Antioxidant Action via p53-mediated Apoptosis

Ming Liu, Jill C. Pelling, Jingfang Ju, Edward Chu, and Douglas E. Brash

Departments of Therapeutic Radiology [M. L., D. E. B.] and Genetics [D. E. B.], Yale University School of Medicine, New Haven, Connecticut 06520; Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas 66160 [J. C. P.]; and Departments of Medicine and Pharmacology, Yale Cancer Center, Yale University School of Medicine and VA Connecticut Healthcare System, New Haven, Connecticut 06520 [J. E. B.]

ABSTRACT

The biological effects of antioxidants are often considered in terms of their effects on oxygen or lipid radicals. However, antioxidants can also exert their effects through altering the cellular redox potential. Herein, we report that sulfur-containing antioxidants such as N-acetylcysteine and dimercaptopropanol induced apoptosis in several transformed cell lines and transformed primary cultures but not in normal cells. In contrast, chain-breaking antioxidants such as vitamin E lacked this activity. An increased glutathione level was not required for apoptosis; however, all apoptosis-inducing antioxidants elevated the total cellular thiol levels. Antioxidant-induced apoptosis required the p53 tumor suppressor gene. N-Acetylcysteine elevated p53 expression posttranscriptionally by increasing the rate of p53 mRNA translation rather than by altering the protein stability. The p53 induction occurred in normal cultures but not in normal cells. These observations indicate a redox sensor for p53 induction in vivo, with additional transformation-specific information being required for apoptosis. Manipulating p53-dependent apoptosis with nontoxic antioxidants may have a direct clinical application.

INTRODUCTION

Antioxidants have a wide range of biochemical activities. These include inhibiting the generation of reactive oxygen species, directly or indirectly scavenging free radicals, and altering the intracellular redox potential (1). Some antioxidants have been used as inhibitors of apoptosis, because apoptosis was at first thought to be mediated by oxidative stress (2). However, it is clear that reactive oxygen species are not always required to induce apoptosis (3, 4). Furthermore, antioxidants have been shown to trigger apoptosis in smooth muscle cells independent of oxidative reactions (5).

Pro-oxidant states have been considered to be contributing factors for tumorigenesis (6). Correspondingly, an increasing body of evidence indicates that antioxidants have anticancer activities. Antioxidants can inhibit tumor initiation, tumor promotion, and cell transformation (7, 8). For example, the antioxidant NAC has antimutagenic and chemopreventive activities in a variety of organs, such as the lung, liver, skin, and colon (9–11). NAC inhibits the transformation of mouse embryo fibroblast; PDTC, pyrrolidinedithiocarbamate; C/EBPβ, CAAT/enhancer binding protein β.

We demonstrate that several sulfur-containing antioxidants selectively induce p53-dependent apoptosis in transformed cells. In contrast, antioxidants whose action is limited to scavenging radicals do not seem to have this activity.

MATERIALS AND METHODS

Cell Lines. Primary MEF cells (passage, <5) and E1A/Ha-ras-transformed MEFs were generously provided by Dr. S. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (12). The 308 papilloma cell line, which has a mutant Ha-ras(allele 24) and wild-type p53 (25), was the gift of Dr. S. Youspa (National Cancer Institute, Bethesda, MD). The (101) MEI cell line was the gift of Dr. A. Levine (Princeton University, Princeton, NJ) (16). BALB/c 3T3 A31 cells were obtained from the American Type Culture Collection (Bethesda, MD). The 308 papilloma cells were maintained in 0.05 mM Ca²⁺ Eagle’s minimal essential medium with 10% fetal bovine serum, and all other cells were grown in DMEM with 10% heat-inactivated fetal bovine serum. Cell culture clonality was maintained below 80%.

Chemicals and Cell Treatments. All chemicals were obtained from Sigma, except for Trolox, which was purchased from Aldrich. These compounds were freshly dissolved in medium and adjusted to a neutral pH, if necessary, or (for vitamin E acetate and BHA) first dissolved in ethanol and added to the medium. Cell viability was determined by trypsin blue exclusion as a measure of cell death independent of any growth suppression by p53.

Apoptosis Assays. For fixed cells, apoptosis-associated DNA strand breaks were visualized by fluorescent in situ end labeling as described previously (17). For isolated DNA, DNA fragmentation analysis was performed (18).

For flow cytometry analysis, approximately 10⁶ cells/sample were washed with ice-cold PBS and fixed in 95% ethanol. Cells were then resuspended in 1 mg/ml RNase (Sigma) for 30 min at 37°C and stained with 0.05 mg/ml propidium iodide (Sigma) for 1 h on ice. Flow cytometric analysis was performed with a fluorescence-activated cell-sorting Vantage flow cytometer (Becton Dickinson). Cells were excited at 488 nm, and the emission was detected through a 630/22-nm band pass filter. A minimum of 10,000 cells were analyzed for each sample. Cell cycle analysis was performed using ModFit 5.2 software (Verity Software House). Cells were considered to be in apoptosis if they exhibited sub-G₁ DNA fluorescence and a forward angle light scatter the same as or slightly lower than that of cells in G₁ (28). Cellular debris was gated out using the electronic threshold.

Northern and Western Blot Analysis. Northern and Western blot analyses were performed as described previously (19).

Analysis of p53 Protein Synthesis. Cultures of 308 cells were treated in the absence or presence of 20 mM NAC. At 4.5 h posttreatment, cells were incubated with methionine-free medium containing 2% dialyzed and chelated fetal bovine serum for 0.5 h. At 5 h posttreatment, biosynthetic labeling was initiated by adding 200 μCi of [³⁵S]methionine per milliliter of methionine-free medium. The labeling was terminated at 5, 10, or 15 min. Throughout the experiment, 20 mM NAC was included in the group of NAC-treated cells. Cells were then washed twice with 10 ml of ice-cold PBS, scraped, and pelleted by centrifugation at 1500 rpm for 4°C for 5 min. The supernatant was removed, and the cell pellet was lysed in ice-cold cell lysis buffer [0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride]. Aliquots of cell lysate containing equal therapy-induced apoptosis (16, 17). There is also a good correlation between a tumor’s p53 functional status and its response to some chemotherapeutic agents (18–20). In the past few years, p53 function has been reported to be redox-regulatable in vitro through its cysteine residues (21–23). In the present report, we extend the previous observations on the redox regulation of p53 protein to the cellular level. We demonstrate that several sulfur-containing antioxidants selectively induce p53-dependent apoptosis in transformed cells. In contrast, antioxidants whose action is limited to scavenging radicals do not seem to have this activity.
amounts of protein (30 μg) were subjected to immunoprecipitation analysis with anti-p53 antibody PAbl22 (25) and protein A-agarose (Life Technologies, Inc.). The immunoprecipitated proteins were resolved on a 10% SDS-PAGE gel. The levels of synthesized p53 protein were then determined by densitometric scanning using a Hewlett-Packard ScanJet 4P scanner and NIH Image 1.59 analysis software.

**Analysis of p53 Protein Half-Life.** Cultures of 308 cells were treated in the absence or presence of 20 mM NAC. After a 3.5-h incubation, cells were incubated with methionine-free medium containing 2% dialyzed and chelated fetal bovine serum for 0.5 h. The cells were then labeled by adding 100 μCi of [35S]methionine per milliliter of methionine-free medium for 1 h. At 5 h posttreatment, cells were washed with PBS and incubated with a chase medium containing a 2-fold excess of unlabeled methionine (45 μg/ml) and cysteine (72 μg/ml) for 0, 20, or 40 min. Throughout the experiment, 20 mM NAC was included in the group of NAC-treated cells. Aliquots of each sample lysate were subjected to immunoprecipitation analysis as described in the measurement of the p53 protein synthesis rate.

**Measurement of GSH and Total Thiols.** Cells (4 × 10⁶) were harvested from each sample. The GSH-400 kit (R&D Systems) was used following the manufacturer's instructions.

**RESULTS**

**p53-dependent Apoptosis by NAC.** Treatment of the murine papilloma 308 cell line with the chemopreventive agent NAC led to dose-dependent cell death (Fig. 1A). Death was apoptotic, with cells showing *in situ* end labeling of DNA strand breaks after 24 h of treatment with 20 mM NAC, but not at 6 h (data not shown), as well as morphological changes such as cell shrinkage and nuclear condensation (Fig. 1B). Morphological changes were minimal in cells treated with doses of NAC associated with high cell viability (data not shown).

In view of the fact that 308 cells contain a mutant Ha-ras allele and wild-type p53 (24, 25), we sought a matched pair of normal and transformed cells for comparison. We compared normal primary MEF cells to a matched line of MEF cells transformed by Ha-ras plus E1A (18). As shown in Fig. 2, A and B, the transformed fibroblasts (tMEF p53+/−) were sensitive to NAC-induced apoptosis, but their normal counterparts (MEF p53+/+) were strikingly resistant. In contrast, both transformed and normal primary cells from p53−/− null mice were deficient in apoptosis induced by NAC (Fig. 2A). A specificity of apoptosis toward transformed cells has been observed previously with chemotherapeutic agents and with hypoxia (18, 29). The specificity of apoptosis toward transformed cells was not due to the level of p53 induction alone, because p53 was induced in their normal counterparts (MEF p53+/+) without causing apoptosis (Fig. 2, A and C). An additional transformation-related signal is evidently also required for apoptosis. Apoptosis in response to transformation or other heritable abnormalities has been observed in other systems (18, 29—32).

**p53 Induction by NAC via Increased p53 Translation Rate.** We next investigated the molecular mediator of antioxidant-induced apoptosis. The tumor suppressor protein p53 is required for the induction of apoptosis in response to DNA-damaging agents such as γ or UV-irradiation (18, 27) and after hypoxia (29). As shown in Fig. 3A, treatment of 308 cells with NAC resulted in a dose-dependent 5–10-fold increase of p53 protein levels within 3–8 h. Northern blot analysis revealed no major difference in the steady-state level of p53 mRNA between control and NAC-treated cells (Fig. 3A), indicating...
Fig. 2. p53-dependent apoptosis by NAC in selective to transformed cells. A, viability of normal (MEF) and transformed (tMEF) cells measured by trypan blue exclusion after treatment with NAC for 24 h. □, MEF p53+/+; ■, MEF p53−/−; ○, tMEF p53+/+; △, tMEF p53−/−. B, analysis of DNA fragmentation from tMEF cells exposed to NAC. C, immunohistochemical staining of p53 protein in p53+/+ MEF cells after 5 h of exposure to 20 mM NAC.
that p53 induction was controlled at the posttranscriptional level. NAC also induced p53 in the murine fibroblast cell line BALB/c 3T3 A31 (data not shown).

Most p53-inducing agents damage DNA (15). Some of these agents increase p53 posttranscriptionally (33–35). In some cases, the induction has been shown to be due to increased p53 protein stability (34–37). NAC, in contrast, does not induce DNA damage (38–40). To determine the precise molecular mechanism(s) for the induction of p53 in response to NAC treatment (Fig. 3A), we directly measured both the biosynthetic rate of p53 protein and the p53 protein half-life in 308 cells. As shown in Fig. 3, B and C, the biosynthetic rate of p53 protein was elevated by nearly 5-fold after NAC treatment. In contrast, the half-life of p53 protein was not altered in the presence of NAC. These results indicate that enhanced translation of p53 mRNA, and not increased protein stability, accounts for the induction of p53 protein after NAC exposure.

Apoptosis by Other Sulfur-containing Antioxidants. Because NAC is well known to ameliorate oxidative stress (12, 41), we investigated the capacity of other antioxidants (42–44) for transformation-specific apoptosis. As demonstrated in Fig. 4, A–C, the sulfur-containing reducing agents DMP and OTC also selectively induced apoptosis in E1A/Ha-ras-transformed cells, but not in their normal counterparts. Lipic acid behaved similarly (data not shown). DMP was active at doses as low as 50 μM. DMP, OTC, and lipic acid also required p53 (Fig. 4A; data not shown). These agents, as well as NAC, all induced apoptosis in the human p53++ colorectal carcinoma cell line RKO (data not shown).

In contrast, the non-sulfur-containing antioxidants vitamin E acetate (tocopherol acetate), BHA, and the water-soluble analogue of vitamin E, Trolox (4), had little effect on the cell viability of p53++ tMEFs for at least 48 h (Fig. 5). The chosen antioxidant concentrations here were basically the highest soluble or nontoxic doses to p53++ tMEFs. DNA analysis also confirmed that no apoptosis occurred in p53++ tMEFs cells treated with these chain-breaking antioxidants (data not shown). Thus, the significant feature of these sulfur-containing compounds seems to be their effect on the intracel-
DISCUSSION

Previous mechanisms proposed for the beneficial effects of antioxidants have involved the prevention of oxidative damage to cellular DNA or membrane lipids. Indeed, antioxidants do block apoptosis and cell cycle arrest when these are mediated by reactive oxygen species or oxidative stress (47, 48). The present study identifies a novel cellular mechanism for NAC and other sulfur-containing antioxidants that involves the induction of p53-mediated apoptosis selectively in transformed cells. The two mechanisms seem to use different properties of antioxidant compounds, radical scavenging activity and alteration of intracellular redox potential.

In this study, we demonstrated that BSO cannot block NAC-induced cell death in tMEF p53−/− cells. Antioxidants were dissolved in ethanol and then added to the medium. The final concentration of ethanol in the medium is 1:2000 (v/v). At 48 h, cell viability was measured by trypan blue exclusion. All data represent two independent experiments. 0, ethanol; I, Trolox; 2, BHA; 3, vitamin E acetate.

Fig. 6. Analysis of GSH, total thiols, and viability in tMEF p53−/− cells treated with BSO and/or NAC. Cells treated with both compounds were preincubated with medium containing 20 mM BSO for 1 h and then treated with medium containing 20 mM BSO and 20 mM NAC for 5 h (GSH and thiols) or for 24 h (viability).

GSH Independence of NAC-Induced Apoptosis. A major intracellular pathway of NAC metabolism is deacetylation to the thiol cysteine, the limiting amino acid precursor for synthesis of GSH (45). GSH, in turn, is the major cellular antioxidant (46). To test the possibility that NAC acts by increasing the level of GSH, we pre-treated and co-incubated cells with BSO; this agent inhibits all GSH synthesis by inactivating γ-glutamylcysteine synthetase (46). As expected, Fig. 6 shows that BSO completely blocked the induction of cellular GSH by NAC, whereas it only partially blocked the induction of total thiols. However, BSO did not block NAC-induced apoptosis (Fig. 6), implying that NAC exerts its redox effect by a means other than by increasing GSH.

lular redox potential rather than their effect on radical species. In fact, all of the apoptosis-inducing agents tested above elevate the cellular thiol level (Fig. 6; data not shown).

GSH-Independence of NAC-Induced Apoptosis. A major intracellular pathway of NAC metabolism is deacetylation to the thiol cysteine, the limiting amino acid precursor for synthesis of GSH (45). GSH, in turn, is the major cellular antioxidant (46). To test the possibility that NAC acts by increasing the level of GSH, we pre-treated and co-incubated cells with BSO; this agent inhibits all GSH synthesis by inactivating γ-glutamylcysteine synthetase (46). As expected, Fig. 6 shows that BSO completely blocked the induction of cellular GSH by NAC, whereas it only partially blocked the induction of total thiols. However, BSO did not block NAC-induced apoptosis (Fig. 6), implying that NAC exerts its redox effect by a means other than by increasing GSH.

Fig. 5. Sulfur-free antioxidants tocopherol acetate (200 μM), Trolox (200 μM), and BHA (100 μM) do not induce cell death in tMEF p53−/− cells. Antioxidants were dissolved in ethanol and then added to the medium. The final concentration of ethanol in the medium is 1:2000 (v/v). At 48 h, cell viability was measured by trypan blue exclusion. All data represent two independent experiments. 0, ethanol; I, Trolox; 2, BHA; 3, vitamin E acetate.

Fig. 4. p53-dependent apoptosis induced by 50 μM DMP and 20 μM OTC in tMEFs. A, cell viability. B, DNA fragmentation analysis at 24 h post-treatment. C, flow cytometry analysis at 48 h post-treatment. Box R1 represents viable cells. Box R2 shows apoptotic cells, defined as having sub-G1 DNA fluorescence (Y axis) and a forward angle light scatter (X axis) ≤ to that of cells in G1. All data represent two to three independent experiments.
apoptosis, although it inhibits cellular GSH elevation by NAC (Fig. 4). This finding indicates that the present apoptosis differs from the BSO-sensitive biphasic toxicity (Fenton reaction) of some antioxidants other than NAC (49). Furthermore, we found that penicillamine (50 μM), a potent chelator of copper, had no effect on NAC-induced apoptosis (data not shown). Similarly, it has been reported that apoptosis in smooth muscle cells by the antioxidant PDTC was not attributable to the Fenton reaction through autoxidation of PDTC (5).

The induction of p53 is often associated with the cellular response to DNA-damaging agents (14), and the trigger is believed to result from strand breaks in nuclear DNA (50). However, we do not expect that strand breaks are the mechanism of antioxidant action in the present case. The 5–10-fold induction of p53 observed after exposure to NAC exceeds that produced by 5–10 Gy of X-ray (33, 51, 52). Alkaline elution and nucleoid sedimentation routinely detect DNA strand breaks produced by doses 10-fold smaller (53, 54). However, alkaline elution studies have indicated no DNA-damaging activity of NAC (38–40), nor are the requisite numbers of strand breaks seen by nucleoid gradients (55). On the contrary, NAC blocks DNA strand breaks and mutagenesis by DNA-damaging agents (12, 38, 39). Our results also show that NAC induces the expression of p53 protein through enhanced p53 mRNA translational efficiency. The increased protein stability that has been previously described after DNA strand breaks (34–37) is apparently not the mechanism here. In 308 cells, for example, UVB irradiation increases the p53 protein half-life by 7-fold (35).

Three mechanisms for translational regulation of p53 expression have been reported. In mouse cells, p53 expression seems to be autoregulated by a negative feedback loop involving p53 protein, the 5′ untranslated region, and some 280 nucleotides of the coding region of p53 mRNA (56). In contrast, it has been suggested that human p53 mRNA translation is regulated by a RNA binding factor(s) that acts on the 3′ untranslated region of p53 mRNA (57). Lastly, p53 mRNA translation can be regulated by thymidylate synthase. This protein has been demonstrated to bind to p53 mRNA, thereby decreasing p53 protein synthesis in vitro (58). Thymidylate synthase has recently been shown to regulate the p53 protein level in vivo, as well.4 The activity of thymidylate synthase, in turn, is redox regulatable (58).

The agents that induced p53 and apoptosis in this study increased cellular thiol levels, directly or indirectly. Thus, p53 induction may be an important biological effect of increasing thiols or increasing the reducing potential. p53 function has itself been reported to be redox regulatable through its cysteine residues (21–23). Antioxidants may therefore also induce p53 DNA binding activity. p53 transactivation activity can be activated by redox enzyme Ref-1 (59). Redox regulation of other transcription factors, such as AP-1 and nuclear factor κB, by NAC and other thiols is well established (reviewed in Ref. 60).

Sulfur-containing antioxidants might find cancer-related clinical uses. p53-mediated apoptosis is an important determinant of a tumor’s response to chemotherapy (18, 19) and is a critical natural regulator of tumorigenesis (30, 31). Hypoxia, which creates a milieu similar to that of antioxidants, induces p53-dependent apoptosis in tumors in vivo (29). Sulfur-containing antioxidants, including those studied here, are already in clinical use for other purposes. NAC is used for acetaminophen poisoning and chronic bronchitis at a whole-body distribution of approximately 1.5 mm (61). DMP, which was active here at 50 μM, is used clinically for metal poisoning at a dose corresponding to a whole-body distribution of approximately 60 μM (62). Therefore, antioxidants may provide a practical nongenotoxic route to cancer prevention or therapy of the 50% of human cancers that retain wild-type p53. Even for p53−/− tumors, use of antioxidants may eliminate normal cells mutated during chemotherapy.

While this paper was under review, the antioxidants PDTC and a water-soluble vitamin E analogue, Trolox, were reported to induce apoptosis by inducing p21 via C/EBPβ (particularly in the presence of chemotherapeutic drugs; Ref. 63). This process differs from the present mechanism in two respects: (a) It seems to be p53 independent, because apoptosis was not preceded by an increase in p53 protein. Although p53 independence cannot be determined from the p53−/− HCT-15 cell line used (20, 63), our own unpublished results indicate that PDTC is equally toxic in p53−/− and p53+/− transformed MEFs. (b) This non-p53 pathway is activated by a different set of antioxidants. Chain-breaking antioxidants such as vitamin E acetate and Trolox did not induce p53-dependent apoptosis of transformed MEFs (Fig. 5), although we have not investigated the high Trolox concentrations used in Ref. 63. Whereas the p53-dependent mechanism seems to reflect changes in the redox potential only, the non-p53 apoptosis mechanism seems to involve radical species (63, 64). A possible pathway relating the two mechanisms is shown in Fig. 7.

ACKNOWLEDGMENTS

We are very grateful to Drs. Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and A. Levine (Princeton University, Princeton, NJ) for providing cell lines and thank A. Jonason for technical assistance.

---

4 Jingfang Ju and Edward Chu, unpublished data.
REFERENCES


Antioxidant Action via p53-mediated Apoptosis

Ming Liu, Jill C. Pelling, Jingfang Ju, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/58/8/1723

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/58/8/1723. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.