Role of p53 in antioxidant defense of HPV-positive cervical carcinoma cells following H_2O_2 exposure

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Summary

In HPV-positive cervical carcinoma cells, p53 protein is functionally antagonized by the E6 oncoprotein. We investigated a possible role of p53 in antioxidant defense of HPV-positive cervical cancer cell lines. We found that SiHa cells containing integrated HPV 16 had higher expression of p53 and exhibited the greatest resistant to H_2O_2-induced oxidative damage, compared with HeLa, CaSki and ME180 cell lines. Downregulation of p53 resulted in the inhibition of p53-regulated antioxidant enzymes and elevated intracellular ROS in SiHa cells. By contrast, the ROS level was not affected in HeLa, CaSki and ME180 cell lines after inhibition of the p53 protein. Under mild or severe H_2O_2-induced stress, p53-deficient SiHa cells exhibited much higher ROS levels than control SiHa cells. Furthermore, we analyzed cell viability and apoptosis after H_2O_2 treatment and found that p53 deficiency sensitized SiHa cells to H_2O_2 damage. Inhibition of p53 resulted in excessive oxidation of DNA; control SiHa cells exhibited a more rapid removal of 8-oxo-7,8-dihydro-2′-deoxyguanosine from DNA compared with p53-deficient SiHa cells exposed to the same level of H_2O_2 challenge. These data collectively show that endogenous p53 in SiHa cells has an antioxidant function and involves in the reinforcement of the antioxidant defense.

Key words: p53, SiHa, H_2O_2, ROS, Oxidative stress, RNA interference (RNAi)

Introduction

Reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals, and hydrogen peroxide (H_2O_2) are more reactive than molecular oxygen (Thannickal and Fanburg, 2000) and therefore can indiscriminately oxidize biological molecules, providing a constant threat to cells in an oxygen-rich environment (Storz and Imlay, 1999). In living cells, endogenous ROS are generated as byproducts of cellular metabolism and through leakage of electrons from the mitochondrial electron transport chain (Nohle et al., 2003); they are also derived from exogenous sources such as UV radiation, γ-irradiation and chemicals. Present in all aerobic cells, ROS exist in a physiological balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted, increasing intracellular ROS levels. To maintain intracellular redox homeostasis and protect against oxidative damage, cells have developed a sophisticated antioxidant defense system by enlisting functional antioxidant buffers through redox-coupled enzymatic networks that regenerate oxidized substrates (Martindale and Holbrook, 2002; Waris and Ahsan, 2006). In this system, superoxide dismutase (SOD) converts the superoxide anion radical (O_2^-) to H_2O_2, which is subsequently eliminated by catalase (CAT), glutathione peroxidase 1 (Gpx1) and peroxiredoxin (Prx). Gpx1 – a selenoprotein – is considered the primary enzyme responsible for the removal of H_2O_2 and may have a role in the regulation of cellular redox status (Burdon, 1995; de Hann et al., 1998). Likewise, Prx is a major reductant of endogenously produced peroxides in eukaryotes and catalyzes the conversion of H_2O_2 into H_2O by using reducing equivalents provided by thioredoxin (Trx) (Rhee et al., 2001; Chae et al., 1994a; Chae et al., 1994b). During the peroxiredoxin catalytic cycle, peroxidatic cysteine is oxidized to a sulfenic acid form, which typically reacts with a proximal thiol to form a disulfide bond (Georgiou and Masip, 2003; Woo et al., 2003). This intermolecular disulfide bond is subsequently reduced by Trx; however, because the formation of the resolving disulfide bond is slow, high concentrations of ROS cause further oxidation of the peroxidatic cysteine to sulfinic acid, yielding an inactive form of Prx that cannot be reduced by typical cellular reductants such as glutathione or thioredoxin (Rabilloud et al., 2002; Wood et al., 2003; Yang et al., 2002). Sestrins are a family of cysteine sulfinyl reductases and are essential for reactivating Prx by reducing the cysteine sulfinic acid to thiol, thus reestablishing the antioxidant firewall (Budanov et al., 2004).

The tumor suppressor protein p53 is an important sensor of cellular stress and is involved in regulating cellular responses to DNA damage. Depending on the cell type and the context of stimuli, p53 can either induce cell growth arrest to allow repair or alternatively triggers apoptosis to prevent DNA damage in abnormal or stress-exposed cells from becoming fixed as a mutation (Lane, 1992). Additional evidence exists suggesting that p53 works in a more positive way, participating in the maintenance of intracellular redox homeostasis and protection of the genome from oxidative damage. First, there

are several p53-regulated genes such as *GPX1*, *SOD2* and aldehyde dehydrogenase 4 family member A1 (*ALDH4A1*), which encode products that act as antioxidants (Hussain et al., 2004; Tan et al., 1999; Yoon et al., 2004). Second, reactivation of overoxidized Prx is mediated by two p53-regulated sestrins, namely, PA26 and Hi95 (encoded by *SESN1* and *SESN2*, respectively), which are required for regeneration of Prx containing cysteine sulfinic acid. Third, in the absence of stress or after mild stress, a relatively low level of p53 is sufficient to upregulate several antioxidant genes that decrease ROS levels and protect cells from DNA damage (Sablina et al., 2005).

Decreasing intracellular levels of ROS has long been a goal for cancer prevention. Owing to the effects of many cancer therapeutics, cancerous cells can be subjected to oxidative stress (Renschler, 2004), increasing the understanding of how ROS homeostasis is achieved in cancer cells, which may be crucial for the development of more effective cancer therapies. Cervical cancer is the second most common cancer among women in the world in both incidence and mortality, and high-risk human papillomavirus (HPV) – mainly serotypes 16 and 18 – is present in more than 90% of such tumors (Lazo, 1999; zur Hausen, 1996). Most HPV-positive cervical carcinoma cells possess the wild-type p53 gene, but it is often rendered non-functional by the E6 oncoprotein, which complexes with cellular proteins E6-AP and p53 to facilitate p53 degradation via a ubiquitin-dependent proteolytic system (Scheffner, 1998). When studied in cervical carcinoma cells expressing E6 in the context of its natural promoter, endogenous p53 protein could exert transcriptional function following DNA damage, despite coexpression of the viral E6 protein, suggesting that the amount of expressed HPV E6 is proportionate to the inhibition of p53 activity. Basal p53 activity, as measured with luciferase reporter assays in HPV-positive cervical cancer cells, showed that these cells have residual p53 activity and that p53 is not completely inactivated in cervical cancer cells (Butz et al., 1995; Butz et al., 1999; Hietanen et al., 2000).

To provide new insight into the possible role of endogenous p53 in the antioxidant defense of HPV-positive cervical cancer cells, we used a plasmid-mediated short hairpin RNA (shRNA) to knock down p53 expression in SiHa, HeLa, CaSki and ME180 cells (all of which integrated with high risk HPV sequences) and investigated whether p53 could modulate intracellular ROS levels under both nonstressed and stressed conditions. We found that endogenous p53 in SiHa cells was involved in the modulation of ROS levels, and may participate in maintaining ROS homeostasis. These findings might help to facilitate future clinical studies of HPV-positive cervical cancer therapy.

Results

Analysis of p53 and antioxidant enzymes in several cervical carcinoma cell lines

Levels of p53 protein were examined in several cervical carcinoma cell lines and in HFKs by immunoblot analysis with the mouse monoclonal antibody DO1 to human p53. HFK cells were included in this analysis as a negative control because they do not carry HPV and the p53 level in HFKs is low, as it is in most primary cells. SiHa cells, which contain only one copy of HPV 16 per cell, exhibited higher amounts of p53 than CaSki cells containing 600 copies of HPV 16, HeLa cells with 25 copies of HPV 18, and ME180 cells with HPV 68. C33A cells, which contain mutant p53 and no HPV, also served as a negative control in this analysis. The p53 level detected in the C33A cell line was extremely high (Fig. 1A). Further, we investigated the expression of p53-regulated antioxidant enzymes GPX1, SESN1 and SESN2, and found very high levels of GPX1 protein, SESN2 protein and *SESN1* mRNA in SiHa cells, compared with the three other HPV-positive cell lines examined (Fig. 1A,B). Because a suitable anti-SESN1 antibody for quantification of SESN1 protein level is not available, no western blot analysis could be done for the SESN1 protein expression. We also tested the expressions of SOD1 and SOD2, the similar level of each protein were found in these six cell lines studied.

Sensitivity of cervical carcinoma cells to H_2O_2 challenge

Having shown the status of p53 and its regulated antioxidant enzymes in HPV-positive cervical carcinoma cell lines, we next analyzed the sensitivity of these cells to H_2O_2-induced oxidative damage. As shown in Fig. 2, we observed differences in the response to the H_2O_2 challenge in the four HPV-positive cell lines. The SiHa cell line was the most resistant to H_2O_2 whereas the other three cell lines displayed similarly reduced survival. The sublethal doses of H_2O_2 to SiHa, HeLa, CaSki and ME180 were 0.8 mM, 0.2 mM, 0.2 mM and 0.1 mM, respectively. We also found that HFK and C33A cells exerted a similar resistance to H_2O_2 challenge, the sublethal dose of H_2O_2 to HFK and C33A was 0.4 mM.

Downregulation of p53 elevates intracellular ROS in SiHa cells

As SiHa cells had a higher expression of p53 and were much more resistant to H_2O_2 challenge than other HPV-positive cell lines, we attempted to analyse the impact of p53 RNAi on intracellular ROS. We used the human U6 promoter to drive the expression of shRNA targeting p53 in a plasmid-based system (pSuppressorNeo p53 plasmid). A negative control plasmid, which contained a scrambled sequence, was also included in experiments. Each construct produced a shRNA composed of two 19-nucleotide repeats in an inverted orientation as shown in Fig. 3A.

When the pSuppressorNeo p53 plasmid and negative control plasmid were transfected into SiHa cells, the p53 protein level

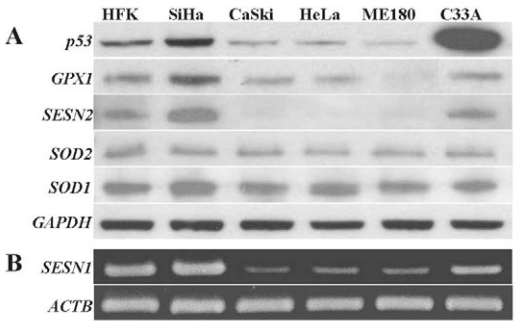

Fig. 1. Expression of p53 and antioxidant enzymes in human cervical carcinoma cell lines and in HFKs. (A) Western blot analysis for p53, GPX1, SESN2 (Hi95), SOD2 and SOD1. GAPDH was used as a loading control. (B) RT-PCR for *SESN1* and *ACTB* (β-actin).

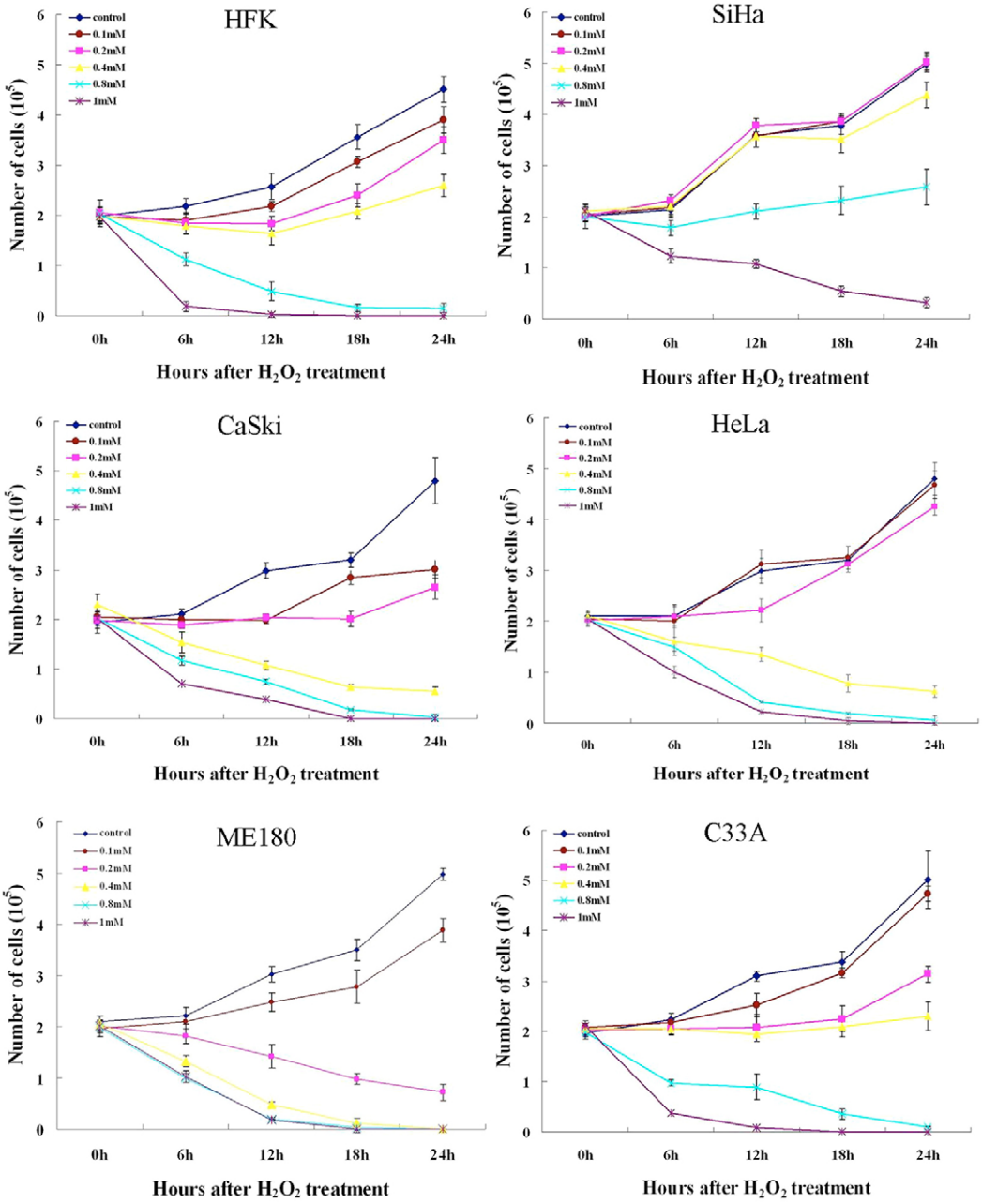

Fig. 2. Cell viability of HFK, SiHa, CaSki, HeLa, ME180 and C33A cells after H_2O_2 treatment as detected by Trypan Blue assay. The data represent the means of pooled results from five independent experiments.

was markedly decreased after 24-72 hours, compared with the normal level of expression of p53 in the control SiHa transfection (Fig. 3B). In contrast to p53, GAPDH was not affected with either construct, indicating that the reduction of p53 by applying U6-driven shRNA constructs was specific. We further tested how p53 deficiency affects the levels of p53-regulated antioxidant enzymes. Inhibition of p53 in SiHa cells 24-72 hours after transfection resulted in a notable decrease in the p53-inducible transcript T2 (Woo et al., 2003) of *SESN1* and a virtual disappearance of GPX1 and SESN2, whereas there were no differences in *SOD1* and *SOD2* expression after p53 knockdown in SiHa cells (Fig. 3B).

To show the effects of p53 on ROS levels in nonstressed cells, SiHa cells were labeled with the cell-permeable fluorescent dye CM-H_2DCFDA, the fluorescence of which increases following oxidation by H_2O_2 and hydroxyl radicals in the cells. Fluorescent signals were analyzed by flow cytometry. CM-H_2DCF staining indicated that there was an approximately twofold increase in ROS levels 24 hours after inhibition of p53, and the high levels of ROS remained for 72 hours (Fig. 4A,B), in agreement with the above observations that the expressions of *GPX1*, *SESN1* and *SESN2* were markedly decreased after p53 inhibition by siRNA. Next, we examined whether N-acetyl cysteine (NAC, a potent antioxidant) inhibits ROS accumulation in SiHa cells. As shown in Fig. 4C, the increase in ROS induced by p53-specific siRNA 48 hours after transfection was almost completely reversed by incubation with 5 mM NAC. To test whether p53 levels affect superoxide levels in SiHa cells, we employed DHE as a cytosolic superoxide indicator and MitoSOX RED as a

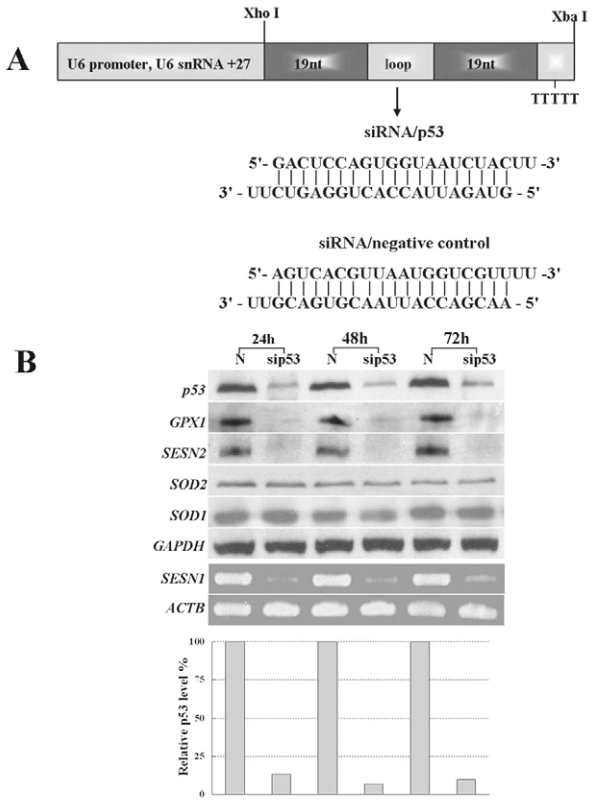

Fig. 3. Reduction of p53 levels in SiHa cells transfected with p53 siRNA or nonspecific siRNA. (A) Schematic illustration of the U6 promoter-driven shRNA constructs containing the sequence targeting p53 and the scrambled sequence. (B) Upper panel: Expression levels of p53, GPX1, SESN2, SOD2 and SOD1 in control or in si-p53-expressing cells, as detected by western blot analysis. GAPDH was used as a loading control. Middle panel: Expression of *SESN1* (T2) and *ACTB* as detected by RT-PCR. Lower panel: Quantification of p53 in western blots above normalized to GAPDH expression.

mitochondrial superoxide indicator. Fluorescent intensities of both DHE and MitoSOX RED were not changed before and after p53 knockdown in SiHa cells (Fig. 4D,E), which was consistent with the observations that the expression of SOD1 and SOD2 were not affected by p53 deficiency in SiHa cells. When tested in cell culture, there was no notable change in the growth rate (Fig. 5A) or cell cycle distribution (Fig. 5B) of SiHa cells with inhibited p53, and addition of NAC (5 mM) did not affect cell proliferation. We also found the expression of p53-specific siRNA did not increase ROS levels in HeLa, CaSki and ME180 cell lines, similar to results with the negative control plasmid (Fig. 6).

p53 deficiency promotes intracellular ROS level in SiHa cells under oxidative stress

The results presented above suggest that p53 in the SiHa cell line may provide a protective function by participating in antioxidant defense. To test the role of p53 in antioxidant defense according to the severity of oxidative stress, we compared ROS levels in control and p53-deficient SiHa cells stimulated with different concentrations of H_2O_2. 48 hours after transfection with either the negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were mock treated or treated with different concentrations of H_2O_2 for 12 hours and intracellular levels of ROS were analyzed. Compared with SiHa/N cells with negative siRNA, both untreated and H_2O_2-treated SiHa/sip53 cells (in which p53 was inhibited by siRNA) exhibited markedly increased ROS levels. Intriguingly, we also observed that even at a lethal dose of H_2O_2 (1 mM), ROS level in SiHa/sip53 cells was much higher than that in SiHa/N cells (Fig. 7). Taken together, the data strongly suggest that endogenous p53 in HPV-positive SiHa cells functions as an antioxidant and may play an important role following oxidative stress.

Inhibition of p53 sensitizes SiHa cells to H_2O_2-induced oxidative damage

Since the reduction of p53 induced a marked increase in the level of ROS under both unstressed conditions and oxidative challenge, we investigated whether p53 deficiency could sensitize SiHa cells to oxidative damage. To address this question, we transfected SiHa cells with a negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53). After 48 hours, cells were treated with varying concentrations of H_2O_2 for either 12 hours or 24 hours. As expected, the proliferation in SiHa/sip53 cells by 0.5 mM H_2O_2 was reduced to 57.5% and 56.5%, 12 and 24 hours after treatment, respectively, compared with control SiHa/N cells. Likewise, treatment of control SiHa/N cells with 0.5 mM H_2O_2 had almost no effect on cell viability. When tested at a lethal dose of H_2O_2 (1 mM) for 12 and 24 hours, we found that SiHa/sip53 cells were more susceptible to H_2O_2-induced damage. For example, treatment with 1 mM H_2O_2 for 12 hours induced death in 68% of SiHa/sip53 cells, whereas the rate was much lower (27%) in SiHa/N cells (Fig. 8A).

Although the XTT assay is a convenient method to measure cell death, it does not discriminate between apoptosis and necrosis. To determine whether apoptosis was involved in H_2O_2-induced cell death, SiHa/sip53 cells were further examined by annexin V and propidium iodide staining. Under defined salt and Ca^{2+} concentrations, annexin V is predisposed towards binding externalized phosphatidylserine (PS) that is present on the cell surface during the early stages of apoptosis. Addition of PI helps to distinguish between early apoptotic cells and late apoptotic or necrotic cells because PI cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact. Cells were treated with H_2O_2 for 12 hours, and apoptosis studies indicated that there was a strong induction in early stage apoptosis (annexin V positive) as well as in late apoptosis (annexin V and PI positive) in SiHa/sip53 cells, as depicted in Fig. 8B. Collectively, the data suggest that the reduction of p53 sensitized SiHa cells to oxidative damage.

p53 deficiency increases DNA oxidation in SiHa cells

To determine whether p53 is required for the protection of DNA from oxidative damage, we monitored the rate of formation of 8-oxoguanine (8-oxo-dG), the major product of DNA oxidation, using an assay kit as described in the Materials and Methods. SiHa cells with siRNA-inhibited p53 displayed a twofold increase in levels of 8-oxo-dG compared with control cells (Fig. 9A). We also examined whether p53 is associated with oxidative DNA damage induced by challenge with H_2O_2,

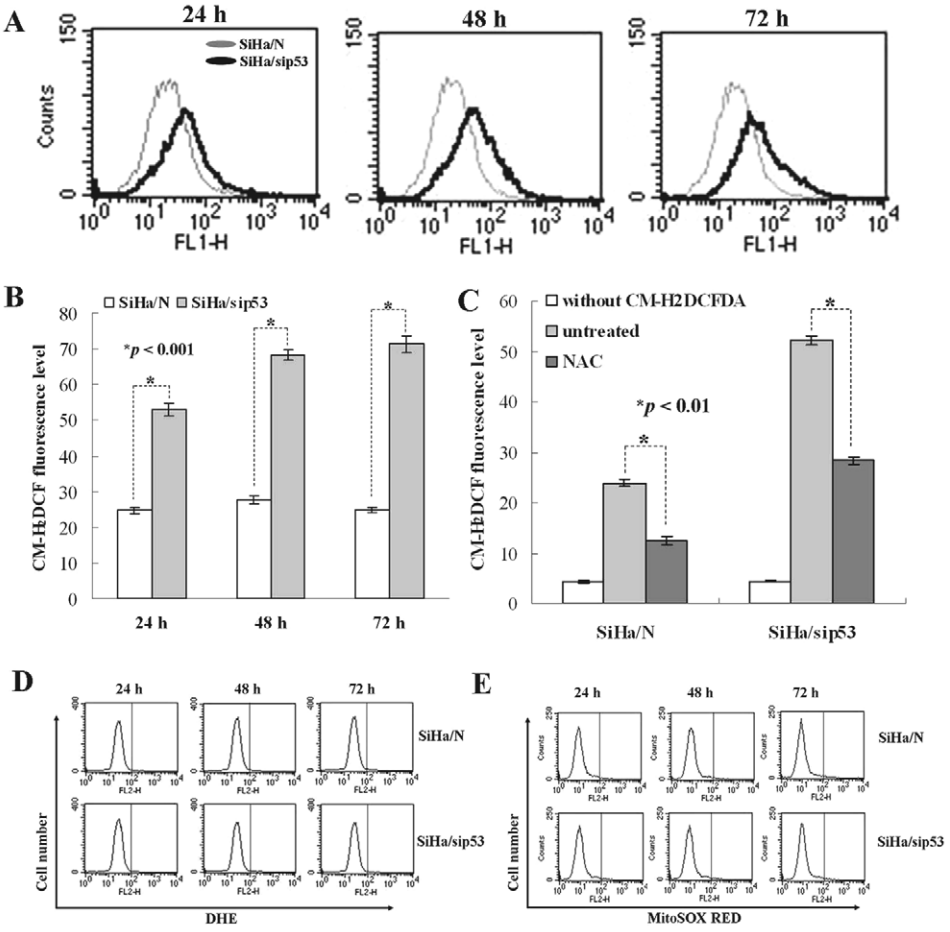

Fig. 4. Effect of deficiency in p53 on intracellular ROS level in the SiHa cell line. (A) Intracellular ROS levels in SiHa cells measured by CM-H_2DCFDA staining and FACS analysis at different time intervals after transfection with p53 siRNA or nonspecific siRNA. (B) ROS levels expressed as the mean ± s.d. intensity of cell fluorescence. **P*<0.001 compared with the corresponding negative control by Student's *t*-test. (C) FACS analysis of CM-H_2DCFCA-stained cells treated with NAC (5 mM, 6 hours) 48 hours after transfection with p53 siRNA or nonspecific siRNA. **P*<0.01 compared with the corresponding untreated group by Student's *t*-test. (D) Cytosolic superoxide levels in SiHa cells transfected with p53 siRNA or nonspecific siRNA measured by DHE staining and FACS analysis. (E) Mitochondrial superoxide levels in SiHa cells transfected with p53 siRNA or nonspecific siRNA measured by MitoSOX RED staining and FACS-analysis.

and evaluated the possible role of p53 in the removal of such DNA damage in SiHa cells. Treatment of cells with 0.1 and 0.5 mM H_2O_2 for 1 hour resulted in an increase in 8-oxo-dG levels, as indicated by the increase in fluorescence of the 8-oxo-dG signal. However, there was a significant difference in the levels of 8-oxo-dG in SiHa/N and SiHa/sip53 cells. SiHa/sip53 cells consistently exhibited higher 8-oxo-dG content than SiHa/N cells (Fig. 9B). Therefore, these data suggest that in the SiHa cell line, p53 may participate in protecting DNA from oxidative damage.

p53 facilitates the removal of 8-oxo-dG residues from DNA in whole SiHa cells

Finally, we performed pulse-chase experiments to test whether p53 facilitates the removal of 8-oxoG residues from DNA in the SiHa cell line. 48 hours after transfection with negative

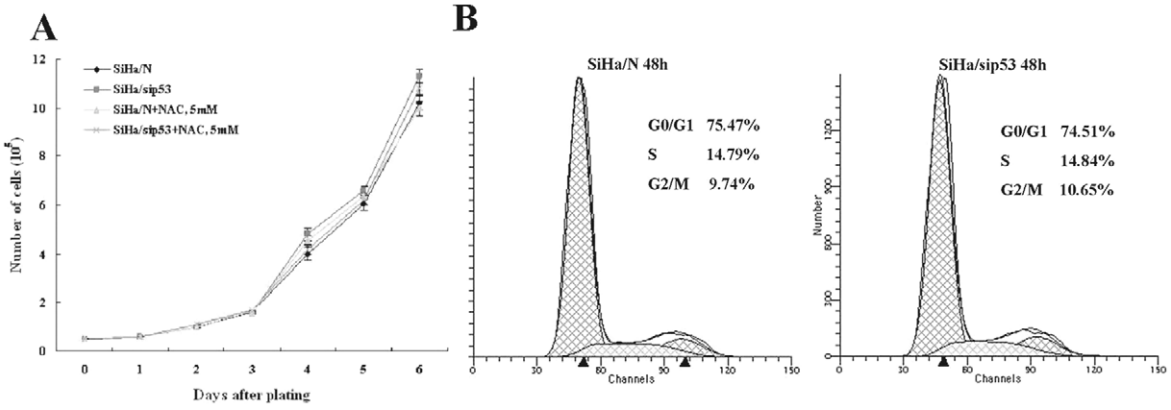

Fig. 5. Characterization of SiHa cells with inhibited expression of p53. (A) Growth curves of SiHa/N and SiHa/sip53 cells untreated or treated with 5 mM NAC. **P*>0.98 by Student's *t*-test. (B) Cell cycle distribution of SiHa/N and SiHa/sip53 was analyzed 48 hours after transfection.

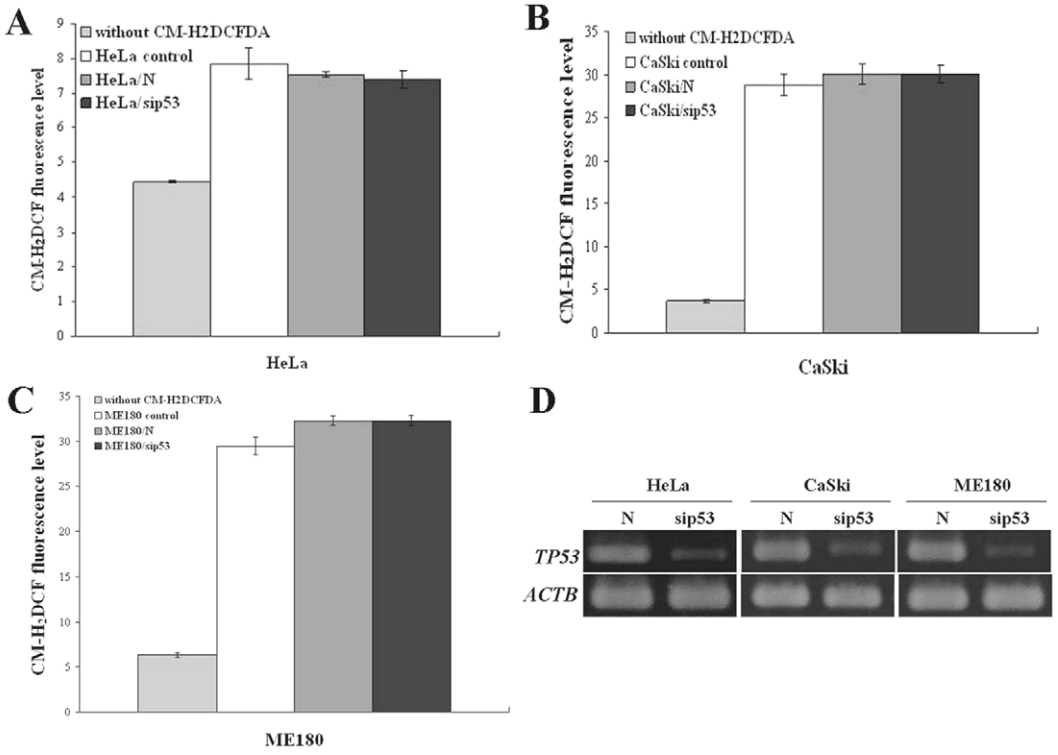

Fig. 6. ROS levels in HeLa (A), CaSki (B) and ME180 (C) cells 48 hours after inhibition of p53 with siRNA (sip53). For a negative control we used nonspecific siRNA. **P*>0.95 compared with the corresponding cells with negative control vector (Student's *t*-test). (D) Expression levels of p53 mRNA in both control and si-p53-expressing cells 48 hours after transfection with corresponding plasmid, as detected by RT-PCR.

control plasmid or p53 shRNA plasmid, cells were treated with 0.5 mM H_2O_2 for 1 hour to induce 8-oxo-dG accumulation. After treatment, the medium was changed and the cells were incubated without H_2O_2 for various times. To evaluate the rate of 8-oxo-dG elimination, the level of 8-oxo-dG remaining was detected at each time point. SiHa/sip53 cells were slower than SiHa/N cells to remove 8-oxo-dG from the DNA, with the time for the level of 8-oxo-dG to decrease by 50% ($t_{1/2}$) was 3.1 hours and more than 6 hours, respectively (Fig. 10).

Discussion

Persistent infection by high-risk types of HPV has been associated with the development of human cervical cancer. Based on epidemiological and experimental evidence, it is widely accepted that carcinogenesis of HPV-infected cells is a process involving integration of the viral genome in cancer cells, resulting in the loss of expression of the viral E2 gene and the persistence of E6 and E7 oncoprotein expression (Jeon et al., 1995). The oncogenic activity of high-risk HPVs is explained in part by the ability of the viral E6 oncoprotein to

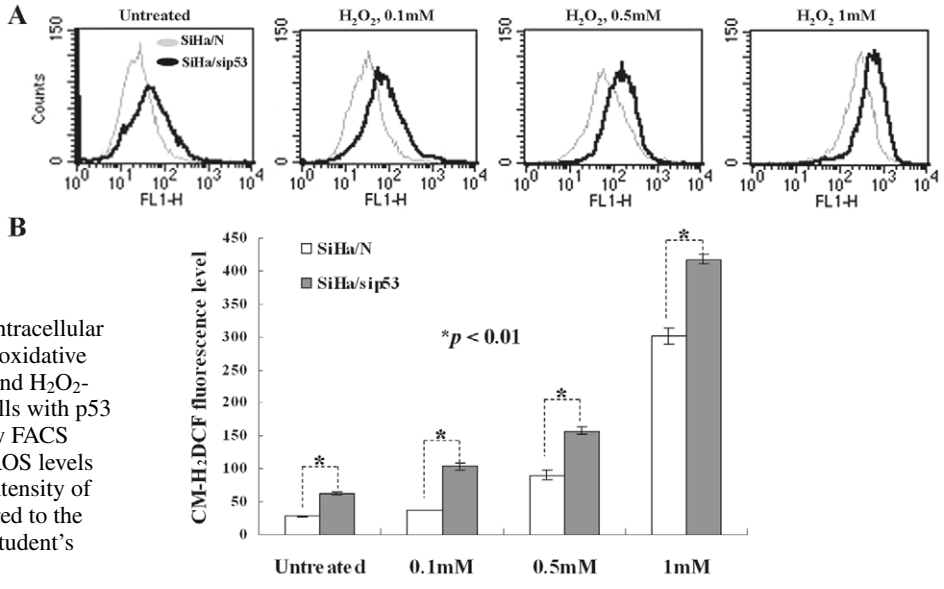

Fig. 7. Effect of p53 deficiency on intracellular ROS level in SiHa cells undergoing oxidative stress. (A) ROS levels in untreated and H_2O_2-treated (12 hours) SiHa and SiHa cells with p53 inhibited with siRNA, as detected by FACS after CM-H_2DCFCA-staining. (B) ROS levels were expressed as the mean ± s.d. intensity of cell fluorescence. **P*<0.001 compared to the corresponding negative control by Student's *t*-test.

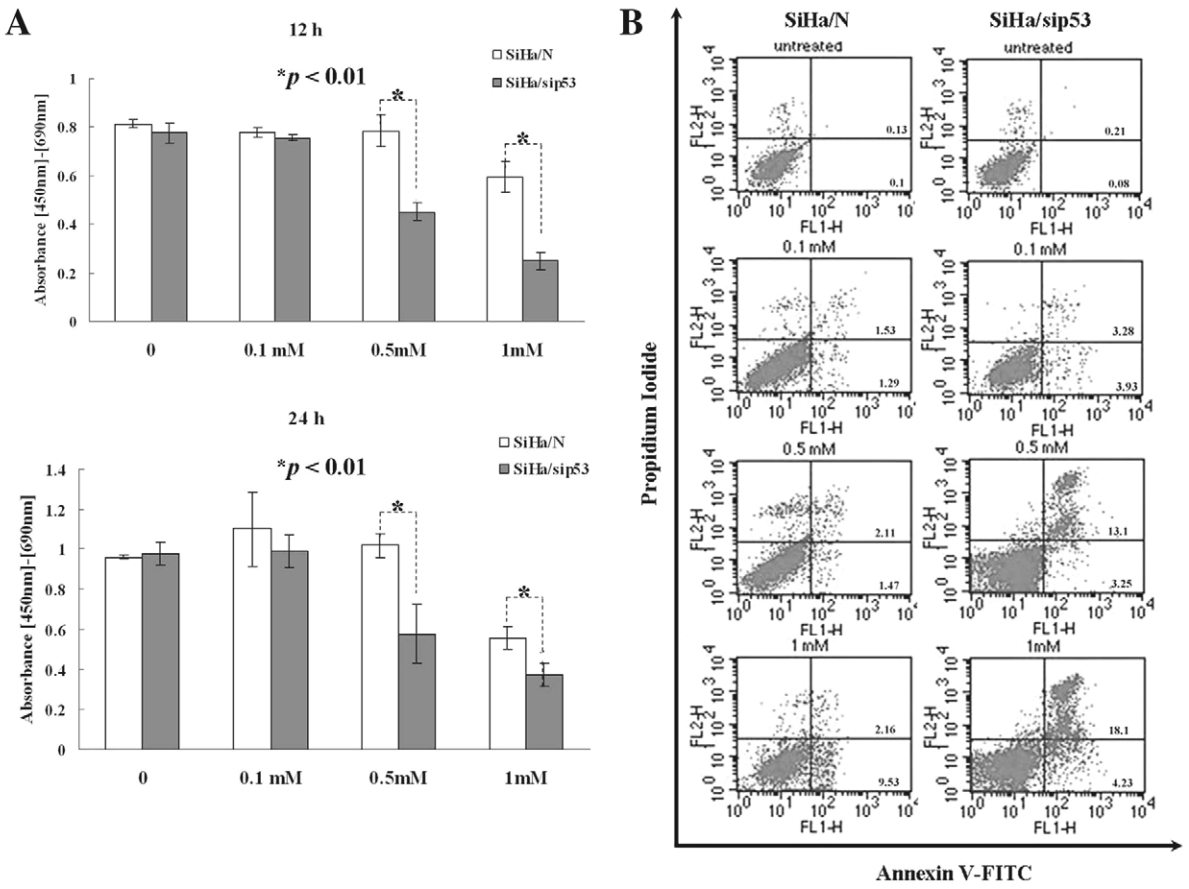

Fig. 8. Inhibition of p53-sensitized SiHa cells to H_2O_2-induced oxidative damage. (A) 48 hours after transfection with negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were treated with various concentrations of H_2O_2 for either 12 hours or 24 hours, after which time cell viability was measured with an XTT assay. Results shown are expressed as mean ± s.d. of triplicate microcultures. *P<0.01 compared with the corresponding negative control using Student's t-test. (B) Apoptosis and death levels of SiHa/N and SiHa/sip53 cells 12 hours after treatment with 0.1, 0.5 and 1 mM of H_2O_2 as detected by FACS after Annexin V/PI staining. Apoptotic (Annexin V^+/PI^-), dead (Annexin V^+/PI^+) and alive (Annexin V^-/PI^-) populations were readily identified. The rates of apoptotis and death are shown in upper right and lower right panels, respectively. *P<0.01 compared with the corresponding untreated cells (Student's t-test).

target p53 for degradation and thus to inhibit p53-mediated transcription (Hoppe-Seyler and Butz, 1993). However, studies investigating the functional status of p53 in a series of HPV-positive cancer cells expressing the E6 gene from chromosomally integrated viral sequences under its natural promoter have produced differing results, indicating that despite co-expression of the viral E6 oncogene, there is residual p53 activity, indicating that endogenous p53 is not completely inactivated in HPV-positive cancer cells (Abdulkarim et al., 2002; Butz et al., 1995; Butz et al., 1999; Hengstermann et al., 2001; Hietanen et al., 2000). To date, there are no suitable anti-E6 antibodies available for reliable quantification, and thus the presence of viral E6 protein has not been unambiguously demonstrated in HPV-positive cancers. Therefore, it is currently widely assumed that E6 protein levels are very low in HPV-positive carcinoma cell lines and might be limited in their capacity to interfere with p53 function. In the present study, we found that endogenous p53 in SiHa cells is involved in the modulation of intracellular ROS levels, participates in maintaining ROS homeostasis in response to oxidative stress and protects DNA against oxidative damage.

Direct comparison of p53 protein levels between HPV-positive cervical carcinoma cell lines, as examined by immunoblot analysis, indicated that SiHa cells had the highest steady-state level of p53. This result is in full agreement with previous findings Scheffner et al. (Scheffner et al., 1991) who showed that p53 level in SiHa cells is 3-5 times higher than that in other HPV-positive cervical cancer cells. A recent study (Sablina et al., 2005) provided evidence that even the very low levels of p53 present in normal tissues in the absence of stress are sufficient enough to drive the expression of several antioxidant genes. Therefore, we next addressed the hypothesis that high levels of p53 in SiHa cells may contribute to some antioxidant enzymes. Indeed, among the series of HPV-positive cancer cell lines investigated in this study, SiHa cells exhibited the highest expression of *GPX1*, *SESN1* and *SESN2*, implying that SiHa cells may have a powerful antioxidant defense system and are thus tolerant of severe oxidative insults. By using H_2O_2 to induce oxidative stress, we found that HeLa, CaSki and ME180 cells were severely impaired in their antioxidant capacity, whereas SiHa cells, as expected, exhibited a higher resistance to H_2O_2 damage. Given the

evidence that p53 is a major regulator of the H_2O_2 response in human cells (Desaint et al., 2004), the above findings raised the question whether the presence of a high steady-state level of p53 in SiHa cells has an antioxidant function.

Expression of p53 was knocked down by transfecting U6 promoter-driven shRNA constructs into SiHa cells. We were able to demonstrate that p53 was reduced 24-72 hours post transfection, compared with that in the control. Likewise, the levels of GAPDH protein and *ACTB* (encoded β-actin) mRNA remained unchanged, indicating that the reduction of p53 was specific. Interestingly, we also noted that the expression of *GPX1*, *SESN1* and *SESN2* were also inhibited following p53 RNAi in SiHa cells. These observations further supported the idea that p53 is transcriptionally competent despite co-expression of the viral E6 protein and could induce antioxidant gene expressions in SiHa cells in the absence of stress. Elevated levels of ROS were also found in p53-deficient SiHa cells, although this could be reversed by NAC supplementation. These data strengthened the idea that the antioxidant function of p53 is mediated through a set of antioxidant gene products, and the depletion/downregulation of p53 damped down the antioxidant defense in SiHa cells.

Intriguingly, although p53 deficieny promoted ROS levels in SiHa cells, we did not observe a similar correlation between p53 protein and ROS levels in the other three HPV-positive cervical cancer cells HeLa, CaSki and ME180. Different scenarios could be envisaged to explain the differences in the modulation of ROS by p53 in the HPV-positive cancer cells investigated in the present study. The transcriptional activity of p53 to activate antioxidant genes is necessarily correlated with p53 protein abundance, and a steady-state level of p53 may be required for its transcriptional activity in HPV-positive cells under physiological conditions. Consistent with this notion, the p53 levels in HeLa, CaSki and ME180 cells were much lower than that in normal HFK cells. It has been reported that p53 levels detected in HeLa, CaSki and ME180 cells ranged from 0.4- to 0.6-times that found in secondary cultures of HFKs (Scheffner et al., 1991). Furthermore, the levels of p53-regulated antioxidant enzymes, GPX1, SESN1 and SESN2, were very low, and even undetectable in the three cell lines. In addition, there may also be cell-specific differences in the regulation of cellular antioxidant abilities between these cell lines. It will also be interesting to study the role of HPV in the differential response of these HPV-positive cell lines to oxidative stress because these cell lines harbor different subtypes and copies of HPV.

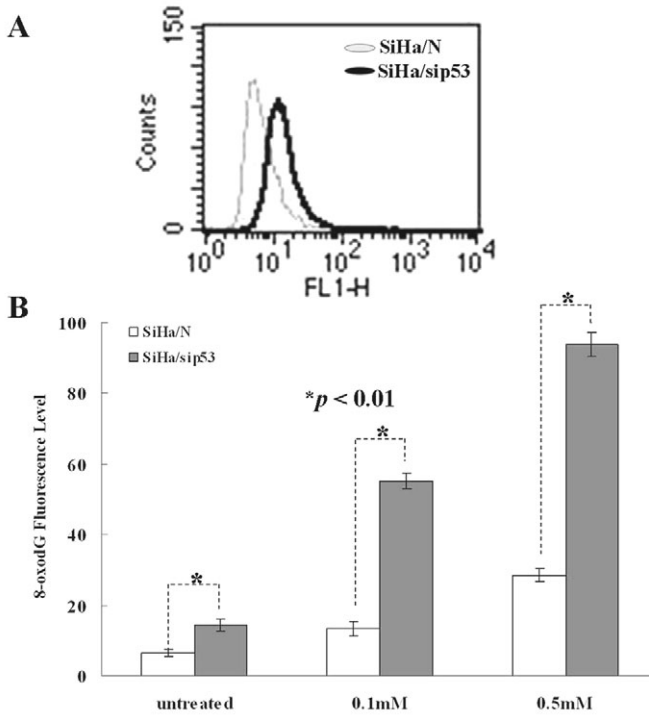

Fig. 9. Reduction of p53 expression increased DNA oxidation in SiHa cells. (A) 8-oxo-dG level in SiHa cells expressing negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53) 48 hours after transfection. (B) 48 hours after transfection with negative control plasmid or p53 shRNA plasmid, cells were treated with the indicated concentrations of H_2O_2 for 1 hour. Quantification of 8-oxo-dG staining was performed after FACScan analysis using CellQuest software. 8-oxo-dG levels are expressed as the mean ± s.d. intensity of cell fluorescence. **P*<0.01 compared with the corresponding cells with negative control vector by Student's *t*-test.

In concordance with the notion that the p53 protein is functional in SiHa cells, we observed a much higher level of ROS in p53-deficient SiHa cells following H_2O_2 exposure than in SiHa cells containing wild-type p53. Indeed, it has been shown previously that p53 induces ROS accumulation and a number of genes induced by p53 are associated with the metabolism of ROS (Macip et al., 2003; Polyak et al., 1997). Understanding how p53 stimulates the accumulation of ROS level is complicated by the opposite finding that p53 also regulates the expression of antioxidant enzymes. The study from by Sablina et al. extended such observations that (1) in the absence of stress or after mild stress, p53 induces the expression of antioxidant genes that decrease ROS levels and protect cells from DNA damage; (2) severe or extended stress leads to stronger activation of p53, and thus induces the pro-oxidant genes, results in elevated ROS levels and cell death (Sablina et al., 2005; Bensaad and Vousden, 2005). In the present study, the p53-deficient cells showed a higher ROS level than control cells did, even at lethal concentrations of H_2O_2 (1 mM). These findings were in contrast with a previous study (Sablina et al., 2005) in which p53 showed pro-oxidant activity and thus promoted ROS levels in HPV-negative cell types under severe oxidative damage. One possible explanation may be that the antioxidant and pro-oxidant function of p53 was temporally separated (Bensaad and Vousden, 2005; Sengupta and Harris, 2005) and the pro-oxidant function of p53 is severely impaired in SiHa cells.

Furthermore, cells containing reduced p53 were more susceptible to H_2O_2-induced cell death. As shown in this study, a more rapid and greater magnitude of cell death was observed in SiHa cells with p53 deficiency after H_2O_2-challenge, indicating that the antioxidant defense of the SiHa cell line was achieved, at least in part, by endogenous p53, and therefore depletion of p53 followed by inhibition of the expression of some antioxidant enzymes disintegrated the antioxidant firewall and sensitized cells to oxidative stress.

The p53 tumor suppressor, a 'guardian of the genome', restricts abnormal or stress-exposed cells before DNA damage becomes fixed as a mutation (Lane, 1992). A key target of ROS

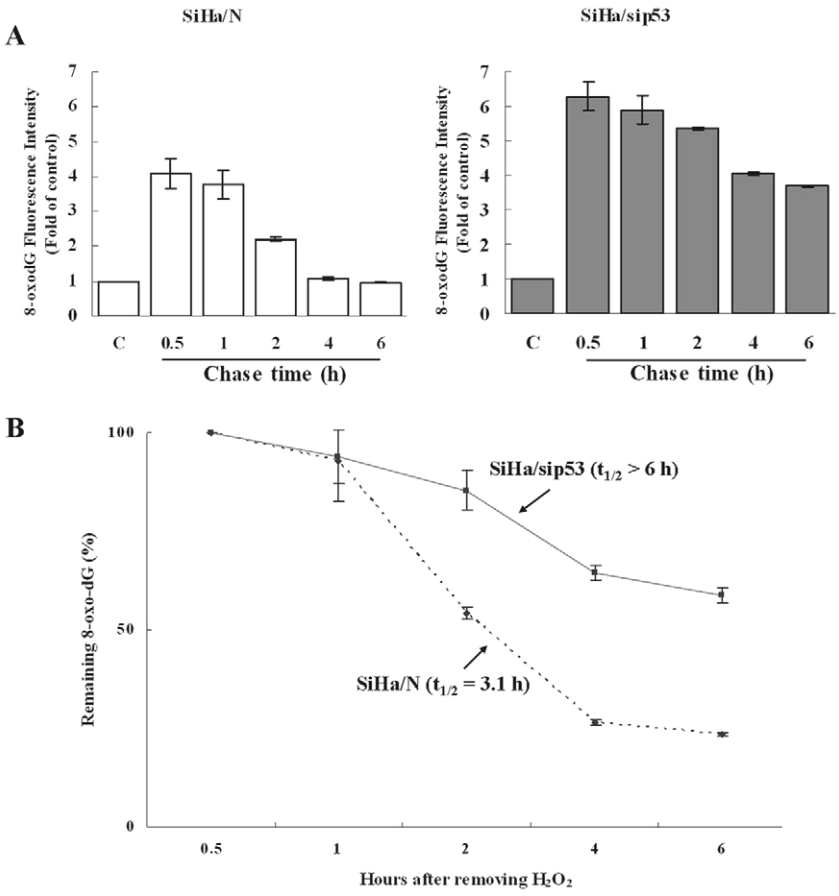

Fig. 10. p53 facilitated the removal of 8-oxo-dG residues from DNA in whole SiHa cells. (A) 48 hours after transfection with negative control plasmid (SiHa/N) or p53 shRNA plasmid (SiHa/sip53), cells were treated with 0.5 mM H_2O_2 for 1 hour. The cells were washed with fresh medium and then incubated in fresh medium for the indicated times. 8-oxo-dG levels were measured at each time point as indicated and expressed as fold of the control sample. (B) The percentage of remaining 8-oxo-dG residues in cellular DNA was plotted as a function of chase time. The $t_{1/2}$ value reflects the estimated time for levels of 8-oxo-dG to decrease to 50% of its original level.

in cells is the DNA, and under physiological conditions the endogenous ROS that are byproducts of normal respiration modify approximately 20,000 bases of DNA per day in a single cell (Beckman and Ames, 1997; Droge, 2002; Finkel, 2003). Thus, endogenous ROS are a major source of DNA damage and a substantial factor contributing to chromosomal instability, accumulation of mutations (Jackson and Loeb, 2001; Klungland et al., 1999) and deletions that may lead to cancer (Griffiths et al., 1997; Havre et al., 1995). A frequent oxidative modification of DNA is the hydroxylation of guanine at C-8, leading to the formation of 8-oxo-dG) (Breen and Murphy, 1995; Wiseman and Halliwell, 1996). In the present study, we showed that p53 protein in the SiHa cell line has a protective role against DNA oxidation. SiHa cells deficient in p53 displayed significantly increased 8-oxo-dG levels under both non-stressed and oxidative stressed conditions, a finding that was consistent with the observation that 8-oxo-dG residues were removed more rapidly from cellular DNA in whole SiHa cells with wild-type p53. Together, these results suggested that the endogenous p53 in the SiHa cell line plays a role in protecting DNA and facilitating cellular responses to ROS-induced DNA damage.

In conclusion, we have provided evidence that endogenous p53 in SiHa cells has an antioxidant function and is involved in the reinforcement of the antioxidant defense. Depletion of p53 by RNAi in the SiHa cell line resulted in disintegration of the antioxidant firewall and increased oxidative damage. As ROS are also involved in cancer chemoradiotherapy, these findings may help to facilitate clinical studies of HPV-positive cervical cancer therapy.

Materials and Methods

Cell lines and cell culture

Five cervical carcinoma cell lines, SiHa, CaSki, HeLa, ME180 and C33A, were used in this study. SiHa and CaSki contain integrated HPV 16; HeLa cells carry integrated HPV 18; and ME180 cells contain HPV 68 sequences; C33A cells are HPV-negative, and contain mutant p53. SiHa, HeLa and C33A cells were grown in DMEM (Gibco-BRL, Grand Island, NY), CaSki and ME180 cells were maintained in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Normal human foreskin keratinocytes (HFKs) were obtained from Cambrex Bio Science (Walkersville, USA) and grown in KGM Bulletkit (Cambrex). Cultures were maintained at 37°C in a 5% CO_2 atmosphere at 100% humidity.

Western blot analysis

Cell lysates were made using the PRO-PREP protein extraction solution (Intron Biotech, Korea). Lysates were clarified by centrifugation at 15,000 ***g*** at 4°C for 20 minutes and the protein concentration of the supernatant was determined with a Bradford assay (BioRad). For SDS-PAGE protein lysates were mixed with Laemmli sample buffer and boiled for 20 minutes. Total protein was separated on a 12% gel and transferred to nitrocellulose (Amersham, Germany). After blocking in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6) with 5% skimmed milk, membranes were incubated with the primary antibodies diluted in TBST with 3% skimmed milk for 2 hours at room temperature. Next, membranes were washed three times with TBST, and incubated for an additional 2 hours with horseradish peroxidase-linked secondary antibody (1:2000) diluted in TBST with 3% skimmed milk. Labeled protein bands were visualized with the enhanced chemiluminescence reagent (ECL) (Pierce, Rockford). Mouse monoclonal DO1 antibody against p53 (1:2000), goat polyclonal antibody against SOD2 (1:200), rabbit polyclonal antibody against SOD1 (1:200) and goat polyclonal antibody against GAPDH (1:2000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against GPX1 (2 μg/ml) was obtained from MBL (Japan). Rabbit polyclonal antibody against SESN2 (1:1000) was purchased from Proteintech (Chicago, IL). Horseradish peroxidase-labeled secondary antibodies were purchased from Zymed. Immunoblotting for GAPDH was performed to verify equivalent protein loading. Densitometry analysis of western blots was performed using TINA 2.0 Software (Raytest Isotopenmessgerate Gmbh, Straubenhardt, Germany).

RNA isolation and RT-PCR analysis

Cells were collected and total RNA was extracted using an UltraspecTM-II RNA isolation kit (Biotecx, Houston, TX). To prepare cDNA, 500 ng total RNA was mixed with the SuperScript RT-PCR System (Invitrogen). 5 μl of the RT reaction was then used for PCR using the HotStarTaq DNA polymerase (Qiagen). The PCR reactions were carried out under the following temperature profile: (1) denaturation at 95°C for 15 minutes; (2) 35 cycles of 95°C for 40 seconds, 56°C for 1 minute, 72°C for 1 minute; (3) a final extension for 10 minutes at 72°C. To detect the corresponding gene expression, we used the following primers: TP53, 5′-TCCACTACAACTACATGTGTAAC-3′ and 5′-GTGAAATATTCTCCATCCAGTG-3′; SESN1 (T2), 5′-CGACCAGGACGAGGAACTT-3′ and 5′-CCAATGTAGTGACGATAATGTAGG-3′ ACTB, 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′.

RNA interference

To generate a short hairpin RNA specific for p53, we used the pSuppressorNeo p53 plasmid (IMG-803, Imgenex, San Diego, CA). A negative control plasmid (IMG-800) with a scrambled sequence was supplied by Imegenex. The following sequences, representing 19 bp of the mRNAs, were present in the hairpin transcripts: TP53, 5′-GACTCCAGTGGTAATCTAC-3′; negative control, 5′-AGTCACGT-TAATGGTCGTT-3′. Cells were cultured overnight and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Briefly, both the DNA and Lipofectamine 2000 were diluted with serum-free medium. The diluted DNA was added to the diluted Lipofectamine 2000, mixed gently, and incubated at room temperature for 30 minutes. Lastly, the DNA-Lipofectamine 2000 mixture was carefully added to cells at 95% confluency in fresh medium without antibiotics and incubated at 37°C.

Cell proliferation and viability analysis

To measure the number of viable cells, a Trypan Blue exclusion assay was used. Cells were seeded in a 24-well plate and incubated for 24 hours. At the indicated time points, cells were collected and stained with 0.2% Trypan Blue. Cell numbers were determined by direct counting of cells under the microscope, using a standard haemocytometer. Cell viability was measured using a WelCountTM Cell Viability Assay Kit (WelGENE, Seoul, Korea). Briefly, we defrosted the XTT reagent and PMS at 37°C, prepared the reaction mixture (40 μl PMS and 2 ml XTT reagent per plate), added 20 μl of the reaction mixture to each well (each containing 100 μl media) and incubated at 37°C for 4 hours. After observing a change in color of the solution, we measured these samples at a wavelength of 490 nm using a Molecular Devices VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate.

Measurement of ROS accumulation

Intracellular production of ROS was measured using cell-permeable fluorescent dyes, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes), dihydroethidium (DHE, Molecular Probes) and MitoSOX RED (Molecular Probes). When these dyes are oxidized by ROS in cells, their fluorescent signals increase. CM-H_2DCFDA is mainly oxidized by hydrogen peroxide (H_2O_2) and hydroxyl radicals, DHE is oxidized by cytosolic superoxide anion, whereas MitoSOX RED is selectively oxidized by superoxide anion in the mitochondria. For the assay, cells were plated in six-well plates and loaded with CM-H_2DCFDA or DHE for 30 minutes, or MitoSOX RED for 10 minutes in the dark at 37°C. Next, cells were washed twice with PBS, trypsinized and fluorescence was measured using flow cytometry (excitation at 488 nm, emission at 515–545 nm). Data analysis was performed with CELLQuest software and the mean fluorescence intensity was used to quantify the responses. A minimum of 10,000 cells was acquired for each sample.

Cell cycle distribution observation

Flow cytometry analysis of PI-stained cells was performed to demonstrate the progression of the cell cycle. Briefly, cells were harvested, washed and fixed in 70% ethanol overnight at 4°C. Prior to flow cytometry, cells were washed and stained with 1 ml PI (5 μg/ml) containing 0.1 mg/ml RNaseA. DNA content was determined with a FACScan flow cytometer (Becton Dickinson) and the proportion of cells in a particular phase of cell cycle was determined with CellQuest software.

Apoptosis assay by Annexin V/PI staining

An Annexin V-FITC Apoptosis Kit I (BD, San Jose, CA, USA) was used to detect apoptosis. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS). In apoptotic cells, PS is translocated from the inner leaflet of the plasma membrane to the outer leaflet. Propidium iodide (PI), a standard flow cytometric viability probe, was used to distinguish between viable and nonviable cells. Cells that were stained were detected by a flow cytometer. Briefly, 1×10^6 cells were harvested, washed with ice-cold PBS, and resuspended in 500 μl of Annexin binding buffer. A 100 μl aliquot (2×10^5 cells) was taken, 5 μl each of Annexin V-FITC and PI were added and cells were incubated for 15 minutes at room temperature in the dark. 400 μl binding buffer (10 mM HEPES-NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was added and samples were acquired on a FACScan flow cytometer (FACSCalibur, Becton Dickinson) and analyzed using CELLQuest software with in 1 hour. Cells that were Annexin V^-/PI^+ were counted as necrotic, those that showed up as Annexin V^+/PI^+ were counted as late apoptotic or secondarily necrotic, and Annexin V^+/PI^- cells were recognized as apoptotic. All procedures stated above were performed according to the manufacturer's instructions.

Detection of 8-oxo-dG residues in DNA

After transfection with the pSuppressorNeo p53 or negative control plasmids for 48 hours, cells were treated with H_2O_2 at concentrations of 0.1 mM and 0.5 mM for 1 hour. The levels of 8-oxo-dG residues in cellular DNA before and following treatment with H_2O_2 were determined using the OxyDNA assay kit (EMD Biosciences, San Diego, CA). This assay is based upon the direct binding of a fluorescent probe to 8-oxoguanine in the DNA of fixed cells (Achanta and Huang, 2004; Struthers et al., 1998). Cells were harvested, washed with PBS, and fixed with 2% paraformaldehyde on ice for 15 minutes. The samples then were washed with PBS and fixed in 70% ethanol at –20°C overnight. The following day, the cells were washed once with PBS, followed by the wash solution provided by the manufacturer. Next, the cells were incubated with blocking solution, and then treated with the 8-oxo-dG-specific FITC-labeled probe and analyzed by flow cytometry according to the manufacturer's protocol. The intensity of the FITC fluorescent signal was proportional to the level of 8-oxoG residues in DNA.

Analysis of 8-oxo-dG residue removal from DNA

A pulse-chase experiment was used to assay the removal of 8-oxo-dG residues from DNA in whole cells. First, cells were transfected with pSuppressorNeo p53 or negative control plasmids for 48 hours. Following the transfection, cells were treated with 0.5 mM H_2O_2 for 1 hour. Afterwards, cells were rinsed with fresh medium (without H_2O_2) incubated. Cells were harvested before or at various times after incubation in fresh medium. Relative abundance of 8-oxo-dG in cellular DNA was measured as described previously. The decrease of 8-oxoG signal as a function of the chase time was taken as 8-oxoG removal from DNA in whole cells.

Statistical analysis

The Student's *t*-test was used for statistical analysis. Statistical significance was accepted if the null hypothesis was rejected with $P<0.05$.

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