Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1

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Contributed by Leo Sachs, November 22, 2000

The tumor suppressor gene wild-type p53 encodes a labile protein that accumulates in cells after different stress signals and can cause either growth arrest or apoptosis. One of the p53 target genes, p53-inducible gene 3 (PIG3), encodes a protein with significant homology to oxidoreductases, enzymes involved in cellular responses to oxidative stress and irradiation. This fact raised the possibility that cellular oxidation–reduction events controlled by such enzymes also may regulate the level of p53. Here we show that NADH quinone oxidoreductase 1 (NQO1) regulates p53 stability. The NQO1 inhibitor dicoumarol caused a reduction in the level of -irradiation-induced p53 in HCT116 human colon carcinoma cells. This reduction was prevented by the proteasome inhibitors MG132 and lactacystin, suggesting enhanced p53 degradation in the presence of dicoumarol. Dicoumarol-induced degradation of p53 also was prevented in the presence of simian virus 40 large T antigen, which is known to bind and to stabilize p53. Cells overexpressing NQO1 were resistant to dicoumarol, and this finding indicates the direct involvement of NQO1 in p53 stabilization. NQO1 inhibition induced p53 degradation and blocked wild-type p53-mediated apoptosis in -irradiated normal thymocytes and in M1 myeloid leukemic cells that overexpress wild-type p53. Dicoumarol also reduced the level of p53 in its mutant form in M1 cells. The results indicate that NQO1 plays an important role in regulating p53 functions by inhibiting its degradation.

The wild-type p53 gene is a tumor suppressor gene that is often mutated in various tumors (reviewed in refs. 1 and 2). p53 accumulation and activation induces either growth arrest (1, 2) or apoptosis (3–6). p53 is a very labile protein, with a half-life as short as a few minutes (7). The rapid degradation of p53 is largely achieved through the ubiquitin proteasome pathway. The accumulation of p53 in response to DNA damage and other types of stress occurs mainly through posttranslational modifications. Proteins known to alter p53 stability include HPV16-E6 (8), simian virus 40 (SV40) large T antigen (9, 10), adenovirus E1B/E4orf6 (11), WT1 (12), and Mdm2 (13, 14). Whereas association of SV40 T antigen or WT1 with p53 increases p53 stability, the binding of E6 or Mdm2 to p53 accelerates its degradation (8–14). Many biological functions of p53 are attributed to its ability to function as a sequence specific transcriptional activator of selected genes (1, 2). One of the target genes of p53, p53-inducible gene 3 (PIG3), encodes a protein that shares significant homology with oxidoreductases from several species (15), raising the possibility of p53 regulation by oxidoreductases.

NADH quinone oxidoreductase 1 (NQO1) is a ubiquitous cytosolic flavoenzyme that catalyzes two-electron reduction of various quinones, with NADH or NADPH as an electron donor. This NQO1-mediated reduction mechanism is responsible for the cellular defense against various damaging quinones (16); however, some nontoxic quinones such as b lapachone are reduced by NQO1 to become toxic to cells (17). Expression of the NQO1 gene is induced in response to a variety of agents, including oxidants, antioxidants, and ionizing radiation (reviewed in ref. 18), and NQO1 expression is altered in a number of cancers, including breast, colon, and lung cancers (19–22). NQO1 is inhibited by dicoumarol [3,3’-methylene-bis(4-hydroxycoumarin)], which competes with NADH or NADPH for binding to the oxidized form of NQO1 and thus inhibits NQO1 activity (23).

We have now investigated the relationship between NQO1 and p53. Our results show that inhibition of NQO1 activity by dicoumarol caused enhanced p53 proteasomal degradation that can be prevented by overexpression of NQO1. The ability of NQO1 inhibition to enhance p53 degradation resulted in the reduction of p53 accumulation and suppression of p53-dependent apoptosis in -irradiated normal thymocytes and in M1 myeloid leukemic cells that overexpress p53. Our findings indicate the involvement of NQO1 in p53 regulation.

Materials and Methods

Cells and Cell Culture. The cell lines used were HCT116 human colon carcinoma cells, COS 1 monkey kidney cells, normal thymocytes obtained from 2.5-month-old BALB/c mice, and M1-t-p53 mouse myeloid leukemic cells that express a temperature-sensitive mutant p53 (Val-135) protein (3). The p53 in M1-t-p53 cells behaves like a tumor-suppressing wild-type p53 at 32°C and like a mutant p53 at 37°C (24). HCT116 and COS 1 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured at 37°C in a humidified incubator with 5.6% CO2. Normal thymocytes and M1-t-p53 cells were grown in DMEM supplemented with 10% heat-inactivated (56°C, 30 min) horse serum and cultured at 37°C in an incubator with 10% CO2.

Compounds. Dicoumarol (Sigma) was dissolved in 0.13 N NaOH, and MG132 and lactacystin (Sigma) were dissolved in DMSO.

Establishment of Hemagglutinin (HA)-NQO1 Overexpressing Cell Lines. The coding region of human NQO1 (GenBank accession no. J03934), including a 5’ influenza HA tag, was inserted into the pEFIREs expression vector containing a puromycin resistance gene (25). HCT116 cells (2.5 × 106 cells, in 10-cm plates) were transfected with 10 μg of purified pEFIREs-HA-NQO1 plasmid with the use of the Superfect transfection reagent (Qiagen, Chatsworth, CA). Puromycin-resistant colonies expressing HA-NQO1 were identified by immunoblot analysis with the use of anti-HA antibody.

Immunoblot Analysis. Cell extracts were prepared by lysis of PBS-washed cells in RIPA lysis buffer [150 mM NaCl/1% Nonidet P-40 (vol/vol)/0.5% AB-deoxycholate (vol/vol)/0.1% SDS (vol/vol)/50 mM Tris-HCl (pH 8)/1 mM DTT/1 μg/ml each of leupeptin, aprotinin, and pepstatin (Sigma mixture)]. The

Abbreviations: HA, hemagglutinin; NQO1, NADH quinone oxidoreductase 1; SV40, simian virus 40.

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Article published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.021558898.

Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.021558898
...SDS gel. After electrophoresis, proteins were transferred to 0.45-μm cellulose nitrate membranes (Schleicher & Schuell). Loading equivalence and transfer efficiency were monitored by Ponceau S staining, and the membranes were then incubated with appropriate antibodies to proteins of interest followed by horseradish peroxidase-conjugated anti-IgG antibodies. Signals were developed using Super Signal (Pierce) at 20°C for 5 min, and the membranes were then exposed to x-ray film (Fuji) for an appropriate time and developed. Membranes were stripped with 50 mM citric acid before a different primary antibody was used. The antibodies used were monoclonal anti-human p53 (Pab 1801) (26), monoclonal anti-mouse and human p53 (Pab240), monoclonal anti β-tubulin, and anti-HA (Sigma).

Apoptosis and Cell Viability Assays. Apoptosis in normal thymocytes was induced by γ-irradiation at 4 Gy (Co60 source, 0.63 Gy/min) and in M1-t-p53 cells by culture at 32°C. The percentage of apoptotic thymocytes was determined on May–Grünwald–Giemsastained cytopsin preparations by counting 400 cells 5 h after γ-irradiation. Apoptotic cells were scored by their smaller size, condensed chromatin, and fragmented nuclei compared with nonapoptotic cells. Analysis of DNA fragmentation during apoptosis in thymocytes was performed by DNA agarose gel electrophoresis as described (27). Apoptotic M1-t-p53 cells undergo secondary changes, including uptake of Trypan Blue (28). The percentage of viable cells (nonapoptotic and not stained with Trypan Blue) was determined by counting 400 cells in a hemacytometer after 23 h at 32°C.

Results

Regulation of p53 Degradation by NQO1. To determine whether the p53 level is regulated by NQO1 in HCT116 human colon carcinoma cells expressing wild-type p53 (29), cells were treated with the NQO1 inhibitor dicoumarol. Treatment for 90 min resulted in a significant reduction in p53 level, and after 180 min p53 was almost completely eliminated (Fig. 1A). To further test whether dicoumarol can similarly affect the p53 level accumulated upon irradiation, cells were γ-irradiated at 6 Gy and incubated for 4 h without or with dicoumarol. As expected, p53 accumulated upon γ-irradiation (compare Fig. 1B, lanes 1 and 2). Accumulation of p53 was lower in the presence of 200 μM dicoumarol and almost reached the uninduced level at 400 μM dicoumarol (Fig. 1B). Under the same conditions the level of β-tubulin was unaffected (Fig. 1A and B, Bottom), indicating that there is some specificity of this effect. To further establish the specific effect of dicoumarol on the p53 level, we used COS 1 cells in which p53 is stabilized because of expression of SV40 large T antigen (30). In contrast to the results obtained with HCT116 cells, the p53 level in COS 1 cells was not reduced by treatment with dicoumarol, even at 400 μM (Fig. 1C). The results indicate that dicoumarol caused a strong decrease in both basal and induced p53 levels. Because the NQO1 inhibitor could not overcome the p53 stabilization by SV40 large T antigen, we conclude that dicoumarol induces p53 protein destabilization.

It is well documented that p53 accumulation is determined by the rate of its proteasomal degradation (reviewed in refs. 1 and 2). To determine whether the observed p53 destabilization by dicoumarol occurs through protein degradation, cells were treated with dicoumarol together with the proteasome inhibitors MG132 or lactacystin. The results show that dicoumarol-induced p53 elimination was completely blocked by the addition of either MG132 (Fig. 2A) or lactacystin (Fig. 2B). These results indicate that dicoumarol induced p53 proteasomal degradation.

To verify that enhanced p53 degradation by dicoumarol was mediated by inhibition of NQO1 activity, we determined whether overexpression of NQO1 could prevent dicoumarol-induced p53 degradation. A pool of stable clones of cells overexpressing HA-tagged NQO1 was established, and the level of NQO1 protein was verified by immunoblotting. These cells became resistant to p53 degradation by dicoumarol (Fig. 3). Similar results were obtained with three individual stable clones expressing HA-tagged NQO1. The level of HA-NQO1 was not reduced in the presence of dicoumarol (Fig. 3). These results indicate that dicoumarol-induced p53 degradation is most probably the direct outcome of inhibition of NQO1 activity.

Suppression of p53-Mediated Apoptosis by Dicoumarol-Induced p53 Degradation. Induction of wild-type p53 accumulation in various cell types by overexpression of p53 or by γ-irradiation can lead...
to apoptotic cell death (3–6). To determine whether inhibition of p53 protein accumulation by the inhibition of NQO1 activity can also prevent apoptosis, we measured the effect of dicoumarol on p53-dependent apoptosis in γ-irradiated normal mouse thymocytes. Dicoumarol inhibited the induction of apoptosis in 4 Gy γ-irradiated thymocytes in a dose-dependent manner, as determined by morphological analysis of apoptosis (Fig. 4A) and DNA fragmentation at internucleosomal sites (Fig. 4B). Complete inhibition of apoptosis was obtained with 200 μM dicoumarol. The inhibition of p53-dependent apoptosis in γ-irradiated thymocytes by dicoumarol was associated with a decrease in the level of p53 (Fig. 4C). The results indicate that dicoumarol inhibits p53-mediated apoptosis in γ-irradiated normal thymocytes through enhanced p53 degradation.

We also determined the ability of dicoumarol to affect induction of apoptosis in M1-t-p53 myeloid leukemic cells that overexpress a temperature-sensitive p53 transgene. These cells are viable and proliferate at 37°C when p53 behaves like a mutant form, but undergo apoptosis at 32°C when p53 behaves like wild type (3). Dicoumarol also inhibited p53-induced apoptosis in these cells. The addition of 75 μM or 100 μM dicoumarol at 32°C for 23 h resulted in an increase in cell survival compared with cells cultured under the same conditions without dicoumarol (Fig. 5A). Doses of 125 μM dicoumarol or more were toxic to these cells. As in HCT116 cells and normal thymocytes, the p53...
level in M1-t-p53 cells cultured at 32°C was reduced by the
addition of dicoumarol (Fig. 5B). Inhibition of NQO1 activity
thus enhanced the degradation of overexpressed p53 and re-
sulted in reduced p53-dependent apoptosis in these cells.

We previously have shown that IL-6 and the calcium-
mobilizing compound thapsigargin are highly efficient antiapo-
ptotic agents in M1-t-p53 cells (3, 31). The effect of these
compounds on p53 stability was therefore also investigated. In
contrast to dicoumarol, IL-6 and thapsigargin did not cause a
reduction in p53 level in these cells at 32°C (Fig. 6A).

Discussion

We describe here a pathway regulating p53 accumulation. Ac-
cumulation of wild-type p53 after DNA damage (4–6) or
overexpression even without prior DNA damage (3) induces
apoptosis. Therefore, to avoid uncontrolled induction of apo-
pptosis, the endogenous p53 protein is maintained at low levels by
mechanisms that ensure its short half-life and degradation
through the ubiquitin–proteasome pathway (32). A major com-
ponent in this process is Mdm-2, which acts as a ubiquitin ligase
that mediates p53 degradation in the proteasome (13, 14).

Because mdm-2 is a transcriptional target gene of p53 (33, 34),
this system creates an autoregulatory negative loop that main-
tains cellular integrity. After DNA damage or other types of
stress, p53 levels are increased through different posttransla-
tional modifications of both p53 and Mdm-2 that reduce their
association and thus decrease p53 degradation. These modifi-
cations include phosphorylations (35–38), acetylations (39),
and interactions with proteins such as the DNA helicase WRN (40).
In addition, the level and activity of Mdm-2 itself are modulated
by other proteins, including the Mdm-2 antagonist p19ARF (41,
42), or by Ras, which can enhance mdm-2 transcription through
the Raf–MEK–mitogen-activated protein kinase pathway (43).

Our data provide experimental evidence for a role of NQO1
activity in p53 accumulation and thus establish a new pathway
determining the p53 level. It will be interesting to determine
whether this pathway is Mdm2-dependent.

We now show that endogenous and γ-irradiation-induced p53
levels were significantly reduced in different cell types by the
NQO1 inhibitor dicoumarol. The ability of proteasome inhibi-
tors to block this effect indicates that dicoumarol enhanced p53
proteasomal degradation. To confirm that p53 degradation
enhanced by dicoumarol was due to inhibition of NQO1 activity,
we established cell lines that overexpress NQO1. These cells became resistant to dicoumarol-induced p53 degradation, indicating that the effect of dicoumarol was indeed due to inhibition of NQO1 activity. However, even inhibition of NQO1 by dicoumarol could not cause degradation of p53 that had already been stabilized by SV40 T antigen. This finding indicates that the NQO1 pathway for the regulation of the cellular p53 level is subject to regulation by other pathways that control p53 stability.

As shown with p53, NQO1 inhibition by dicoumarol also enhanced the degradation of p73, and c-Myc (data not shown). In contrast, NQO1 inhibition did not cause degradation of β-tubulin, NQO1, IκB, or poly(ADP-ribose) polymerase [data not shown for poly(ADP-ribose) polymerase]. These data attribute a certain level of specificity to this regulatory pathway of protein degradation. The proteins that were destabilized by dicoumarol, p53, p73, and c-Myc, share the capacity to induce apoptosis (3–6, 44–47). However, NQO1 inhibition also destabilized a mutant p53, which does not induce apoptosis. It will be interesting to determine whether the stability of other apoptosis-regulating proteins is also subject to regulation by NQO1 activity and whether dicoumarol can inhibit p73-mediated and c-Myc-mediated apoptosis pathways.

NQO1 is a ubiquitous flavoenzyme that catalyzes two-electron reduction of various quinones, with NADH or NADPH as an electron donor. The involvement of NQO1 in p53 accumulation suggests that redox reactions controlled by oxidoreductases may be an important factor in determining the p53 intracellular level. The recent finding that overexpression of another oxidoreductase, WOX1, can increase the p53 protein level in L929 cells (48) supports this suggestion. p53 accumulates in cells under hypoxic conditions when the cellular level of reactive oxygen species is low (reviewed in ref. 49). p53 also accumulates after DNA damage that is associated with increased production of reactive oxygen species (reviewed in refs. 2 and 49). Because both oxidants as well as antioxidants up-regulate the expression of NQO1 (18), it can be suggested that one mechanism that regulates p53 accumulation under irradiation or hypoxic conditions is mediated by increased NQO1 activity. Some p53-inducible genes encode proteins that may participate or respond to oxidative stress, including p53-inducible gene 3 (15) and glutathione peroxidase (50). Our sequence analysis of the NQO1 promoter has revealed a putative p53-binding element. We therefore suggest that NQO1, glutathione peroxidase, and p53-inducible gene 3 may be part of a positive autoregulatory loop that regulates the level of p53.

The oxidoreductase activity of NQO1 is mediated by the conversion of NADH to NAD+. Therefore, inhibition of NQO1 by dicoumarol might cause a substantial NAD+ loss. NAD+ serves as a substrate for poly(ADP-ribose) polymerase, a DNA-binding protein that catalyzes the transfer of ADP-ribose residues from NAD+ to itself and to other proteins, including p53 (reviewed in ref. 51). Human breast, skin, and lung cells with reduced NAD level due to the use of nicotinamide-deficient medium, as well as cells deficient in poly(ADP-ribose) polymerase, exhibit decreased basal levels of p53 protein (52, 53). In addition, cells with defective NAD/poly(ADP-ribose) polymerase metabolism showed resistance to DNA damage-induced p53 accumulation and subsequent apoptosis (53). Therefore, dicoumarol-induced loss of NAD+ can provide a mechanism for p53 regulation by NQO1. Recently, NAD has been shown to play an important role in the regulation of gene expression and life span in yeast (54–56). A major gene in this process is Sir2p, which possesses ADP-riboseyltransferase activity and promotes silencing of gene transcription at selected loci (54, 55). In yeast increased longevity can be induced by calorie restriction, and this effect requires Sir2p and one of the two major pathways of NAD synthesis (56). Sir2p and p53 are structurally and functionally distinct, and our results suggest that they share the capacity to regulate cell fate via NAD (Fig. 7).

Mutations in p53 are found in more than 50% of cases of human cancer (1, 2). Some p53 mutants are gain-of-function mutants, which can suppress apoptosis (57–59). The ability of dicoumarol to induce the degradation of p53 in its mutant form and the fact that dicoumarol is already in clinical use as an anticoagulant raises the possibility of combining dicoumarol with cytotoxic agents in therapy against cancer cells that express high levels of mutant p53.

We thank R. Kama and S. Budilovsky for their assistance, Dr. S. Hobbs for the pEFires expression vector, and Dr. M. Oren for monoclonal anti-human p53 (PAb 1801) antibody. This work was supported by the Israel Academy of Sciences and Humanities and by grants from the Benoziyo Institute of Molecular Medicine and the Dolfi and Lola Ebnner Center for Biomedical Research at the Weizmann Institute of Science.
