Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2442–2447, March 1996
Medical Sciences

Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53

(tumor suppressor gene/mutagenesis/carcinogenesis)

KATHLEEN FORRESTER*, STEFAN AMBS*, SHAWN E. LUPOLD*, RACHEL B. KAPUST*, ELISA A. SPILLARE*, WENDY C. WEINBERG†, EMANUELA FELLEY-BOSCO‡, XIN W. WANG*, DAVID A. GELLER§, EDITH TZENG§, TIMOTHY R. BILLIAR§, AND CURTIS C. HARRIS*¶

*Laboratory of Human Carcinogenesis and †Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255; ‡Pharmacology and Toxicology Institute, Bugnon 27, 1007 Lausanne, Switzerland; and §Department of Surgery, University of Pittsburgh, Pittsburgh, PA 15261

Communicated by Theodore T. Puck, University of Colorado Health Sciences Center, Denver, CO, December 18, 1995 (received for review October 15, 1995)

ABSTRACT **The tumor suppressor gene product p53 plays an important role in the cellular response to DNA damage from exogenous chemical and physical mutagens. Therefore, we hypothesized that p53 performs a similar role in response to putative endogenous mutagens, such as nitric oxide (NO). We report here that exposure of human cells to NO generated from an NO donor or from overexpression of inducible nitric oxide synthase (NOS2) results in p53 protein accumulation. In addition, expression of wild-type (WT) p53 in a variety of human tumor cell lines, as well as murine fibroblasts, results in down-regulation of NOS2 expression through inhibition of the NOS2 promoter. These data are consistent with the hypothesis of a negative feedback loop in which endogenous NO-induced DNA damage results in WT p53 accumulation and provides a novel mechanism by which p53 safeguards against DNA damage through p53-mediated transrepression of NOS2 gene expression, thus reducing the potential for NO-induced DNA damage.**

Nitric oxide (NO) is an important bioregulatory agent involved in a variety of biological processes including the immune response, cytotoxicity, neurotransmission, and vasodilation (1). NO is synthesized *in vivo* from L-arginine by isoforms of nitric oxide synthase (NOS) (2). The activities of the constitutive forms neuronal NOS (NOS1) and endothelial NOS (NOS3) are dependent on elevated concentrations of calcium (3). The inducible NOS isoform (NOS2) is active at resting concentrations of calcium in the cell (3). NOS2 is synthesized in a variety of cell types from multiple mammalian species and can produce consistent, high concentrations of NO upon induction with cytokines and/or bacterial lipopolysaccharide (LPS) (3). Biochemical reactions involving NO may also lead to DNA damage (4–6). High concentrations of NO have been shown to cause deamination of deoxynucleotides and bases within intact DNA *in vitro* and are mutagenic *in vivo* (4, 7). Exposure of human cells to NO under aerobic conditions results in DNA strand breakage and nitrosative deamination of DNA bases (5). Therefore, NO can cause DNA damage as well as mutation in human cells. NO may also play a role in cytokine-induced (8) and activated macrophage-mediated (9) apoptosis of tumor cells.

p53 plays an important role in the cellular response to DNA damage (10). DNA damage caused by exposure to ionizing radiation, UV, or some exogenous chemical mutagens that result in DNA strand breakage can trigger an accumulation of p53 (11, 12). p53 can activate transcription of growth regulatory genes such as p21WAF1/Cip1 (13), GADD45 (14), and cyclin G (15), resulting in G_1 growth arrest (16), presumably to allow for repair of damaged DNA. In addition, p53 can induce programmed cell death through the process of apoptosis (17, 18) to remove cells unable to repair damaged DNA.

Since p53 plays an important role in the cellular response to DNA damage from exogenous chemical and physical mutagens (10), we hypothesized that p53 performs a similar role in response to putative endogenous mutagens such as NO and that DNA damage resulting from overproduction of NO induces wild-type (WT) p53 accumulation and subsequent down-regulation of NO synthesis through p53-mediated repression of NOS2 gene expression.

MATERIALS AND METHODS

Cell Lines and Plasmids. Primary normal human fibroblasts (NHFs) were obtained from the Coriell Cell Repository and grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS). THLE-5b cells, a simian virus 40 T-antigen immortalized normal human liver epithelial cell line grown in LCM medium (19), were infected with the retroviral vector DFG-iNOS containing the human NOS2 gene or with Bag-lacZ, containing the β-galactosidase gene as a vector control (20), and were selected on G418 (50 μg/ml) to generate clonal cell lines. DLD-1, a human colon carcinoma cell line grown in A50 medium (Biofluids, Rockville, MD) supplemented with 10% FBS contains a mutant p53 (codon 241, C → T). AKN-1, a spontaneously immortalized human liver cell line (21) grown in LCM medium contains a functionally inactive p53 (data not shown). Calu 6, a human lung adenocarcinoma cell line grown in HUT medium supplemented with 10% FBS, lacks endogenous p53 expression. Murine fibroblasts were isolated from dermis of 1- to 4-day-old p53-deficient mice (GenPharm, Mountain View, CA) to identify the p53 status after genotyping (22, 23) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The p53 expression vectors contain a human (24) or murine (23) WT (pC53-WT) or mutant p53 under the control of the cytomegalovirus (CMV) promoter. pNOS2(1.3)Luc and pNOS2(7.0)Luc reporter constructs start at +33 and extend the indicated length of the human NOS2 5′ flanking region (25) (21) liked to the luciferase gene in the pXP2 vector (26).

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: NOS2, nitric oxide synthase 2; LPS, lipopolysaccharide; WT, wild type; NHF, normal human fibroblast; CMV, cytomegalovirus; L-NMA, *N*-methyl-L-arginine; rh, recombinant human; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; RLU, relative light unit(s); S-NO-GS, *S*-nitrosoglutathione.

¶To whom reprint requests should be addressed at: Laboratory of Human Carcinogenesis, National Cancer Institute, Building 37, Room 2C05, 37 Convent Drive, MSC 4255, Bethesda, MD 20892-4255.

The murine NOS2 reporter construct was obtained from C. J. Lowenstein (Johns Hopkins University, Baltimore) and contains a 1.7-kb fragment of the murine NOS2 regulatory region linked to the luciferase gene in the pGL2 vector (Promega) (27).

Immunocytochemical Analysis. NHFs were treated with 1 mM *S*-nitrosoglutathione (S-NO-GS) (Calbiochem) or a mixture of 0.5 mM oxidized glutathione and 1 mM $NaNO_2$. As a control, NHFs were treated with 20 μM cisplatin for 24 hr. THLE-5b cells were cultured in the presence or absence of 2 mM *N*-methyl-L-arginine (L-NMA; Calbiochem) for 48 hr. Cells were fixed with 2% paraformaldehyde (in PBS) and postfixed with methanol, immunolabeled with a 1:200 dilution of the anti-p53 polyclonal CM-1 antibody (Signet Laboratories, Dedham, MA), and incubated with a 1:300 dilution of a fluorescein isothiocyate or Texas Red-coupled secondary antibody (Vector Laboratories).

Transient Transfection Assays and Cytokine Treatment. For NOS2 protein synthesis and activity determination, confluent DLD-1 cells were treated with lipofectin (GIBCO/BRL) with 1 μg of human pC53-WT or pCMVneo vector control and 4 hr later cells were treated with a mixture of recombinant human (rh) interferon γ (IFN-γ) (100 units/ml) (Boehringer Mannheim, Indianapolis, IN), rh interleukin 1β (IL-1β) and rh tumor necrosis factor α (TNF-α) (10 ng/ml) (R&D Systems) and cultured with or without 2 mM L-NMA. After 48 hr, supernatant was analyzed for nitrate and nitrite production and cell lysates were prepared. For luciferase assays, cells at $\approx$80% confluency were treated with lipofectin with 1–3.5 μg of the pNOS2-Luc reporter and 1–10 μg of human or murine WT p53 expression vectors or the pCMVneo control vector. Following transfection, human cells were treated with IFN-γ (100 units/ml), IL-1β (1.2 ng/ml), and TNF-α for 8 hr (2 ng/ml), and cell extracts were prepared. Murine fibroblasts were treated with IFN-γ (80 units/ml), IL-1β (1 ng/ml), and TNF-α (2 ng/ml) for 8 hr and cell extracts were prepared.

Western Blot Analysis. Total cellular extract was prepared using RIPA buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS/1% aprotinin). Protein samples (120 μg) were electrophoresed through a SDS/6% polyacrylamide gel and electrophoretically transferred to an Immobilin-P membrane (Millipore). The membrane was probed with ANTI-macNOS (Transduction Laboratories, Lexington, KY) monoclonal NOS antibody. Blots were developed using Renaissance Western blot chemiluminescence and exposed to reflection autoradiography film (DuPont/NEN).

Determination of NOS2 Activity. *Escherichia coli* ATCC 25922 (Baxter Diagnostics, McGaw Park, IL) was cultured overnight in anaerobic heart brain infusion (Becton Dickinson) supplemented with 0.1 M KNO_3, 0.4% glucose, and 5 ml of a salt solution (pH 2) per 1-liter final vol containing 80 mM $MgSO_4$, 1.4 mM $FeSO_4$, 0.9 mM $CaCl_2$, 8 mM $MnCl_2$, and 0.2 μM ammonium molybdate. Bacteria were harvested and washed with PBS, and the final pellet was resuspended 1:10 in 50 mM potassium phosphate buffer (pH 7.4) (EC-mix). KNO_3 standards (5–50 μM) or cell supernatants were mixed 1:1 with the EC-mix. After 4 hr at 37°C, the bacteria were pelleted and the supernatant was mixed 1:1 with Griess reagent and the absorbance was determined at 546 nm.

Luciferase Assays. Cell extracts were prepared 24–48 hr after transfection and 20 μl of lysate was used for determination of luciferase activity, which was quantified on a Monolite 2010 (Analytical Luminescence Laboratory, San Diego) luminometer and expressed as resonance light units (RLU) per μg of protein extract. The reported results represent at least three separate transfections.

RESULTS

NO Induces p53 Accumulation in NHFs. Since p53 accumulates in response to DNA damage (10, 12), we examined whether exposure of cells to NO would also result in induction in the p53 protein. NHFs were treated with an NO donor, S-NO-GS for increasing amounts of time. Treatment with 1 mM S-NO-GS resulted in an increase in nuclear localized p53 immunocytochemical staining, with a peak at 16 hr (Fig. 1 *B, D, F,* and *H*). Although the intensity of p53 staining in untreated cells diminished with increased time, which is most likely due to variations in staining patterns or to instability of the WT p53 protein in normal cells, unexposed NHFs exhibited a lesser degree of p53 staining than the NO exposed cells at each of the various time points (Fig. 1 *A, C, E,* and *G*). As a positive control for DNA damage-induced p53 accumulation (28), NHFs were also treated with chemotherapeutic drug (20 μM) cisplatin (Fig. 1 *I* and *J*). Therefore, exposure of cells to exogenous NO can result in induction of p53 accumulation in human cells.

Overexpression of NOS2 Induces p53 Accumulation in Human Liver Epithelial Cells. We then wished to determine whether endogenous overproduction of NO would also result

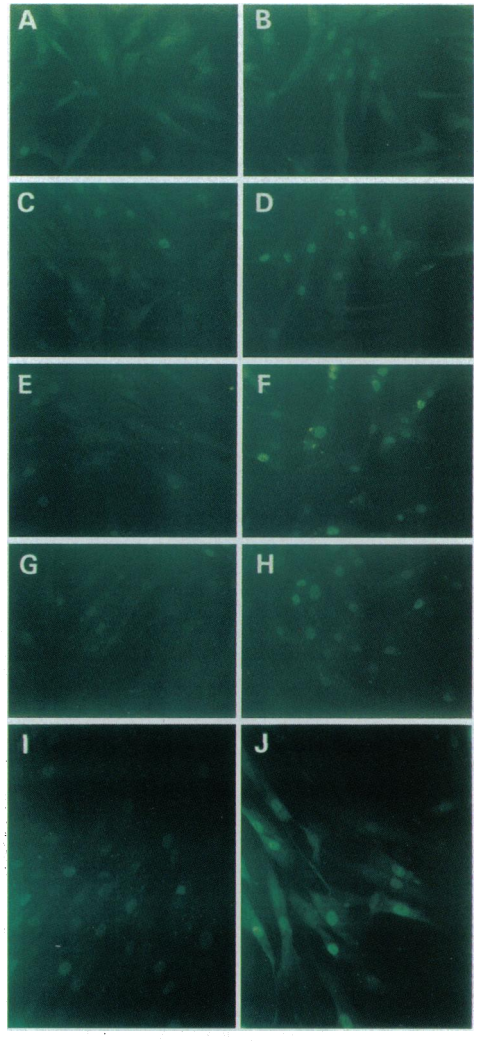

FIG. 1. Induction of p53 accumulation in normal human fibroblasts exposed to the NO donor S-NO-GS. Cells were grown in the absence (*A, C, E,* and *G*) or presence (*B, D, F,* and *H*) of 1 mM S-NO-GS for 4, 8, 16, or 24 hr followed by staining for p53 protein with the CM-1 antibody. As a control for DNA damaged-induced p53 accumulation, cells were grown in the absence (*I*) or presence (*J*) of 20 μM cisplatin for 24 hr.

in accumulation of p53 in human cells overexpressing the NOS2 gene. THLE-5b cells, a simian virus 40 T-antigen immortalized human liver epithelial cell line (19), were infected with the retroviral vector DFG-iNOS, which contains the human hepatocyte NOS2 cDNA (29) linked to the murine Moloney leukemia virus promoter (20). These cells were shown to produce high concentrations of nitrate and nitrite, a measure of NOS2 enzymatic activity, as well as an increase in NOS2 immunocytochemical staining (data not shown). As a control, cells were infected with the vector Bag-lacZ. As was observed in cells exposed to exogenous NO, there is an accumulation of p53 protein after 24 hr in THLE-5b cells overexpressing human NOS2 (Fig. 2*C*). This induction, however, is not seen in cells infected with the vector control (Fig. 2*A*), which expressed only low levels of p53 protein. This increase in p53 protein in THLE-5b-NOS2 cells corresponds to an increase in NOS2 staining (data not shown). To determine whether the observed induction of p53 accumulation is specific for NO production, THLE-5b-NOS2 or vector control cells were grown in the presence of 2 mM L-NMA, an inhibitor of NOS2 enzymatic activity. Accumulation of p53 protein in NOS2 expressing cells is effectively diminished by L-NMA incubation, indicating that the observed increase in p53 protein is specific for production of NO (Fig. 2*D*).

WT p53-Mediated Repression of Human NOS2 Expression and Enzymatic Activity in DLD-1 Human Colon Carcinoma Cells. In cells exposed to NO through an NO donor or through NOS2 overexpression, we did not observe an increase in cells undergoing programmed cell death, as determined by chromatin condensation, nuclear fragmentation, and apoptotic bodies, characteristic features of apoptosis (data not shown). Therefore, since p53 can function as a transactivator and transrepressor, we examined if p53 can control NO overproduction through p53-mediated regulation of NOS2 expression and enzymatic activity. Since NOS2 protein synthesis and enzymatic activity are induced 50-fold in DLD-1 human colon carcinoma cells upon cytokine treatment (30), we used these cells to transiently transfect the human WT p53 expression vector under the control of the CMV promoter. Overexpression of WT p53 resulted in a decrease in NOS2 protein synthesis as well as a significant decrease in NOS2 enzymatic activity in the cytokine-induced DLD-1 cells, as measured by the accumulation of nitrate and nitrite in the culture medium (Fig. 3). Therefore, WT p53 can inhibit production of potentially mutagenic NO through repression of NOS2 expression and enzymatic activity.

WT p53-Mediated Repression of Basal and Cytokine-Induced NOS2 Promoter Activity in Human and Murine Cells. To investigate the mechanism of p53-mediated down-regulation of NOS2 expression, we transiently cotransfected the WT p53 expression vector and either pNOS2(1.3)Luc or pNOS2(7.0)Luc reporter constructs into AKN-1 cells, a human liver cell line known to express NOS2 by increased transcription (21). The 7.0-kb NOS2 promoter construct has been shown to be induced 5-fold upon treatment with cytokines in these cells, while the 1.3-kb construct is unresponsive, indicating that transcriptional control is dependent on elements within the 7.0-kb region (21). WT p53 repressed basal activity of the 1.3-kb and 7.0-kb NOS2 promoter fragments 2-fold and 6-fold, respectively, in AKN-1 cells (Fig. 4*A*). Similar to what has been observed previously, 8-hr cytokine treatment resulted in a 3.3-fold induction of the 7.0-kb NOS2 promoter construct; however, the 1.3-kb construct was unresponsive. WT p53 was able to significantly repress NOS2 promoter activity of the 7.0-kb reporter construct 9-fold in cytokine-stimulated AKN-1 cells (Fig. 4*A*). Therefore, p53 can transrepress both basal and cytokine-induced human NOS2 promoter activity.

Since AKN-1 cells contain an endogenous mutant p53 (data not shown), which may influence the activity of the transfected WT p53 through dominant negative effects (31), we also examined the effects of WT p53 on the 1.3-kb NOS2 promoter construct in human tumor cell lines lacking endogenous p53 expression. WT p53 repressed NOS2 promoter activity in a dose-dependent manner, in the human lung cancer cell line Calu 6 (Table 1; data not shown). Cotransfection of the 175^{his} dominant negative p53 mutant abolished this repression, suggesting that down-regulation of NOS2 expression is WT p53 specific. In addition, the 175^{his} mutant did not affect NOS2 promoter activity in the absence of WT p53. A similar dose-

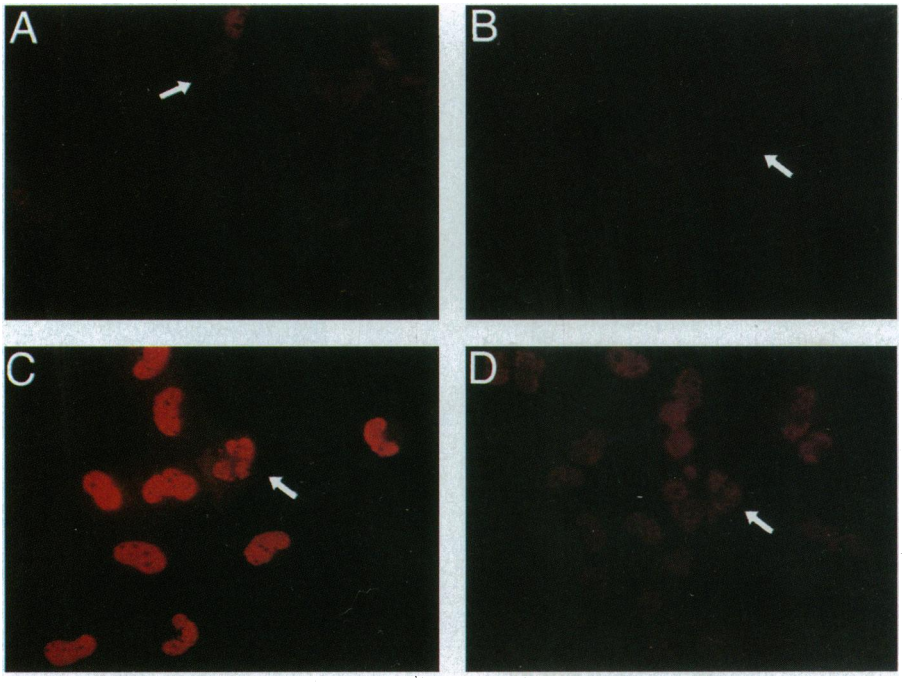

FIG. 2. p53 accumulation in NOS2 infected simian virus 40 T-antigen immortalized normal human liver epithelial cells. THLE-5b cells infected with the retroviral vector expressing human NOS2 (*C* and *D*) or the vector control (*A* and *B*) were grown in the absence (*A* and *C*) or presence (*B* and *D*) of 2 mM L-NMA followed by p53 immunocytochemistry.

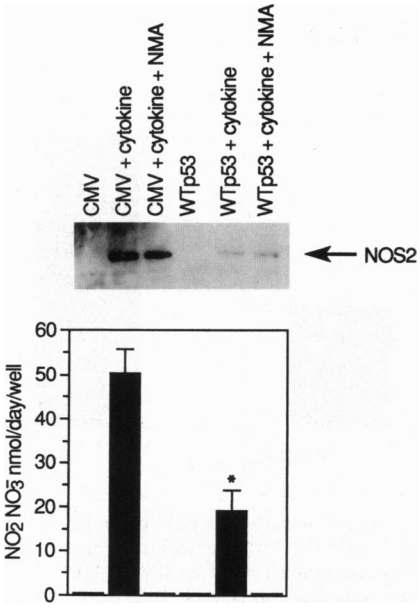

FIG. 3. Decrease in NOS2 protein expression and enzymatic activity in DLD-1 human colon carcinoma cells expressing WT p53. Total cell extract and cell supernatants from confluent DLD-1 cells transfected with pCMVneo or pC53-WT and treated with or without 2 mM L-NMA and cytokines were used for determination of NOS2 protein expression and enzymatic activity. NOS2 protein expression was analyzed by immunoblotting using the anti-macNOS monoclonal antibody. NOS2 enzymatic activity is expressed as mean ± SD of nmol nitrite (NO_2) and nitrate (NO_3) formed per day per well, *, Student's t test comparing NOS2 activity in the presence of WT p53 ($P < 0.01$).

dependent repression of NOS2 promoter activity by WT p53 was also observed in other human tumor cell lines lacking p53 expression (SK-OV-3, derived from an ovarian carcinoma; PC-3, derived from a prostate carcinoma) (data not shown). However, since Calu 6 cells did not respond to cytokine treatment (data not shown), analysis of the effects of p53 on the activity of the 7.0-kb construct was not examined.

Studies analyzing the murine NOS2 promoter have localized regions that are functionally important for regulating NOS2 expression in response to LPS and cytokines (27, 32). The human NOS2 promoter <50% homologous to the murine promoter but does contain motifs for binding of cytokine-related transcription factors (25). Interestingly, whereas a 1.7-kb fragment of the murine NOS2 promoter readily responds to cytokine stimulation (27, 32), an analogous fragment of the human NOS2 regulatory region is nonresponsive, indicating intrinsic differences in the two promoter regions (21). We therefore analyzed the effects of WT p53 overexpression on the basal and cytokine stimulated activity of the murine NOS2 promoter in primary dermal fibroblasts isolated from mice deficient in p53 expression. Introduction of a murine WT p53 expression vector into p53-null murine fibroblasts resulted in a 10-fold reduction in the basal activity of a 1.7-kb murine NOS2 promoter luciferase reporter construct (Fig. 4*B*). The activity of this reporter was induced 2.5-fold upon treatment of the fibroblasts with a cytokine mixture for 8 hr (Fig. 4*B*). Overexpression of WT p53 in cytokine-induced murine fibroblasts resulted in a 5-fold decrease in activity of the murine NOS2 promoter (Fig. 4*B*). Therefore, similar to the results observed in human cells, p53 can down-regulate both basal and cytokine-induced murine NOS2 promoter activity.

DISCUSSION

NO-induced DNA damage may occur through several mechanisms including nitrosative deamination (4), DNA strand breakage by NO_2 (33), oxidative damage by peroxynitrite (34), and DNA modification by metabolically activated N-nitrosamines (35). The inducible NOS isoform (NOS2) can produce high, persistent concentrations of NO upon induction with cytokines in many cell types (3) and is expressed in the resting state in other cells (36), potentially resulting in cytotoxicity, tissue damage, or DNA damage. p53 plays an important role in safeguarding the genomic integrity of mammalian cells in response to DNA damage (10). DNA damage can trigger an accumulation of p53 (11, 12), resulting in p53-mediated increases in expression of growth regulatory genes (13–15) and G_1 growth arrest (16). In addition, p53 can interact with the DNA repair machinery (37) to allow for repair of damaged DNA or induce apoptosis (17, 18) to remove cells unable to repair damaged DNA. Since p53 plays an important role in the cellular response to DNA damage from exogenous chemical and physical mutagens, we hypothesized that p53 performs a similar role in response to putative endogenous mutagens (38), such as NO. We have examined if p53 can accumulate in response to NO and subsequently control the production of NO, and therefore the potential for NO-induced DNA damage, by regulating expression of NOS2. Our results indicate that p53 accumulates in cells exposed to NO generated either from an NO donor or from overexpression of NOS2. In addition, overexpression of WT p53 in a variety of human tumor cell lines, as well as in murine fibroblasts, results in down-regulation of NOS2 expression, as well as enzymatic activity, through inhibition of the NOS2 promoter. DNA damage-induced p53 accumulation has been generally examined in cells exposed to exogenous physical and chemical mutagens that result in DNA strand breakage. The data presented here indicate that p53 accumulation in human cells can occur in response to a putative endogenous mutagen.

Recent results have shown that NO can stimulate p53 expression and apoptosis in rodent macrophage, pancreatic cell lines, and murine thymocytes, suggesting that NO-induced apoptosis results from DNA damage and subsequent accumulation of p53 (39, 40). However, we did not observe an induction in apoptosis in normal human cells treated with an NO donor or overexpressing NOS2. Instead, p53 controls overproduction of NO, and therefore the potential for NO-induced DNA damage, through repression of NOS2 promoter activity. This is a novel mechanism, in addition to monitoring of the G_1 checkpoint and the induction of apoptosis, by which p53 controls for potential DNA damage. In addition, these data are consistent with the hypothesis of a negative feedback loop in which NO-induced DNA damage results in p53 accumulation and a subsequent down-regulation of NO synthesis through p53-mediated repression of the NOS2 promoter.

Recent studies have begun to examine the expression and activity of NOS in human tumor samples. An increased level of NOS expression and/or activity was observed in human gynecological (41), breast (42), and central nervous system (43) tumors. In the case of human gynecological and breast cancers, this increased level of expression was inversely associated with the differentiation grade of the tumor (41, 42). Inactivation of p53 through mutation occurs in most tumor types and is the most commonly identified molecular alteration detected in human cancer (44). For these particular tumor types in which NOS levels are increased, p53 is mutationally inactivated in 25–40% of cases (44). One contributing factor to the observed increased level of NOS expression in human tumor samples may be a loss of p53-mediated NOS gene regulation due to functional inactivation of WT p53.

The p53 tumor suppressor protein can activate transcription of genes containing p53-specific responsive elements (45, 46). Conversely, p53 can repress transcription from a variety of cellular and viral promoters (47). WT p53 repressed the basal as well as cytokine-induced activity of the human NOS2 promoter in a variety of human tumor cell lines of multiple

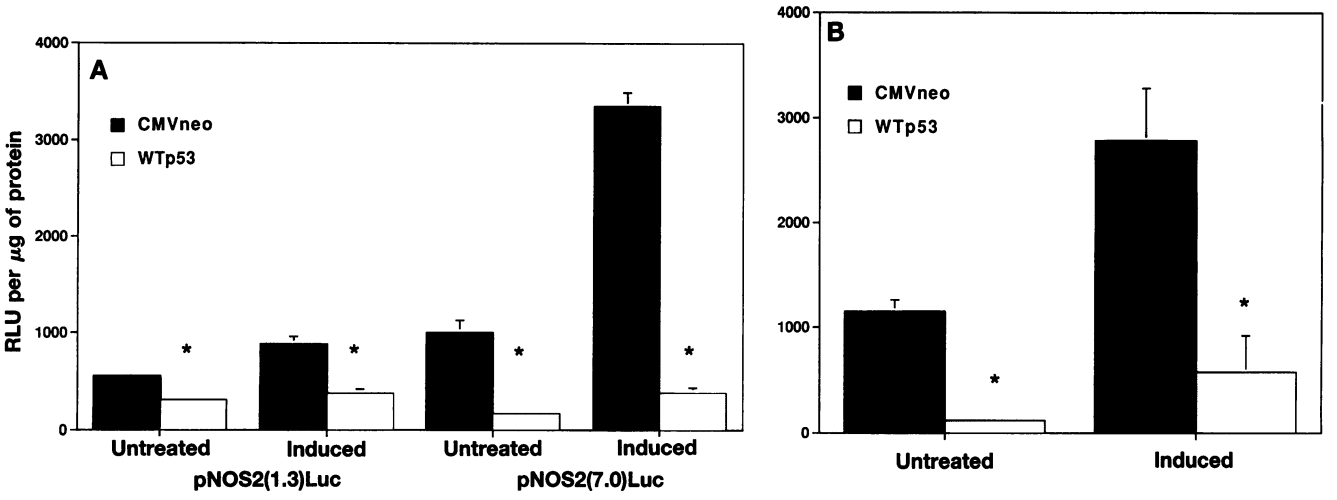

FIG. 4. WT p53-mediated repression of cytokine-induced NOS2 promoter activity in human and murine cells. (*A*) Subconfluent AKN-1 human liver cells were cotransfected with pC53-WT and equimolar amounts of the pNOS2(1.3)Luc or pNOS2(7.0)Luc luciferase reporter constructs and treated or untreated cytokines. *, Student's *t* test comparing basal or cytokine-induced NOS2 promoter activity of the 1.3-kb or 7.0-kb construct in the presence or absence of WT p53 ($P < 0.04$, $P < 0.058$, $P < 0.004$, or $P < 0.000$, respectively). (*B*) Murine fibroblasts isolated from p53-null mice were cotransfected with the murine NOS2 promoter construct and murine pCMV-p53-WT or pCMVneo control vector. *, Student's *t* test comparing untreated and cytokine-induced NOS2 promoter activity in the presence or absence of WT p53 ($P < 0.008$ or $P < 0.017$, respectively). In each case, luciferase activity is expressed as RLU per μg of protein extract. Means ± SEM of three independent experiments are shown.

histological subtypes, suggesting that p53-mediated repression of NOS2 expression may be a mechanism for regulation of NO overproduction in several different cell types. In addition, this down-regulation is specific for WT p53, since a dominant negative mutant of p53 abrogates this repression and itself has no effect on NOS2 promoter activity. The reproducible 4- to 10-fold repression of NOS2 promoter activity by WT p53 was similar to that observed for other p53 down-regulated cellular promoters such as *bcl-2* (48), PCNA (49), and *hsp70* (50), and viral promoters such as CMV (49, 51). We have localized the region required for WT p53-mediated repression to ≈400 bp upstream of the transcription start site of the human NOS2 gene (data not shown). This region contains consensus sequences for NF-κB and IFN-γ as well as a TATAA element (25). p53-mediated transcriptional repression is thought to be related to the interaction with the N- and/or C-terminal domains of p53 with the TATA binding protein (TBP) (52–54), the DNA binding subunit of the TFIID transcriptional initiation complex. Therefore, the observed p53-mediated down-regulation of NOS2 expression may occur through interaction of p53 with the TBP.

A low level of human NOS2 promoter activity was observed in DLD-1 colon carcinoma cells, AKN-1 liver cells, and Calu 6 lung carcinoma cells as well as in pulmonary epithelial cells (36), which were not stimulated with cytokines. This result suggests that at least a small degree of NOS2 gene expression may occur in unstimulated cells. DLD-1 cells have been shown to express NOS2 mRNA in the absence of cytokine treatment (55). In addition, basal transcription of the NOS2 gene in unstimulated AKN-1 cells has been detected by nuclear run-on analysis (21). We have found that WT p53 can repress basal as well as cytokine-induced NOS2 promoter activity. Therefore, p53 may play a role in the regulation of NOS2 expression in both resting and cytokine-stimulated cells.

Table 1. WT p53-specific repression of NOS2 promoter activity in Calu 6 lung carcinoma cells

Expression vector	Relative luciferase activity ± SEM
CMVneo	4.3 ± 0.3
WT p53	2.9 ± 0.2*
WT p53 + 175^{his}	4.0 ± 0.3*
175^{his}	3.9 ± 0.6

Calu 6 cells were cotransfected with 2 μg of pNOS2(1.3)Luc or pXP2 reporter construct and 3 μg of the 175^{his} mutant either alone or with 3 μg of pC53WT or pCMVneo control vector. Luciferase activity was normalized to the activity of the pXP2 reporter construct lacking the NOS2 promoter, which was set at 1 and is expressed as mean ± SEM of at least six independent experiments.

*Student's *t* test comparing activity in the presence or absence of WT p53 or in the presence or absence of the 175^{his} mutant ($P < 0.05$).

Characterization of the murine NOS2 gene promoter region has elucidated functional regulatory elements located within a 1.7-kb fragment of the 5′ flanking region that are responsive to IFN-γ and LPS (27, 32). There is <50% homology between the sequence of the first 1.5-kb of the murine and human NOS2 promoter regions (data not shown), suggesting that the regulation of these two genes may be different. While induction of the 1.7-kb fragment of the murine NOS2 promoter readily responds to cytokine stimulation (27, 32), an analogous fragment of human NOS2 is nonresponsive, indicating intrinsic differences in these two promoters (Fig. 4; ref. 21). In the experiments presented here, murine WT p53 was able to repress activity of the 1.7-kb murine NOS2 promoter fragment in untreated and cytokine-stimulated murine fibroblasts lacking endogenous p53 expression. Therefore, while cytokine-mediated induction of the human and murine NOS2 gene promoters differ, WT p53 may still play a role in regulating the expression of both genes. In addition, this result suggests a common mechanism for p53-mediated repression of NOS2 expression, perhaps through the common TATAA sequence located in both promoters.

Our results indicate that p53 plays a role in regulation of NOS2 gene expression and therefore the potential mutagenic and carcinogenic activity of NO. NO concentrations are elevated in chronic hepatitis (56) and ulcerative colitis (57, 58), which are predispositions for increased cancer risk, suggesting that overproduction of NO may play a role in human carcinogenesis. The hepatitis B virus X antigen can interact with and functionally inactivate WT p53 (59). In addition, in human liver and colon cancer, p53 is frequently inactivated through mutation (44), raising the possibility that loss of p53-mediated regulation of NOS2 activity can result in an increase in NO and NO-related DNA damage.

p53 plays an important role as a sensor and guardian of genomic integrity at times of genomic stress resulting from

exposure of cells to exogenous physical and chemical mutagens (10). The control of NO overproduction by p53 suggests that p53 may also be actively involved in the cellular response to endogenously produced mutagens. Furthermore, a negative feedback loop in which NO-induced DNA damage results in WT p53 accumulation and subsequent repression of NOS2 expression may be a novel mechanism, in addition to cell cycle regulation and/or direct interaction with DNA repair proteins, or apoptosis, by which p53 safeguards against DNA damage from exogenous and endogenous mutagens.

We thank B. Vogelstein for the pC53-WT and mutant p53 expression plasmids and C. J. Lowenstein for the murine NOS2 reporter construct. We are grateful to M. E. de Vera for helpful information on the human NOS2 promoter constructs and the NOS2 retroviral vector. This work was supported in part by National Institutes of Health Grants GM-44100 (T.R.B.) and GM-52021 (D.A.G.).

1. Moncada, S., Palmer, R. M. & Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142.
2. Nathan, C. (1992) *FASEB J.* **6,** 3051–3064.
3. Nathan, C. & Xie, Q. W. (1994) *Cell* **78,** 915–918.
4. Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuri, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S. & Keefer, L. K. (1991) *Science* **254,** 1001–1003.
5. Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S. & Tannenbaum, S. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3030–3034.
6. Fehsel, K., Jalowy, A., Qi, S., Burkart, V., Hartmann, B. & Kolb, H. (1993) *Diabetes* **42,** 496–500.
7. Arroyo, P. L., Hatch-Pigott, V., Mower, H. F. & Cooney, R. V. (1992) *Mutat. Res.* **281,** 193–202.
8. Xie, K., Huang, S., Dong, Z. & Fidler, I. J. (1993) *Int. J. Oncol.* **3,** 1043–1048.
9. Cui, S., Reichner, J. S., Mateo, R. B. & Albina, J. E. (1994) *Cancer Res.* **54,** 2462–2467.
10. Lane, D. P. (1992) *Nature (London)* **358,** 15–16.
11. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. & Craig, R. W. (1991) *Cancer Res.* **51,** 6304–6311.
12. Lu, X. & Lane, D. P. (1993) *Cell* **75,** 765–778.
13. El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W. & Vogelstein, B. (1994) *Cancer Res.* **54,** 1169–1174.
14. Hollander, M. C., Alamo, I., Jackman, J., Wang, M. G., McBride, O. W. & Fornace, A. J., Jr. (1993) *J. Biol. Chem.* **268,** 24385–24393.
15. Okamoto, K. & Beach, D. (1994) *EMBO J.* **13,** 4816–4822.
16. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B. & Fornace, A. J., Jr. (1992) *Cell* **71,** 587–597.
17. 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.
18. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362,** 847–849.
19. Pfeifer, A. M. A., Cole, K. E., Smoot, D. T., Weston, A., Groopman, J. D., Shields, P. G., Vignaud, J.-M., Juillerat, M., Lipsky, M. M., Trump, B. F., Lechner, J. F. & Harris, C. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5123–5127.
20. Tzeng, E., Billiar, T. R., Robbins, P. D., Loftus, M. & Stuehr, D. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 11771–11775.
21. de Vera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M., Jr., Billiar, T. R. & Geller, D. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1054–1059.
22. Weinberg, W. C., Azzoli, C. G., Kadiwar, N. & Yuspa, S. H. (1994) *Cancer Res.* **54,** 5584–5592.
23. Weinberg, W. C., Azzoli, C. G., Chapman, K., Levine, A. J. & Yuspa, S. H. (1995) *Oncogene* **10,** 2271–2279.
24. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. & Vogelstein, B. (1990) *Science* **249,** 912–915.
25. Chartrain, N. A., Geller, D. A., Koty, P. P., Sitrin, N. F., Nussler, A. K., Hoffman, E. P., Billiar, T. R., Hutchinson, N. I. & Mudgett, J. S. (1994) *J. Biol. Chem.* **269,** 6765–6772.
26. Nordeen, S. K. (1988) *BioTechniques* **6,** 454–458.
27. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W. & Murphy, W. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9730–9734.
28. Fritsche, M., Haessler, C. & Brandner, G. (1993) *Oncogene* **8,** 307–318.
29. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H. & Billiar, T. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3491–3495.
30. Sherman, P. A., Laubach, V. E., Reep, B. R. & Wood, E. R. (1993) *Biochemistry* **32,** 11600–11605.
31. Vogelstein, B. & Kinzler, K. W. (1992) *Cell* **70,** 523–526.
32. Xie, Q. W., Whisnant, R. & Nathan, C. (1993) *J. Exp. Med.* **177,** 1779–1784.
33. Gorsdorf, S., Appel, K. E., Engeholm, C. & Obe, G. (1990) *Carcinogenesis* **11,** 37–41.
34. Beckman, J. S., Chen, J., Crow, J. P. & Ye, Y. Z. (1994) in *Progress in Brain Research*, ed. Seil, F. J. (Elsevier, Amsterdam), Vol. 103, pp. 371–380.
35. Marletta, M. A. (1988) *Chem. Res. Toxicol.* **1,** 249–257.
36. Kobzik, L., Bredt, D. S., Lowenstein, C. J., Drazen, J., Gaston, B., Sugarbaker, D. & Stamler, J. S. (1993) *Am. J. Respir. Cell Mol. Biol.* **9,** 371–377.
37. Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J. M., Wang, Z., Friedberg, E. C., Evans, M. K., Taffe, B. G., Bohr, V. A., Hoeijmakers, J. H., Forrester, K. & Harris, C. C. (1995) *Nat. Genet.* **10,** 188–195.
38. Ames, B. N., Gold, L. S. & Willett, W. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5258–5265.
39. Messmer, U. K., Ankarcrona, M., Nicotera, P. & Brune, B. (1994) *FEBS Lett.* **355,** 23–26.
40. Fehsel, K., Kroncke, K.-D., Meyer, K. L., Huber, H., Wahn, V. & Kolb-Bachofen, V. (1995) *J. Immunol.* **155,** 2858–2865.
41. Thomsen, L. L., Lawton, F. G., Knowles, R. G., Beesley, J. E., Riveros-Moreno, V. & Moncada, S. (1994) *Cancer Res.* **54,** 1352–1354.
42. Thomsen, L. L., Miles, D. W., Happerfield, L., Bobrow, L. G., Knowles, R. G. & Moncada, S. (1995) *Br. J. Cancer* **72,** 41–44.
43. Cobbs, C. S., Brenman, J. E., Aldape, K. D., Bredt, D. S. & Israel, M. A. (1995) *Cancer Res.* **55,** 727–730.
44. Greenblatt, M. S., Bennett, W. P., Hollstein, M. & Harris, C. C. (1994) *Cancer Res.* **54,** 4855–4878.
45. El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. & Vogelstein, B. (1992) *Nat. Genet.* **1,** 45–49.
46. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E. & Shay, J. W. (1992) *Mol. Cell Biol.* **12,** 2866–2871.
47. Ginsberg, D., Mechta, F., Yaniv, M. & Oren, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9979–9983.
48. Miyashita, T., Harigai, M., Hanada, M. & Reed, J. C. (1994) *Cancer Res.* **54,** 3131–3135.
49. Subler, M. A., Martin, D. W. & Deb, S. (1992) *J. Virol.* **66,** 4757–4762.
50. Agoff, S. N., Hou, J., Linzer, D. I. & Wu, B. (1993) *Science* **259,** 84–87.
51. Jackson, P., Bos, E. & Braithwaite, A. W. (1993) *Oncogene* **8,** 589–597.
52. Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A. J. & Shenk, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 12028–12032.
53. Mack, D. H., Vartikar, J., Pipas, J. M. & Laimins, L. A. (1993) *Nature (London)* **363,** 281–283.
54. Horikoshi, N., Usheva, A., Chen, J., Levine, A. J., Weinmann, R. & Shenk, T. (1995) *Mol. Cell. Biol.* **15,** 227–234.
55. Chu, S. C., Wu, H. P., Banks, T. C., Eissa, N. T. & Moss, J. (1995) *J. Biol. Chem.* **270,** 10625–10630.
56. Liu, R. H., Jacob, J. R., Hotchkiss, J. H., Cote, P. J., Gerin, J. L. & Tennant, B. C. (1994) *Carcinogenesis* **15,** 2875–2877.
57. Middleton, S. J., Shorthouse, M. & Hunter, J. O. (1993) *Lancet* **341,** 465–466.
58. Boughton-Smith, N. K., Evans, S. M., Hawkey, C. J., Cole, A. T., Balsitis, M., Whittle, B. J. & Moncada, S. (1993) *Lancet* **342,** 338–340.
59. Wang, X. W., Forrester, K., Yeh, H., Feitelson, M. A., Gu, J. R. & Harris, C. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2230–2234.