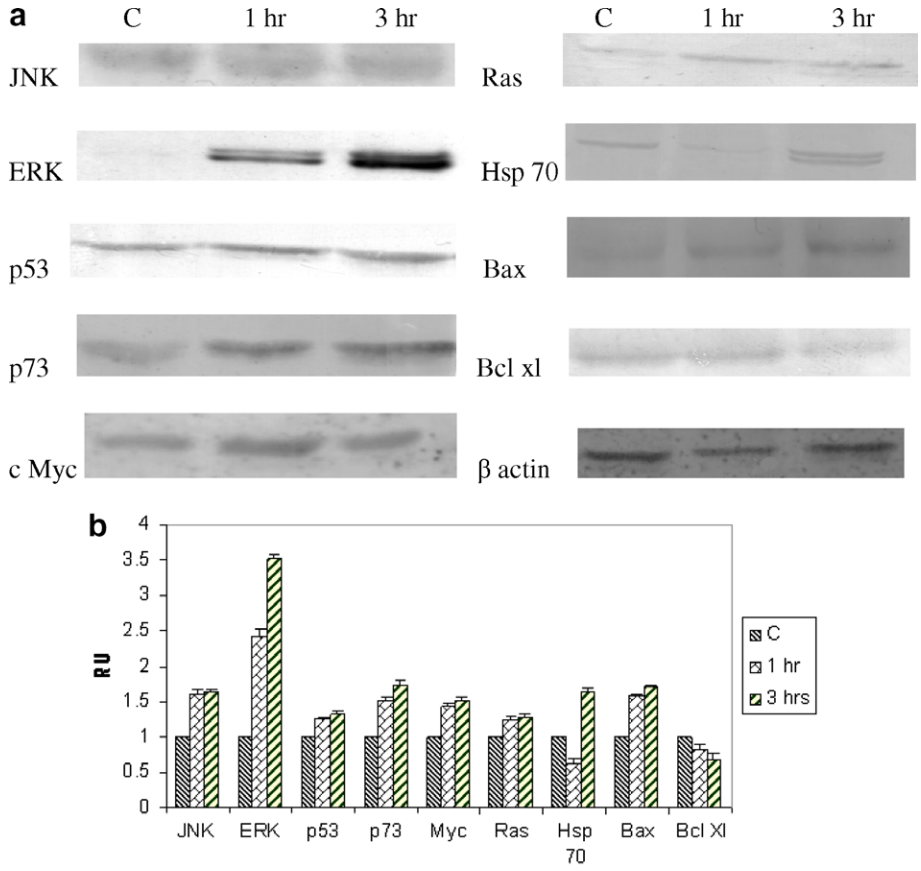

Fig. 2. Expression level of apoptosis related proteins in HeLa cells on treatment with 125 μM H_2O_2. (a) Bands of a representative Western blot. (b) The results are means ± SD of three individual experiments.

interplay decides the fate of cell following stress conditions. We monitored the protein expression of Bax a pro-apoptotic member and Bcl-XL an anti-apoptotic member, following treatment of HeLa cells by hydrogen peroxide. As shown in Fig. 2, H_2O_2 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 (Fig. 2).The results indicate that the expression levels of Bax and Bcl-XL proteins modulate apoptosis induced by H_2O_2 in HeLa cells. HeLa showed a 57% and 71% increase in expression of Bax while a simultaneous 17% and 32% decrease in expression of Bcl XL was obtained after treatment for 1 h and 3 h.

3.5. Hydrogen peroxide increases the protein expression of c-Myc and H-Ras

Expression of c-Myc transcription factor sensitizes cells to diverse apoptotic stimuli. c-Myc induces apoptosis via mitochondrial release of cytochrome *c* by cooperating with other proteins like Max, Mad. As shown in Fig. 2 sustained increase in expression of c-Myc was seen. The role of c-Myc protein has also been implicated in malignant transformation of cells along with another oncoprotein Ras. So we also probed the effect of hydrogen peroxide on Ras. A slight time dependent increase of Ras was obtained (Fig. 2). HeLa showed a 42% and 51% increase in expression of c-Myc while 24% and 28% increase in expression of Ras was obtained after treatment for 1 h and 3 h.

3.6. Hydrogen peroxide induces activation of Hsp-70

Hsp-70 molecular chaperone plays a critical role during cell stress to prevent appearance of misfolded proteins. While an initial decrease in expression of Hsp-70 was observed after 1 h, it was followed by an increase at 3 h (Fig. 2). HeLa showed an initial 37% decrease in expression of Hsp70 after 1 h followed by 65% increase in its expression after 3 h (Fig. 2).

3.7. Hydrogen peroxide induces release of cytochrome c from the mitochondria to the cytosol

Cytochrome *c* release from mitochondria is a critical step in the apoptotic cascade as this activates downstream caspases. To examine the release of cytochrome *c* in H_2O_2 treated HeLa cells, we conducted Western blotting in both the cytosolic and mitochondrial fractions. These experiments demonstrate a consistent increase in cytochrome *c* in cytosol after treatment with hydrogen peroxide. Simultaneously, there was a decrease in cytochrome *c* in mitochondrial fraction, indicating that there is a time dependent

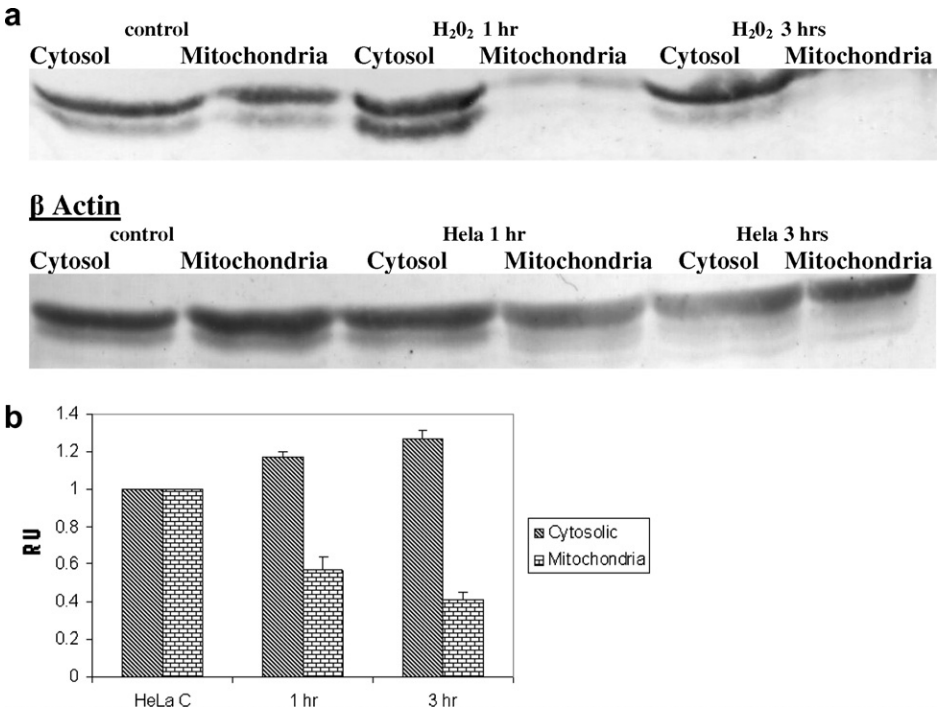

Fig. 3. Effect of 125 μM hydrogen peroxide on release of cytochrome *c* from mitochondria. (a) Representative blot. (b) Data shown are means ± SD of three individual experiments.

release of cytochrome *c*, and suggesting the involvement of mitochondria in H_2O_2 mediated apoptosis. There was a 17% and 27% decrease in the mitochondrial cytochrome *c* level at 1 h and 3 h. This was accompanied by a simultaneous increase in cytochrome *c* level in the cytosol of 48% and 59%, respectively (Fig. 3).

3.8. Caspases-9 and -3 mediate H_2O_2 induced apoptosis

Activation of caspases by cytochrome *c* is a key event during apoptosis caused by various toxic agents. To confirm whether caspases are activated after cytochrome *c* release we measured the changes in caspases-9 and -3 activity in HeLa cells after H_2O_2 treatment. As shown in Fig. 4, a time dependent increase in activity of caspase-9 and caspase-3 was observed in H_2O_2 treated cells. There was a significant increase in caspase-9 and -3 activity after 3 h suggesting that hydrogen peroxide is inducing apoptosis in HeLa cells by the mitochondrial pathway. There was an initial 10% increase in caspase-3 activity at 1 h followed by an 80% increase after 3 h. Caspase 9 showed an initial 28% increase followed by 100% increase in activity after 3 h.

4. Discussion

Reactive oxygen species can induce lesions in the DNA, thereby activating the cellular apoptotic machinery (Schwartz and Osborne, 1995). Protein damaging stresses activate ERK and JNK kinase pathways which are involved in controlling the cellular decision to proliferate, arrest or die,

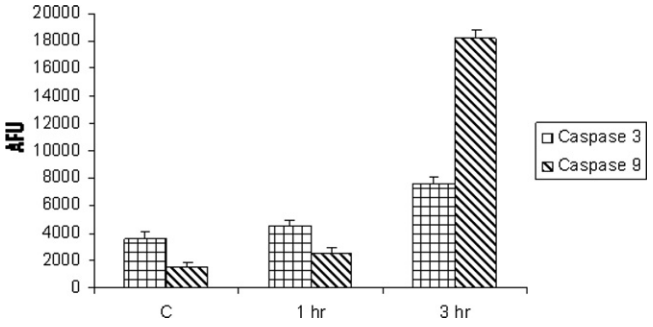

Fig. 4. Caspase-9 and -3 activity in HeLa cells treated for the indicated times with 125 μM H_2O_2. Data shown are mean ± SD of three independent experiments.

depending on severity of stress. The decision to live or die depends on the relative strengths of cellular survival and apoptotic signals. The MAP kinase module which consists of three protein kinases is conserved and has emerged as an important membrane to nucleus signaling pathway in eukaryotes. We observed a distinct activation of both JNK and ERK on treatment with hydrogen peroxide. This was accompanied by activation of Ras. ERK, JNK/ SAPK and p38 pathway are implicated in oncogenesis as well as in apoptosis (Ishikawa and Kitamura, 2000). The pro-apoptotic function of JNK/SPK pathway has been noted in PC 12 cell lines (Xia et al., 1995). ERK induces autocrine expression of growth factors that act back on surface receptors that signal through Ras. Here we must stress that though ERK along with Ras has been implicated in tumor progression, it along with JNK has also been shown to induce apoptosis (Raffeles et al., 2004). Clearly the role

of ERK in determining fate of cell is controversial. JNK has been established as an apoptotic inducer, it induces apoptosis by phosphorylation of Bcl-2 family of proteins, including anti-apoptotic Bcl-2, Bcl-XL and proapoptotic Bax, Bak and Bid. This phosphorylation cascade activates pro-apoptotic proteins and inactivates anti-apoptotic proteins, thereby activating apoptotic pathway (Davison et al.,2004), as observed in this study.

The p53 family of tumor suppressors consists of closely related genes comprising of p53, p63 and p73. Wild type p53 is capable of acting as a transcription factor and inducing the genes involved in cell cycle arrest and apoptosis. HeLa cells contain integrated form of HPV-18 which codes for E6 protein, which in turn causes ubiquitin mediated degradation of p53 protein (Werness et al., 1990; Du et al., 2000). 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 (Bergamaschi, 2003; Toshinori and Akira, 2005; Gerry et al., 2003). However, the molecular mechanism remains unclear, though it has been shown that p73 induces apoptosis via mediation of PUMA protein, which in turn activates Bax, which induces mitochondrial translocation and cytochrome *c* release (Melino et al., 2004). The observed increase in p73 in a time dependent manner suggests that perhaps p73 is taking over the function of p53 in HeLa cells, as these have low level of p53 because of ubiquitin mediated degradation of p53. It appears that it is p73 which is causing downstream activation of Bax and inactivation of Bcl-XL.

Members of the Bcl-2 family of proteins have been demonstrated to be associated with regulating the mitochondrial membrane permeability (Adams and Cory, 1998). Bcl-2 family of genes code for membrane channel forming proteins which have critical role in apoptosis. Proapoptotic proteins like Bax by translocation from the cytosol to the mitochondria, induce cytochrome *c* release, whereas Bcl-XL exerts its anti-apoptotic activity, at least in part by inhibiting the translocation of Bax to the mitochondria (Normura et al., 1999; Murphy et al., 2000). By downregulating Bcl-XL levels in HeLa cells, H_2O_2 may promote the translocation of Bax from cytosol to the mitochondria, leading to release of cytochrome *c*. Thus, H_2O_2 induced oxidative stress which leads to release of cytochrome *c* from mitochondria, further initiates the activation of execution caspases, leading to apoptotic cell death (Reed, 1997). The anti-apoptotic members of Bcl-2 family have been proposed to be connected to caspases in two ways. Firstly they maintain cell survival by dragging caspase to intracellular environment (mitochondrial membrane) and thus prevent their activation. Secondly they may act by regulating the release of caspase activators i.e. cytochrome *c* and AIF. We observed an increase in expression of Bax in a time dependent manner and a simultaneous decrease in Bcl-XL. Thus, it appears that hydrogen peroxide is inducing apoptosis via activation of pro-apoptotic Bcl-2 family of proteins and inhibiting the anti-apoptotic proteins.

c-Myc as a transcriptional factor is able to repress or activate transcription and is an important regulator of cell cycle progression. Its expression alone can push the quiescent cells into DNA synthesis phase of cell cycle (Eilers et al., 1991). In addition it can cause cells to undergo apoptosis. Interestingly both over and under expression of c-Myc can induce apoptosis depending on circumstances (Xia et al., 1998). We obtained a sustained time dependent increase in c-Myc expression after 3 h treatment of hydrogen peroxide, suggesting its involvement.

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 (Jolly and Morimot 2000; Mayer and Bukau 2005). 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 signal and commit cells to apoptosis by releasing cytochrome *c* (Liu et al., 1996), Smac/Diablo(Du et al., 2000; Wu et al., 2000), AIF (Joza et al., 2001). Once released into cytosol during apoptosis cytochrome *c* binds to Apaf thus forming a complex called apoptosome which recruits and activates procaspase-9 (Li et al., 1997, 1996). We conclude that in HeLa cervical carcinoma cell line, apoptosis is triggered by H_2O_2 by upregulation 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, 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 HeLa cells. As cervical carcinoma cell lines are relatively low in p53 content due to E6 mediated degradation, p73 may be acting as DNA damage sensor and triggering apoptosis via mitochondrial pathway.

Acknowledgements

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