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Reactive oxygen species are downstream mediators of p53-dependent apoptosis

(adenovirus/antioxidants/atherosclerosis/restenosis/smooth muscle cell)

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ABSTRACT **Reactive oxygen species (ROS) have been implicated as potential modulators of apoptosis. Conversely, experiments under hypoxic conditions have suggested that apoptosis could occur in the absence of ROS. We sought to determine whether a central modulator of apoptosis, p53, regulates the levels of intracellular ROS and whether a rise in ROS levels is required for the induction of p53-dependent apoptosis. We transiently overexpressed wild-type p53, using adenoviral gene transfer, and identified cell types that were sensitive or resistant to p53-mediated apoptosis. Cells sensitive to p53-mediated apoptosis produced ROS concomitantly with p53 overexpression, whereas cells resistant to p53 failed to produce ROS. In sensitive cells, both ROS production and apoptosis were inhibited by antioxidant treatment. These results suggest that p53 acts to regulate the intracellular redox state and induces apoptosis by a pathway that is dependent on ROS production.**

Deficiency of p53-mediated apoptosis is thought to play an important role in the transformed phenotype (1, 2). In addition to its role in neoplastic cells, apoptosis may also regulate proliferative disorders in normal cells. The role of apoptosis in the proliferative vascular disorders of restenosis and atherosclerosis is an area of increased interest. The smooth muscle cell (SMC) accumulation characteristic of these vascular diseases is thought to be the result of the net balance of proliferation and apoptosis (for review, see 3–5). Consistent with such a mechanism, significant levels of SMC apoptosis have been documented in both animal and human atherosclerotic and restenotic lesions (6–8). In addition, a recent study linking human cytomegalovirus infection and restenosis suggested that inhibition of p53 by viral proteins might block apoptosis in arterial SMC and thereby increase SMC accumulation and restenosis (9). Therefore, it has been postulated that deregulation of p53-mediated apoptosis in SMC may contribute to vascular diseases (3–5), suggesting that an understanding of the mechanism of apoptosis in SMC could lead to the development of new therapeutic approaches.

The downstream mediators of p53-dependent apoptosis are poorly characterized, but some evidence suggests a role for reactive oxygen species (ROS) (10, 11). Bcl-2, which blocks p53-mediated apoptosis, was initially thought to function as an intracellular antioxidant enzyme (12), but recently that hypothesis has been challenged (13, 14). To begin to resolve the role of ROS in different apoptotic pathways, we examined apoptosis mediated solely by p53. We used a recombinant adenoviral construct containing the human p53 cDNA under the control of the cytomegalovirus immediate early promoter (Adp53) (15, 16). This construct was employed to determine whether increased levels of p53 induces apoptosis in normal human (HSMC) or rat (RSMC) vascular SMC and, if so, whether ROS constitute a critical component of the downstream signaling pathway.

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MATERIALS AND METHODS

Adenoviral Infections and Detection of Protein Expression. Primary HSMC were obtained from Clonetics (San Diego). Primary RSMC were isolated from thoracic aorta as described (17). The adenovirus Adp53 contains the p53 human cDNA inserted into an Ad5 vector that is E1 deleted (15). Two different control adenoviruses were used; dl312 is an Ad5 vector that is E1 deleted, and Adβgal is an Ad5 vector that is E1 deleted and contains the *Escherichia coli LacZ* gene under the control of the cytomegalovirus promoter. Both viruses have been previously described (18, 19) and had indistinguishable effects on cells. Adenoviral stocks were prepared and titered on 293 cells (19). For immunoblotting, 30 μg of protein lysates was loaded in each lane, and filters were probed with antibodies to p21 (C-19; Santa Cruz Biotechnologies), p53 (clone DO-1; Santa Cruz Biotechnologies), and α-tubulin (clone DM1A; Oncogene Science). Antibody binding was visualized by enhanced chemiluminescence (Tropix, Bedford, MA).

Assays for Cell Death. Growth curves were obtained from cells trypsinized the day after infection and subsequently plated onto 24-well plates. Initial plating density was varied (HSMC, 8000 cells per well; RSMC, 1000 cells per well) to allow for the faster growth and higher confluent density of RSMC. On the indicated day, cells were trypsinized and counted. For DNA ladder analysis, 10 μg of genomic DNA was separated by electrophoresis through a 2% agarose gel containing ethidium bromide at 1 mg/ml. The terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay was performed on HSMC using an ApopTag kit (Oncor) according to the manufacturer's recommendations.

Detection of ROS. Cells were rinsed once and then incubated for 5 min with 3 ml of Hank's buffered saline containing 5 mg/ml 2′-7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes). ROS-induced fluorescence of intracellular DCF was visualized with a Leica Laser (Heidelberg) confocal scanning microscope. Fluorescence was measured on an arbitrary gray scale from 0–256 units as described (20). Levels of DCF fluorescence represent the mean ± SD of approximately 60 cells from two duplicate plates and assessed from six separate fields.

Abbreviations: ROS, reactive oxygen species; SMC, smooth muscle cell(s); HSMC, human SMC; RSMC, rat SMC; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; DCF-DA, 2′-7′-dichlorofluorescein diacetate; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetyl-L-cysteine; pfu, plaque-forming units.

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Antioxidant Treatments. HSMC were pretreated with the indicated doses of antioxidants for 4 days and replenished every 24 h. On day 5, to determine the extent of antioxidant treatment on cell growth, cells subjected to each treatment were counted. The values obtained were used to infect treated and untreated cells with identical viral doses of 200 plaque-forming units (pfu) per cell of either Adp53 or dl312. ROS levels and protein expression were determined 24 h after infection. Three to four days after infection, cells were scored for survival by the method of trypan blue exclusion, and data were expressed as a ratio of remaining viable Adp53 cells to dl312 infected cells. As previously shown, pyrrolidine dithiocarbamate (PDTC) (Sigma) treatment slowed the growth of SMC (21). Therefore, control cells receiving no PDTC were plated at a lower density on day 1 to achieve a density on day 5 similar to that of PDTC-treated cells (approximately 50,000 cells per well). Treatment with *N*-acetyl-L-cysteine (NAC; United States Biochemical) and human erythrocyte catalase (Calbiochem) had minimal effects on cell growth; higher doses showed an approximately 10% reduction in cell number after 5 days of treatment.

RESULTS

Differential Sensitivity of HSMC and RSMC to p53-Induced Cell Death. Infection of both HSMC and RSMC with Adp53 resulted in increased levels of p53 expression, as demonstrated by immunoblotting (Fig. 1*a*). To test for function of human p53 in both species, we performed immunoblotting for p21/waf-1/cip-1 following Adp53 infection, as p21 has been shown to be transcriptionally up-regulated by p53 (22). Increased levels of human p53 resulted in increased endogenous p21, demonstrating that p53 produced by the Adp53 vector was transcriptionally active in SMC from both species (Fig. 1*a*). Despite similar levels of p53 and similar increases in p21, there was a striking difference in the biological effect of Adp53 infection in the two cell types. The viability of HSMC markedly declined, with a 95% decrease in cell number at 8 days after infection (Fig. 1*b*). This suggested that p53 may induce apoptosis in normal HSMC. RSMC, however, were resistant to p53 overexpression, showing a slight inhibition of growth compared with that of control infected cells (Fig. 1*c*).

Induction of Apoptosis in Normal Cells by p53 Overexpression. To establish that Adp53 induced apoptosis in HSMC and that RSMC were resistant, we assayed for DNA ladder formation. DNA from Adp53-infected HSMC showed the approximately 200-bp multimers generated by the endonucleolytic digestion that occurs in apoptotic cells (Fig. 2*a*). Ladder formation was not evident in HSMC infected with a control adenovirus or in RSMC infected with Adp53.

To further confirm the ability of Adp53 to induce apoptosis in HSMC, cells were infected for 2 days with Adp53 or a control virus and stained by the TUNEL assay for DNA fragmentation. Positive, pyknotic nuclei were evident in the Adp53-infected cells, whereas no staining was detected in cells infected with control virus (Fig. 2 *b* and *c*). Additionally, cells were infected with varying amounts of pfu of virus per cell to establish whether p53 expression induced apoptosis in a dose-dependent manner. There was a clear increase in the percentage of TUNEL-positive cells after infection with increasing pfu of Adp53 (Fig. 2*d*). We also demonstrated an increase in TUNEL-positive cells with increased time after infection (Fig. 2*d*).

These results demonstrated that p53 overexpressed in non-transformed HSMC and RSMC was transcriptionally active and that such overexpression in HSMC, but not RSMC, caused apoptosis. Therefore, these results provided us with a model to study the mechanism of p53-mediated apoptosis.

Generation of ROS During p53-Mediated Apoptosis. There has been increasing evidence that ROS act as second messengers to mediate apoptosis and proliferation in response to a variety of stimuli (20, 23). Therefore, we tested whether p53-mediated apoptosis was associated with generation of ROS using the fluorophore DCF-DA. DCF-DA diffuses into cells, where it is deacetylated to DCF, which fluoresces upon reaction with a variety of ROS (24). HSMC and RSMC were infected with Adp53 or a control virus 24 h before the DCF-DA assay. Adp53-infected HSMC showed strong DCF fluorescence (Fig. 3*A*). Conversely, the p53-resistant RSMC showed no DCF fluorescence, indicating a lack of production of ROS (Fig. 3*C*). No DCF fluorescence was exhibited by either cell type when infected with the control virus (Fig. 3 *B* and *D*).

A time course was performed to assess the correlation between ROS production and p53 expression. In Adp53-infected HSMC, DCF fluorescence was evident at 4 h and increased through 12 and 24 h after infection (Fig. 4*b*). This

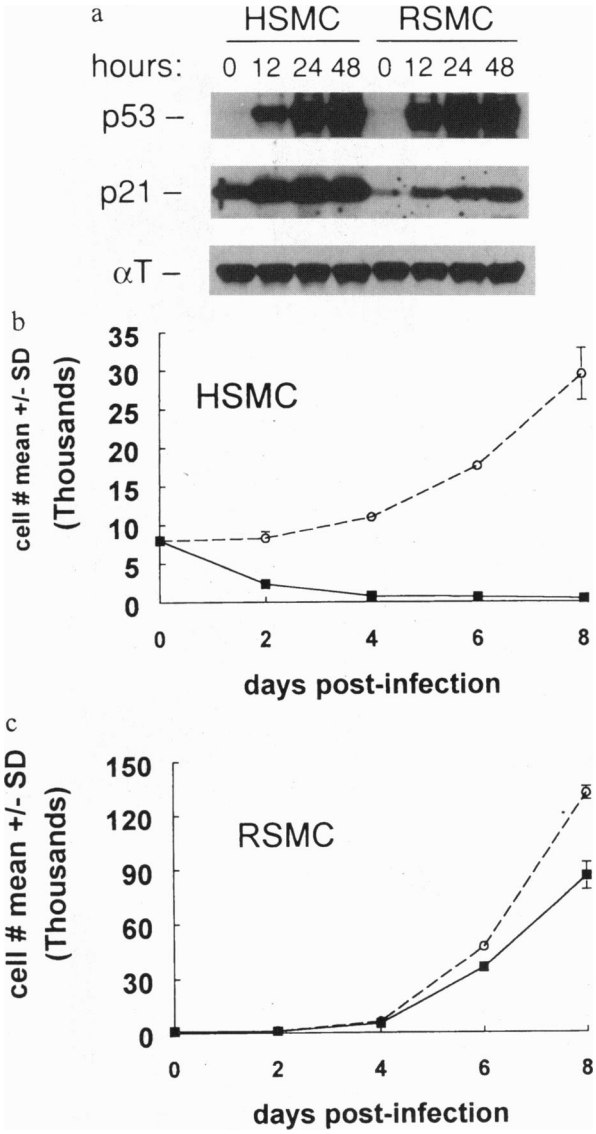

FIG. 1. Differential sensitivity of HSMC and RSMC to p53-induced cell death. (*a*) Immunoblot for levels of p53, p21, and α-tubulin (αT) (to confirm equal protein loading) in HSMC and RSMC infected with 200 pfu per cell of adenovirus expressing human p53 (Adp53) for the indicated timepoints (0–48 h). (*b*) Growth curve of HSMC infected with Adp53 (■) or a control virus dl312 (○) at 200 pfu per cell. Values are mean ± SD from triplicate cultures. (*c*) Growth curve of RSMC infected with Adp53 (■) or a control virus dl312 (○). Values are mean ± SD from triplicate cultures.

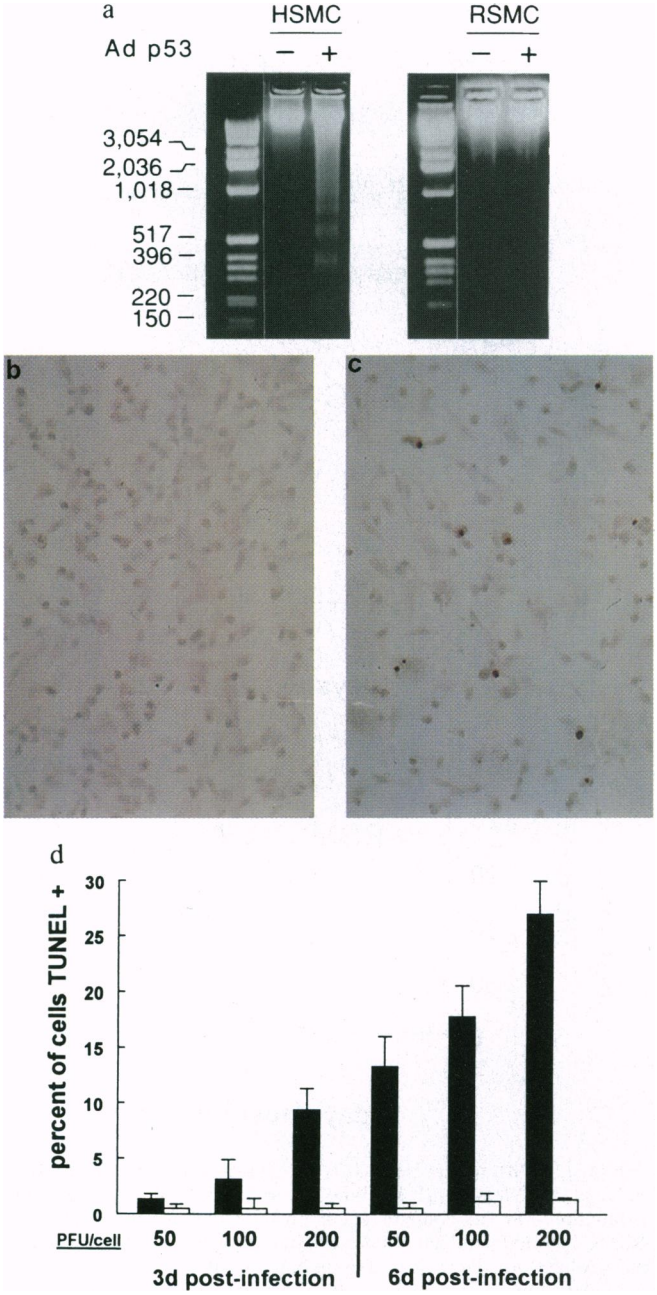

FIG. 2. Apoptosis in normal HSMC overexpressing p53. (*a*) Ethidium bromide-stained agarose gel containing genomic DNA isolated from HSMC and RSMC infected 3 days before harvest with Adp53 (+) or control virus (−) at 200 pfu per cell and demonstrating internucleosomal DNA fragments produced in HSMC infected with Adp53. (*b* and *c*) TUNEL assay of HSMC 2 days after infection with 200 pfu per cell of either a control virus dl312 (*b*) or Adp53 (*c*). (*d*) Quantitation of TUNEL assay performed on HSMC infected with Adp53 (solid bar) or a control virus Adβgal (open bar) at indicated pfu per cell. Each bar represents the percentage of adherent TUNEL-positive cells (mean ± SD) counted (n = 100) in three random high-power fields.

increase paralleled the increase in p53 protein levels, as demonstrated by immunoblotting (Fig. 4*a*). No DCF fluorescence was observed either in HSMC infected with control virus or in RSMC infected with Adp53 (Fig. 4 *a* and *b*). To confirm that ROS production correlated with p53 levels, we measured ROS production in response to differing steady-state levels of p53. Levels of ROS, as measured by intensity of DCF fluorescence, paralleled the incremental increases in p53 levels obtained after infection with 0, 10, and 200 pfu of virus per cell (Fig. 4*c*). Immunoblot for p21 levels confirmed the incremental increases in p53 transcriptional activity achieved by increasing viral pfu per cell (Fig. 4*c Inset*).

Inhibition of p53-Mediated Apoptosis by Antioxidant Treatment. To determine whether p53-induced ROS were required for apoptosis, HSMC infected with Adp53 were treated with two chemically distinct antioxidant compounds, PDTC and NAC (25). In addition, cells were also treated with the enzyme catalase, which rapidly degrades hydrogen peroxide to water and molecular oxygen, and has been shown to be actively taken up by vascular SMC (20). Each of the three antioxidant treatments resulted in increased survival of Adp53-infected HSMC in a concentration-dependent manner (Fig. 5*a*). PDTC exhibited the most potent effect with near complete inhibition of p53-mediated apoptosis at 200 μM (Fig. 5*a*). The inhibition of apoptosis by three chemically distinct antioxidants indicated that ROS, produced concomitantly with rising levels of p53, were required for the induction of apoptosis.

Because of the potent anti-apoptotic effect in Adp53-infected HSMC, we further investigated the effects of PDTC in this system. In parallel with the effect on cell survival, PDTC treatment resulted in decreased DCF fluorescence in Adp53-infected cells, indicating a reduction in ROS production in the presence of PDTC (Fig. 5*b*). A similar decrease in DCF fluorescence was obtained with NAC and catalase (data not shown). Antioxidant treatment had no effect on expression of p53 (Fig. 5*b Inset*), or on up-regulation of p21 (data not shown).

DISCUSSION

Although a number of studies have shown that ROS are required for apoptosis (1–3), others have shown that apoptosis could still be induced under hypoxic conditions, in which ROS generation is inhibited (4, 5). Whether apoptosis induced in each of these latter studies was p53-dependent was not determined. In this investigation, we determined whether overex-

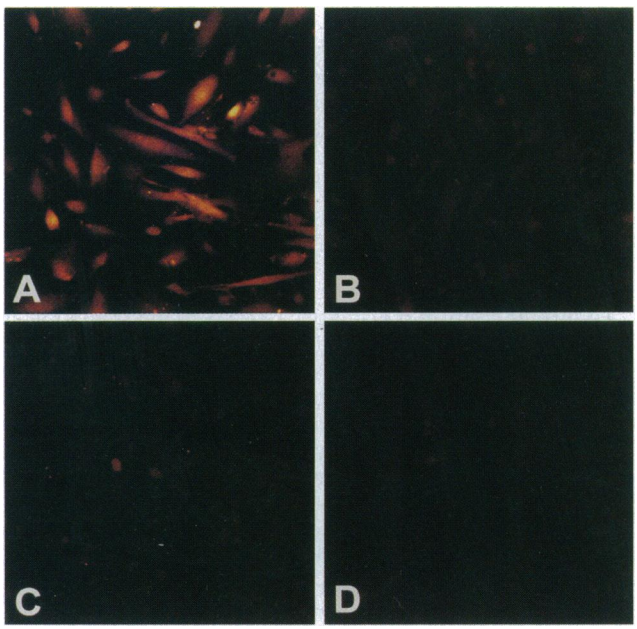

FIG. 3. Differential production of ROS in HSMC and RSMC in response to overexpression of p53. Cells were assayed for ROS production using the ROS-sensitive fluorophore DCF-DA. Assays were performed 24 h after infection with 200 pfu per cell of Adp53 or a control virus, dl312. (*A*) HSMC infected with Adp53. (*B*) HSMC infected with dl312. (*C*) RSMC infected with Adp53. (*D*) RSMC infected with dl312. Shown are representative fields from one of three independent experiments with similar results.

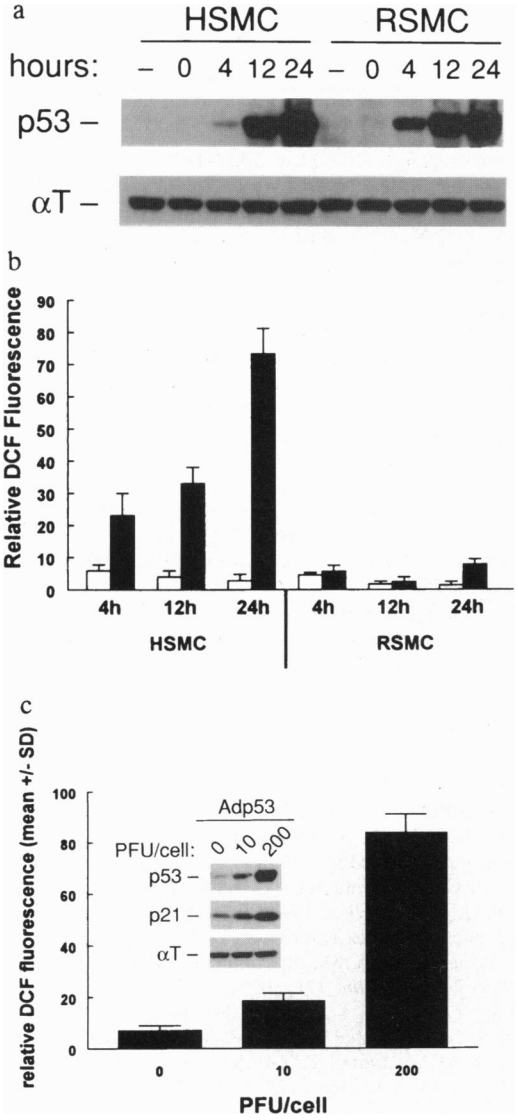

FIG. 4. ROS production correlates with p53 expression. (*a*) Immunoblot for p53 levels in HSMC and RSMC at indicated times after infection with Adp53: uninfected cells (0) and cells 24 h after infection with the control virus, dl312 (–). (*b*) ROS production in HSMC and RSMC at indicated times after infection: Adp53 (solid bars) or control virus dl312 (open bars). (*c*) ROS production correlates with varying levels of p53 expression. HSMC were infected with the indicated pfu per cell, and 12 h later, they were assayed for DCF fluorescence intensity. (*Inset*) Immunoblot for the indicated proteins in HSMC after infection 12 h earlier with Adp53.

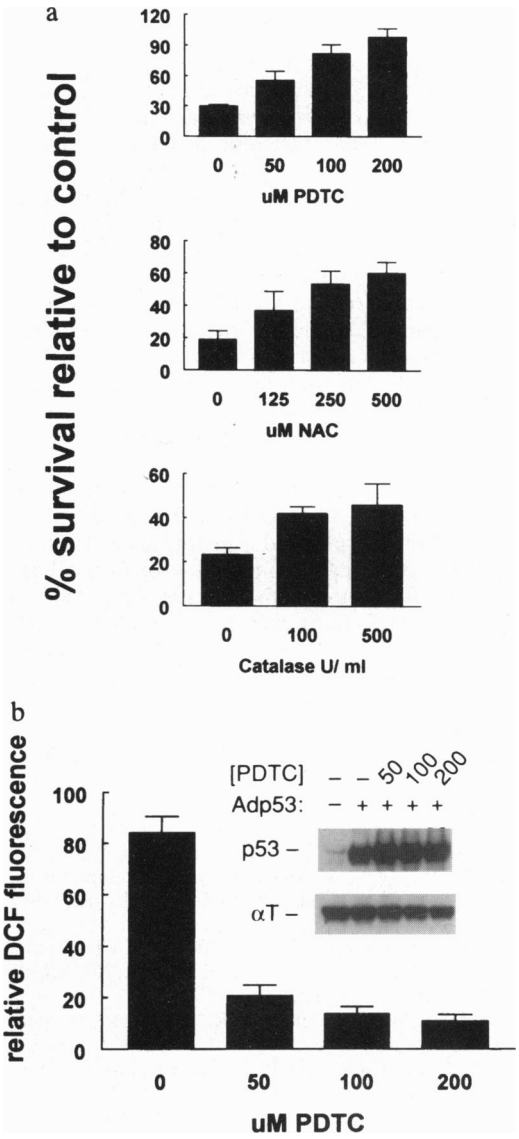

FIG. 5. Inhibition of p53-mediated apoptosis and ROS production by antioxidant treatment. (*a*) Inhibition of apoptosis by the antioxidants PDTC, NAC, and catalase in Adp53-infected HSMC. Values are mean ± SD from triplicate cultures. (*b*) PDTC treatment decreases ROS levels in Adp53-infected HSMC. (*Inset*) Immunoblot showing p53 levels in PDTC-treated cells.

pression of p53 was sufficient to induce apoptosis in normal vascular SMC. We then used this system to determine whether a central modulator of apoptosis, p53, regulates the levels of intracellular ROS and whether a rise in ROS levels is required for the induction of p53-dependent apoptosis. The results showed that ROS were generated concomitantly with increasing levels of p53 and the onset of apoptosis. Furthermore, the functional role of ROS production was supported by the ability of three unrelated antioxidants to rescue cells from p53-dependent apoptosis.

Of the antioxidants tested, PDTC appeared the most effective, followed by NAC and then catalase (Fig. 5*a*). The increased efficacy of PDTC over other antioxidants may relate to its previously demonstrated additional effects on superoxide dismutase activity, copper metabolism, or gene transcription (26–28). Nonetheless, the ability of three chemically different antioxidants to protect cells supports a role for ROS in p53-dependent apoptosis. In addition, the protective effect of the enzymatic peroxide scavenger catalase, suggests that hydrogen peroxide is directly involved. However, one interpretation of the increased efficacy of PDTC and NAC over catalase is that the generation of other ROS not metabolized by catalase, such as hydroxyl radicals, may also participate in the induction of apoptosis.

Our results provide the first link between two known mediators of apoptosis, p53 and ROS, and indicate the order in the pathway by which these mediators exert their effects—ROS downstream to p53. Consistent with this model, ROS production in response to p53 occurred as early as 4 h after infection, when increased p53 levels were first detected by immunoblotting. At that timepoint, cells showed no overt evidence of apoptotic death, indicating that p53-generated ROS may constitute a signal for apoptosis rather than being a consequence of the cellular changes associated with apoptosis.

Our results also suggest that overexpression of wild-type p53 is sufficient to induce apoptosis in normal cells. The proliferation of vascular SMC is thought to contribute to both

atherosclerosis and restenosis following balloon angioplasty. Recently, several laboratories have demonstrated that adenoviral-mediated gene transfer, using either a cytotoxic (19, 29, 30) or cytostatic (31, 32) gene product, can significantly reduce SMC accumulation following vascular injury. However, concern has been raised that such approaches, especially cytotoxic approaches, may provoke an inflammatory response. The ability to induce apoptosis in vascular SMC by adenoviral-mediated gene transfer of p53 may, therefore, provide an additional therapeutic approach in which SMC accumulation is reduced but inflammation is limited.

In addition to its role in vascular disease, the loss of p53 function is clearly a barrier to successful chemotherapeutic treatment of many tumor cells, and restoring p53 function to such resistant cells is of great potential therapeutic interest (1, 2). Although we have concentrated on the effects of Adp53 in normal SMC, we have observed similar increases in ROS levels after Adp53 infection of transformed cells harboring mutant p53, and after transient transfection of cells with a p53 expression plasmid. This consistent effect of p53 on ROS generation in both normal and transformed cells suggests that one function of p53 may be to regulate the intracellular redox state. Further elucidation of the specific mechanism by which p53 generates ROS to induce apoptosis might offer new approaches for the development of therapeutic agents capable of selectively inducing apoptosis in normal or neoplastic cells.

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