Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9166–9171, August 1996
Medical Sciences

Cellular oxidative stress and the control of apoptosis by wild-type p53, cytotoxic compounds, and cytokines

JOSEPH LOTEM, MIRA PELED-KAMAR, YORAM GRONER, AND LEO SACHS

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Contributed by Leo Sachs, May 28, 1996

ABSTRACT **Apoptosis induced by wild-type p53 or cytotoxic compounds in myeloid leukemic cells can be inhibited by the cytokines interleukin 6, interleukin 3, granulocyte–macrophage colony-stimulating factor, and interferon γ and by antioxidants. The antioxidants and cytokines showed a cooperative protective effect against induction of apoptosis. Cells with a higher intrinsic level of peroxide production showed a higher sensitivity to induction of apoptosis and required a higher cytokine concentration to inhibit apoptosis. Decreasing the intrinsic oxidative stress in cells by antioxidants thus inhibited apoptosis, whereas increasing this intrinsic stress by adding H_2O_2 enhanced apoptosis. Induction of apoptosis by wild-type p53 was not preceded by increased peroxide production or lipid peroxidation and the protective effect of cytokines was not associated with a decrease in these properties. The results indicate that the intrinsic degree of oxidative stress can regulate cell susceptibility to wild-type p53-dependent and p53-independent induction of apoptosis and the ability of cytokines to protect cells against apoptosis.**

The tumor suppressor gene wild-type p53 is a mediator of apoptotic cell death in malignant and normal cells of different lineages (1–7), but there are also p53-independent pathways for apoptosis (3–9). Induction of apoptosis by different agents mediated by p53-dependent or p53-independent pathways can be inhibited by certain viability inducing cytokines including interleukin 6 (IL-6), granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), and interferon γ (IFN-γ) (1, 6–11). One of the factors that can influence cell susceptibility to induction of apoptosis is the extent of intracellular oxidative stress (for reviews, see refs. 12–14), and antioxidants can reduce the extent of apoptosis induced by a variety of cytotoxic agents in different cell types (for reviews, see refs. 13 and 14). We have now examined the involvement of oxidative mechanisms in the regulation of apoptosis by wild-type p53-dependent and p53-independent pathways in myeloid leukemic cells with a high or a low degree of oxidative stress. It is shown that reducing oxidative stress by the antioxidant butylated hydroxyanisole (BHA) and other antioxidative compounds inhibited induction of apoptosis by wild-type p53 and by different cytotoxic compounds, and increased the antiapoptotic effect of IL-6, IL-3, GM-CSF, and IFN-γ. The results link oxygen free-radicals and p53-mediated apoptosis, and indicate that the intrinsic degree of oxidative stress in the cells can regulate the ability of wild-type p53 and cytotoxic compounds to induce apoptosis and the ability of cytokines to protect cells from apoptosis.

MATERIALS AND METHODS

Cells and Cell Culture. The cells used were M1 myeloid leukemic cells that do not express p53 transfected with plasmids containing either the neomycin-resistance gene (M1-*neo*) or both the *neo* gene and a temperature-sensitive mutant p53 gene (M1-t-p53) (1, 11). The 7-M12 myeloid leukemic cells (15) that do not express *bcl*-2 (16) were also used. The temperature-sensitive p53 codes for a protein [Val^{135}] that behaves like a tumor-suppressing wild-type p53 at 32°C and like a mutant p53 protein at 37°C (17). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% heat-inactivated (56°C, 30 min) horse serum (GIBCO) at 37°C, unless otherwise stated, in a 10% CO_2 atmosphere and multiplied in suspension as myeloblasts to promyelocytes.

Assays for Apoptosis and Cell Viability. The percent of apoptotic cells was determined on May–Grünwald Giemsa-stained cytospin preparations by counting 400 cells. Apoptotic cells were scored by their smaller size, condensed chromatin, and fragmented nuclei compared with nonapoptotic cells (see Fig. 1) (1, 3, 11, 18). Nonapoptotic and apoptotic cells exclude trypan blue, but the apoptotic cells then undergo additional changes, including staining with trypan blue and loss of internal morphology (18). The number of viable cells was determined by counting the trypan blue-excluding cells in a hemocytometer after adding 0.5% trypan blue, and subtracting the number of apoptotic cells that was calculated from the percent apoptotic cells determined in the cytospin preparations. The percent of cell viability was calculated from the ratio of the number of viable cells (trypan blue-excluding and nonapoptotic cells) divided by the total number of cells (including trypan blue-stained cells). The compounds used to determine their effect on induction of apoptosis were the antioxidants BHA, cimetidine (CIM), *N*-*t*-butyl-α-phenylnitrone (BPN), and *N*-acetylcystein (NAC) (Sigma), the apoptosis-inducing compounds cycloheximide and doxorubicin (Sigma), and the recombinant mouse cytokines IL-6 (from J. Van Snick, Ludwig Institute for Cancer Research, Brussels), IL-3 (PeproTech, Rocky Hill, NJ), GM-CSF (Immunex), and IFN-γ (Genzyme).

Detection of p53 and Bcl-2 Proteins by Western Blotting. Whole-cell extracts from 10×10^6 M1-neo or 7-M12 cells were prepared in ice-cold RIPA buffer (150 mM NaCl/0.1 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/5 mM EDTA) containing a mixture of protease inhibitors, and the protein concentration was determined by the Bradford assay (19). For Western blotting, 40 μg (for Bcl-2) or 100 μg (for p53) protein was electrophoresed in an SDS/12% polyacrylamide gel and electroblotted to a nitrocellulose filter. The blots were blocked for 1 hr at room temperature in TBS blocking buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl) containing 0.2% Tween 20 and 5% low fat milk and were incubated for 1 hr at room temperature with a 1:2000 dilution of polyclonal hamster anti-mouse Bcl-2 antibody (3F11) (PharMingen) or a 1:100 dilution of monoclonal anti-p53 antibody (Pab 240) (Santa Cruz Biotechnology) in

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BHA, butylated hydroxyanisole; BPN, *N*-*t*-butyl-α-phenylnitrone; CIM, cimetidine; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon γ; IL-3 and IL-6, interleukins 3 and 6, respectively; NAC, *N*-acetylcystein; Cu/Zn SOD; Cu/Zn superoxide dismutase.

blocking buffer. Blots were washed five times (10 min each wash) in blocking buffer and incubated for 1 hr with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-hamster IgG (Kirkegaard & Perry Laboratories) (for detecting Bcl-2) or horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) (for detecting p53). The blots were washed five more times in TBS containing 0.2% Tween 20 and developed with the Enhanced Chemiluminesence detection kit (Amersham). Bands of Bcl-2 and p53 were visualized after exposing of the blots to Fuji RX Medical x-ray film.

Fluorescent Measurement of Intracellular Peroxides and Lipid Peroxidation. A flow cytometric method was used to detect intracellular peroxide production in the myeloid leukemic cells as described (20). Cells were washed twice with phosphate-buffered saline (PBS) and resuspended at 1.5×10^6 cells per ml in 4 ml phenol red-free Hepes-buffered DMEM and incubated with 5 μM 2′,7′-dichlorofluorescin diacetate (DCFDA) (Molecular Probes), a nonfluorescent compound that freely permeates cells. When inside cells, DCFDA is hydrolyzed to 2′,7′-dichlorofluorescin (DCFH) and trapped. When DCFH interacts with peroxides, it gives rise to the fluorescent compound 2′-7′-dichlorofluorescin (DCF), which can be detected by fluorescence-activated cell sorter (FACS) analysis (21, 22). Fluorescent DCF was analyzed in 5000 cells using a Becton Dickinson FACStar^{PLUS} with excitation and emission settings of 500 ± 15 nm and 535 ± 15 nm, respectively. Mean fluorescent peak height was determined during 30–45 min incubation at 37°C or 32°C in different experiments. Lipid peroxidation was determined as described (20) using the fluorescent polyunsaturated fatty acid probe *cis*-parinaric acid (23, 24). This probe is incorporated readily into cell membranes and its fluorescence intensity decreases upon lipid peroxidation. Cells were suspended in PBS at 1×10^6 cells per ml and 5 μM *cis*-parinaric acid (Molecular Probes) was added. Fluorescence was determined during 60-min incubation at 37°C or 32°C using a Perkin–Elmer LS-5B luminescence spectrometer with excitation and emission settings of 307 and 418 nm, respectively (slit width, 5 nm).

RESULTS

Protection Against Wild-Type p53-Induced Apoptosis in M1 Myeloid Leukemic Cells by Antioxidants. The tumor suppressor gene wild-type p53 can induce apoptosis in M1 myeloid leukemic cells (1). In leukemic cells transfected with the Val 135 temperature-sensitive p53 (M1-t-p53) gene, the cells undergo apoptosis (Fig. 1*a*) at the permissive temperature (32°C) (1, 11). At this temperature, the p53 protein has the conformation and activity of wild-type p53 (17). This induction of apoptosis can be inhibited to different extents by IL-6 (1) and IFN-γ (11). Neither of these cytokines down-regulated expression of the p53 transgene nor did they inhibit activation of the wild-type p53 inducible cyclin-dependent kinase inhibitor p21/WAF1 (11).

To examine the possible involvement of oxidative mechanisms in induction of apoptosis by wild-type p53 and in the suppression of apoptosis by the cytokines, we first used the antioxidant BHA. Addition of 0.1 mM BHA to M1-t-p53 cells at the time of transfer to 32°C resulted in a 3-fold reduction in the percent of apoptotic cells recorded 23 hr later (Fig. 1*b*). Similar results were obtained with 0.2 mM BHA. At 0.05 mM, BHA was still effective and decreased the percent of apoptotic cells from 28 ± 1% to 18 ± 3% but at 0.02 mM BHA there was no detectable inhibition of apoptosis. All further experiments were therefore carried out with 0.1 mM BHA. This inhibitory effect of 0.1 mM BHA on induction of apoptosis was similar in its extent to that obtained with the optimum concentration of IFN-γ but weaker than with the optimum concentration of IL-6 (Fig. 1*b*). Apoptotic cells initially still exclude trypan blue like nonapoptotic cells, but this property is lost within a few hours. The cells then undergo other changes, stain with trypan blue, and lose internal morphological features (for review, see ref. 18). At 23 hr after transfer to 32°C, the cultures of M1-t-p53 cells contained cells with these other changes in addition to the apoptotic cells and cells that had not yet become apoptotic (Fig. 1*a*). Data on the percent of viable cells that were trypan blue-excluding and nonapoptotic showed that BHA was somewhat less effective than IFN-γ and much less effective than IL-6 at all time points after culturing M1-t-p53 cells at 32°C (Fig. 2*a*). After 40 hr at 32°C (Fig. 2*a*), the percent of viable cells was only 3 ± 1% without any additions compared with 10 ± 3%, 15 ± 3%, and 72 ± 4% with BHA, IFN-γ, or IL-6, respectively.

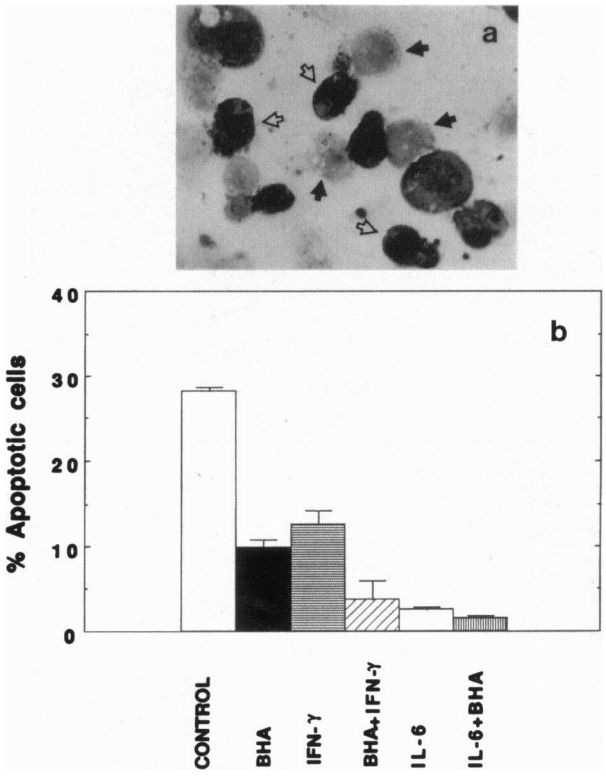

FIG. 1. Induction of apoptosis in M1-t-p53 cells by activation of wild-type p53 at 32°C. Cells were cultured at 3×10^5 per ml at 32°C for 23 hr and then stained with May–Grünwald Giemsa for determination of apoptosis. (*a*) Apoptotic cells, three of which are indicated by open arrowheads, are smaller in size and have condensed and fragmented chromatin compared with nonapoptotic cells. Three cells that have lost internal morphological features (black arrowheads) and that stain with trypan blue are also featured. (*b*) Cells were cultured at 32°C without (control) or with 0.1 mM BHA, 1 ng/mlIFN-γ, 5 ng/ml IL-6, or a combination of BHA and IFN-γ or BHA and IL-6. The percent of apoptotic cells was determined 23 hr after culturing at 32°C.

We determined whether BHA and IFN-γ or IL-6 can cooperate to inhibit apoptosis and enhance cell viability in cultures of M1-t-p53 cells at 32°C. The addition of BHA together with IFN-γ was more effective in reducing the percent of apoptotic cells at 23 hr compared with each compound alone (Fig. 1*b*) and increased the total percent of viable cells at 40 hr (Fig. 2*a*). At 40 hr after transfer to 32°C, the percent of viable cells was increased to 45 ± 8% in the presence of BHA and IFN-γ compared with only 10–15% with each compound alone (Fig. 2*a*). The cooperative protective effect of BHA on cell viability ranged over three logs of IFN-γ concentrations, and was found even at the optimum concentration (10 ng/ml) of IFN-γ (Fig. 2*b*). This indicates that the antioxidant BHA can increase the effectiveness of IFN-γ. The protective effect of BHA together with IFN-γ was still weaker than the protection found with an optimal amount of IL-6 alone (Fig. 2). However,

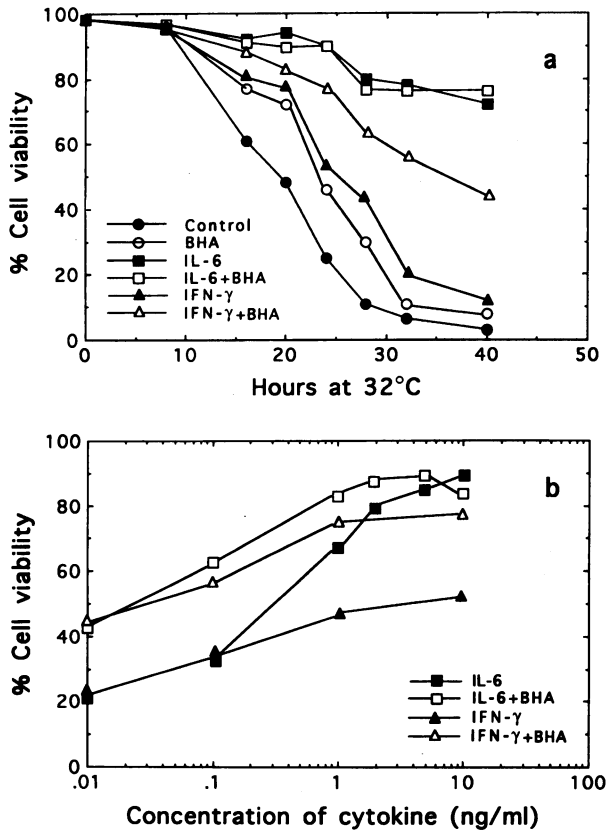

FIG. 2. The protective effect of BHA, IL-6, and IFN-γ on cell viability of M1-t-p53 cells at 32°C. (*a*) Time course of percent cell viability. Cells were cultured at 3×10^5 per ml at 32°C in the absence (control) or presence of 0.1 mM BHA, 5 ng/ml IL-6, 1 ng/ml IFN-γ, or their combinations and the percent of viable cells (trypan blue-excluding and nonapoptotic) was determined. (*b*) Concentrations of cytokines required for protection against loss of cell viability and for cooperation with 0.1 mM BHA after 23 hr at 32°C.

experiments at suboptimal concentrations of IL-6 showed that BHA also increased the effectiveness of IL-6 (Fig. 2*b*).

Several other compounds that inhibit different oxidative reactions were tested for their ability to protect M1 myeloid leukemic cells against wild-type p53-induced apoptosis. Although less protective than BHA, the antioxidants CIM, BPN, and NAC also showed a protective effect against wild-type p53-induced apoptosis after 23 hr at 32°C and increased the protective effect of IFN-γ (Table 1). Increasing oxidative stress by adding 50 μM H_2O_2 reduced cell viability from 64 ± 4% to 21 ± 6% in cells cultured at 32°C for 16 hr. When cultured at 37°C for 16 hr, cell viability was 93 ± 5% without and 88 ± 3% with this concentration of H_2O_2. The extent of wild-type p53-induced apoptosis can thus be modulated by decreasing or increasing the extent of oxidative stress.

Table 1. Protective effect of antioxidants and IFN-γ against wild-type p53-mediated apoptosis

Compound added	% cell viability at 23 hr*	
	− IFNγ	+ IFNγ
None	24.7 ± 7.2	48.7 ± 7.3
BHA	42.1 ± 13.7†	70.3 ± 6.9‡
CIM	34.1 ± 4.3†	60.2 ± 6.5§
BPN	34.1 ± 2.5†	58.6 ± 4.5¶
NAC	33.8 ± 4.5†	57 ± 3.6†

M1-t-p53 cells were cultured at 32°C for 23 hr either with no additions (None) or with 0.1 mM BHA, 2 mM CIM, 1 mM BPN, or 10 mM NAC without or with 1 ng/ml IFN-γ.

*Seven experiments were carried out ($n = 7$) and the P values (Student's t test) given are for the statistical significance of the difference between each of the added compounds and the control (None) group. †, $P < 0.02$; ‡, $P < 0.001$; §, $P < 0.01$; ¶, $P = 0.01$.

Protection by Antioxidants Against Induction of Apoptosis in 7-M12 Myeloid Leukemic Cells by γ Irradiation, Doxorubicin, and Cycloheximide. Clone 7-M12 myeloid leukemic cells do not express *bcl*-2 mRNA (16) or Bcl-2 protein (Fig. 3*a*). These leukemic cells are more sensitive to induction of apoptosis by γ-irradiation and different cytotoxic agents including doxorubicin and cycloheximide compared with M1 myeloid leukemic cells (16), which express high levels of *bcl*-2 mRNA (16) and protein (Fig. 3*a*). As with M1 leukemic cells (1), p53 protein was not detected in untreated 7-M12 cells (Fig. 3*b*). However, in contrast to M1 cells, treatment of 7-M12 cells with γ-irradiation, doxorubicin, or cycloheximide resulted in a rapid accumulation of p53 protein (Fig. 3*b*), which preceded the appearance of morphologically detectable apoptotic cells. This accumulation of p53 protein suggests that apoptosis induced in 7-M12 cells by these agents may be wild-type p53-dependent. Induction of apoptosis in 7-M12 cells was inhibited by cytokines such as GM-CSF, IL-3, and IFN-γ (7–11), which did not decrease the accumulation of p53 protein (Fig. 3*b*).

Induction of apoptosis in 7-M12 myeloid leukemic cells is more rapid than in M1 cells and can be scored in a few hours. Apoptosis induced in 7-M12 cells by cycloheximide was effectively inhibited by BHA, CIM, and BPN, but not by NAC (Fig. 4). After 5 hr in culture, the addition of 1 μg/ml doxorubicin or 300R γ-irradiation (a Co^{60} source, 63R per min at a source-sample distance of 120 cm) led to 35 ± 3% and 32 ± 5% apoptotic cells, respectively, and BHA, BPN, or CIM reduced this percent apoptotic cells 2- to 3-fold. The protective effect of BHA against γ-irradiation-induced apoptosis, as in the case of the cytokines, was not associated with reduced accumulation of p53 protein (Fig. 3*b*). The combined treatment with IFN-γ, GM-CSF, or IL-3 together with BHA showed a better protection from apoptosis induced by cycloheximide than treatment with each compound alone (Fig. 4). The results with the antioxidants suggest that induction of apoptosis in 7-M12 leukemic cells by γ-irradiation, doxorubi-

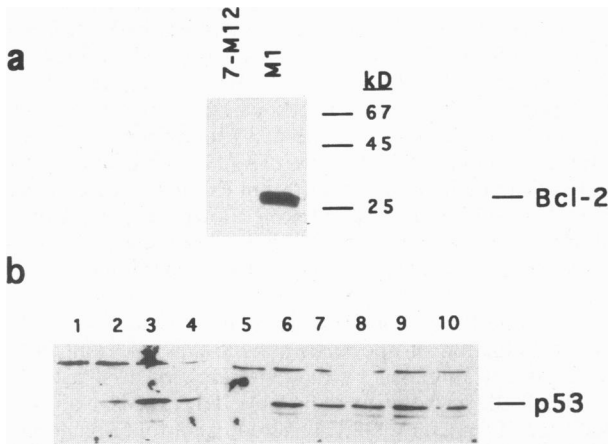

FIG. 3. Expression of Bcl-2 and p53 proteins in M1 and 7-M12 myeloid leukemic cells. (*a*) Cell extracts from M1-neo or 7-M12 cells were analyzed for Bcl-2 protein. M1-t-p53 cells had similar amounts of Bcl-2 as M1-neo cells. (*b*) Cell extracts from 7-M12 cells without any treatment (lanes 1 and 5), 2 hr after γ-irradiation at 300R (lanes 2 and 6–10), 2 hr after addition of 1 μg/ml doxorubicin (lane 3), or 1 hr after addition of 0.5 μg/ml cycloheximide (lane 4) were analyzed for p53 protein. After γ-irradiation, cells were cultured either without any additions (lane 6) or with 5 ng/ml GM-CSF (lane 7), 5 ng/ml IL-3 (lane 8), 1 ng/ml IFN-γ (lane 9), or 0.1 mM BHA (lane 10). The positions of the Bcl-2 and p53 proteins are indicated.

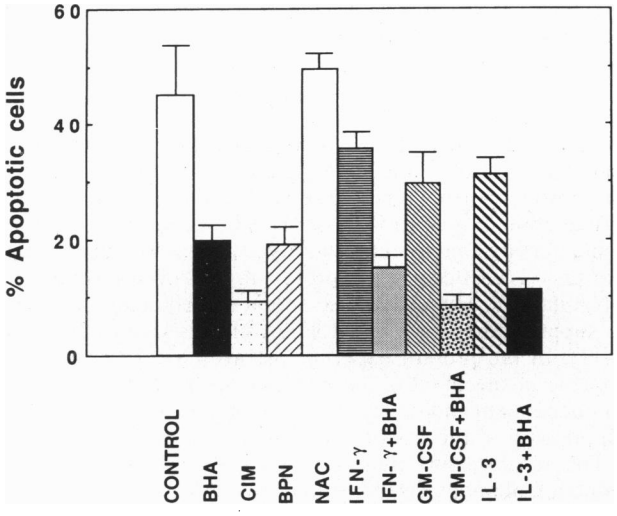

FIG. 4. Protective effect of antioxidants and IFN-γ against induction of apoptosis in 7-M12 cells. Cells were treated with 0.5 μg/ml cycloheximide for 3 hr without or with 0.1 mM BHA, 2 mM CIM, 1 mM BPN, 5 mM NAC, 1 ng/ml IFN-γ, 5 ng/ml GM-CSF, 5 ng/ml IL-3, or these cytokines together with BHA and the percent of apoptotic cells was determined.

cin, and cycloheximide, which may be mediated at least in part by wild-type p53, also involves an oxidative mechanism.

Protective Effect of BHA Against Induction of Apoptosis by a p53-Independent Pathway in M1 Myeloid Leukemic Cells. To determine whether an oxidative mechanism also participates in induction of apoptosis by a p53-independent mechanism, we used M1 myeloid leukemic cells that did not express any p53 protein either before or after γ-irradiation or treatment with doxorubicin. Induction of apoptosis in M1-neo cells incubated with doxorubicin for 16 hr was inhibited by BHA and IFN-γ, and there was a cooperative protection when BHA and IFN-γ were added together (Table 2). The results with BHA indicate involvement of an oxidative mechanism also in apoptosis induced by a p53-independent pathway in M1 leukemic cells.

Determination of Peroxide Production and Lipid Peroxidation During Activation of the Apoptotic Pathway and in its Suppression by Cytokines. The above results have indicated the involvement of an oxidative mechanism in induction of apoptosis by wild-type p53-dependent and p53-independent pathways in the myeloid leukemic cells. One of the oxidative mechanisms in cells involves generation of peroxide, which can be further used to generate other reactive oxygen species (12) that can lead to apoptosis (for reviews, see refs. 12–14). We therefore determined production of peroxide in M1-t-p53 and 7-M12 myeloid leukemic cells under conditions associated with induction of apoptosis or its suppression by cytokines and the antioxidant BHA. FACS analysis of the rate of oxidation of DCFDA and generation of the intracellular fluorescent product DCF has shown that before any treatment 7-M12 cells generated, in different experiments, 5- to 15-fold higher amounts of peroxide compared with M1 or M1-t-p53 leukemic cells grown at 37°C (Figs. 5 and 6). This shows that the basal degree of oxidative stress in 7-M12 leukemic cells was higher than in M1 leukemic cells. Moreover, increasing the degree of oxidative stress by adding 5 mM paraquat, a superoxide-generating compound, induced a much higher degree of apoptosis in 7-M12 cells than in M1 cells (42% versus < 1%, respectively, at 8 hr). Similar results were obtained by adding 50 μM H_2O_2.

It was previously shown that thymocyte suspensions containing apoptotic cells have an increased production of peroxide and that apoptotic cells had higher levels of peroxide than the nonapoptotic cells (25). This suggests that an increased production of peroxide found in cell populations already containing apoptotic cells may be the result rather than the cause of the apoptotic process. We therefore tested whether an increase in peroxide production, as measured by oxidation of DCFDA to DCF, precedes the appearance of apoptotic cells so as to assign a possible causative role for peroxide in the process leading to apoptosis. Peroxide production was determined in M1-t-p53 cells cultured at 32°C for 6 hr, which is sufficient for activation of the wild-type conformation of p53 (17) and for activation of the wild-type p53 inducible gene p21/WAF1 (11), and in 7-M12 cells 30 min after adding cycloheximide or 1 hr after γ-irradiation. At these time points, there were still no detectable apoptotic cells in the cultures. The level of peroxide production in M1-t-p53 cells cultured for 6 hr at 32°C was similar to M1-neo cells cultured under the same conditions and to M1-t-p53 cells at 37°C before transfer to 32°C (Fig. 5). There was also no change in peroxide

Table 2. Protective effect of BHA and IFN-γ against p53-independent apoptosis induced in M1 cells by doxorubicin

Material added	% cell viability at 16 hr*
None	47.6 ± 6.2
BHA	64.8 ± 4.3†
IFN-γ	69.2 ± 7.3‡
IFN-γ + BHA	84.9 ± 6.1†

M1-neo cells that do not express p53 were cultured for 1 hr with no additions (None) or with 0.1 mM BHA, 1 ng/ml IFN-γ, or with IFN-γ and BHA together and were then cultured for 16 hr with 1 μg/ml doxorubicin.

*Five experiments were carried out ($n = 5$) and the P values (Student's t test) given are for the statistical significance of the difference between each of the added compounds and the control (None) group. †, $P < 0.001$; ‡, $P = 0.001$.

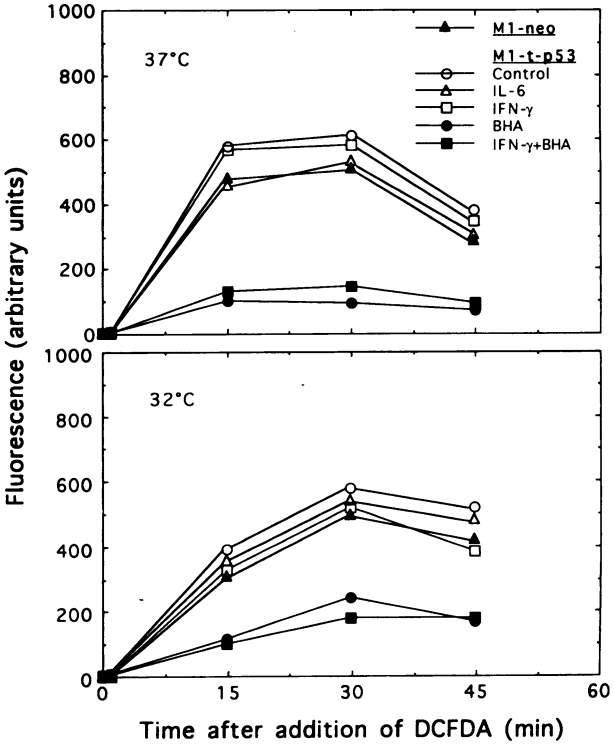

FIG. 5. Peroxide production in M1-neo and M1-t-p53 cells. Cells were cultured at 37°C (*Upper*) or 32°C (*Lower*) for 6 hr with no additions (control) or with 0.1 mM BHA, 5 ng/ml IL-6, or 1 ng/ml IFN-γ and analyzed for peroxide production using DCFDA as described.

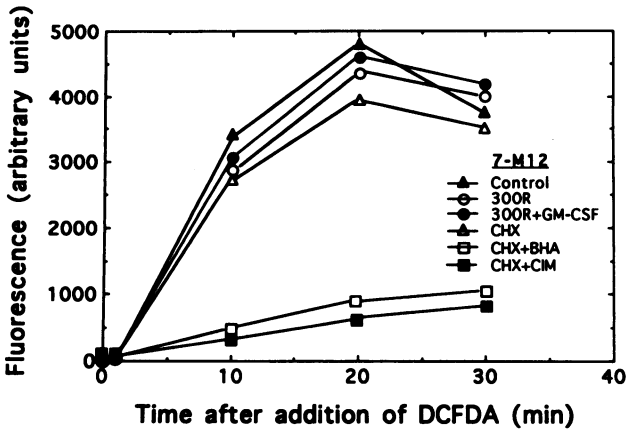

FIG. 6. Peroxide production in 7-M12 cells. Cells were cultured at 37°C. The 7-M12 cells were cultured with no additions (control) or treated with: γ-irradiation (300 R) followed by culture without or with 5 ng/ml GM-CSF; 0.5 μg/ml cycloheximide (CHX); CHX and 0.1 mM BHA; CHX and 2 mM CIM. Cells were analyzed for peroxide production using DCFDA as described, 1 hr after γ-irradiation at 300 R or 30 min after adding 0.5 μg/ml CHX.

production in M1-t-p53 cells after 15 min or 1 hr at 32°C. In 7-M12 leukemic cells, peroxide production was not increased by induction of apoptosis with cycloheximide or γ-irradiation (Fig. 6). Furthermore, addition of IL-6 or IFN-γ to M1-t-p53 cells at 32°C or addition of GM-CSF to 7-M12 cells after γ-irradiation, which protect the cells from induction of apoptosis (1, 3, 7–11), did not reduce peroxide production (Figs. 5 and 6). In contrast, BHA (Figs. 5 and 6) and CIM (Fig. 6) strongly decreased oxidation of DCFDA to DCF. These results indicate that activation of the apoptotic pathway in leukemic cells by wild-type p53, γ-irradiation, or cycloheximide and suppression of the apoptotic pathway by cytokines were not associated with an increased or decreased peroxide production.

Measurement of lipid peroxidation, which determines accumulated oxidative damage to membrane lipids, has shown that there was no increase in the rate of lipid peroxidation in M1-t-p53 cells cultured for 6 hr at 32°C and suppression of the apoptotic pathway by IL-6 or IFN-γ did not decrease lipid peroxidation (Fig. 7). There was, however, some protection from lipid peroxidation when the apoptotic pathway was inhibited by the antioxidant BHA (Fig. 7). Due to the toxicity of the fluorescent probe *cis*-parinaric acid to 7-M12 cells, we

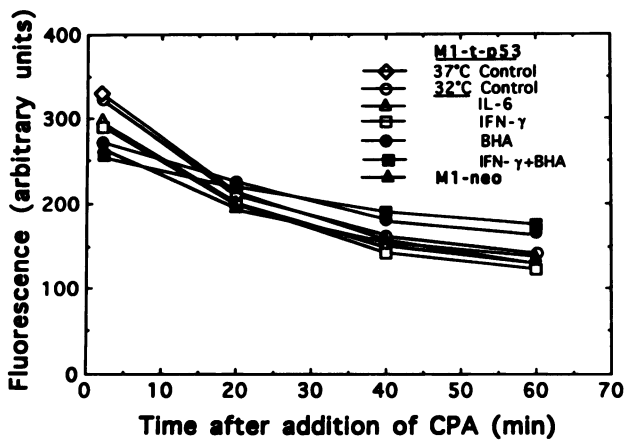

FIG. 7. Lipid peroxidation in M1-t-p53 cells. Cells were cultured at 37°C or 32°C for 6 hr with no additions (control) or with 0.1 mM BHA, 5 ng/ml IL-6, or 1 ng/ml IFN-γ and analyzed for lipid peroxidation using the fluorescent probe *cis*-parinaric acid (CPA) as described.

were unable to determine lipid peroxidation in 7-M12 cells by the method used with M1 cells.

DISCUSSION

In cells with wild-type p53, treatment with a variety of DNA damaging agents resulted in a rapid accumulation of wild-type p53 protein (for reviews, see refs. 26 and 27), which can lead to induction of apoptosis (1–9). This induction of apoptosis is dependent on expression of wild-type p53 (1–5). Furthermore, expression of wild-type p53 protein in cells even without prior DNA damage can also induce apoptotic cell death, which can be suppressed by certain cytokines such as IL-6 or IFN-γ (1, 6–11). In the present experiments, we have determined the possible involvement of an oxidative mechanism in wild-type p53-dependent and -independent induction of apoptosis and in suppression of this apoptosis by cytokines.

The results show that different antioxidants can inhibit induction of apoptosis in myeloid leukemic cells by expression of a wild-type p53 transgene, and by cytotoxic compounds via a p53-independent pathway. There was also a cooperative protection against induction of apoptosis by the antioxidants and viability-inducing cytokines and the antioxidants increased the effectiveness of the cytokines. The results indicate that oxidative mechanisms are involved in p53-dependent and p53-independent induction of apoptosis and in the protection from apoptosis by different cytokines. Bone marrow granulocyte–macrophage colony-forming cells and thymocytes from transgenic-Cu/Zn superoxide dismutase (Cu/Zn SOD) mice, which have a higher degree of oxidative stress than cells from normal mice, are more sensitive to induction of apoptosis (20). There was also in this case a better protection by IL-3 and GM-CSF against induction of apoptosis in the bone marrow colony-forming cells from normal mice than in cells from transgenic-Cu/Zn SOD mice (20). In other cell types, a relationship between enhanced oxidative stress and sensitivity to induction of apoptosis was shown in neurons from transgenic-Cu/Zn SOD mice (28) and from Down syndrome patients that overexpress Cu/Zn SOD (29) and in patients with the familial neurodegenerative disease amyotrophic lateral sclerosis (30), which is caused by mutations in the Cu/Zn SOD gene (31). In our experiments, the higher degree of oxidative stress in 7-M12 compared with M1 myeloid leukemic cells was associated with the higher susceptibility of 7-M12 cells to induction of apoptosis by different cytotoxic compounds (16) and the lower expression of *bcl*-2. It has been shown in other cell types that *bcl*-2 expression may (32, 33) or may not (34, 35) be associated with changes in the extent of oxidative stress.

Oxidative mechanisms that have been implicated in induction of apoptosis in some cell systems involve increased production of peroxide (13, 14, 20, 36, 37) and increased lipid peroxidation (20, 32). However, under our assay conditions we did not detect an increased peroxide production or lipid peroxidation following wild-type p53 induction of apoptosis and there was no decrease in these properties in cells protected from apoptosis by IL-6 or IFN-γ. This indicates that unlike thymocytes from mice injected with bacterial endotoxin (20), fibroblasts treated with tumor necrosis factor (36) or neurons treated with β-amyloid (37), the oxidative mechanism involved in induction of apoptosis by wild-type p53 in myeloid cells, does not involve increased peroxide production. It can be suggested that wild-type p53-induced apoptosis requires oxidative activation of certain targets downstream from p53 and that this is controlled by the intrinsic oxidative level within the cells. Decreasing the intrinsic oxidative level by antioxidants thus protected cells from wild-type p53-induced apoptosis, whereas increasing this intrinsic level by adding H_2O_2 enhanced apoptosis. Our results are in agreement with those obtained with an IL-3-dependent cell line induced to undergo apoptosis by withdrawal of IL-3 or treatment with dexamethasone (32).

Also in this system, despite the apoptosis-protective effect of antioxidants, there was no increase in peroxide production after IL-3 withdrawal or addition of dexamethasone, and protection from apoptosis by overexpressing *bcl*-2 did not reduce the level of peroxide production (32). The results indicate that the degree of oxidative stress, which can be decreased by antioxidants and increased by H_2O_2, can regulate cell susceptibility to induction of apoptosis and protection from apoptosis by cytokines.

We thank Nurit Dorevitch and Rachel Kama for skillful technical assistance. This work was supported by the National Foundation for Cancer Research (Bethesda), by the Esther Mazor Family (Washington, DC), by the National Institutes of Health Grant HD 21229 (Bethesda), and by the Ebner Family Biomedical Research Foundation at the Weizmann Institute of Science in memory of Alfred and Dolfi Ebner.

1. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A. & Oren, M. (1991) *Nature (London)* **352,** 345–347.
2. Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B. & Costa, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4495–4499.
3. Lotem, J. & Sachs, L. (1993) *Blood* **82,** 1092–1096.
4. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362,** 847–849.
5. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. & Wyllie, A. H. (1993) *Nature (London)* **362,** 849–852.
6. Sachs, L. & Lotem, J. (1993) *Blood* **82,** 15–21.
7. Sachs, L. & Lotem, J. (1995) in *Apoptosis and the Immune Response*. ed. Gregory, C. D. (Wiley, New York), pp. 371–403.
8. Sachs, L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4742–4749.
9. Lotem, J. & Sachs, L. (1996) *Leukemia* **10,** 925–931.
10. Lotem, J. & Sachs, L. (1992) *Blood* **80,** 1750–1757.
11. Lotem, J. & Sachs, L. (1995) *Leukemia* **9,** 685–692.
12. Halliwell, B. (1994) *Lancet* **344,** 721–724.
13. Buttke, T. M. & Sandstrom, P. A. (1994) *Immunol. Today* **15,** 7–10.
14. Slater, A. F. G., Nobel, C. S. I. & Orrenius, S. (1995) *Biochim. Biophys. Acta* **1271,** 59–62.
15. Lotem, J. & Sachs, L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5554–5558.
16. Lotem, J. & Sachs, L. (1993) *Cell Growth Differ.* **4,** 41–47.
17. Michalovitz, D., Halevy, O. & Oren, M. (1990) *Cell* **62,** 671–680.
18. Arends, M. J. & Wyllie, A. H. (1991) *Int. Rev. Exp. Pathol.* **32,** 223–254.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
20. Peled-Kamar, M., Lotem, J., Okon, E., Sachs, L. & Groner, Y. (1995) *EMBO J.* **14,** 4985–4993.
21. Cathcart, R., Schwiers, E. & Ames, B. N. (1983) *Anal. Biochem.* **134,** 111–116.
22. Royall, J. A. & Ischiropoulos, H. (1993) *Arch. Biochem. Biophys.* **302,** 348–355.
23. Kuypers, F. A., Van den Berg, J. J. M., Schalkwijk, C., Roelofsen, B. & Op den Kamp, J. A. F. (1987) *Biochim. Biophys. Acta* **921,** 266–274.
24. Hedley, D. & Chow, S. (1992) *Cytometry* **13,** 686–692.
25. Fernandez, A., Kiefer, J., Fosdick, L. & McConkey, D. J. (1995) *J. Immunol.* **155,** 5133–5139.
26. Levine, A. J., Momand, J. & Finlay, C. A. (1991) *Nature (London)* **351,** 453–456.
27. Oren, M. (1992) *FASEB J.* **6,** 3169–3176.
28. Bar-Peled, O., Korkotian, E., Segal, M. & Groner, Y. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8530–8535.
29. Busciglio, J. & Yanker, B. A. (1995) *Nature (London)* **378,** 776–779.
30. Wiedau-Pazos, M., Goto, J. J., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S. & Bredesen, D. E. (1996) *Science* **271,** 515–518.
31. Brown, R. H., Jr. (1995) *Cell* **80,** 687–692.
32. Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. & Korsmeyer, S. J. (1993) *Cell* **75,** 241–251.
33. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Butler Gralla, E., Selverstone Valentine, J., Örd, T. & Bredesen, D. E. (1993) *Science* **262,** 1274–1277.
34. Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H. & Tsujimoto, Y. (1995) *Nature (London)* **374,** 811–813.
35. Jacobson, M. D. & Raff, M. C. (1995) *Nature (London)* **374,** 814–816.
36. Goossens, V., Grooten, J., DeVos, K. & Fiers, W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8115–8119.
37. Behl, C., Davis, J. B., Lesley, R. & Schubert, D. (1994) *Cell* **77,** 817–827.